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The Electrophoretic Profile Myofibrillar Proteins Extracted From Camel Muscles, Kept in Various Modes

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Abstract— *Changes in electrophoretic profiles of myofibrillar protein (MFP) in the Longissimus thoracis (LD) of young camels (2 to 4 years), preserved by refrigeration has been treated or not by lactic acid solution 4% or citric acid 1%, were followed during the post-mortem time at the following times: 1, 2, 4, 6, 8, 10, 12, 24 and 48 hours. The cold preservation for 48 hours has not shown any particular distinctions in the protein profiles of this muscle. Changes related to the type of treatment were recorded during the storage time. Proteolysis of the myofibrillar fraction was earlier in this muscle in the case of treatment with one of two solutions of organic acids used, particularly in the case of using lactic acid. Indeed, these changes have affected at the first hour after slaughter the proteolysis of the myofibrillar proteins. Fragments of low molecular weight (42, 36, 33, 26, 23, 18, 16, 14 and 13 kDa) have been identified. The electrophoretic analysis showed that during refrigeration, LD treated with a solution of lactic acid is more sensitive to disruption phenomena and muscle protein proteolysis that lots of this muscle that even in the case of preservation by refrigeration only or by refrigeration after treatment with a solution of citric acid.*

Keywords— Camel, citric acid, lactic acid, Longissimus thoracis, refrigeration, protein electrophoresis.

I. INTRODUCTION

Meat is the product of transformation of muscle after animal slaughter [1]. Organoleptic quality is extremely important in the red meat industry [2, 3]. Tenderness is the first criteria for selection by consumers of meat and they are willing to pay more for this quality [4, 5, 6]. The main problem facing meat industry are the variabilities due to complex factors rising from variations during animal growth as well as factors ante and *post mortem*.

Tenderness is the most difficult criterion to control or predict [7, 8], the tenderness of the meat depends on two tissue structures, the myofibrillar proteins and connective tissue. The first is strongly influenced by meat's storage conditions while the second is directly linked to livestock characteristics of the animal at time of slaughter [9]. Indeed, after slaughter, myofibrillar muscle structure undergoes profound changes that are largely dependent on enzymatic activity and physicochemical characteristics of the fibers.

After the death of the animal, the tenderizing processes are started. They are the cause of the rupture of the myofibrils and leads to tenderizing meat [10, 11]. There are few studies on the comparison of the effect of the refrigeration or refrigeration after treatment with a solution of lactic or citric acid on the myofibrillar proteins of dromedary muscle. The objective of this work is to study the evolution of Longissimus thoracis myofibrillary profiles (LD) of camels aged two to four years within 48 hours of refrigeration. These are subjected to a prior treatment with one of two organic acid solutions (the 4% lactic acid or citric acid 1%). And searching for the effect of variation of the method of preservation on these profiles.

II. MATERIALS AND METHODS

1.1. Biological material

To study the evolution of proteolysis of the myofibrillar proteins, the *Longissimus thoracis* (LD) from camels ages two to four years of the Sahrawi race were used. The animals were slaughtered according to the Muslim rituals at the slaughter house of Ouargla, Algeria.

Longissimus thoracis (dorsal Long) is the larger muscles of the body. It forms the most important muscle of the thoracolumbar region; it extends along the vertebral gutter bro-costal from the sacrum to the base of the neck (Figure 1).

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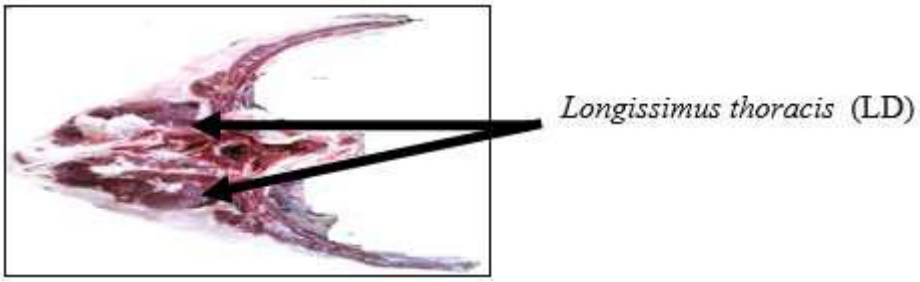

Fig.1: Location of the studied muscle at the last vertebra.

1.2. Preservation (organic acids)

Conservation of the muscles studied, is accomplished by the combination of two methods, one physical (refrigeration), while the other is chemical using two organic acid solutions (lactic acid and citric acid, at concentrations of 4% and 1%, respectively).

Samples of muscle were taken after the gutting and cutting of carcasses. Triplicate samples of each muscle were individually wrapped in polythene bags and then transported to the laboratory where they were boned and trimmed of external fat. The removal of lipids before protein extraction is desirable to avoid formation of an emulsion preventing protein extraction [12, 13]. These were divided into three groups; a control, one treated with 4% lactic acid, the third was subjected to treatment with 1% citric acid. Each sample was individually packaged in sealed sterile plastic bags and placed in a refrigerator at 4°C. These muscles were followed up proteolysis myofibrillar proteins at specific time intervals.

1.3. Extraction of myofibrillar proteins

The extraction of myofibrillar proteins was achieved using the method described by Gagaoua et al., [14]. To avoid activation of proteases, samples were maintained on ice throughout the extraction procedure. 200 mg of sample was incubated on ice with an extraction buffer for 10 minutes with constant agitation. These were then ground and homogenized by polytron for 15 to 20 seconds and reincubated under the same conditions for a further 5 minutes. The mixture was then centrifuged for 15 min at 5000g. The supernatant containing the sarcoplasmic proteins was removed. The myofibrils pellet obtained was reconstituted in the extraction buffer and homogenized using a vortex. These samples were stored at -20 ° C for ulterior use.

1.4. Electrophoresis on polyacrylamide gel in the presence of SDS (SDS-PAGE)

Myofibrillar protein electrophoresis was performed under denaturing conditions as described by Laemmli, [15]. Protein separation was based solely on their molecular weights. The estimate of the degree of proteolysis of the myofibrillar proteins was performed at different time points post mortem by electrophoresis on polyacrylamide gel in the presence of sodium dodecyl Sulfate (SDS-PAGE) from myofibrillar proteins obtained at the end of extraction. The protein sample was treated with a reducing agent, the β-mercaptoethanol, a compound which has a denaturing effect on proteins by disruption of their three dimensional structure. Migration in the gel is thus affected only by molecular weight [16]. Samples were fractionated by electrophoresis on 12% polyacrylamide gel. All protein fragments separated according to their molecular weight which has been determined by protein markers (reference protein). The standard proteins used are low molecular weight markers containing the β phosphorylase (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30kDa), trypsin inhibitor (20, 1 kDa), and α-lactalbumin (14,4 kDa). Protein bands were revealed using the stain with Coomassie blue R-250. This colouration starts with a fixing step in a solution containing 30% methanol, 5% acetic acid for 20 min, followed by staining in the same solution containing 0.12% Coomassie Blue R-250 for 1 hour. Discoloration was carried out by the same solution as that used for fixing, it may last overnight. Finally, the gels were scanned to be studied.

III. RESULTS AND DISCUSSION

According to Jia et al., [17]; Zapata et al., [18]; Kemp et al., [19] and Ouali et al., [20], *post-mortem* maturation of muscles is an enzymatic process that leads to degradation of myofibrillar structures and to lesser extent collagens by endogenous proteolytic enzymes, which will condition tenderizing red meat. Proteolysis of the myofibrillar proteins was evaluated in the LD muscle preserved by refrigeration or treated with citric acid or 1% lactic acid 4% before being refrigerated. The effect of different treatments was well highlighted. The electrophoretic profiles of myofibrillar fraction muscle protein in *Longissimus thoracis* from camels aged 2 to 4 years preserved by refrigeration having undergone previous treatment with a citric acid solution 1%, lactic acid 4%, are represented respectively in figures 2, 3, 4.

According to the electrophoretic profile of the myofibrillar fraction in refrigerated LD or previously treated by a solution of citric acid 1%, several bands were observed in protein extracts (Figures 2, 3 and 4). The

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bands identified one hour after slaughter were had molecular weights of 66, 53, 42, 36, 33, 18, 16 and 14 KDa. According to the work of Delbarre-Ladrat et al., [21] and Bond et al., [22], these bands were identified as tropomyosin, desmin, actin (AC), tropomyosin (TMP), Troponin I, Troponin C and two light chains myosin (MLC), respectively. Starting from hours post-mortem, the strips 66 and 48 kDa disappeared as well as the bands of relative molecular weights of the order of 42, 36, 33, 26 and 23 kDa, have been identified and the molecular weight bands 13, 14, 16, 18 and 97 kDa. The intensity of these bands is variable. Observing these protein profiles showed no major differences 6 hours after slaughter for LD preserved by refrigeration having ,or not, undergone previous treatment with a 1% citric acid solution (Figures 2 and 3). This is probably due to a similar proteolysis during this period. Similar results were observed in sheep [23]. However, the small differences in band intensity can be attributed to the difference in the relative amount of protein loaded on the gel electrophoresis as was noted by Martinez et al., [24].

Pretreatment with a 4% lactic acid solution strongly influenced the proteolysis of the myofibrillar proteins, the disappearance of the bands corresponding to molecular weight fragments greater than 66 kDa was observed starting from the first hour following slaughter (Figure 4). The disappearance of high molecular weight protein in citric acid treated samples took at least eight hours. The appearance of bands at 33 kDa indicator of tenderness were also reported by Ho et al., [25] and Zamora et al., [26] on beef and Smili, [27] on camel meat. The lower molecular weight bands at 23 kDa for the muscles having undergone or not before refrigeration acid treatment consistent with the results of Chobert et al., [28]; Cho, [29]; Barany et al., [30], Delbarre-Ladrat et al., [21] and Ouali et al., [20] on beef. The use of organic acids (citric and lactic acids) accelerated proteolysis myofibrillar proteins, thus leading to early proteolysis, which can induce a tenderizing meat. The same effect of the organic acids has been demonstrated by Cannon et al., [31] and Ertbjerg et al., [32]. Early onset fragments whose molecular weight varies between 33 and 30 kDa, associated with the maturation of meat confirms that the rate of maturation of the treated muscles was faster than the muscles which did not undergo any treatment before refrigeration . This suggests an early proteolysis, which can be explained by lower pH thus promoting the activity of proteases. Bands whose molecular weight is in the order of: 13, 14, 16, 18, 23, 26, 33, 36, 42 and 53kDa, persisted in the electrophoretic profile of refrigerated muscles or having previously been treated by one organic acid solutions used, between 24 hours and 48 hours *post-mortem*. Intensity was more remarkable at the end of the experiment (48 hours) (Figure 2).

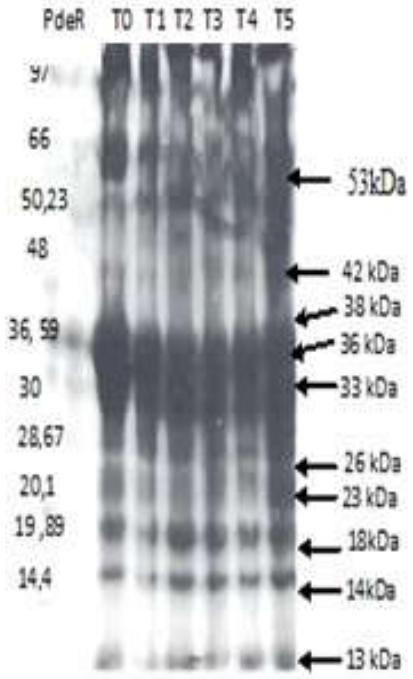

Fig.2: Electrophoretic profile of the myofibrillar fraction in refrigerated LD

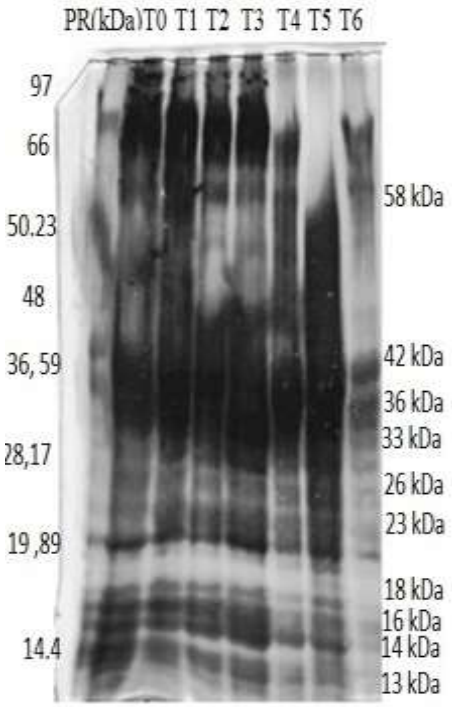

Fig.3: Electrophoretic profile of the myofibrillar fraction in refrigerated LD previously treated by a solution of citric acid 1%

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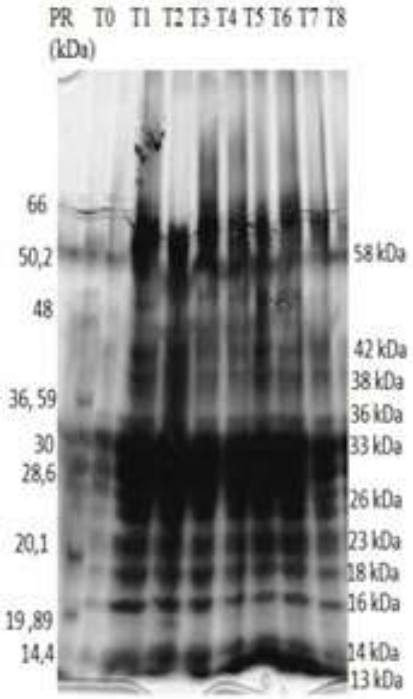

Fig.4: Electrophoretic profile of the myofibrillar fraction in refrigerated LD previously treated by a solution of lactic acid 4%

IV. CONCLUSION

During refrigeration for 48 hours, the profiles of myofibrillar proteins of muscle *Longissimus*

During refrigeration for 48 hours, the profiles of myofibrillar proteins of muscle *Longissimus thoracis* (LD) of dromedary have shown relative stability and have displayed only small changes in the intensities of several protein bands for the same treatment suffered. This indicated a low proteolysis of myofibrils. However, variations affecting these proteins were detected between different lots of the muscle according to the retention mode. Art refrigeration supplemented with a treatment with an organic acid has proven adequate to accelerate the maturation of muscle LD dromedary. But other analyzes of other qualitative parameters, including the enzyme level is needed to better determine the chilling period and the dose of organic acids which allow preserving the nutritional quality of the meat.

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Chemical Composition of the Biomass of *Saccharomyces cerevisiae* - (Meyen ex E. C. Hansen, 1883) Yeast obtained from the Beer Manufacturing Process

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Abstract**—** *Brewer's yeast was subjected to analytical studies to determine the chemical composition of its biomass. To this end, traditional methods of analysis were used to determine ribonucleic acid (RNA), mineral elements, amino acids and fatty acids. The results showed that proteins (49.63%), carbohydrates (31.55%), minerals (7.98%), RNA (8.12%) and total lipids (4.64%) predominate in the biomass composition. The amino acid profile of the protein is suitable for human nutrition, exceeding the recommendations from the FAO/WHO/UNU for essential amino acids. It is particularly rich in lysine and could be recommended as protein supplement in cereals. It was also observed that the yeast was an excellent source of some microelements, such as selenium, chromium, nickel and lithium; that it is also a good source of dietary fiber, particularly soluble fibers; and that the content of lipids was low, with a predominance of saturated and mono-unsaturated fatty acids with 10, 16 and 18 carbon atoms.*

Keywords**— Brewer's yeast, Biomass, Chemical Composition, Protein Value.**

I. INTRODUCTION

The spent yeast used in the fermentation for the production of beer is an industrial residue that represents the second largest waste product generated in this sector, second only to brewers' spent grain. This residue has a high BOD (biological oxygen demand), which may represent up to 60% of the value of this indicator in the effluent (Andreis, 2012). Since Brazil is a major producer of beverages and fuels through alcoholic fermentation (fifth largest beer producer in the world) (Cetesb, 2005), the amount of yeast produced is high.

The micro-organism used in the beer production process is *Saccharomyces cerevisiae.* During the fermentation process, the yeast uses 90% of the available fermentable sugars to produce alcohol, and only 10% for the production of biomass (Cetesb, 2005). From an industrial and national perspective, however, 10% represents a large amount of residue.

The spent yeast used in the alcoholic fermentation process has been used in various sectors, such as the production of animal feed because it is an excellent source of protein (Huige, 2006); as fish feed, potentially offsetting up to 50% of the protein in the feed without any negative effect (Ferreira et al., 2010); and also as input in the biotechnology industry, e.g. for the production of pulp, in the food industry to obtain flavors for certain foods and in pharmaceutical industry (Nasseri *et al*., 2011; Lin *et al.*, 2013).

Studies with yeasts have stood out not only because they are traditionally associated with the preparation of fermented food and beverages, but also because of their versatility and ability to grow quickly on a wide variety of substrates (Guzmán-Juarez, 1983; Sgarbieri, 1987; Giec and Skupin, 1988). Yeast as a source of proteins has been studied mainly after the 1960s, and so far there is no comprehensive and suitable technology for its extensive application (Nasseri *et al.*, 2011).

The production of protein isolates and concentrates from micro-organisms such as yeast, algae and bacteria, has received considerable attention in recent decades. Due to the high protein content (45-65%), they are considered great non-conventional sources of protein (Halàsz; Baráth and Màtri, 1988).

Brewer's yeast has been poorly studied for nutritional purposes, perhaps because of its bitter taste, which results from the beer fermentation process. Some studies performed with the excess yeast from beer production, used the intact inactive cells for functionality tests,

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without addressing the nutritional aspects (Roshkova; Dukiandjiev; Pavlov, 1986). The two main factors cited as limiting factors for the biological use of yeast nutrients are its high content of nucleic acid (RNA) and the very thick and resistant cell wall, which interferes with its digestibility (Kihlberg, 1972; Nasseri *et al.*, 2011).

Considering the nutritional potential of waste arising from the beer manufacturing process, the objective of this work was to evaluate the influence of the mechanical rupture of the cell walls for the biological use of the protein biomass from yeast cells obtained from a craft beer brewery in the southwestern region of the state of Paraná, Brazil.

II. MATERIALS AND METHODS

Yeast:

The yeast *Saccharomyces* sp., from a brewery located in the southwestern region of the state of Paraná, Brazil, was obtained in the form of fresh, already debittered cells suspended in water.

Analytical Methods:

Proximate composition - total protein, moisture and ashes were determined in accordance with the AOAC procedures (1975; 1990). Total carbohydrates were determined through the colorimetric method from Dubois *et al.*, (1958). Total lipids were extracted through the procedure of Blight and Dyer (1959) and determined gravimetrically. Soluble and insoluble fibers were quantified through the method from Asp *et al.*, (1983).

Nucleic Acid (RNA):

The nucleic acids in yeast, which consist mainly of RNA, were determined through the method from Hebert et al., (1971). The RNA was extracted with 0.5m perchloric acid at a temperature of 37° C for 2 hours. It was then hydrolyzed with 0.5m perchloric acid at a temperature of 100° C for 15 minutes.

The quantification of ribose was done with the orcinol reagent, which produces a greenish color and absorbs at 670 nm. The readings were compared with those of the standard curve made with the purified RNA of yeast (Sigma).

Mineral Elements:

The samples were first burned and left in the oven at 450°C for several days, until they were completely white. They were then dissolved in nitric acid at 5%. Aliquots were injected in an Argon Plasma Emission Spectrometer (ICT 2000 Baird). The operating conditions were: radio frequency, 40.68 Mhz; concentric pneumatic nebulizer; entry flow of the sample to be nebulized, 4 mL/min; cooling gas flow, 70 mL/min; position of the vertical torch, 9.8 mm; power applied, 100W.

The quantification of minerals was performed using the standard curve constructed based on a solution (100 µg/mL) of the BAIRD analytical grade in nitric acid at 5%.

Amino Acids:

Amino acids were determined in a laboratory analyzer with a cation exchange column and post-column derivatisation with ninhydrin. The samples were hydrolyzed in advance with HCl 6N at 110°C for 22h, with the exception of tryptophan, for which the hydrolysis was performed with LiOH 4N for 24 h at the same temperature.

Fatty Acids:

The fatty acid composition was determined by gas chromatography of the methyl esters of the fatty acids, obtained according to the method described by Hartman and Lago (1973). A Variam 3400/3300 gas chromatograph was used with a flame ionization detector (FID), OV 275-15% Carbowax (1/8" x 2m) column. The fatty acids were identified by comparing the retention time with the standards (Sigma) and quantified through the automatic calculation of the area with the Perkin-Elmer-100 integrator.

Statistical Analysis:

The statistical analysis was performed with the SANEST software (Statistical Analysis System), submitting the experimental results to analysis of variance and Tukey's test at a confidence interval of 95% (Gomes, 1982).

III. RESULTS AND DISCUSSION

The proximate composition of the brewer's yeast biomass of *Saccharomyces cerevisiae* is shown in table 1.

For the calculation of the crude protein (49.63%), the conversion factor of 5.8 was used, calculated after subtracting the non-protein nitrogen corresponding to the RNA of the biomass. The composition is characterized by high levels of protein, ashes, RNA and soluble fiber. The total lipid content is low and total carbohydrates represent approximately one third of the biomass.

When the data presented in Table 1 is compared with other studies, one can see that the data is similar to those obtained by Farnun and Cleland (1975); Guzmán-Juarez (1983) and Caballero-Córdoba *et al.*, (1997). The results of these studies are very similar and are very close to those found in the study presented here.

In Table 2 , the data of the amino acid composition of the yeast biomass as compared with the reference standard of FAO/WHO/UNU (1985), is presented.

One can see that all essential amino acid profiles of the yeast cells exceed the recommended amino acid quantities by the three organizations of the United Nations. It should be noted that the high concentrations of lysine (LYS) and threonine (Thr) in the yeast biomass, turn it into an exceptional material for the supplementation of cereals,

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since the protein content of cereals is commonly deficient in these two amino acids and also in tryptophan.

The fatty acids profile of the lipid fraction of the biomass of *Saccharomyces cerevisiae* is shown in Table 3.

After the performed analysis, 11 fatty acids (C8-C18) could be identified, including palmitic acids (34.33), oleic acids (11.02), stearic acids (9.56), capric acids (6.26), linoleic acids (4.37) and palmitoleic acids (2.99). Fatty and mono-unsaturated acids, therefore. The linoleic acid content (C18:3) was low, as it was only 0.63%. It should be emphasized that the data obtained in this study are in line with the data from Caballero-Córdoba *et al.*, (1997), who analyzed fatty acids levels in yeast using the same methodology, presenting very similar rates, but they are not in agreement with the results from Halász and Lástity (1991), both from a quantitative and qualitative perspective.

Accordig to Halász and Lástity (1991), the lipid content of yeast varies from 7 to 15% and the fatty acid composition is characterized by a high content of unsaturated fatty acids, with the oxygen flow during the cultivation of the cells being the parameter that most influences the fatty acid composition.

In Table 1, the total lipid content is 4.64% lower than the range of 7-15%, but very similar to the data presented by Farnun and Cleland (1977); Guzmán-Juarez (1983) and Caballero-Cordoba *et al.*, (1997) with values of 3.44; 4.91 and 4 to 7%, respectively.

In the data observed in Table 3, on the other hand, the saturated and mono-unsaturated fatty acids predominated instead of the poly-unsaturated fatty acids. It is likely that this difference reflects the different physiological and nutritional conditions of the different biomasses. In the case of the results reported in this work, the biomass was more spent after several recyclings in the fermentation process. In the example of the literature, the biomass used was grown in ideal conditions for nutrient concentration in the medium and oxygen supply.

The mineral composition of the biomass is presented in Table 4. Considering the high content of nucleic acid in the yeast (Table 1), which limits its daily intake to a maximum of 20-30 g of dry yeast per day, the yeast will not be able to contribute with very significant quantities of macro-elements. With respect to the micro-elements, it can be considered an excellent source of selenium, manganese, chromium, nickel and lithium, with observed values of 25.12; 14.98; 10.11; 9.05, 8.23 and 6.13 mg for every 100 grams of biomass evaluated.

The data presented here are in line with the data obtained by Caballero-Cordoba *et al.*, (1997) in studies that also considered the yeast biomass of the same species and in which they observed the presence of the same chemical elements in the performed quantifications. In the two works compared here, the macro-elements phosphorus, potassium, sodium, magnesium, aluminum, calcium and iron were observed. The micro-elements obtained in this work are the same observed in the work described here.

Yeast is considered an excellent source of selenium and chromium, with its intake being recommended as a dietary supplement to prevent deficiencies of these elements, which are characterized by hair loss, growth retardation, reproductive deficiency, heart diseases, necrosis and degeneration of the liver and pancreas (Levander, 1989).

The presence of lithium should also be emphasized, because it has been used in the treatment of various problems associated with neuropsychiatric disorders in the past three decades. It is particularly beneficial for the acute treatment of mania, and usually for the prophylaxis and treatment of depression in bipolar patients. Currently, lithium is the treatment of choice for bipolar disorder, preventing relapses and suicide attempts. Its use is successful in dramatically reducing depressive and manic symptoms in 70% to 80% of patients (Muller-Oerlinghausen *et al.*, 2002).

IV. CONCLUSION

With the data obtained, the conclusion can be drawn that the biomass of the brewer's yeast of *Saccharomyces cerevisiae* - (Meyen ex E. C. Hansen, 1883) obtained from the beer production process is a rich source of protein with good nutritional value, taking into account the various levels of biological evaluation.

With a high content of lysine, this protein source proves to be of special interest as a protein supplement for cereals. The rupture of the cell wall significantly improved the digestibility and use of the net protein from the biomass.

It was also observed that the yeast proved to be an excellent source of some microelements, such as selenium, chromium, nickel and lithium; that it is also a good source of dietary fiber, particularly soluble fibers; and that the content of lipids was low, with a predominance of saturated and mono-unsaturated fatty acids with 10, 16 and 18 carbon atoms.

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Table 1: Proximate composition of the yeast biomass obtained from breweries - Saccharomyces cerevisiae.

Components	Yeast[1]	Yeast[2]	Yeast[3]	Yeast[4]
Protein	49.63±2.43	48.51	49.80	45-49
RNA	8.12±1.54	7.52	8.40	8-12
Lipids	4.64±0.52	3.44	4.91	4-7
Ashes	7.98±0.76	8.33	5.10	5-10
Total Carbohydrates	31.55±4.32	32.86	-	26-27

Soluble Fibers	9.12±1.22	9.59	-	-
Insoluble Fibers	2.87±0.87	2.60	-	-

Yeast[1] – Conversion factor to protein = at 5.8.
Yeast[2] - Farnun and Cleland (1975).
Yeast[3] - Guzmán-Juarez, (1983).
Yeast[4] - Caballero-Córdoba et al., (1997).

Table 2: Composition of amino acids (g/100g of protein) of the brewer's yeast biomass - Saccharomyces cerevisiae.

Not Essential	BI	Essential	BI	PR
Cys	1.24±0.21	Lys	6.73±1.21	5.8
Tyr	4.12±0.32	Leu	8.75±0.67	6.6
Glu	8.56±0.45	Ile	4.63±0.32	2.2
Asp	10.05±0.56	Thr	6.09±0.57	3.4
Ser	5.45±0.31	Try	0.96±0.04	1.1
Pro	5.11±0.67	Val	5.34±0.43	3.4
Ala	6.89±0.12	Met+Cys	3.55±0.64	2.5
Gly	5.23±0.87	Phe+Tyr	8.36±0.89	6.3
Arg	4.02±0.64	His	2.78±0.06	1.9
Phe	5.57±0.10	Met	3.12±0.21	-

BI = whole biomass; PR = reference standard of the FAO/WHO/UNU (1985).

Table 3: Composition of fatty acids and total lipids of the brewer's yeast biomass - Saccharomyces cerevisiae.

Fatty Acids	Structure	Total Concentration (%)
Caprylic	C8:0	0.29
Capric	C10:0	6.26
Lauric	C12:0	1.26
Myristic	C14:0	0.78
Myristoleic	C14:0	0.39
Palmitic	C16:0	34.33
Palmitoleic	C16:1	2.99
Stearic	C18:0	9.56
Oleic	C18:1	11.02
Linoleic	C18:2	4.37
Linolenic	C18:3	0.63

Table 4: Mineral composition (macro and micro-elements) of the brewer's yeast biomass - Saccharomyces cerevisiae.

Macro-elements	Mg/100g	Micro-elements	Mg/100g
Phosphorus	17.31	Selenium	25.12
Potassium	14.21	Manganese	14.98
Sodium	9.13	Lead	10.11
Magnesium	3.02	Chromium	9.05
Aluminum	1.12	Nickel	8.23
Calcium	0.87	Lithium	6.13
Iron	0.17	Zinc	4.89
		Copper	4.19
		Vanadium	0.56
		Cadmium	0.45

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Evaluation of Land Suitability for Stone Pine (*Pinus pinea*) plantation in Lebanon

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Abstract— *Stone pine (Pinus pinea) is a typical Mediterranean tree well adapted to drought and high temperatures. It is a species of great interest and economical importance in Lebanon and has a strong beneficial impact on the local communities from the marketing of its edible nuts. This tree is threatened by human activities and fire that are leading to its degradation. Therefore, the aim of this study is to delineate and map the suitability of soils for the plantation and extension of the stone pine. For this purpose, the adopted research methods were composed of the following three steps: (1) identifying through available data and traditional methods the ability of the lands to be planted with stone pine (2) identifying the various factors influencing the growth and fruiting of the treeand (3) transforming and integrating all the data into geo-referenced thematic maps and introducing them into the Geographic Information System (GIS) suitable for delimiting Lebanese areas suitable for planting stone pines. The obtained results were presented in a form of 10 thematic maps (GIS layers) that represent the influence of each ecological factor on the land suitability for afforestation by stone pine. A final thematic map that illustrates the most suitable areas for Pinus pinea plantations was generated by superimposing the 10 GIS layers.*

Keywords—Pinus pinea, forest plantation, suitability of soil, GIS, Lebanon.

I. INTRODUCTION

Stone pine (*Pinus pinea* L.) is a Mediterranean tree. It is well suited to high temperatures and drought (Montero et al. 2004), characteristic of Mediterranean climates. The total area covered by the forest of stone pine is about 380,000 hectares (75% in Spain, 9% in Portugal, 9% in Turkey, 5% in Italy), and lower percentages in Greece, Lebanon and France (Moussouris&Regato 1999, Rego 2007).

In Lebanon, the stone pine forests cover the northern and southern Mediterranean facades exposure of Mount Lebanon to an altitude of 1200 m and represents 18% of total forest cover and 36% of coniferous forests or nearly 14 000 ha (Baltaxe 1996). It is considered as one of the essential tree species that form the botanical richness of the Mediterranean phyto-sociology stages of the country. The multi-usage of its light and soft wood (carpentry, framing and especially in marine construction), the landscape value of this species and the use of its seeds in a wide variety of sweets, give the stone pine a high economic value (Calama et al. 2007).

In Lebanon and according to the Ministry of Agriculture, the total annual production was estimated at 1200-1500 tons for the period 2003 to 2005, with a total annual value of 16.5 to 25.8 millions of dollars. Most of the production comes from the Mount Lebanon region, especially Rass al-Metn and from the region of Jezzine. The price of one kilogram of seeds in the Lebanese market reached $ 37 per kg (Nehme& Johnson 2010).However, forest stands of pine trees in the country are threatened by deforestation, overgrazing, and urban development and fires (MOE / UNDP, FNRCBD2009). References show that during the past 40 years a decrease of 58% in the forest cover of the inner region of the Bekaa and 5 to 10% in southern and northern Lebanon (Masri et al. 2002).Currently, the priority in Lebanon is to promote afforestation and reforestation activities. Many actions and programs have been undertaken by the Ministry of Agriculture to compensate for the lossesin the forest cover over, and extends the next 20 years, including the program "40 million trees" (MoA 2012). The country has never had such ambitious reforestation movements. The choice of species is made after consultation and in collaboration with municipalities and local stakeholders. It turns out that stone pine is considered one of the most attractive species. The International Institute of Genetic Resources (IPGRI 1998), classified pine pinion among the top three most valued forest species in Lebanon. This classification is based on three main parameters: (1) the economic value of the tree, (2) the ecological value and (3) the risks that threaten it.However, favourable places to grow this tree were not geographically delineated by IPGRI (1998) or by reforestation plans adopted by the

Ministries in Lebanon. Reforestation of stone pine implies knowledge of the available free surfaces, the soil types and the favourable climate conditions for the development of this tree. A Scientifically unjustified reforestation means loss of seedlings, repeated and costly work.Identifying proper planting locations requires; an up-to-date land use map, the availability of a database on soil types and a detailed understanding of the behaviour of the tree in relation to the environmental conditions. Most of the maps that were needed for this work were be reproduced, updated and digitized.

The purpose of this study is to classify the lands according to their ability to reforestation. This ability will be illustrated geographically in the form of a coloured thematic map with 5 classes of hierarchy from the most to the least favourable. This thematic map will become a key tool for the development of a reforestation plan where priority areas will be designated as well as the number of neededseedlings. The calculation of the total cost of the operation at the national level will be even possible.

II. MATERIALS AND METHODS

2.1. Study zone

Lebanon is located on the eastern coast of the Mediterranean Sea, between 36 ° 66 'and 38 ° 60' latitude (Figure 1). Its area is 10,452 km2 and spans over 210 km length from southwest to northeast, with a width not exceeding 85 km. It is characterized by a mountainous topography formed by two chains running from north to south parallel to the coast separated by the internal plain of Bekaa. This topography has divided Lebanon into four geographical zones (Murtada 2003):

1- The coastal area that forms a narrow border between the sea and the Mount Lebanon chain. It expands to reach 30 km in the plain of Akkar.

2- Mount Lebanon, a mountain chain ranging from 600 m in the south to 3080 m north. It gradually overcomes the sea to the peaks, while it forms a steep slope to the opposite side to the plain of Al Bekaa. At Beirut, this chain is crossed from east to west by the collar of Dahr al-Baydar which influences the climate and biodiversity of the Mount Lebanon eastern exposure. This area is divided into two main bioclimatic stages. The first is of average altitude ranging between 500 and 1200 m, divided by a series of narrow valleys. The second, a high altitude chain, reaching up to 3080 m of altitude at Kornet El-Sawda.

3-The Anti-Lebanon is the eastern chain of the country with a peak of 2616 m at Tallat Moussa. The upper zone is a high and dried plateau. It is lower than Mount Lebanon and decreases gradually from south to north.

4- The interior plain Al Bekaa separates the two mountain ranges. It is a high plateau of 800 m of altitude and 133 km in length. Lebanon is characterized by a temperate climate with hot dry summer and mild and wet winter influenced by the cold winds of the west and north and sub-desert from the east and south. Five climatic units are present (Abi Saleh & Safi 1988): the Thermomediterranean, the Eumediterranean, the Supramediterranean, the Montane Mediterranean and the Oromediterranean.

According to the soil map of Lebanon (Darwish et al. 2005), a high variability of soil types was observed. Stone pine is abundant on sandy soil. The soil has greatly influenced the landscape determining its floristic composition. Stone pine is associated with other vegetation, mainly *Erica spiculifora*, *Gallipoli rose*, *Calicotomespinosa*, *Lavandulastoechas* and *Juniperus oxycedrus*.

2.2. Methodology

The methodology is based on three main steps: (1) identify and characterize through available data and traditional methods the ability of the soil for stone pine plantation, (2) identify the various factors that influence the growth and the fruiting of the tree, and (3) transform and integrate all the data into geo-referenced thematic maps and introduce them into the Geographic Information System (GIS) suitable for delimiting areas suitable for stone pine plantation pine (Figure 2).

2.3. Land Classifications

The classification of lands suitable for the stone pine reforestation takes into account the topographical, soil and climatic factors (FAO 1984). Different information is derived from several maps from the National Remote Sensing Centre. Several field investigations have allowed us to update and validate the map information and identify all of the factors influencing the growth and regeneration of stone pine.

30 field trips were carried out according to the budget available and taking into consideration the unstable political situation in some areas of the country. A data set was collected: geomorphologic parameters (slope, exposure, altitude), pedologic (drainage, soil depth, CaCO3, pH, texture, organic matter) and climate (precipitation, sum of annual temperatures, extreme temperatures) that affect the growth and fruiting of the tree.

The purpose is to arrange all these spatial and geometric data into an integrated system where they can be organized, analyzed and transformed into a map. Each spatial unit, referring to the soil and geo-climatic data, will advise us on reforestation potential of stone pine in Lebanon.

2.4.Gradation of the ecological parameters

The choice of appropriate evaluationof parameters is essential to successfully develop an evaluation method for

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land suitability (Olivas 2007). For the stone pine, we focussed on the biological and ecological characteristics of the lands rather than parameters that can be used in a more general framework for assessing lands suitability(Deng et al. 2014).

2.4.1. Determination of criteria influencing land suitability

The parameters of evaluation selected in this study are based on a large literature review taking into account soil and climatic requirements of the species.Soil properties such as soil type, pH, organic matter and soil depth significantly influence the growth and existence of stone pine. A soil with a pH between 4 and 9 is the most suitable for the growth of pine. Values lower than 4 and greater than 9 cause inhibition of the extension of roots. Stone pine grows poorly on heavy, compact, clay or marl soils and prefers sandy loam and sandy soils and supports also stony and dry soils (Alexandrian 1982). A maximum of 50% of total limestone and 15% of active limestone is tolerated by the pine trees (Boisseau 1993). The organic matter content is also important because it improves the growth of the tree by increasing soil fertility (Becker et al., 1994). Weather conditions are also important to the growth of the species. The optimum rainfall varies from 550-1500 mm (Alexandrian 1982). In addition, this tree is sensitive to temperature above its absolute minimum (Rapp &Cabanette 1981), in which the effect varies with altitude and exposure (Alexandrian 1982,Adili2012).The species can endure low temperatures between -2 ° C and 7 ° C on average during the coldest month, and high temperatures between 27 ° C and 30 ° C on average of the hottest months (Zaki 1978). The species comes into winter dormancy when low temperatures become unfavourable for its growth. During harsh winters, it is the least resistant among all conifers (Vazhov et al. 1988). For this reason, Boisseau (1993) considered the correlation of temperature with altitude as an indicator of upper limits to the Mediterranean expansion of this species. According to Labadie (1983), stone pines prefer humid climate (> 70%). In addition, topographic factors (i.e. slope and elevation) play an important role in the distribution of moisture and temperature (Deng et al. 2014). The tree manifests itself best on flat lands and with a slope of less than 8 degree (Alexandrian 1982).

2.4.2. The weight (pi) of the ecological parameters

Based on the literature reviews, it is confirmed that several environmental factors have different effects on the growth of stone pine. Therefore, the weight determination of parameters is an important step for the development of a model equation. There are many methods for determining the weight of parameters (Deng et al. 2014). To assign weights to the parameters used in this analysis, comparisons were made between these parameters. In result of these comparisons, each parameter was attributed a weight from 1 to 5 depending on its importance (Partoune&Quoilin2002). It varies in ascending order from 1 (limiting factor) to 5 (least influencing) by providing significant value to the best environmental conditions. For example, calcium carbonate will have a low weight of around 2 (limiting factor), while the factor that ensures better conditions of the tree, like the rate of organic matter in soil (fertility) or exposition will have a higher weight equivalent to 4. These weights were awarded following an extensive literature review and in collaboration with 5 forestry specialists taking into account local conditions of the study area.

2.4.3. Gradation and standardization of the ecological parameters

In the context of Geographic Information System (GIS), it was necessary to calculate land suitability based on the ecological factors specific to the development of *Pinus pinea* trees (J Malczewski 2006). An equation model was established to enable the GIS to filter the data. This equation 1 is used to classify a given location (i) using the coefficients and weights assigned to each environmental parameter retained.

$$\text{Classe} = \frac{\sum_1^n \text{Cipi}}{\sum_1^n \text{pi}} \text{ x Y} \qquad \textbf{(}\text{equation 1)}$$

Where, Ci is the coefficient value allocated to each rank of parameter gradations; Pi is the weight given to each parameter; and Y is the coefficient of the most important limiting factor for a given location.

The coefficient (Ci) was estimated on the basis of bibliographic data and several field observations (Table 1). The coefficient (Ci) varies from 0.1 (worst) to 1 (optimal). However, the values of some parameters cannot be segregated because of their weak variations (like in the case of pH, which almost always oscillates around 7); they will be considered homogeneous over the entire study area and will not be taken into account. Also, all values of Ci are not represented for all parameters. A deliberate choice was made for every variation of the parameter considered in light of the ecological constraints observed in the field.Therefore empty boxes (N/A) appear in the weighting table (Table 1). Each parameter is spatially represented in the form of a thematic map to enable visualization and analysis. Secondly, and in order to maintain the values ranging from 0 to 1, the sum of the values (Ci.pi) of each station will be divided by the sum of their weight (pi). The resulting number will always be less than 1 classifying the sitesfrom “most suitable” to the “non-suitable” for *Pinus pinea*reforestation.In order to prevent a non-suitable sitefrom having a high rating, in cases when all other conditions are favourable, a factor (Y) was added at the end of the calculation to eliminate

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the non-suitable sites. The multiplicative factor (Y) will differentiate between the classes of suitability emphasizing the importance of the limiting factor. The factor (Y) represents the most restrictive parameters of the coefficient (Ci)gradation in cases when it is not suitable to plant the pines even if the other parameters are suitable.

Based on land suitability characteristics for different crops, FAO (1976) proposed a land suitability evaluation by differentiating five classes: highly suitable, moderately suitable, marginally suitable, currently not suitable and permanently not suitable.In our study, five classes are differentiated by the land's ability for stone pine reforestation:

- Class 1: Highly Suitable > 0.8
- Class 2: Suitable 0.6 to 0.8
- Class 3: Moderately Suitable or Average 0.4 to 0.6
- Class 4: Low suitable 0.2 to 0.4
- Class 5: Not suitable <0.2

2.5.Generating the database in a ArcGIS platform

To illustrate these parameters in the form of thematic maps, we used the computerized database of the National Remote Sensing Centre. The maps used are:

- The soil map of Lebanon (Gèze 1956),
- The Digital Model of the Land (M.N.T.) with contour lines every 50 meters,
- The rainfall map of Lebanon (Plassard 1972),
- The Climatic Atlas of Lebanon Tom. II (Blanchet 1976),
- The land cover map to scale 1/20000 recently prepared with the IRS satellite images 5 meter spatial resolution (CNRS, MoA, MoE 2002).

These maps helped to constitute the various thematic layers (GIS Layers) according to the weighting table (Table 1). We worked under the ArcGIS - ESRI. The crossing of different layers, generated from the equation 1, in GIS resulted in the final map of the ability of Lebanese land for stone pine reforestation.

III. RESULTS

3.1.Identification of constraints to reforestation of pinion pine

3.1.1. Soil occupation

The first step undertaken begins by identifying favourable areas for reforestation according to land availability. So, it was excluded the territories occupied by urbanization, agriculture and dense forests, as well as water surfaces and beaches according to Lebanese soil occupation map at the scale 1:20 000 (CNRS, MoA, MoE 2002).This card includes 4 levels of classification. The first level consists of 9 classes which then detailed in 86 subclasses. In order to transform this map into thematic map layer (Layer GIS), this map was simplified into 5 classes (Figure 3). First class is bare and rocky soils that are considered in principle as priority areas for reforestation with the highest coefficient of 1 (best station), followed by fire and shrublands.Then come the free surfaces in sparse forests, since the vegetation of these forests does not exceed 60% of their total area. In contrast, the dense forests are not completely excluded in the reforestation of stone pine, due to the free surfaces that sometimes exceed 40%. The given coefficient to the latter had a significant weight (Table 1). Similarly, for agricultural and urban land, afforestation by the stone pine is not excluded. It takes sometimes the form of hedges around houses and gardens as well as along highways.

3.1.2. The climate

Climatic factors are not limiting factors, but they usually play a negative role in the growth and fruiting of stone pine. These factors are numerous, such as: precipitation, extreme low temperatures of the coldest month, the rate of evapo-transpiration, wind etc.The data for these parameters are sometimes available, sometimes incomplete or require additional work to their finding and compilation. In this work, we have selected only the two thematic maps for extreme low temperatures of the coldest month (January) and the amount of annual rainfall in order to define the most favourable areas for stone pine reforestation.

3.1.3.The Minimum temperatures

The Mediterranean region characterized by a mild spring with a maritime influence (moisture), is most favourable to this species. Most continental regions where extreme low average temperature of the coldest month is less than 0 or 5°C are considered moderately favourable (Figure 4). In the inner regions of Beqaa valley where temperatures are below 0°C, vegetative growth of the tree is low. In the mountains high altitudes where the average temperatures are negative, stone pine is damaged by the snow that causes breakage of branches. However, the afforestation by this species remains possible given the pine forests which are at 1300 and 1400 meters above the sea. The coefficient assigned to these regions in the weighting table is 0.6. On the other hand, mountain regions where temperatures drop below -5°C (extreme limiting factor) fall into the category of inappropriate places for reforestation by this tree.

3.1.4. Annual precipitations

With the exception of the semi-arid northeastern Lebanese zone where annual rainfall is less than 300 mm considered unfavourable, all Lebanese regions are favourable for growth and fruiting of stone pine where rainfall is often higher than 700 mm. However, the most favourable places are those with rainfall greater than 900 mm (Figure 5).

3.2.The topography

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3.2.1. The altitude

The altitudinal parameter reflects indirectly several clues at once: temperature, rainfall, evapotranspiration and soil moisture. The two Mediterranean stages thermo and Meso (between 0 and 1000 meters above sea level) are considered the most favourable to the stone pine (Figure 6). Beyond this elevation, snowfall causes breakage of branches and acts as a limiting factor in the production of the tree. Altitude between 1000 and 1300 meters corresponds to the coefficient 0.5 regions that exceed 1300 meters are unfavourable.

3.2.2. The slope

The influence of the slope is seen in two complementary negative aspects: first, as an index of erosion, which weakens the soil fertility; the second, as an obstacle to the mechanization of work since the stone pine can be planted as a fruit tree. However, it manifests in crusts and rocky escarpments. It is recommended for reforestation of degraded areas (Figure 7). The coefficient given to over 30% steep slopes is not so weak, it is of the order of 0.4 but a strong weight is assigned in the order of 4.

3.2.3. The exposition

Stone pine is reforested for profits as a fruit tree. The availability of water is a requirement for good production. Evapotranspiration is always less accentuated on the northern exposure than the sunniest southern exposure that will be assigned a lower coefficient. In the weighting table (Table 1), only three classes are distinguished because of the proximity of values and low variation of this factor. The values range from 0.8 for the northern exposure and 0.6 for the South exposure. The stations of all exposition, considered as plateaux facilitating the access to light, soil moisture and work mechanization, are the most favourable (Figure 8).

3.3. The pedology

3.3.1. The Soil depth

The soil physical characteristics are more important to the stone pine that its chemical characteristics. The tree can adapt to different soil types, from the poorest to the most profound. But fertility affects productivity and vigour of the tree. All soils with a depth exceeding 10 cm are considered suitable for reforestation (Figure 9). However, the coefficient assigned to different depths is not the same. It is given the maximum value 1 for soils with a depth exceeding 1 meter and minimum 0.6 for those with depths ranging between 10 and 50 cm (Table 1).

3.3.2. Organic Matter

The majority of the Lebanese soils are poor in organic matter. The amounts found throughout the country do not exceed usually 2%. The contribution of manure from poultry that is free in the mountain areas has been successful. The tree tolerates sandy soil and can also adapt to the rocky soils (Figure 10). The fertility index an important factor in the production of the tree, which is manifested by the percentage of organic matter in the soil is low. This is reflected in the weighting table with high weight of 4, but the coefficients that is maintained at 0.5. These coefficients are not far from the poor and rich soils. They range from 1 for fertile land and 0.5 for soils with less than 1% organic matter.

3.3.3.Calcium carbonate $CaCO_3$

Although the stone pine adapts to different types of soil, the vigour of the tree and its fruit depends largely on the lime content in the soil. The forests that grow on soils formed based sandstone can regenerate spontaneously, unlike the planted forests on calcareous soils where human intervention is necessary. For this reason, the minimum weight given to this parameter is 2. In the other hand, the best coefficient 1 is assigned to the soil completely devoid of CaCO3. However, soils with an amount that exceeds 30% are not considered tolerable by this species (Figure 11) and their ratio remains above 0.4.

3.3.4. Soil texture

For this tree, soil texture is a major limiting factor especially for seedlings germination (Figure 12). In general, the tree tolerates light and well drained soils with the presence of deep groundwater and stony soils. For extreme cases, in heavy, clay or overly compact marl soils, this species grows poorly due to poor structuring and aeration in the soil (Boisseau 1993, 1996). For these reasons, the weight given to this parameter is the one closest to the limiting factor 2, and the coefficients of land having a compact texture (i.e. fine and very fine) have minimum values of respectively 0.2 and 0.4.

3.4. Map of stone pine reforestation opportunities in Lebanon

We took into account the influence of each parameter according to the weighting table (Table 1) and generated 10 thematic maps (GIS layers). The intersection of all the information in the form of thematic layers allow us to reach a final map (Figure 13). Unlike previous maps, where the classification depended on the weight given to each station, the classification of potential areas for stone pine even more delicate since the spatial units no longer carry weight, but the result of repeated multiplication of the weight inherited by all factors.In order not to lead to a fragmentation of stations following this intersection, the stations having the same final weight after each intersection were merged together to form a homogeneous spatial unit (USH). Similarly, the area of stations less than 4 ha were fused with the largest terraced station. Based on this logic crossing cards and multiplying the respective weight for each station, we have reached the final card of the rank of land according

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to their potential for reforestation of pinion pine (Figure 13).

IV. DISCUSSION

The final map is obtained using the geographic information system (GIS) on the ERDAS software. Figure 13 provides a clear delineation of very favourable land for reforestation of stone pine; they occupy an area of 25,365 hectares. This category also adds an area of 21,765 hectares representing favourable land with a total area of 47 130 hectares. These two classes that are distinguished are considered priority for reforestation.They represent nearly 5% of the entire Lebanese territory. This percentage is considerable given that the total forest area in Lebanon according to NFA (2010) is nearly 13% (136 000 hectares). It is observed that the concentration of favourable land (to the state) reforestation by this tree are not only those based on geological basalt (the plateau of Akkar) or lower Cretaceous (Upper Metn and Jezzine) that means devoid of limestone carbonate but also those who are training on compact limestone (the Lebanese south case). However, it appears that the region of Jezzine-Nabatieh is currently the least provided by a leafy forest cover, although the Mediterranean climatic conditions are favourable for reforestation by this forest species. The agricultural vocation of the internal Bekaa and its dry climate pre-steppe appears as an obstacle to reforestation by stone pine. This constraint can be considered as the limiting factor for Mount Lebanon and eastern exposure to the eastern chain (Anti-Lebanon) which now is placed in the category of land with little or unfavourable reforestation by the stone pine. The moderately favourable land reforestation by this tree occupies 56,300 hectares, or approximately 6% of the entire Lebanese territory.It ultimately appears that the classification of these lands is a compromise between several factors and parameters. The results were validated from the land use map. However, an update can be seen from the data and maps and now renewed by the Remote Sensing National Center with better resolution. In addition, several aspects were not addressed in this study, including the logistics (roads), the forestry aspects (density and planting methods, thinning, production and operating age) and status land (state, municipalities, individuals ...). But many constraints did not allow us to have access to this information.However, this work has well demonstrated the relevance of GIS for making synthesis maps that can be very useful for field managers. Nevertheless, it is important to stress that constant updating is expected following the changes that can take place before the complete planting of all land identified suitable for reforestation with the species. Also, other parameters (roads, water etc.) can be integrated according to the assigned objectives.It would also be interesting to extend this methodology to other important species for reforestation in Lebanon. A synthesis map that helps to identify the suitability of soils for reforestation according to the species is a decisive factor for the development of forests in Lebanon.

V. CONCLUSION

The Lebanese government has set the goal of achieving forest coverage of 20%, or 70 000 hectares must be reforested in the coming years. So this work has focused primarily on mapping soil and climatic factors, component necessary, even essential, to achieve a plantation program.Our goal was primarily to result in operational maps for the land manager. The results were presented to officials and decision makers from the Ministry of Agriculture, who are very interested in the methodology and wish to invest in such an approach to facilitate the identification of areas to be reforested.It is clear that this cartographic base is only a starting point for such an ambitious goal. The field manager will implement a strategy of planting where the proposed maps can find all their interest as they allow delineating the Lebanese territories according to their potential. But we must not lose the supply of seeds that will be provided by the Ministry of Agriculture for each region from the elite trees and orchards in pre-identified seed.However, in the future, all this information must be supplemented by field experiments and a network of permanent plots (not currently available in Lebanon). This will enable closer monitoring of stone pine (or other species) to establish indicators of fertility and yield tables for each zone and validate the results.

ACKNOWLEDGMENT

This article is a tribute to the late Professor Talih El Masri a member of the CNRS remote sensing team, he was the initiator of this work. He supervised, facilitated, and never hesitated to share his knowledge. We also wish to express our sincere thanks to the Lebanese Remote Sensing Centre that provided us with databases, research means and the technical nature of Geographic Information System (GIS) for the realization of this work.

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Figures and tables

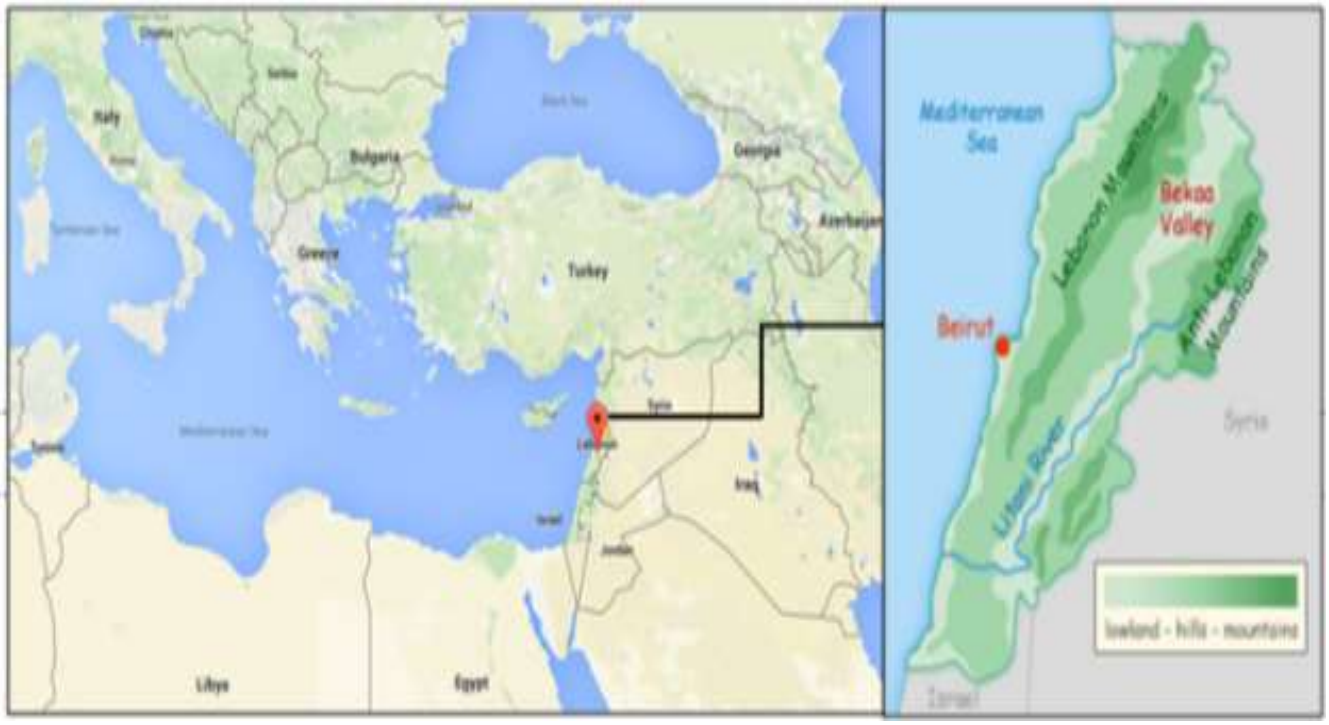

Fig.1: Geographical location of Lebanon according to Mediterranean basin (Google map, www.globaleye.org.uk), and the various topographical zones.

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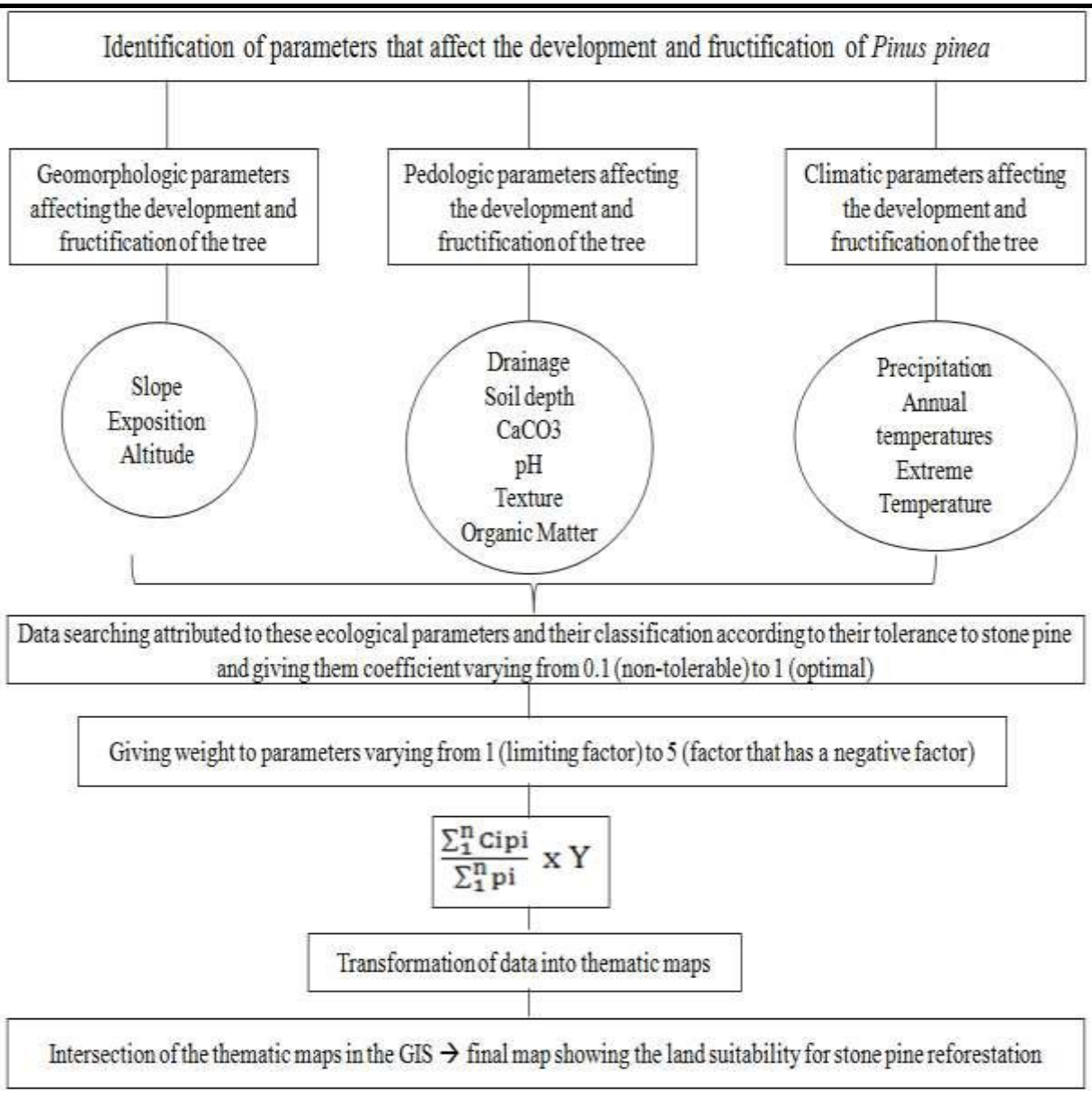

Fig.2: Flowchart of the methodology adopted in this study.

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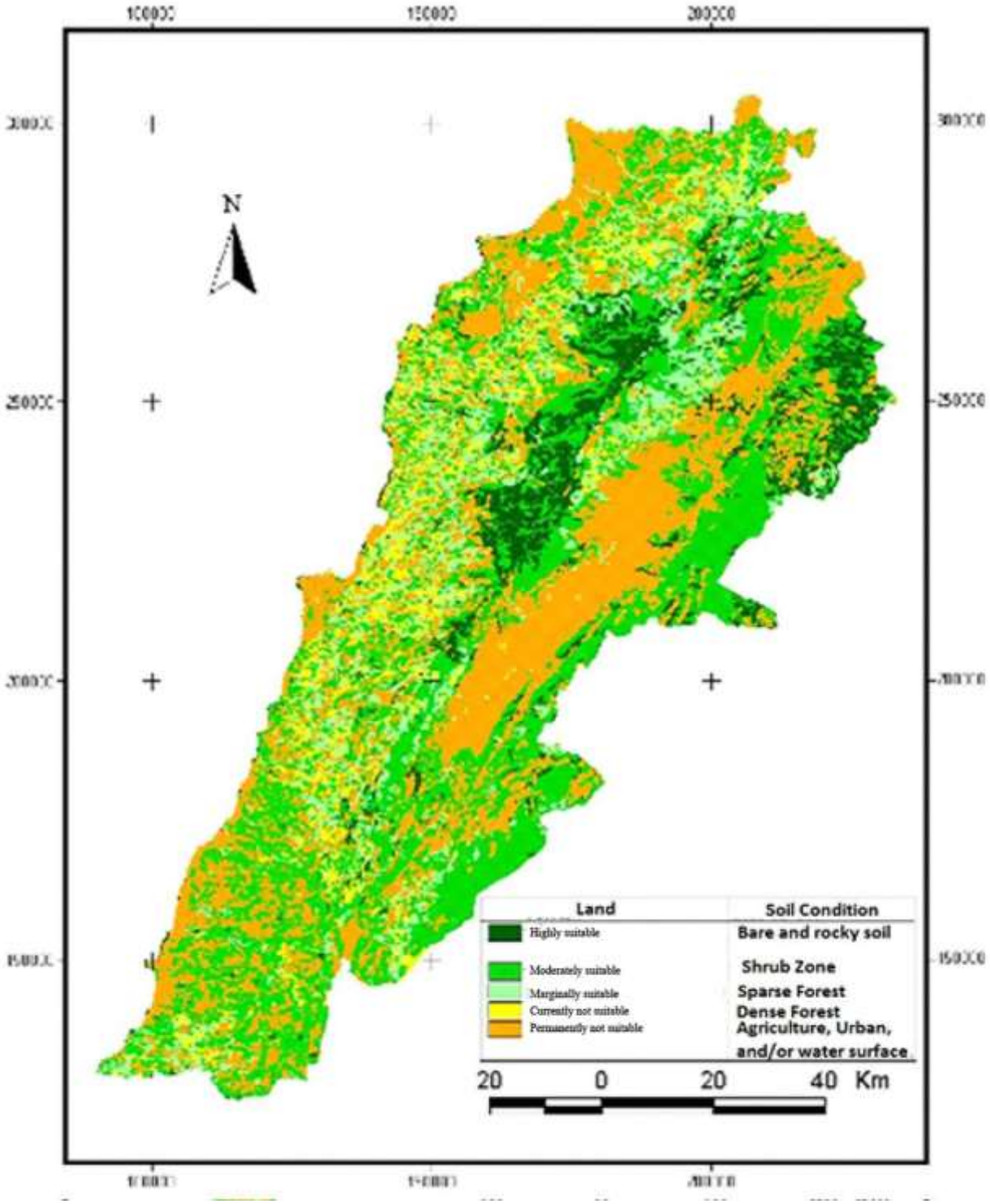

Fig.3: Ability of the Lebanese territory for stone pin reforestation according to land's occupation.

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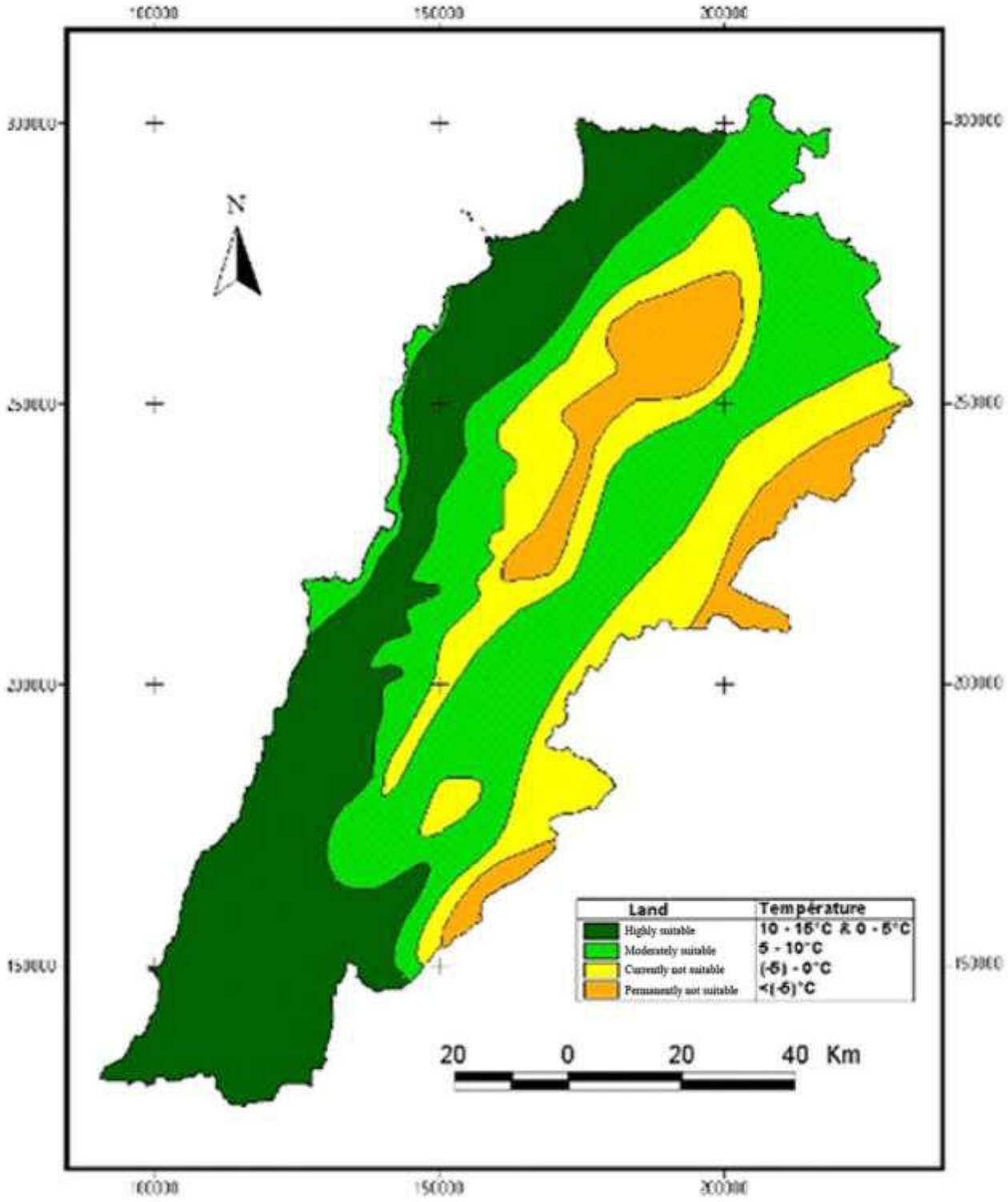

Fig.4: Ability of the Lebanese territory for stone pin reforestation according to the extreme minimum temperatures.

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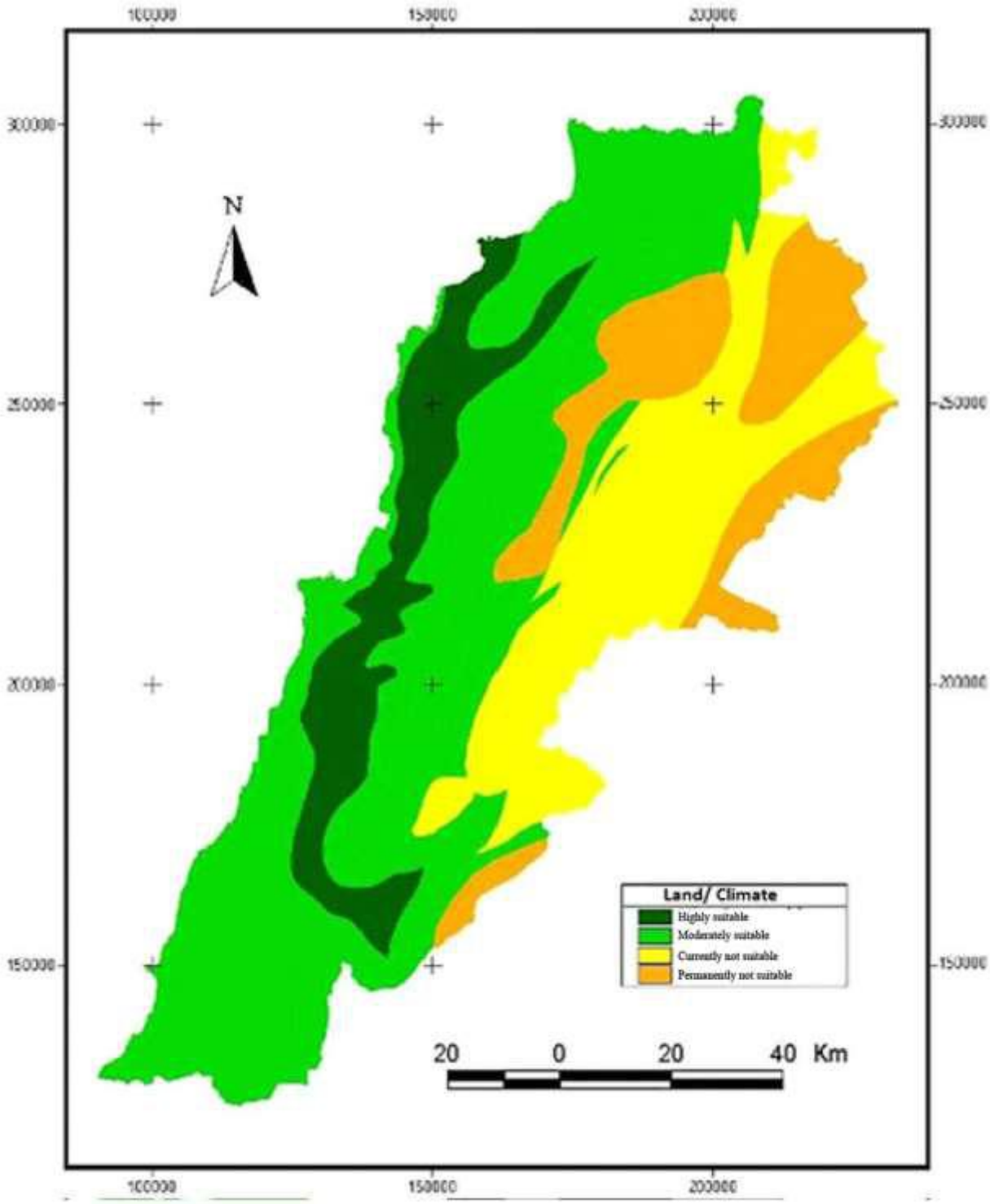

Fig.5: Ability of the Lebanese territory for stone pin reforestation according to climate.

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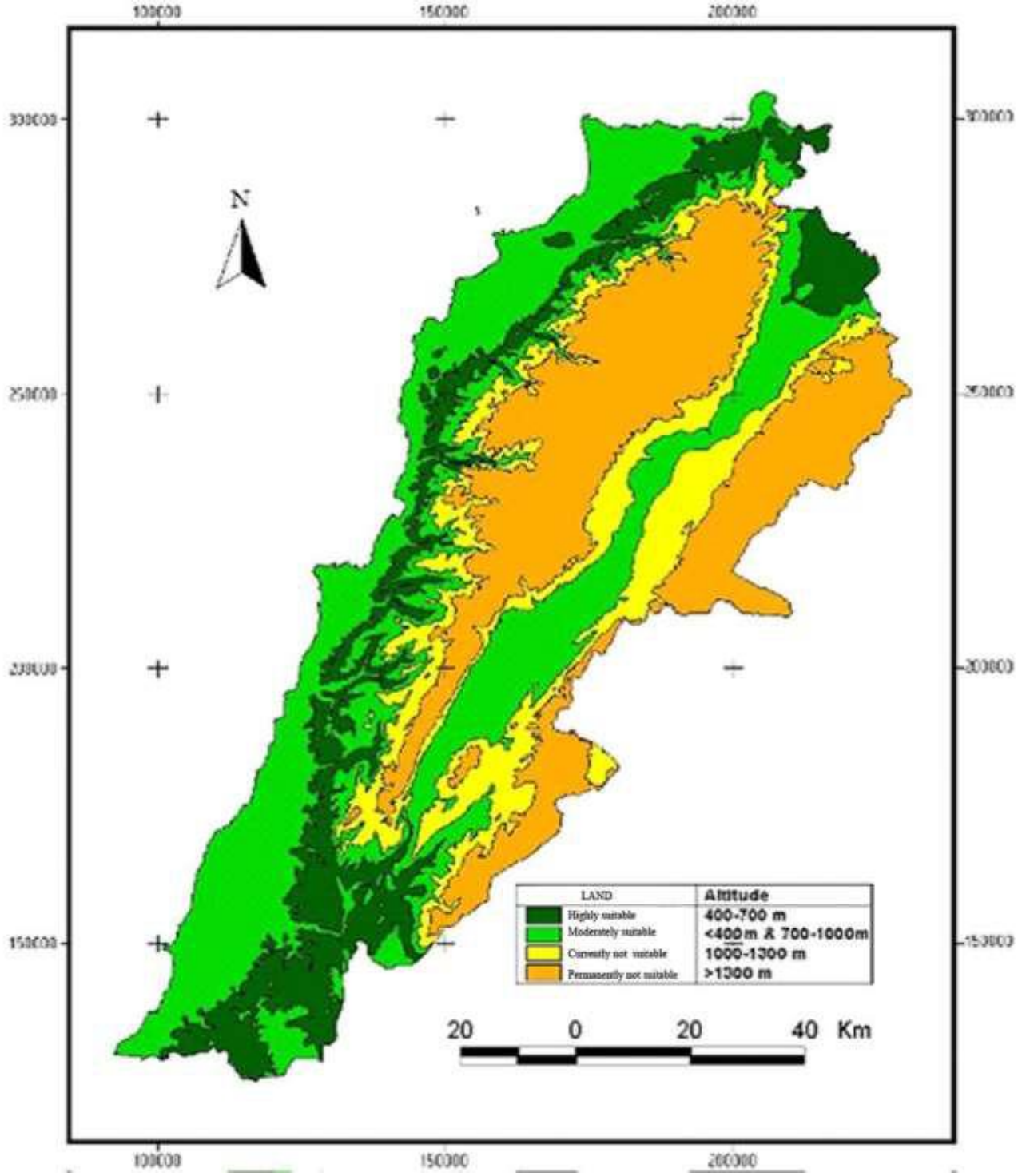

Fig.6: Ability of the Lebanese territory for stone pin reforestation according to altitude.

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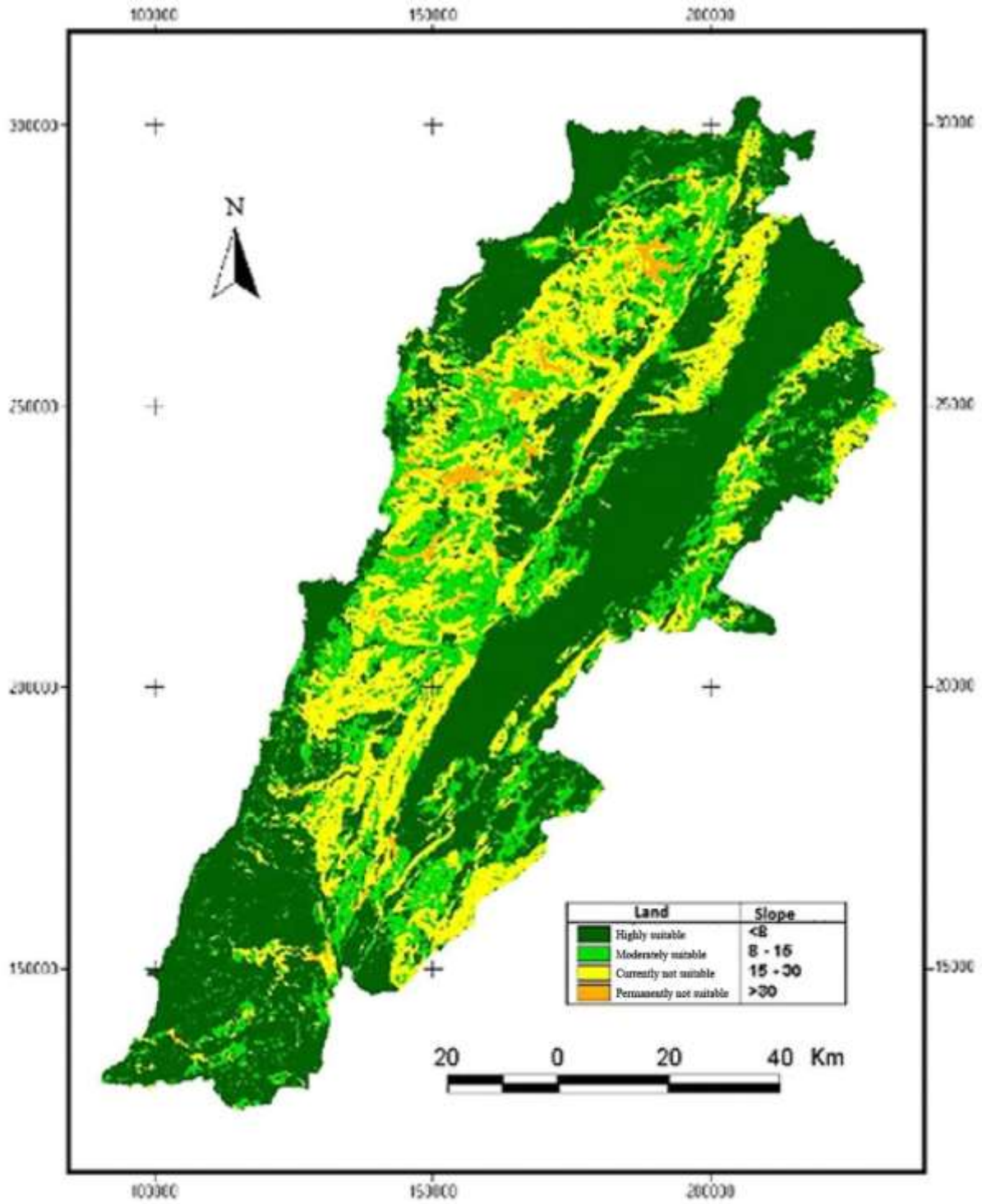

Fig.7: Ability of the Lebanese territory for stone pin reforestation according to slopes.

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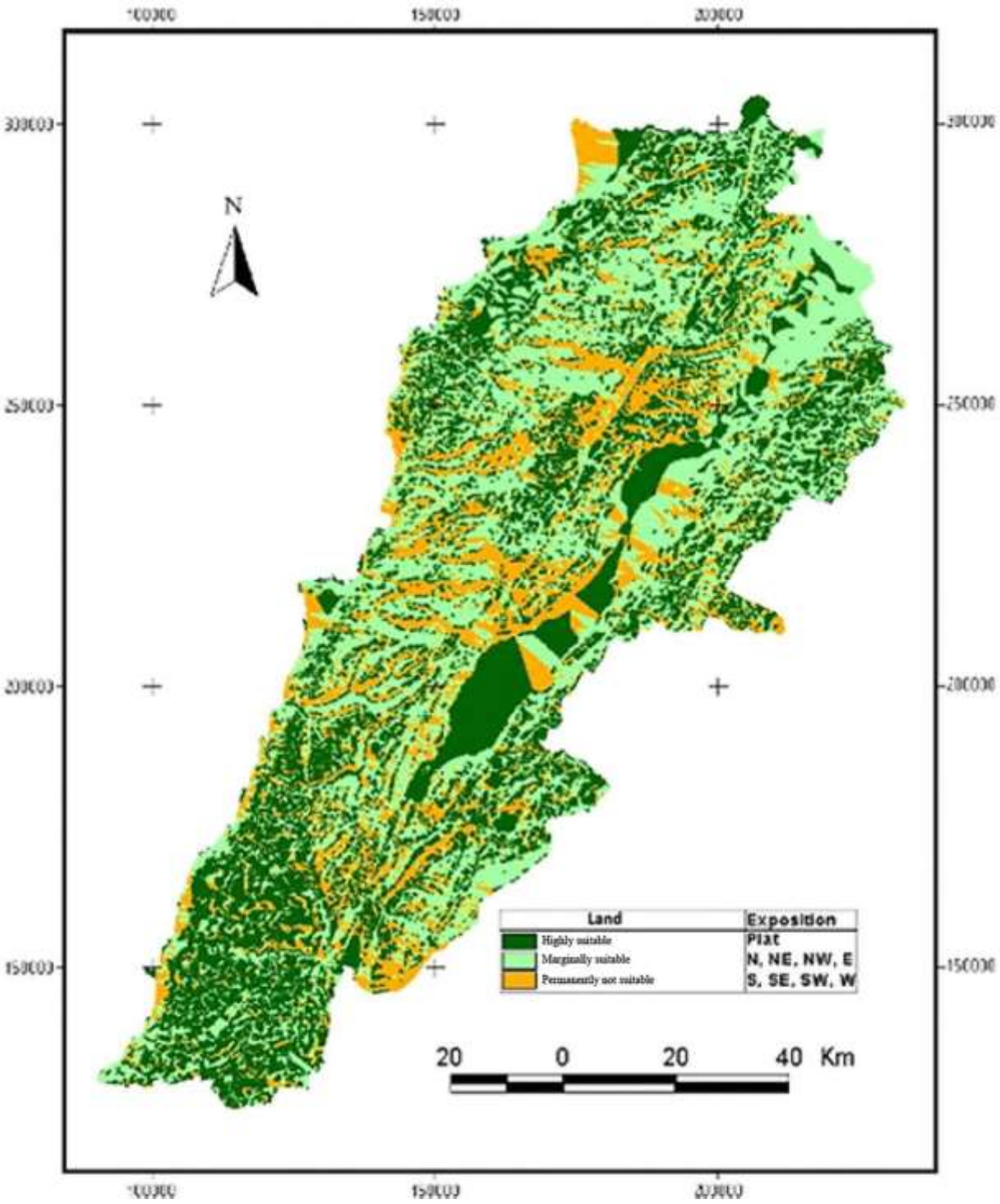

Fig.8: Ability of the Lebanese territory for stone pin reforestation according to exposition.

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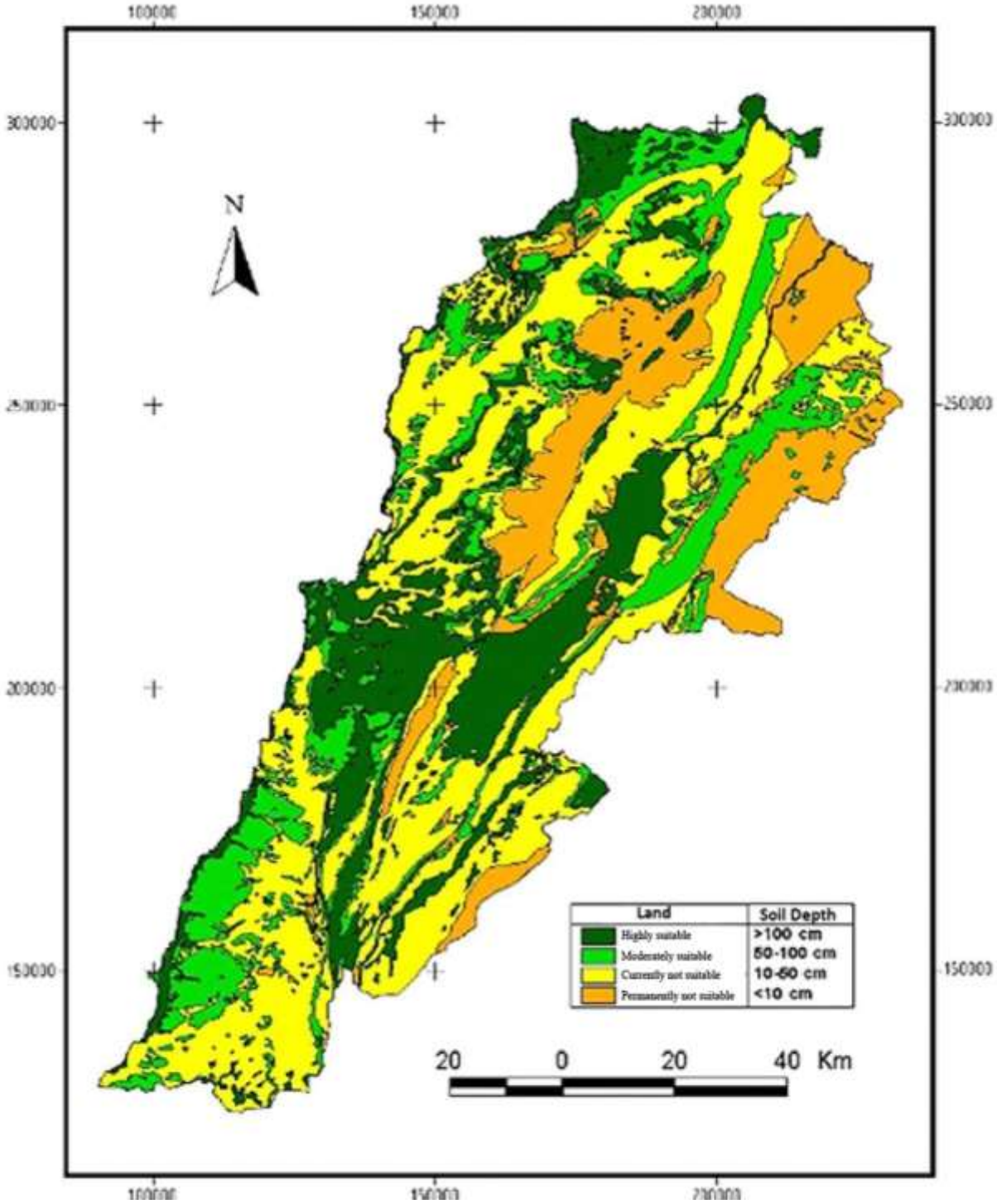

Fig.9: Ability of the Lebanese territory for stone pin reforestation according to soil depth.

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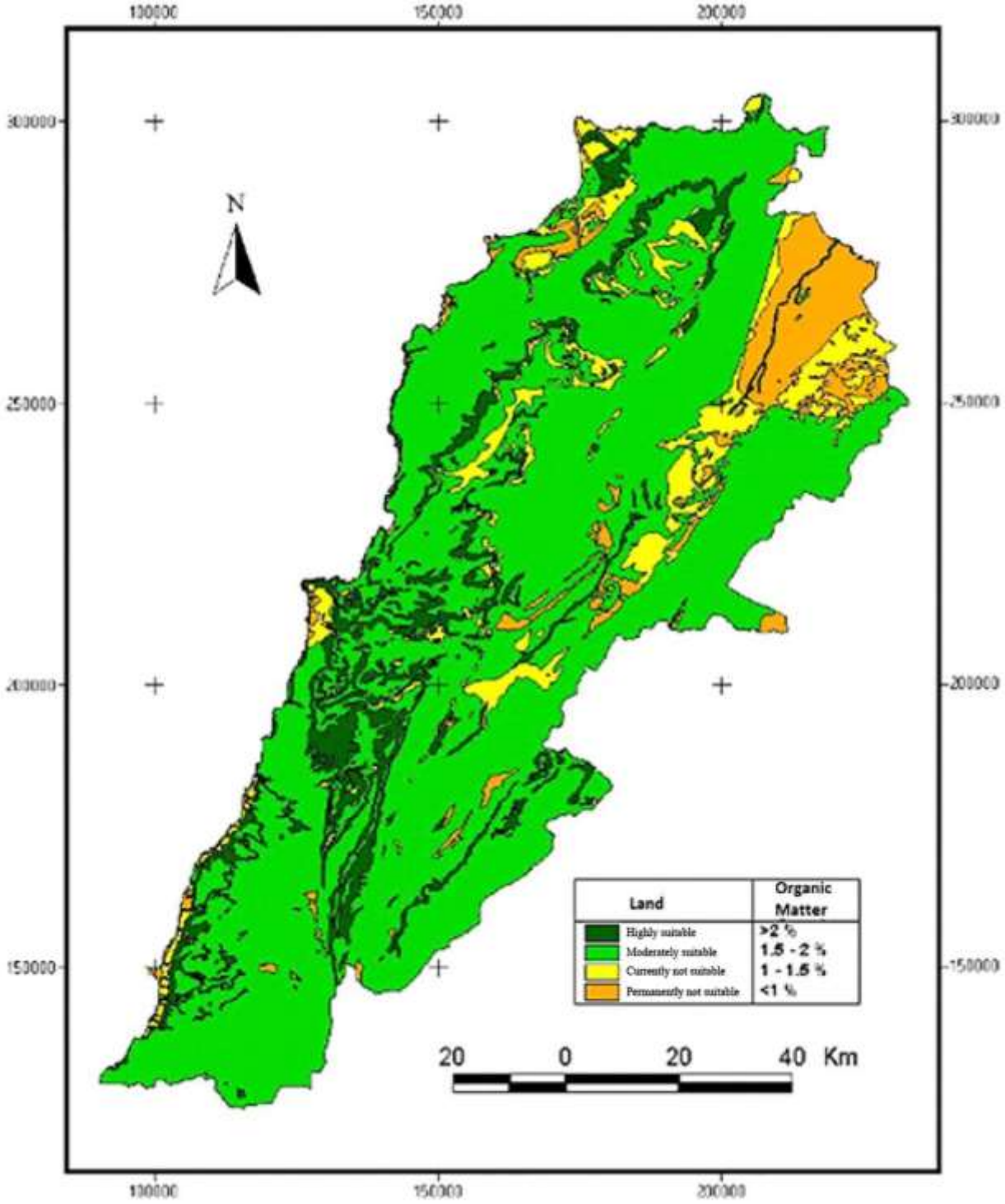

Fig.10: Ability of the Lebanese territory for stone pin reforestation according to soil organic matter.

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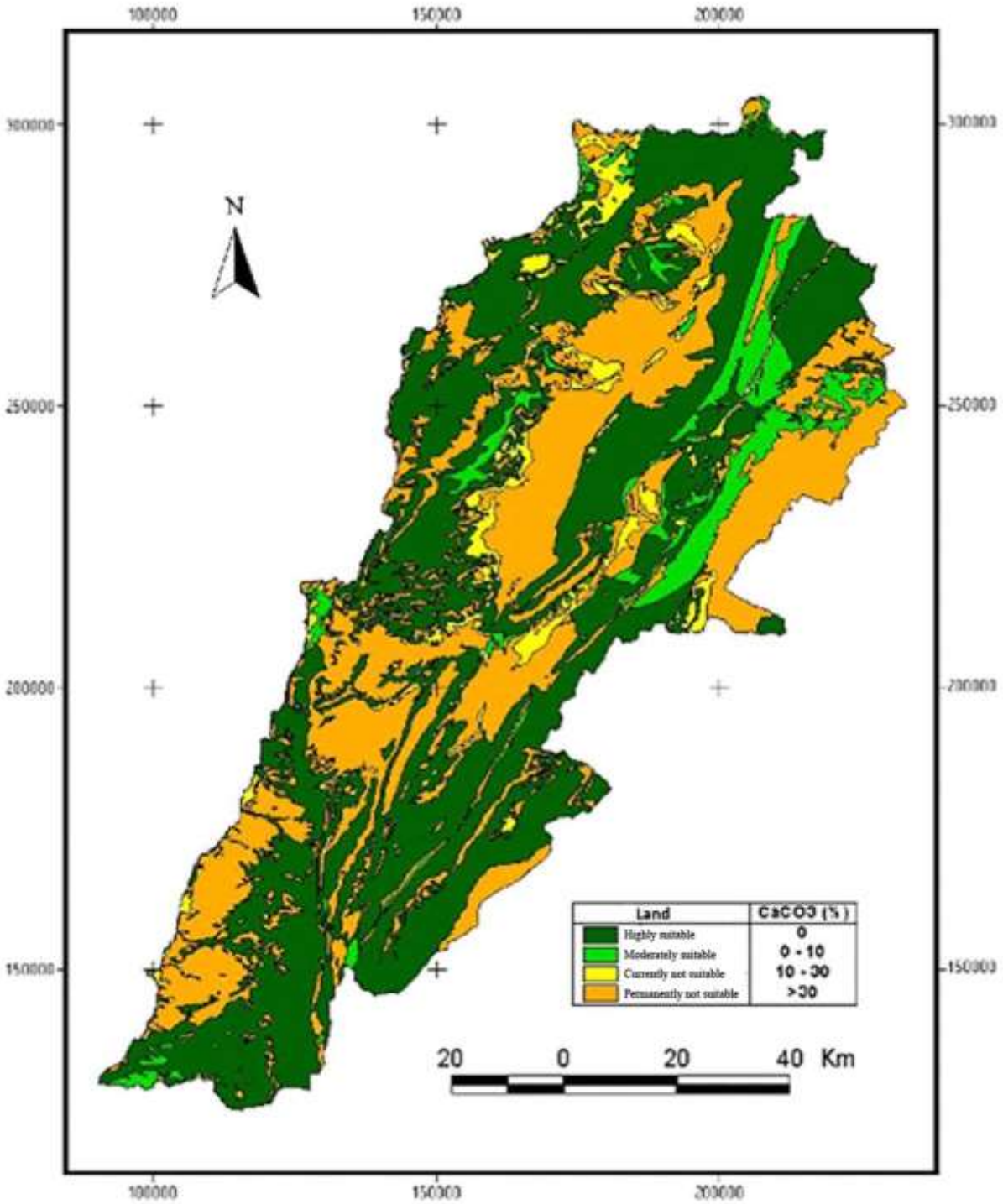

Fig.11: Ability of the Lebanese territory for stone pin reforestation according to CaCO3 in the soil.

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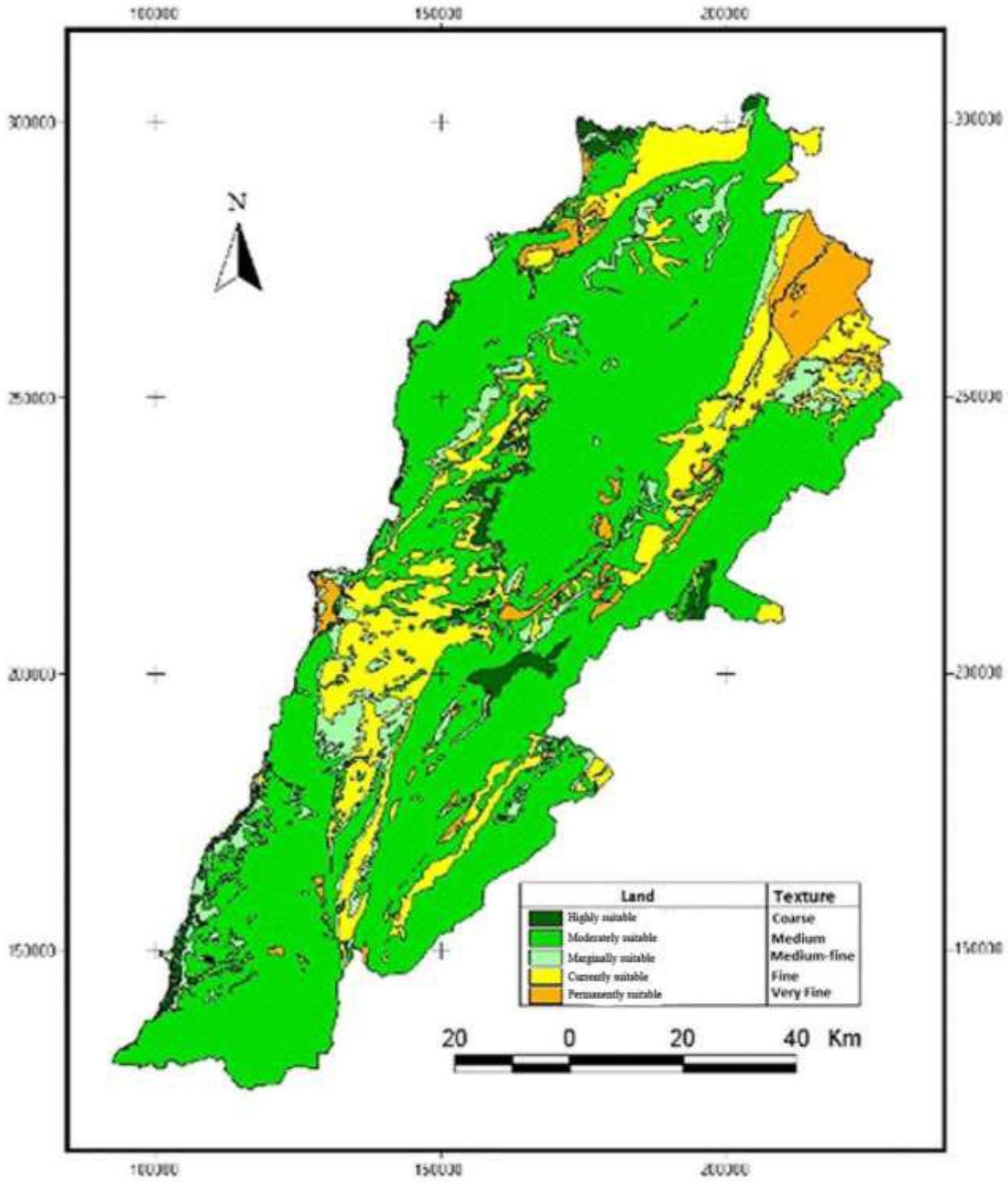

Fig.12: Ability of the Lebanese territory for stone pin reforestation according to soil texture.

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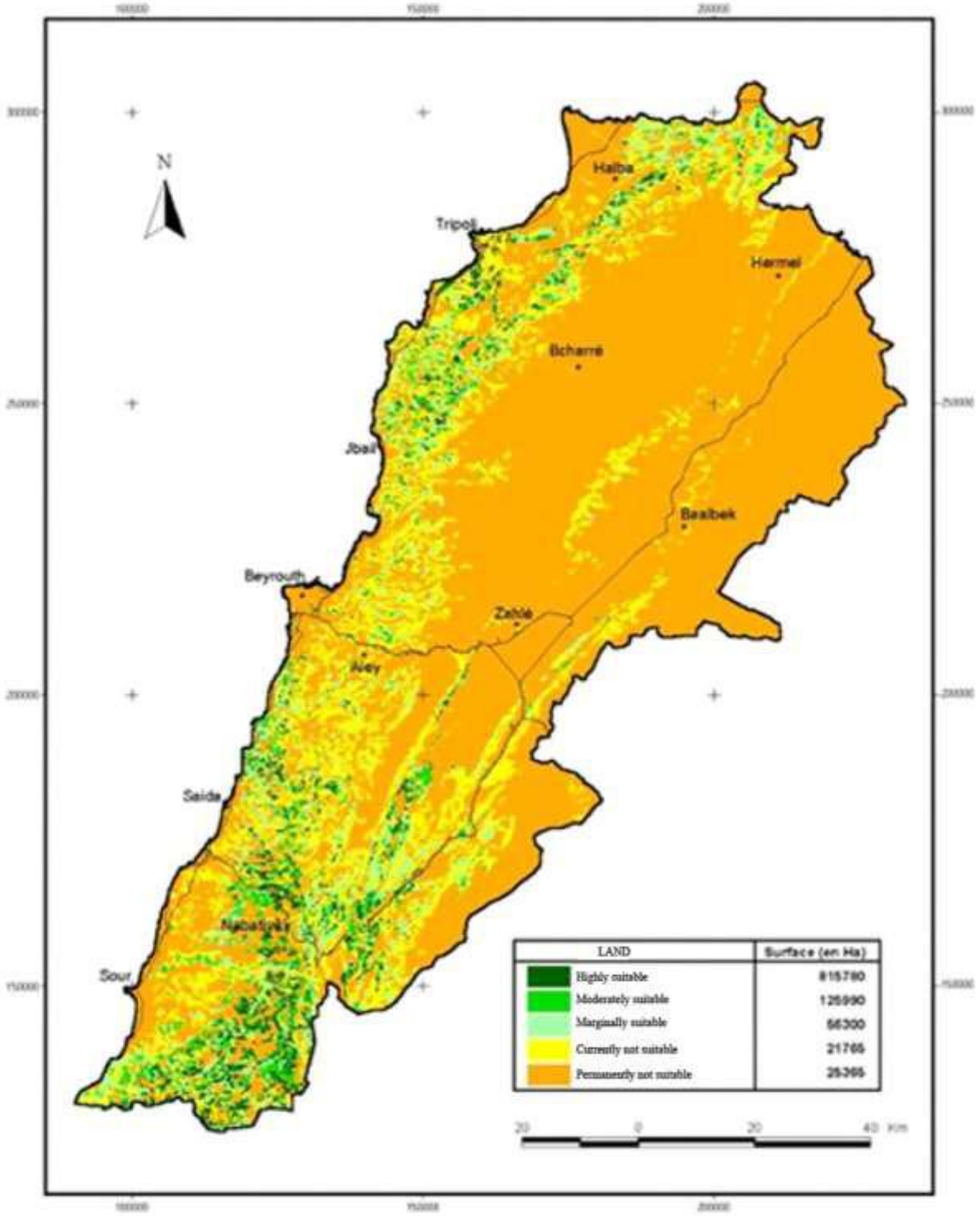

Fig.13: Final map of the ability of the Lebanese territory for stone pine reforestation.

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Table.1 : Evaluation Table.

Parameters		Coefficients (Ci)							Poids (Pi)
		1.0	**0.8**	**0.6**	**0.5**	**0.4**	**0.2**	**0.1**	
	Altitude (m).	400-700	<400 & 700-1000	N/A	1000-1300	N/A	N/A	>1300	3
	Slope (°).	<8	8-15	15-30	N/A	>30	N/A	N/A	4
	Exposition.	Plat.	N, N-E. N, N-W.	S, S-E. S, S-W.	N/A	N/A	N/A	N/A	4
	Soil Depth (cm).	>100	50-100	10-50	N/A	N/A	<10	N/A	3
	Soil Organic Matter (%).	>2.0	1.5-2.0	1.0-1.5	<1.0	N/A	N/A	N/A	4
	Soil $CaCO_3$ (%).	0	0-10	10-30	N/A	>30	N/A	N/A	2
	Soil Texture	Coarse	Medium	medium-fine	N/A	Fine	Very fine	N/A	2
	Precipitation (mm/year).	>900	700-900	500-700	N/A	300-500	<300	N/A	4
	Extreme minimal Temperature (°C).	10-15 & 0-55	-10	0-(-5)	N/A	N/A	< (-5)	N/A	3
	Soil Occupation	Bare and rocky soil	Shrub and herbaceous zones	Sparse Forests	N/A	Dense Forests	Agriculture, rural and/or water surface	N/A	3

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Environmental Changes and Effects on a Population of Smooth Newt *Lissotriton meridionalis* (Boulenger, 1882) (Amphibia, Urodela) in a Mediterranean Woodland

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Abstract — *The population of Lissotriton meridionalis in the area of "Bosco di Palo" Natural Park are monitored since 1995. From 2004 to 2005 in the area it was carried out a massive cutting of dead trees with evidence of alteration of the undergrowth. The study aims to verify, through the index of the population estimate, if the species has suffered changes in the size of the population following environmental changes. For the research were chosen three ponds in the wood and the data collection took place from the breeding season of 1995 – 1996 to 2014 – 2015, in each of the seasons was made an estimation of the population density. The data obtained are been compared in order to make assessments on the conservation status and persistence of the species in the site, also as a result of environmental changes suffered by "Bosco di Palo" Natural Park. The analysis of the population estimate, used in this work as an index of the conservation status of the species in the Park, confirms that, in the previous period and in the period following the die-off of trees and cutting plant health, we have substantially the same values of population size.*

Keywords — Biscogniauxia mediterranea, Lissotriton meridionalis, "Bosco di Palo" Natural Park, population estimate, temporary pond, terrestrial phase, wood cutting.

I. INTRODUCTION

The present work aims to contribute to know the dynamics of the population of Smooth Newt *Lissotriton meridionalis* (Boulenger, 1882) as a result of environmental alteration due to human activities.

The Smooth Newt *Lissotriton meridionalis* (Boulenger, 1882) is an Amphibian distributed in the Italian peninsula, with the exclusion of the southern regions (RAZZETTI & BERNINI, 2006). Its ecology, in the Mediterranean, is closely influenced by local environmental parameters. The vitality of the populations is closely linked to the conservation of small wetland, often temporary, that allow egg laying and larval development (BELL & LAWTON, 1975; ACCORDI & NOBILI, 1999; PIZZUTI PICCOLI, 2008).

The smooth newt breeds in both temporary and perennial waters (ponds, lakes, fountains), never in the flowing waters (BELL, 1977; RAZZETTI & BERNINI, 2006). Given the absence of fish, temporary ponds have the advantage of significantly reducing the number of predators present. The temporary ponds, on the other hand, are extremely unpredictable habitats and often a premature drying can destroy a whole generation of larvae.

Metamorphosed individuals spend about two years in the undergrowth before reaching sexual maturity and return for reproduction in the ponds. The adults make terrestrial life outside of the breeding season (GRIFFITHS, 1984; AGREEMENTS et al., 1990).

Actually their habits in the terrestrial phase are still little known; in particular smooth newts seem to use habitats characterized by old tall forests with undergrowth.

The individuals mostly remain in the vicinity of area of deposition (50% within 100 meters, 100% within 700 meters), though they are rarely found, during terrestrial phase, at less than 30 meters from the ponds (RITTENHOUSE & SEMLITSCH, 2007; SEMLITSCH, 2008)

In the "Bosco di Palo" Natural Park, populations of *Lissotriton meridionalis* are monitored since 1995 and we have seen how their status and their reproductive biology are closely correlated with rainfall, temperatures and seasonal filling of temporary ponds (PIZZUTI PICCOLI, 2008; PIZZUTI PICCOLI, 2010).

Since the 90s, the water table of "Bosco di Palo" has suffered a significant decrease, as underlined by the irregularity of filling of the temporary ponds present. Because of the soil drying and the consequent state of water stress of the trees, since 1999, the mushrooms *Phytophtora sp*. and *Biscogniauxia mediterranea* (De Not.) O. Kuntze have given rise to an epidemic that lead to the death of a high percentage of the trees of the forest (FRATICELLI, 2003; PETRICCIONE, 2003; SCARNATI & ATTORRE, 2014; SOLOMOU et. Al., 2017).

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The pathogen has spread through the vessels of wooden fibers cavity, large and empty due to the lack of water so as to colonize the woody tissues, killing the tree in a single growing season.

Consequently, in 2004 and then in 2005 it was carried out a massive cutting of trees in order to eliminate the fungal pathogens. The cut and the removal of the timber was operated mechanically (with the use of a bulldozer), with evidence of alteration of the undergrowth.

The cut has produced the creation of a large clearing in the middle of the forest, characterized by the presence of scattered trees and a uniform layer of sclerophyllous shrubs. The average percentage of coverage of the tree layer, considering the coverage of the upper layer to 8 meters in height, is lower (42%) compared with the computed values for the same site in 1983 (75%) (FRATICELLI & SARROCCO, 2012)

Habitat alteration is now considered one of the possible causes of the decline of amphibian populations in Italy and Europe (D'AMEN & BOMBI, 2009).

The study aims to verify, through the index of the population estimate, if the *Lissotriton meridionalis*, given the importance of the terrestrial habitat during the non reproductive phase of adults and in the period of growth of metamorphosed individuals, has suffered changes in the size of the population following damage to vegetation.

II. STUDY AREA

The "Bosco di Palo" Natural Park (Figure 1) is located 37 km to the north of Rome (Central Italy - IGM Topographic Map Sheet 149 NE IV) and is situated between the sea and the Via Aurelia in locality of Palo Laziale, in the town of Ladispoli (41 ° 56 'N, 12 ° 05 'E). The study area is part of a narrow coastal plain that extends from the delta of the Tiber River and that was formed during the Quaternary period.

Fig. 1: The "Bosco di Palo" Natural Park.

The territory was divided into three longitudinal strips parallel to the sea, a band made up of silt deposits and marshy black lands, an intermediate band characterized by ancient fossil dunes and a third more recent band formed by coastal dune and beach (currently in strong erosion). The soil wooded area is characterized by clay.

The climate is part of the type mesomediterranean with mild winter, a summer period of about three months of dryness and rainfall regime of maritime type.

The environments that we find in the Park are the Mediterranean scrub, planitial wood and grassland. The planitial wood, characterized by the presence of temporary ponds, consists of a mixed forest of deciduous oaks of about 60 hectares, with the dominance of *Quercus ilex* L., *Quercus cerris* L., *Quercus pubescens* Willd. and *Ulmus minor* Miller (LUCCHESE 1990). The amphibians of the study area are represented by four species: *Bufo bufo* (Linnaeus, 1758), *Hyla intermedia* Boulenger, 1882, *Pelophylax bergeri (Günther, 1986) / Pelophylax klepton hispanicus (Bonaparte, 1839)* and *Lissotriton meridionalis* (Boulenger, 1882).

The temporary ponds are temporary water basins whose depth varies between 20 and 150 cm. These environments are extremely precarious because they are influenced by the seasonal weather patterns. Because of the shallow, thermal stratification is absent; the temperature of the water, from surface to bottom, is under the direct influence of the sun and reflects the seasonal and daily variations in air temperature, even if it remains always few degrees below respect to it. The ponds undergo a drying period, from June to September, and freezing at the surface for few days during negative peaks of temperatures in the months of January and February. The oxygen concentration is subject to daily and annual fluctuations and also varies vertically; it is higher in surface for the presence of photosynthetic organisms and less abundant on the bottom for the presence of organisms decomposers. The water pH decreases with the onset of warm weather (GATTA, 1990; MURA & BRECCIAROLI, 2003).

The bottom of the ponds is characterized by a strong decomposing activity; the half-submerged trees growing around the ponds and directly into the water (mainly *Fraxinus oxycarpa* Bieb.) release a considerable mass of leaves on the bottom of ponds. Within the ponds, the vegetation is very scarce and characterized by terrestrial grasses that withstand periods of immersion. After the phytosanitary cut the vegetation composition was altered.

For the research were chosen three ponds in the wood that have the following characteristics: Pond 1, called "pond of *Emys*", with a maximum diameter of 20 m, a maximum area of 62.8 sq. m. and a maximum depth of 120 cm; Pond 2, called "pond of newts", with a maximum diameter of 4 m, maximum area of 12.56 sq. m. and a maximum depth of 81 cm; Pond 3, called "pond of reeds", with a diameter of 22 m, maximum area of 69 sq. m. with a maximum depth of 83 cm. The Pond 3 is characterized

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by the coverage of rushes, *Juncus sp.* and *Typha sp*. in about a third of the surface (LUCCHESE,1990).

III. MATERIAL AND METHODS

The data collection took place from the breeding season of 1995 – 1996 until the breeding season 2014 - 2015, the breeding season is considered the beginning of the filling of temporary ponds until they are completely drained. Samples were taken every fifteen days. The capture of the specimens was performed by dipnetting, according to pre-established transects, by using a net square shape with side of 36 sq. cm, with square mesh of 0.5 cm side.

For each sampling has been established dipnetting mode according to the size of the pond (HEYER, 1988); in the pond of reeds the research was carried out with an average of 80 dipnetting for sampling, in the pond of *Emys* the research was carried out with an average 80 dipnetting for sampling and newts in the pond of newts the research was carried out with an average of 30 dipnetting for sampling.

During the breeding seasons each exemplar of *Lissotriton meridionalis*, after being captured and measured, has been marked by photograph of the ventral pattern and then released.

In each of the seasons, in the two consecutive sampling in which was recorded the highest seasonal presence of individuals, was made an estimation of the population density.

The estimated population density was performed by the Lincoln - Petersen Method modified by Bailey, suitable for small populations of temporary ponds (ACCORDI & NOBILI, 1999). The method assumes that the total population size to be estimated contains N individuals. From this population, take a sample of M individuals, mark and return them to the population. Later, take a second sample of n individuals from the population. This second sample contains R recaptured animals. The Lincoln-Petersen equation for estimating population size, N, is:

$$N = \frac{Mn}{R}$$

This equation overestimates the actual population size. This bias can be reduced by using Bailey's modification of the Lincoln-Petersen equation:

$$N_B = \frac{M(n+1)}{R+1}$$

Bailey's modification is thought to yield a better estimate when sample size is small (less than circa 20) (BAILEY, 1951; GREENWOOD & ROBINSON, 2006).

The long term monitoring allows to compare the population estimate obtained during all the years of study in order to make assessments on the conservation status and persistence of the species in the site, also as a result of environmental changes suffered by "Bosco di Palo" Natural Park.

The field monitoring was conducted in accordance with applicable laws and authorizations provided for this kind of studies. Handling of individuals was made in compliance with the standards necessary to prevent transmission of pathogens between individuals (RAZZETTI & BONINI, 2001).

IV. RESULTS

In Table 1 are shown the number of individuals caught per breeding season and the population size estimated for the site. The data produced can be considered an underestimation of the population of "Bosco di Palo" for the presence of other breeding sites besides those investigated, certainly has been identified the range size of the population.

Comparing the results obtained with the breeding seasons as reported in Figure 2, it is noted that there is not a significant change in the estimated size of the population, the trend line is almost horizontal, with mean values of 284 individuals.

V. DISCUSSION AND CONCLUSIONS

The work aims to highlight if the cutting action and the damage to the undergrowth may have influenced the survival of populations of newts. This habitat alteration may affect adult population during terrestrial phase, and the juvenile population during the sexual maturity development phase, which takes place on the ground in the undergrowth. In particular, the action of tree cutting it was assumed to have altered the component of the understory (low bushes, litter, bark and rotting logs) that are the refuge microhabitat for this species (VILLE VUORIO et al., 2015).

The causes of extinction of the species in many sites of its distribution could be caused by a set of more environmental factors, such as changes in water chemical component and the increase in temperatures (GALLOY & DENOEL, 2010); however, there seems to be more correlation between the disappearance of the species with the destruction of breeding sites that with destruction of sites where the population spends the terrestrial phase.

Smooth newts live on average 6 years old, and a newt newly metamorphosed spends approximately 2 to 3 years in the undergrowth to reach sexual maturity (GRIFFITHS,

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1984). The cutting of dead trees occurred in the course of years 2004 and 2005 and it was assumed that if the environmental alteration has affected the fitness of newts in the terrestrial phase, we would have a decrease of presence in breeding sites starting by 2009 – 2010 reproductive season (about 6 years after the cutting).

The analysis of the population estimate, used in this work as an index of the conservation status of the species in the "Bosco di Palo" Natural Park, confirms that, in the previous period and in the period following the die-off of trees and cutting plant health, we have substantially the same values of population size.

In the present work the Author uses the parameter of the population size as an indicator of species stability following the alteration of the ecosystem. Also if declining causes may be various, the result of study doesn't detect a substantial alteration of the size of the population, the Author assumes that even the die-off and cutting of trees can be excluded as a cause of declining of the species in the area.

In conclusion, the environmental alteration occurred at the "Bosco di Palo" Natural Park does not seem to have an effect on the population of *Lissotriton meridionalis*. The present work is thought to contribute to the understanding of the dynamics of the species in locations submitted to anthropogenic disturbance such as forests subjected to practices of wood cutting.

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Table.1: The data of the breeding seasons from 1995 – 1996 to 2014 – 2015.

Breeding season	**Number of caught individuals**	**Males**	**Females**	**Male percentage**	**Female percentage**	**Population estimate** ***(numbers of individuals)***
1995 – 1996	100	43	57	43%	57%	257
1996 – 1997	172	52	120	30%	70%	246
1997 – 1998	80	29	51	36%	64%	221
1998 – 1999	145	52	92	48%	52%	267
1999 – 2000	131	61	70	46%	54%	329
2000 – 2001	104	53	51	51%	49%	295
2001 – 2002	114	55	59	48%	52%	387
2002 – 2003	134	47	87	35%	65%	256
2003 – 2004	-	-	-	-	-	-
2004 – 2005	-	-	-	-	-	-
2005 – 2006	-	-	-	-	-	-
2006 – 2007	154	51	103	33%	67%	294
2007 – 2008	165	63	102	38%	62%	232
2008 – 2009	136	56	80	41%	59%	267
2009 – 2010	145	63	82	45%	55%	360
2010 – 2011	109	46	63	42%	58%	342
2011 – 2012	130	48	82	37%	63%	259
2012 – 2013	112	52	60	46%	54%	212
2013 – 2014	147	71	76	48%	52%	324
2014 – 2015	138	50	88	36%	64%	287

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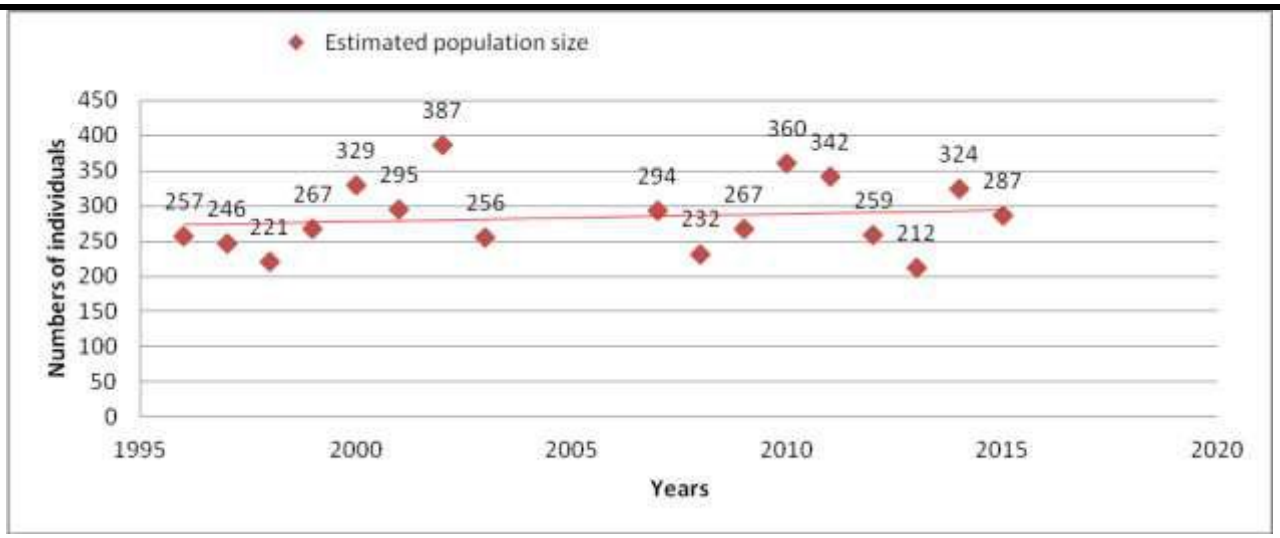

Fig.2: Values of population size from 1995 to 2015.

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Effect of plant growth promoting rhizobacterial (PGPR) inoculation on growth in tomato (*Solanum Lycopersicum* L.) and characterization for direct PGP abilities in Morocco

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Abstract**—** *Plant Growth promoting rhizobacteria are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots. They benefit plants through Production of plant hormones, such as auxins, asymbiotic N_2 fixation, solubilization of mineral phosphates, antagonism against phytopathogenic microorganisms by production of antibiotics, siderophroes, Chitinase and other nutrients ability to effectively colonize roots are responsible for plant growth promotion. An experiment was conducted in the field of National Institute of Agronomic Research of Meknes. Morocco. The experiment was a completely randomized design with six replicates. There were four treatments viz. T1: (control; N0 -PGPR), T2: (N0 +2027-2), T3: (N0 +2066-7) and T_4: (N_0+2025-1). The results indicated that a remarkable increase in root growth, namely length, the diameter of the rod and the total chlorophyll. A total of three different bacteria colonies were isolated and proceed with in vitro screening for plant growth promoting activities; phosphate solubilization, nitrogen fixation, indole acetic acid (IAA), ammonia production and antimicrobial enzymes (cellulose, chitinase and protease) activity. Among the three bacterial strains, all bacterial strains are able to produce ammonia, IAA production and nitrogen fixation activity, one strain phosphate solubilizing activity, two strain are able to produce cellulase syntheses, Protease activity and Chitinase activity.*

Keywords**— Solubilization phosphate, nitrogen fixation, Production antimicrobial enzymes.**

I. INTRODUCTION

Plant nutrients are essential for the production of crops and healthy food for the world's expanding population. Plant nutrients are therefore a vital component of sustainable agriculture. Increased crop production largely relies on the type of fertilizers used to supplement essential nutrients for plants. The nature and the characteristics of nutrient release of chemical, organic and biofertilizers are different, and each type of fertilizer has its advantages and disadvantages with regard to crop growth and soil fertility [1].

Bacteria that are present in the rhizosphere and improve plant growth by some mechanism are called plants growth promoting rhizobacteria (PGPR) [2]. The association between organisms and roots can be beneficial (water uptake, soil stabilization, growth promotion, N_2 fixation, biocontrol, antibiosis, symbiosis), harmful (infection, phytoxicity) or neutral (nutrient flux, free enzyme release, attachment, alleopathy, competition) [6], PGPR most involved are: Pseudomonas, Bacillus, Rhizobium, Burkholderia, Micrococcus, Azotobacter and Erwinia. [7]. Or indirectly via their ability to remove a broad spectrum of bacterial, fungal and parasitic infections, also provide protection against viral disease. Many studies show the diversity of microbial agents involved in the biological control [8].

Some rhizobia strains have the ability to produce siderophores, biomolecules that act as specific iron chelating agents, often unavailable to living organisms and essential for achieving the vital functions such as DNA synthesis, respiration, photosynthesis and Biological nitrogen fixation [9 and 10].

This work was undertaken to evaluate and utilize the potential of rhizobacteria PGPR for plants tomato and characterize three PGPR, for their ability to produce metabolites IAA, solubilization of phosphorus, ammonia production, synthesis of enzymes (chitinase cellulase and protease) and nitrogen fixation.

II. MATERIALS AND METHODS

1. Plant Materials and Experimental Conditions

The experiment was conducted in the field of National Institute of Agronomic Research of Meknes. A field

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experiment was conducted to evaluate the effects of 3 treatments of bacteria (2027-2, 2025-1 and 2066-7) on tomato growth. The experiment was set up as a completely randomized design with six replications, one plantlet in each replicates. There were four treatments viz: T1: (control; N0 -PGPR), T2: (N0 +2027-2), T3: (N0 +2066-7) and T4: (N0+2025-1).

Leaf chlorophyll content of youngest fully expanded leaf (third leaf from the shoot) of each plant was indirectly measured by a chlorophyll meter at harvest, 45 DAI (days after inoculation). Measurements of morphological parameters, namely, the length of the aerial plant, the diameter of the stem, the length of the root system and Average yield of tomato in Kg/Plant were also taken.

2. Study on Plant Growth promoting activity of the Isolate

The isolated bacteria used were taken for studying its plant growth promoting activity. Plant growth promoting activity is studied for its determining it's: Biostimulant Activity, Biofertilization Activity and Biocontrol Activity.

Study on Biostimulant activity: In this the ability production of phytohormones indole acetic acid by the isolate was studied.

Detection of IAA production: For production testing of the AIA, bacterial isolates were plated in Luria Bertani (tubes containing medium supplemented with tryptophan (T1: 0.5g/l and T2: 1g/l). After 72 hours of incubation at 28 ° C, 3 ml of the suspension was removed for the 4000tr to centrifugation at 4 °C for 10min. Then, in an Eppendorf tube 90 µl of supernatant was added to 60 µl of Salkowski reagent, the mixture was incubated in the dark for 30 min. using the spectrophotometer reading the OD at 530 nm were performed to estimate the quantity produced AIA. [11]

Study on Biofertilization activity: Ability of isolate to fix the atmospheric nitrogen and Phosphate solubilization was determined.

Phosphate solubilization: The bacterial strains were evaluated for their ability to solubilize inorganic phosphate. The PVK medium with or without BTB containing tribasic calcium phosphate was used in this trial. Each isolated culture was plated on Petri dishes and the dishes were incubated at 27 ° C for 7 days. The appearance of a clear halo around the colonies after four days has been marked as positive for the solubilization of phosphate [9]. The experiment was performed with three repetitions for each bacterial strain. [12]

PSE= (Solubilization diameter/Colony diameter)*100

Production of ammonia: Isolates were grown in peptone water at 28 ° C for 8 days. At the end of the incubation period, 1 ml of Nessler's reagent was added to each tube. The development of the pale yellow to dark brown said ammonia production. [9]

Nitrogen fixation: For the identification of fixing rhizobacteria nitrogen, nitrogen fixation activity was tested on medium Nfb. The binding activity was tested on both liquid and solid medium 0.5% bromothymol blue was used as a pH indicator. [13]

Study on Biocontrol activity: Ability of isolate to produce cellulase synthesis, protease synthesis and Chitinase synthesis was determined which can act against plant pathogens.

Cellulase synthesis: The ability to produce cellulase was measured on agar plates containing minimal medium with 2% (w/v) 1-carboxymethylcellulose as carbon source. Bacterial strains were grown on this medium and incubated at 30 °C for up to 8 days. The ability of bacterial isolates to hydrolyze cellulose was detected qualitatively by formation of a clear zone after the culturing period by adding 0.1% Congo Reed solution followed by de-staining with 1 M NaCl [14].

Protease synthesis: Protease activity was detected on 3% (w/v) powdered skim milk agar plates. A single bacterial colony of each strain was grown on this medium and incubated at 30 °C for up to 8 days. Protease activity was detected as a clear zone [15].

Chitinase synthesis: Activity of Chitinase was detected on 1% (w/v) colloidal chitin agar plates. A single colony of each bacterial strain was streaked on this medium and incubated at 30 °C for up to 8 days. Chitin hydrolysis was detected qualitatively after the culturing period by pouring 0.1% Congo Reed solution onto culture plates and checking for formation of a clear zone [16].

3. Statistical Analysis

Data from the infectivity and effectiveness tests were analyzed with the Excel2007 software.

III. RESULTS AND DISCUSSION

Effect of the bacterial strains on the parameters of the growth of tomato plants in a field

Agronomic measures namely the length of the overhead system, the diameter of the rod, the total chlorophyll and the length of the root system were used to develop the following figures (Figure 1a, b, c, and d). Indeed the length of the plants was very significantly stimulated by the three strains PGPR 2025-1, 2027-2 and 2066-7 in comparison with the inoculated plants (Figure 1a). For the length of the root system, the three strains were well stimulated length plants in comparison with other plants inoculated with the other strains and compared to the control (Figure 1b, 2).

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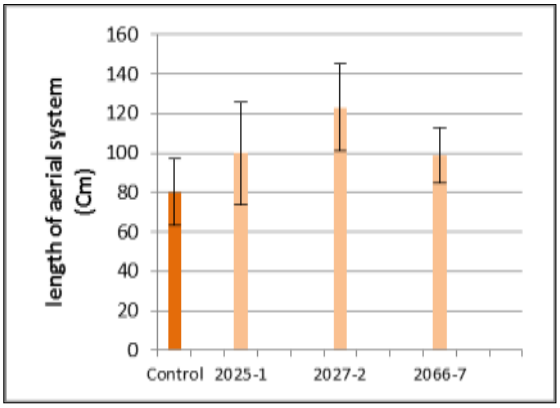
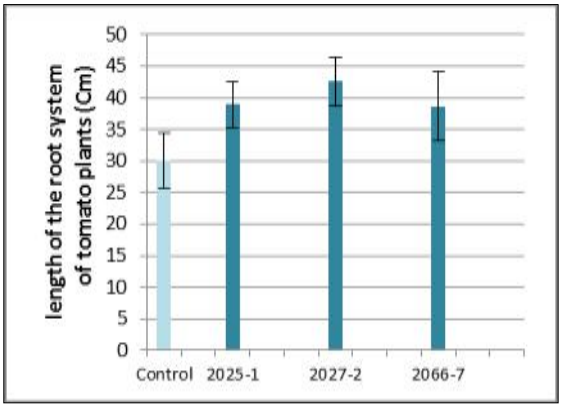
For the collar diameter of the plants, all the tested PGPR have yielded significant results in comparison to the control strain having 2025-1 including well stimulated plants compared to all treatments (Figure 3). While the total leaf chlorophyll was significantly stimulated by the three bacterial strains 2025-1, 2027-2 and 2066-7 strain (Figure 1d).

At the time of harvesting, it was found that the average total yield of tomatoes treated with the bacterial strains was significantly higher than that of the control. In fact, the plants inoculated with the bacterium 2027-2 recorded a yield Doubled compared to the control plants (Figure 1e).

The results of this study, based on inoculation 3 PGPR bacterial strains revealed the stimulatory effect of bacteria belonging to the genus *Bacillus* on the stem height and collar diameter of plants of tomato cultivation this was demonstrated by Huseyin et al., 2007, who experienced significant increase on the growth of apples inoculated with bacteria of the genus *Bacillus*.



(a)



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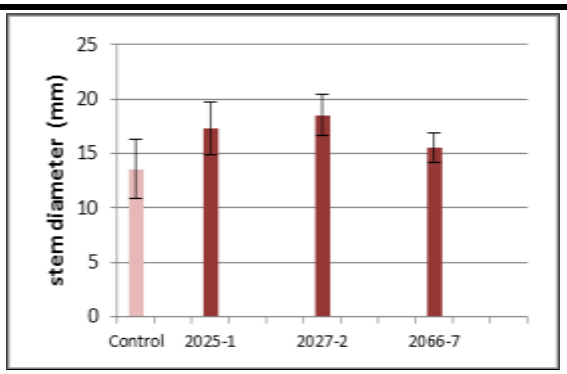

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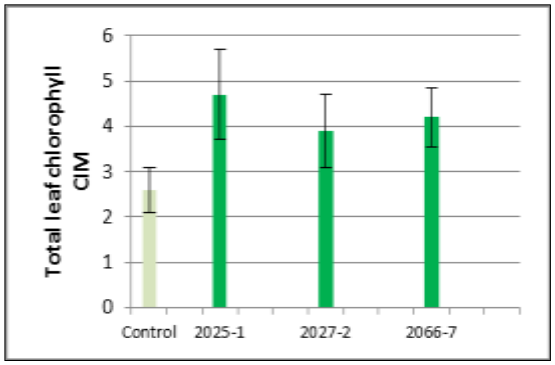
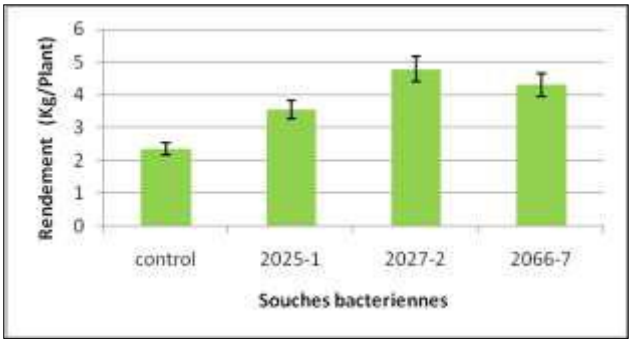


(d)



(e)

Fig.1: Effect of three isolates on the growth of tomato: a. Size of the air part, b. Size root part, c. stem diameter and d. Total leaf chlorophyll and e. Yield of tomato in Kg/Plant

(a) *(b)*

Fig.2: comparison between plant roots inoculated with strain 2027-2 (a) and those control (b)

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In addition, inoculation based on Bacillus bacterial strains resulted in more efficient results in terms of growth and yield compared to other applications (Micobacterium) and compared to control [7]. Similar results were reported in Previous studies show that application of Bacillus can stimulate yield and quality parameters in sugar beet, barley [17], apricot [18], franboise [19] and apple [20].

Inoculation based *Bacillus* bacterial strains has resulted in more effective results in terms of growth and yield compared to other applications (Mycobacterium) and compared to the control [21] .From addition, *Pseudomonas* species, *Bacillus* [22], and other endophytic bacteria such as *Enterobacter*, *Klebsiella*, *Burkholderiaet Stenotrophomonas*, attracted the attention of many researchers in recent years because of their association with important crops and their potential to improve plant growth [23] .

The root growth was stimulated very significantly by the genus *Bacillus* in comparison with other strains and compared to the control. These results corroborate the biological tests performed in 2011 on the cowpea, which showed that its bacteria have the ability to promote root growth by demonstrating a significant increase in the roots of cowpea plants after 21 days [24].

The total chlorophyll content was significantly stimulated by souches *P. agglomeranset Proteamaculansen* comparison with other treatments; these results are similar to those demonstrated by [25]. on the increase in the absorption of nutrients by plants of the rice treated with the compost formulation *Pseudomeunas* resulting in increasing the growth of leaves, stems, roots and the increase in the total chlorophyll content. Also the treatment of rice plants based on the increased strain *pantoeaa* macro-nutrient such as nitrogen, phosphorus and potassium, and increased chlorophyll content [26]. Furthermore, similar results have shown the ability of the strain *Serratia* increased total chlorophyll content relative to other therapies [27].

Since the bacterial strains tested *bacillus cereus* (2027-2), *pantoea agglomerans* (2066-7) and *serratia proteamaculans* (2025-1) gave satisfactory results compared to the control plants in terms of aerial system length, diameter of Stem, root length, chlorophyll content and mean yield. These three bacteria were chosen to study their mode of action on the basis of their characteristics of improvement and promotion of the growth of tomato plants in the field.

Since bacterial strains tested *B.cereus* (2027-2), *P. agglomerans* (2066-7) and *S.proteamaculans* (2025-1) gave satisfactory results compared to control plants in terms of air system length, diameter stem, root length and chlorophyll content these three bacteria were selected to study their mode of action on the basis of their characteristics to improve and promote the growth of tomato plants in a field.

Solubilization of phosphate production of indole acetic acid (IAA), the production of ammonia (NH_3), the biological nitrogen fixation and production of cellulase, chitinase and protease. In our study, among the three strains of *P. agglomerans* a single strain could solubilize phosphate in vitro in Petri dishes as positive the formation of a clear halo around the colony (fig. 6).

Table.1: Characterization of selected bacteria PGPR in different in vitro tests

Tests strains	***solubilization phosphorus***	***production of ammonia (NH_3)***	***Nitrogen fixation***	***Production of cellulase***	***Production of chitinase***	***Production of the protease***
2025-1	-	+	+	-	-	+
2066-7	+	+	+	+	+	+
2027-2	-	+	+	+	+	-

Regarding production of the IAA, all strains are producing indole acetic acid with different ranges for each strain to be the first treatment (0.5 g/l of tryptophan) or to the second treatment (1g/l tryptophan). Products isolates significant amounts of IAA from 4.32 g/l to 6.60 g/l for Serratia, to 4,27g/l to 6,58g/l for Pantoea and 6.37 g/l to 7,84g / l bacillus having shown the highest range of the production of IAA. Similar observations for IAA production have been reported by others [28] (Fig. 3).

Another important feature of the PGPR is the production of ammonia which indirectly affects the growth of plants. All selected isolates were positive for the production of ammonia (Fig. 3).

Nitrogen is an essential nutrient known for the growth and development of plants and its fixation by soil bacteria is considered one of the main mechanisms by which plants benefit from the microbial association. In our study all selected bacteria gave positive results for the nitrogen fixing activity by changing the green color of the medium based on malic acid in a blue environment (Fig. 3). Studies have shown that among the non-fixing bacteria symbiotic nitrogen the most important property of many species: *Azoarcus*sp.,*Gluconacetobacterdiazotrophicus*, *Herbaspirillium*sp., *Azotobacter* sp., *Achromobacter*, *Acetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azomonas*, *Bacillus*, *Beijerinckia*, *Clostridium*,

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Corynebacterium, *Derxia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Rhodospirillum*, *Rhodopseudomonas*et *Xanthobacter*. *Azospirillum* is the representative of PGPR, capacities were evaluated in experiments around the world [29 and 30].

Table.2: phosphorus solubilization efficiency

Strains	***phosphorus solubilization efficiency %***
2025-1	*0*
2066-7	*64,28*
2027-2	*0*

Bacterial strains having protease activity must be highly resistant to environmental stress, mechanical and chemical. All selected strains were positive for the production of the protease enzyme except for *Bacillus cereus* strain that showed negative results.

Furthermore, cellulase activity and chitinase was well observed in both strains and *Pantoea bacillusen* showing a clear zone around the colony on agar colloidal medium, with the exception of *Serratia* strain that showed negative results in production of enzymes chitinase and cellulase.

Isolates producing PGPR plant hormones (indole acetic acid), solubilizing the phosphate and ammonia significantly improve plant growth. The maximum improvement was observed in treatments with the *Bacillus cereus* strain involving the average root length recorded with 42,6 cm. The average length of the plants was recorded with 123 cm compared to control plants.

Table.3: Production of indole acetic acid in g/l by bacterial isolates

Test Strains	***Production AIA in g/L***	
	0,5 g of Tryptophane	*1g of Tryptophane*
2025-1	*4,32*	*6,60*
2066-7	*4,27*	*6,58*
2027-2	*6,37*	*7,84*

IV. CONCLUSION

This study illustrates the importance of rhizobacteria in vitro conditions for several PGPR traits and their evaluation in controlled conditions in a tomato field trial. This led to the selection of effective PGPR namely *Serratia proteamaculans* (2025-1), *P. agglomerans* (2066-7) and *Bacillus cereus* (2027-2) which, because of its multiple Traits PGPR, could prove effective in enhancing the growth and vigor of plants and in the stimulation of plant root system.

This type of study is necessary because it advocates the use of PGPR biofertilizer or as an inoculant is an effective approach to replace chemical fertilizers and isolates of these PGPR can be used as organic fertilizers to improve growth and productivity trade to grow the plants in the local agro-climatic conditions.

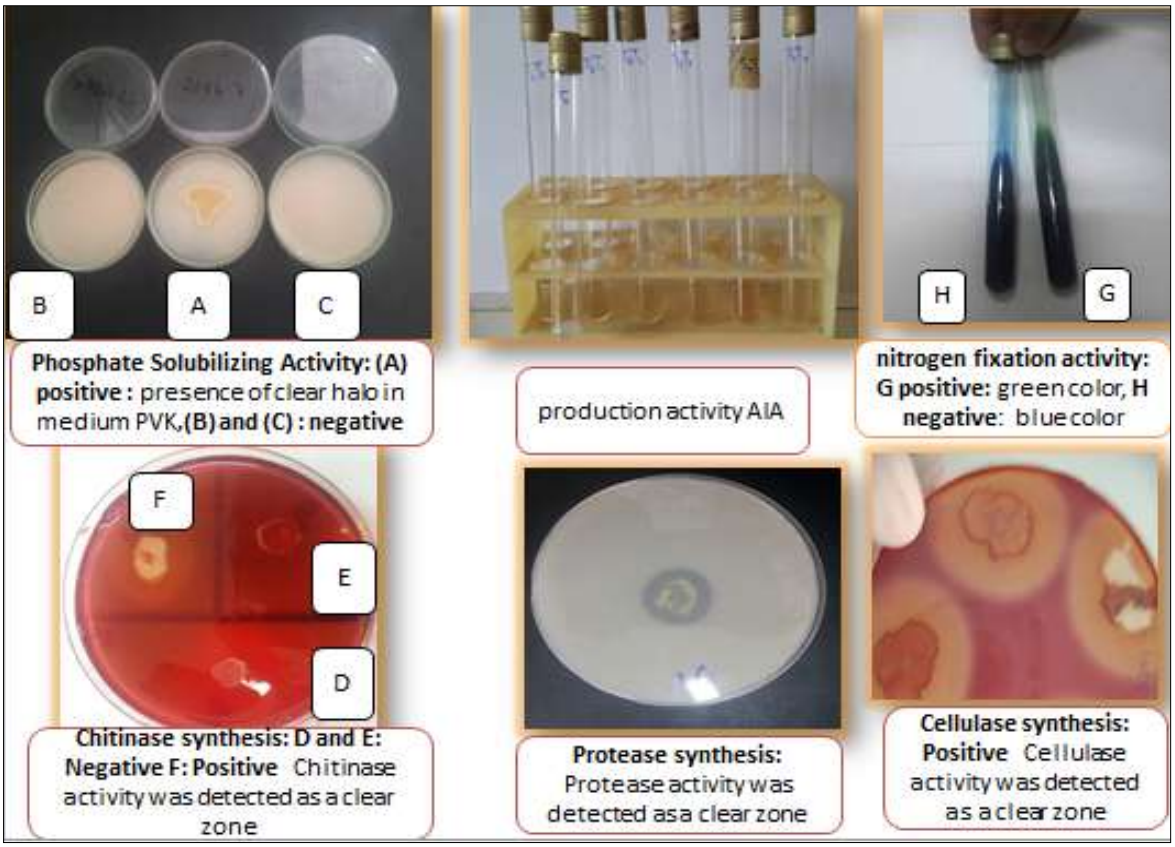

Fig.3: Strains and their PGP traits and production of antimicrobial substances

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Integrated Weed Management Effect on Weeds and Seed Cotton Yield

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***Abstract*—** *Integrated weed management is a system approach where by whole land use planning is done in advance to minimise the very invasion of weeds in aggressive forms and give crop plants a strongly competitive advantage over the weeds. Further, importance is given to involve more than one method of weed control in tackling the weeds so those broad spectrums of weeds are kept under check for longer period. A pre emergence herbicide take care of weeds only for a limited period and do not give long term weed control in a long duration crop like cotton where the problem of late emerging weeds arises and escape killing. So to attain a season long weed control, integration of chemical, mechanical and cultural methods holds a great promise in crop production. Hence, integrated weed management in cotton play important role in increasing crop production. Field experiments were conducted during 2013 and 2014, at Agricultural College and Research Institute, Madurai (Tamil Nadu Agricultural University) to study the effect of integrated weed management in rainfed cotton. The weed management practices consisted of pendimethalin (1.0 kg.ha^{-1}) and (Calotropisgigantea leaf extract spray at three concentrations (10%, 20%, and 30%) in combination with power weeder operation twice and manual weeding twice. From the results of the experiments, it could be recommended that the integrated weed management practices like, application of PE pendimethalin at 1.0 kg ha^{-1} + power weeding on 40 DAS (T_{11}) recorded higher seed cotton yield and economic return.*

***Keywords*— Economic return, Weed density, Weed Dry weight, Yield.**

I. INTRODUCTION

In India, cotton is grown under diverse agro-climatic conditions. Cotton is the most important commercial crop contributing nearly 65% of total raw material needs of textile industry in our country. India ranks first in global scenario occupying about 33 % of the world cotton area but with regard to production it ranks second, next to China. Cotton varieties are cultivated at wider spacing, which in turn invites multiple weed species infestation. Weed competition is severe during its initial growth stages. The increasing cost and unavailability of labour in time has forced to use herbicides for weed control in cotton. Hence, there is a need for selection of pre-emergence herbicides to control early emerging weeds during initial crop growth period. So to attain a season long weed control, integration of chemical, mechanical and cultural methods holds a great promise in crop production. Hence, integrated weed management in cotton play important role in increasing crop production.

Panwar*et al.* (1995) found that the requirement of one hoeing before or after spraying pendimethalin would assist through improved soil moisture conservation and removal of weed population in cotton. Brar*et al*. (1995) stated that pre emergence application of pendimethalin @ 1.5 kg ha^{-1} followed by one hoeing at 30 DAS was effective for the control of annual broad leaved and grassy weeds like *Trianthemaportulacastrum* and *Eleusineindica*. The total weed density was reduced by 60-70 per cent with application of pendimethalin at 1.0 kg ha^{-1} + hand weeding on 30 DAS (Vivek*et al*., 2002). Pendimethalin at 1.0 kg ha^{-1} as pre-emergence herbicide followed by one hand weeding at 30 DAS reduced the weed density and nutrient uptake by weeds (Chander*et al*., 1994). Pre emergence application of pendimethalin 1.0 kg ha^{-1} + one hand weeding resulted in maximum weed control in cotton (AICCIP, 1999). Velayutham(1996) reported that pre-emergence application of pendimethalin at 0.75 kg ha^{-1} followed by one hand weeding resulted in the enhanced kapas yield which was comparable with hand weeding twice. Highest seed cotton yield (2318 kg ha^{-1}) was recorded with pre-emergence application of pendimethalin at 1.50 kg ha^{-1} followed by one hoeing and was 72 per cent higher than the unweeded control (Brar*et al.*, 1999). Rajavel*et al*.(2002) obtained higher seed cotton yield of 1217 kg ha^{-1} under integrated method of herbicide with manual weeding which was comparable with manual weeding twice (1205 kg ha^{-1}). Ali *et al*. (2005) reported that maximum increase in seed cotton yield was obtained with pendimethalin 2.5 kg ha^{-1} in combination with interculturing with hand weeding. The highest seed cotton yield was obtained from application of pendimethalin 1.5 kg ha^{-1} followed by hoeing (Shaikh*et al*. 2006). The higher seed cotton yield

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and benefit: cost ratio were recorded with three hand weedings and three hoeings followed by pre and post-emergence application of pendimethalin and glyphosate with two hand weedings and two hoeings (Deshpande*et al*., 2006).So to attain a season long weed control, integration of chemical, mechanical and cultural methods holds a great promise in cotton production. Hence, integrated weed management in cotton play important role in increasing crop production.

II. MATERIALS AND METHODS

Field experiments wereconducted at Agricultural College and Research Institute, Madurai during 2013 and 2014. Field trials were laid out in randomized block design with fourteen treatments replicated thrice. The weed management practices evaluated in the present study consisted ofPE *Calotropisgigantea* at 30 % + one hand weeding on 40 DAS (T_1), PE *Calotropisgigantea* at 30 % + one power weeding (PW) on 40 DAS (T_2), PE *Calotropisgigantea* at 30 % + EPOE of *Calotropisgigantea* at 30 % (T_3) ,PE *Calotropisgigantea* at 20 % + one hand weeding on 40 DAS(T_4), PE *Calotropisgigantea* at 20 % + one power weeding (PW) on 40 DAS(T_5), PE *Calotropisgigantea* at 20 % + EPOE of *Calotropisgigantea* at 20 % (T_6), PE *Calotropisgigantea* at 10 % + one hand weeding on 40 DAS(T_7), PE *Calotropisgigantea* at 10 % + one power weeding (PW) on 40 DAS(T_8), PE *Calotropisgigantea* at 10 % + EPOE of *Calotropisgigantea* at 10 % (T_9), PE Pendimethalin @ 1.0 kg.ha^{-1}+ one hand weeding on 40 DAS(T_{10}), PE Pendimethalin @ 1.0 kg·ha^{-1}+ one power weeding (PW) on 40 DAS(T_{11}), Two hand weeding at 20and 40 DAS(T_{12}), Two power weeding at 20and 40 DAS (T_{13}) were tested and compared with unweeded control(T_{14}).Leaf extracts of 10, 20 and 30 per cent concentrations were sprayed on 3 DAS as pre emergence (PE) and 10 DAS as early post emergence (EPoE) by using hand sprayer. Weed management practices (hand and power weeding) were done on 40 DAS.

III. RESULTS

3.1. Effect onweeds

Weed flora of the experimental field consisted of fourteen weeds and among these weeds, *Cyanodondactylon*and*Echinochloacolonum*were the dominantgrass, *Cyperusrotundus*was the only sedge,T*rianthemaportulacastrum*, *Corchorustrilocularis*and*Cleome viscose* were the predominantbroad leaved weeds.The results of the experiment revealed that the broad leaved weeds dominated over grasses and sedges in cotton during the initial growth stage. Among broad leaved weeds, *Trianthemaportulacastrum*was the dominant weed flora during both the years. Dominance of broad leaved weeds in early stages was due to their faster growth and deep root system and thus promoted the absorption of soil moisture.

3.1.1. Effect ontotal weed density, total weed dry weight and weed control efficiency

3.1.1.1. Total weed density

Significant variation in total weed density was observed among the weed control methods. At 20 DAS, lesser and comparable level of total weed density was observed in the application of PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}) with 9.17 m^{-2}; 4.68 m^{-2} and application of PE pendimethalin at 1.0 kg ha^{-1} + PW(T_{11}) with 9.18 m^{-2}; 4.31m^{-2} during 2012 and 2013, respectively. At 40 DAS, during 2012 and 2013, lesser density of total weed was observedwith two hand weeding (T_{12}), two power weeding (T_{13}), application of PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}) and PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}) which were comparable with each other(Table 1). At 60 DAS, lesser total weed density was found in two hand weeding (T_{12}) with 17.71 m^{-2}; 6.82 m^{-2}, PE pendimethalin at 1.0 kg ha^{-1}+ HW (T_{10}) with 18.04 m^{-2} ; 7.16 m^{-2}, PE pendimethalin at 1.0 kg ha^{-1}+ PW (T_{11}) with 19.10 m^{-2} ; 7.66 m^{-2} and two power weeding (T_{13}) with 21.35 m^{-2} ; 8.79 m^{-2} which were comparable with each other during 2012 and 2013, respectively. The cotton crop under unweeded check had higher total weed density at all the stages of observation in both the years.

3.1.1.2.Total weed dry weight

Weed management practices imposed to cotton significantly influenced the total dry weight of weed.At 20 DAS, during 2012 and 2013, application of PE pendimethalin at1.0 kg ha^{-1} + HW (T_{10}) and PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}) were comparable and recorded with lesser dry weight of total weed(Table 2). At 40 DAS, during 2012 and 2013, lesser dry weight of total weed was observed with two hand weeding (T_{12}), two power weeding (T_{13}), PE pendimethalin at 1.0 kg ha^{-1}+ HW (T_{10}) and PE pendimethalin at 1.0 kg ha^{-1}+ PW (T_{11}) which were comparable with each other. At 60 DAS, during 2012 and 2013, the lowest dry weight of total weed was registered with two hand weeding (T_{12}), PE pendimethalin at 1.0 kg ha^{-1}+ HW (T_{10}), PE pendimethalin at 1.0 kg ha^{-1}+ PW (T_{11}) and two power weeding (T_{13}) and were comparable. Unweeded check observed with higher density of total weed at all the stages of observation during both the years.

3.1.1.3.Weed control efficiency (WCE)

During 2012, application of PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}) and PE pendimethalin at 1.0 kg ha^{-1} +

PW (T_{11}) registered higher WCE of 74.73 and 74.33 per cent, respectively at 20 DAS(Table 3). During 2012, at 40 DAS, two hand weeding(T_{12}), two power weeding(T_{13}), PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}) and PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}) recorded highest WCE of 68.73, 68.40, 65.94 and 65.65 per cent. At 60 DAS, two hand weeding(T_{12}), PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}), PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}) and two power weeding(T_{13}) were recorded with higher WCE of 88.25, 87.92, 87.66 and 87.32 per cent, respectively. During 2013, at 20 DAS, higher WCE of 89.37 and 89.35 per cent were recorded with the application of PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}) and PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}). At 40 DAS, two hand weeding(T_{12}), two power weeding(T_{13}), PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}) and PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}) recorded highest WCE of 77.84, 77.67,74.73 and 74.44 per cent. At 60 DAS, two hand weeding(T_{12}), application of PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}), PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}) and two power weeding(T_{13}) were recorded with higher WCE.

3.1.2. Nutrient removal by weeds

3.1.2.1. Nitrogen

At 60 DAS, there was significant variation in N depletion by weeds among different weed management practices was found in both the crops(Table 4).In the first and second crop, at 60 DAS, two hand weeding (T_{12}), PE pendimethalin at 1.0 kg ha^{-1}+ HW (T_{10}), PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}) and two power weeding (T_{13}) were comparable and reduced the N removal by weeds markedly from 7.12 to 7.35 kg ha^{-1} in 2012 and 6.94 to 7.46 kg ha^{-1} in 2013 compared to other weed management practices.Unweeded controlrecorded with highest removal of N by weeds by 17.86 and 15.47 kg ha^{-1} during 2012 and 2013.

3.1.2.2. Phosphorus

Weed control methods caused significant variation in P uptake by weeds in cotton.During 2012 and 2013, at 60 DAS, two hand weeding (T_{12}), PE pendimethalin at 1.0 kg ha^{-1}+ HW (T_{10}), PE pendimethalin at 1.0 kg ha^{-1}+ PW (T_{11}) and two power weeding (T_{13}) were comparable and analyzed with reduced P removal by weeds considerably from 3.71 to 4.09 kg ha^{-1} in 2012 and 2.58 to 2.89 kg ha^{-1} in 2013 as compared to control. During 2012 and 2013, at 60 DAS,unweeded control resulted in removal by weeds with 7.34 and 6.12 kg ha^{-1} in 2012 and 2013(Table 4).

3.1.2.3. Potassium

During 2012 and 2013, at 60 DAS, significant variations in K removal by weeds were observed among the weed management practices(Table 4).At 60 DAS, two hand weeding (T_{12}), PE pendimethalin at 1.0 kg ha^{-1}+ HW (T_{10}), PE pendimethalin at 1.0 kg ha^{-1}+ PW (T_{11}) and two power weeding (T_{13}) were found comparable and from 10.74 to 11.14 kg ha^{-1} in 2012 and from 7.96 to 8.32 kg ha^{-1} in 2013 with reduced K removal by weeds compared to other weed management practices.At 60 DAS, removal of potassiumby weeds was highest under unweeded control with 21.06 and 17.13 kg ha^{-1}in 2012 and 2013 respectively.

3. 2. Effect on yield attributes and seed cotton yield

3. 2. 1. Monopodial branches $plant^{-1}$

Weed management practices did not significantly influence the number of monopodial branches $plant^{-1}$ in both the years(Table 5 and 6).

3. 2. 2. Yield characters

The data on number of sympodial branches $plant^{-1}$, number of bolls $plant^{-1}$ and boll weight were recorded and presented under yield characters. Significant variation among the treatments was noticed for all the yield attributes(Table 5 and 6).

3. 2. 3. Sympodial branches $plant^{-1}$

The treatments such astwo hand weeding(T_{12}), PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}), PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}) and two power weeding(T_{13}) were comparable and recorded with sympodial branches $plant^{-1}$ of 19.36,19.11,18.96 and 18.23 in 2012 and 21.53.21.47,21.33 and 20.45 in 2013 (Table 5 and 6).Unweeded control registered lesser number of sympodial branches $plant^{-1}$ 8.41 and 10.37 in 2012 and 2013.

3. 2. 4. Number of bolls $plant^{-1}$

The observation on boll number $plant^{-1}$ showed that the weed management practices had significant effect on the boll production of cotton in the both the years of study.During 2012 and 2013, the treatments *viz.*,two hand weeding(T_{12}), PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}), PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}) and two power weeding(T_{13}) were comparable and recorded with higher number of bolls $plant^{-1}$(Table 5 and 6). Unweeded control registered lesser number of bolls $plant^{-1}$ of 11.60 and 12.90 in 2012 and 2013.

3. 2. 5. Boll weight

In both the years of study, two hand weeding (T_{12}) showed higher boll weight of 3.72 and 3.91 g which were on par with T_{10}, T_{11}, T_{13}, T_1, T_2, T_4, T_5, T_7 andT_8 treatments produced bolls with more weight during 2012 and 2013 respectively(Table 5 and 6). Unweeded control registered

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the lowest boll weight of 2.87 and 2.96 g boll^{-1} in both the years. But it was on par with T_3, T_6 and T_9 also.

3. 2. 6. Seed cotton yield

In the present investigation, significant difference in seed cotton yield was observed among the various weed management practices with chemical, leaf extracts, manual mechanical methods and integrated weed management in both the years of study.During 2012, the maximum seed cotton yield of 2185 kg ha^{-1} was registered with two hand weeding (T_{12}) and the yield under this treatment was comparable with PE pendimethalin at 1.0 kg ha^{-1}+ HW (T_{10}), PE pendimethalinat 1.0 kg ha^{-1} + PW (T_{11}) and two power weeding (T_{13}) with the yield of 2123, 2087, 2045 kg ha^{-1}(Table 5 and 6).During 2013, two hand weeding (T_{12}) was comparable with PE pendimethalin at 1.0 kg ha^{-1}+ HW (T_{10}), PE pendimethalin at 1.0 kg ha^{-1}+ PW (T_{11}) and two power weeding (T_{13}) which registered higher seed cotton yield of 2293, 2232,2196 and 2174 kg ha^{-1} respectively. Unweeded control recorded lesser seed cotton yield of 1356 and 1517 kg ha^{-1} in both the years respectively.

3. 3. Economics

The cost of cultivation was highest in hand weeded twice (T_{12}) with Rs. 50,049 per hectare followed by T_1, T_4 and T_7 with Rs. 49,811 per hectare(Table 7 and 8).In both the crops, PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}), PE pendimethalin at 1.0 kg ha^{-1} + PW(T_{11}) and hand weeding twice (T_{12}) recorded maximum net return. The unweeded control recorded the lowest net return of Rs. 13,156/- ha^{-1} and Rs. 14,268/- ha^{-1} during 2012 and 2013. Highest benefit cost ratio (B: C ratio) was obtained with the application of PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}) with 1.82 and 1.69 during 2012 and 2013. It was followed by PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}) with 1.80 and 1.66 during the two years of study.

IV. DISCUSSION

4. 1. Effect of weed control treatments on weed density, weed dry weight and weed control efficiency

Among the broad leaved weeds, *Trianthemaportulacastrum*was the dominant weed flora during both the years of study. This might be due to the smothering effect of broad leaved weeds on monocots. The leaf area of the weed was more favourable for interception of brighter solar radiation. Nazar*et al*. (2008) reported that dominance of broad leaved weeds during the early stages of cotton was due to their fast growth and deep root system.

In the early stage of the crop growth (20 DAS), total weed density, total weed dry weight , were reduced greatly by the application of PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}) and PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}). Prabhu (2010) pointed out that broad spectrum action of pendimethalin recorded lesser density of grasses at 25 DAS due to the translocative nature of the herbicide. At 20 DAS, the sedge weeds were not satisfactorily controlled by pendimethalin 30 per cent EC formulation. It was supported by Nair *et al*. (1983) stating the failure of pendimethalin to control nutsedge. Pre emergence application of pendimethalin effectively reduced *Trianthemaportulacastrum* which was the predominant weed in the experimental site. This might be possibly due to the effective prevention of seed germination of broad leaved weeds.Nalini (2010) reported that pendimethalin effectively controlled annual weeds than perennial weeds. Das and Duary (1998) reported that the herbicidal effect of pendimethalin might be due to the inhibition of cell division and thus curtailed the density of weeds. The reduced weed dry weight could be due to the reduction in weed density at all the stages of crop growth. This might be attributed to rapid depletion of carbohydrate reserve of the weeds through rapid respiration as pointed out by Prakash*et al*. (1999).At 20 DAS, application of PE pendimethalin at 1.0 kg ha^{-1} + HW and PE pendimethalinat 1.0 kg ha^{-1} + PW recorded the highest WCE of 74.7; 89.35 and 74.33; 89.37 per cent in 2012 and 2013, respectively.

But at later stages of crop growth (40 DAS), total weed density, total weed dry weight, were reduced by manual weeding twice (T_{12}) and power weeding twice (T_{13}). The underground root portions like tubers and stolens were effectively removed by mechanical methods of weed control than the chemical application. This was due to the imposement of first manual weeding on 20 DAS which avoided the competition by weeds with crop for nutrient and moisture (Prabhu, 2010). Shobana (2002) reported that *Cynodondactylon*, was perennial in nature which was not much controlled by pendimethalin application. At this stage, manual weeding twice controlled the grass and sedge weed efficiently and favored the growth of cotton which influenced the crop and covered the field surface area much earlier than the weed.

At 60 DAS, both mechanical methods namely manual weeding twice (T_{12}) and power weeding twice (T_{13}) and integrated weed management *viz.*, application of PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}) and PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}) effectively controlled all the weeds and reduced the dry weight of weedsultimately lead to better weed control efficiency in the above treatments. Shobana (2002) reported that the mechanical methods were better in weed control due to better removal of perennial weeds at 20 and 40 DAS. The early emerging weeds were controlled by first hand weeding and late emerging weeds were removed by second hand weeding with better removal of underground root portions. The integrated weed management practice

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registered the broad spectrum weed control as a result of longer persistence in the soil profile. Similar finding was reported by Balasubramanian (1992) who found that the weed control efficiency was comparatively higher with the application of pendimethalin at 1.0 kg ha^{-1} as compared with 0.5 and 0.75 kg ha^{-1}.

The nutrient (NPK) removal by weeds was greatly reduced by two hand weeding (T_{12}), PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}), PE pendimethalinat 1.0 kg ha^{-1} + PW (T_{11}) and power weeding twice (T_{13}). This might be due to fairly weed free condition at early stages of crop growth and the weed free environment created by the pre emergence herbicide with reduced weed DMP. The dry weight was another factor determining the nutrient removal by weeds. This finding is in line with the reports of Chander*et al*. (1994) who described that application of pendimethalin at 1.25 kg ha^{-1} followed by hand weeding reduced the nutrient removal by weeds which was comparable with hand weeding twice. Such positive effect was due to lower population and dry weight of weeds resulting from better control of the entire weed by two hand weeding.

4. 2. Effect on yield attributes and seed cotton yield

Cotton being a wide spaced and slow growing crop is sensitive to weed competition at early stages of growth than at later stages. Due to heavy infestation of weeds under unweeded check reduction in seed cotton yield was recorded. During both the years, growth character number of monopodial branches $plant^{-1}$ was not significantly influenced by the weed management practices. The yield attributing characters *viz*., number of sympodial branches $plant^{-1}$, number of bolls $plant^{-1}$ and boll weight ultimately decide the seed cotton yield. During both the years, the treatments had significant effect on yield attributes and seed cotton yield. The yield attributes andseed cotton yieldwere more with manual weeding twice (T_{12}), PE pendimethalin at 1.0 kg ha^{-1} + HW (T_{10}), PE pendimethalin at 1.0 kg ha^{-1} + PW (T_{11}) and power weeding twice (T_{13}). This could be due to the enhanced plant height, dry matter production and nutrient uptake of the crop. This might also be due to the season long weed control which was favourable for better growth and enhanced leaf area contributing for the activated photosynthesis and translocation of more photosynthates to sink which increased the boll weight (Nalini, 2010).In the above treatments the yield increasing percentage over control was 61, 57, 54 and 51 per cent during 2012 and 51, 47, 45 and 43 per cent during 2013, respectively. Gnanavel and Babu (2008) also reported maximum seed cotton yield with pendimethalin combined with hand weeding as compared with control.

4. 3. Effect of weed control treatments on economics

Weed management practices showed positive impact on net return and benefit-cost ratio. By considering the cost of cultivation, pre emergence application of pendimethalin at1.0 kg ha^{-1} + power weeding (T_{11}) resulted in higher net return of Rs.37,529/- during 2012 and Rs. 35,895/- during 2013 and benefit cost ratio of 1.82 and 1.69 during both the years, respectively. In the above treatment, the additional income obtained over unweeded control was Rs. 24,373/- and Rs. 21,627/- during 2012 and 2013 respectively.

V. CONCLUSION

From the above study, it could be concluded,that the integrated weed management practices like, application of PE pendimethalin at 1.0 kg ha^{-1} + power weeding on 40 DAS (T_{11}) could keep the weed density and dry weight reasonably at a lower level and recorded higher seed cotton yield and economic net return. The integrated weed management practices also performed equally effective as that of mechanical methods because of good control of early emerging weeds by the pre emergence herbicide application and better removal of late emerging weeds by mechanical methods of weed control.

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Table.1: Effect of different weed management practices on total weed densityin cotton

Treatments	Total weed density(No. m^{-2})					
	2012			2013		
	20 DAS	40 DAS	60 DAS	20 DAS	40 DAS	60 DAS
T_1 - PE Calotropis @ 30 % + HW on 40 DAS	33.75 (5.81)	54.20 (7.36)	44.72 (6.69)	24.89 (4.99)	37.96 (6.16)	27.24 (5.22)
T_2 - PE Calotropis @ 30 % + PW on 40 DAS	34.52 (5.88)	55.36 (7.44)	46.90 (6.85)	25.49 (5.05)	38.56 (6.21)	29.39 (5.42)
T_3 - PE Calotropis @ 30 % + EPoECalotropis @ 30 %	32.02 (5.66)	51.11 (7.15)	109.78 (10.48)	23.66 (4.86)	35.82 (5.99)	82.34 (9.07)
T_4 - PE Calotropis @ 20 % + HW on 40 DAS	46.79 (6.84)	72.23 (8.50)	54.44 (7.38)	31.05 (5.57)	50.57 (7.11)	38.33 (6.19)
T_5 - PE Calotropis @ 20 % + PW on 40 DAS	47.70 (6.91)	72.87 (8.54)	56.92 (7.54)	31.78 (5.64)	51.00 (7.14)	40.19 (6.34)
T_6 - PE Calotropis @ 20 % + EPoECalotropis @ 20 %	44.49 (6.67)	68.81 (8.30)	113.84 (10.67)	29.26 (5.41)	46.85 (6.84)	85.97 (9.27)
T_7 - PE Calotropis @ 10 % + HW on 40 DAS	66.67 (8.17)	93.89 (9.69)	67.17 (8.20)	46.45 (6.82)	69.76 (8.35)	46.81 (6.84)
T_8 - PE Calotropis @ 10 % + PW on 40 DAS	67.96 (8.24)	95.52 (9.77)	69.68 (8.35)	47.24 (6.87)	70.95 (8.42)	48.44 (6.96)
T_9 - PE Calotropis @ 10 % + EPoECalotropis @ 10 %	62.85 (7.93)	91.65 (9.57)	120.44 (10.97)	43.54 (6.60)	65.06 (8.07)	90.20 (9.50)
T_{10} - Pendi. @ 1.0 kg ha^{-1} + HW on 40 DAS	9.17 (3.03)	29.04 (5.39)	18.04 (4.25)	4.68 (2.16)	13.76 (3.61)	7.16 (2.68)

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T_{11} - Pendi. @ 1.0 kg ha^{-1} + PW on 40 DAS	9.18 (3.03)	29.73 (5.45)	19.10 (4.37)	4.31 (2.08)	14.41 (3.65)	7.66 (2.77)
T_{12} - HW on 20 and 40 DAS	81.19 (9.01)	23.36 (4.83)	17.71 (4.21)	58.87 (7.67)	9.74 (3.12)	6.82 (2.61)
T_{13} - PW on 20 and 40 DAS	80.49 (8.97)	25.47 (5.05)	21.35(4.62)	59.15 (7.69)	11.02 (3.32)	8.79 (2.96)
T_{14} - Unweeded control	81.19 (9.01)	109.29 (10.45)	134.17 (11.58)	59.67 (7.72)	79.37 (8.91)	99.00 (9.95)
S. Ed	0.275	0.345	0.360	0.220	0.270	0.295
CD (P = 0.05)	0.55	0.69	0.72	0.44	0.54	0.59

Figures in the parenthesis are transformed values

Table.2:.Effect of different weed management practices on total weed dry weight in cotton

Treatments	**Total weed dry weight (kg ha^{-1})**					
	2012			**2013**		
	20 DAS	**40 DAS**	**60 DAS**	**20 DAS**	**40 DAS**	**60 DAS**
T_1 - PE Calotropis @ 30 % + HW on 40 DAS	146.07 (12.09)	209.29 (14.47)	98.08 (9.90)	112.61 (10.61)	154.40 (12.43)	76.34 (8.74)
T_2 - PE Calotropis @ 30 % + PW on 40 DAS	145.99 (12.08)	209.71 (14.48)	99.41 (9.97)	112.91 (10.63)	154.87 (12.44)	77.16 (8.78)
T_3 - PE Calotropis @ 30 % + EPoECalotropis @ 30 %	144.76 (12.03)	207.60 (14.41)	325.32 (18.04)	111.33 (10.55)	152.87 (12.36)	257.95 (16.06)
T_4 - PE Calotropis @ 20 % + HW on 40 DAS	151.97 (12.33)	226.03 (15.03)	101.99 (10.10)	117.05 (10.82)	163.02 (12.77)	79.99 (8.94)
T_5 - PE Calotropis @ 20 % + PW on 40 DAS	152.65 (12.36)	226.71 (15.06)	104.20 (10.21)	117.81 (10.85)	164.36 (12.82)	80.60 (8.98)
T_6 - PE Calotropis @ 20 % + EPoECalotropis @ 20 %	151.14 (12.29)	221.59 (14.89)	328.86 (18.13)	115.41 (10.74)	160.23 (12.66)	260.90 (16.15)
T_7 - PE Calotropis @ 10 % + HW on 40 DAS	206.03 (14.35)	348.29 (18.66)	110.55 (10.51)	170.10 (13.04)	258.11 (16.07)	83.26 (9.12)
T_8 - PE Calotropis @ 10 % + PW on 40 DAS	209.73 (14.48)	355.56 (18.86)	112.24 (10.59)	171.07 (13.08)	268.40 (16.38)	84.52 (9.19)
T_9 - PE Calotropis @ 10 % + EPoECalotropis @ 10 %	203.78 (14.28)	345.13 (18.58)	332.52 (18.24)	165.88 (12.88)	253.18 (15.91)	266.79 (16.33)
T_{10} - Pendi. @ 1.0 kg ha^{-1} + HW on 40 DAS	63.84 (7.99)	127.31 (11.28)	43.82 (6.62)	22.33 (4.73)	71.46 (8.45)	19.74 (4.44)
T_{11} - Pendi. @ 1.0 kg ha^{-1} + PW on 40 DAS	64.84 (8.05)	128.42 (11.33)	44.76 (6.69)	22.30 (4.72)	72.27 (8.50)	20.34 (4.51)
T_{12} - HW on 20 and 40 DAS	251.87 (15.87)	116.89 (10.81)	42.63 (6.53)	207.78 (14.41)	62.66 (7.92)	18.95 (4.35)
T_{13} - PW on 20 and 40 DAS	252.05 (15.88)	118.14 (10.87)	46.00 (6.78)	208.24 (14.43)	63.15 (7.95)	21.22 (4.61)
T_{14} - Unweeded control	252.61 (15.89)	373.82 (19.33)	377.80 (19.45)	209.70 (14.48)	282.79 (16.82)	377.80 (19.45)
S. Ed	0.54	0.68	0.59	0.43	0.56	0.48
CD (P = 0.05)	1.07	1.36	1.17	0.86	1.11	0.96

Figures in the parenthesis are transformed values

Table.3: Effect of different weed management practices on the weed control efficiency (WCE) in cotton

Treatments	Weed control efficiency(%)					
	2012			2013		
	20 DAS	40 DAS	60 DAS	20 DAS	40 DAS	60 DAS
T_1 - PE Calotropis @ 30 % + HW on 40 DAS	42.17	44.01	72.97	46.30	45.40	73.20
T_2 - PE Calotropis @ 30 % + PW on 40 DAS	42.21	43.90	72.60	46.16	45.24	72.91
T_3 - PE Calotropis @ 30 % + EPoECalotropis	42.69	44.46	10.34	46.91	45.94	9.44

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	@ 30 %						
T_4 -	PE Calotropis @ 20 % + HW on 40 DAS	39.84	39.53	71.89	44.18	42.35	71.92
T_5 -	PE Calotropis @ 20 % + PW on 40 DAS	39.57	39.35	71.28	43.82	41.88	71.70
T_6 -	PE Calotropis @ 20 % + EPoECalotropis @ 20 %	40.17	40.72	9.36	44.97	43.34	8.41
T_7 -	PE Calotropis @ 10 % + HW on 40 DAS	18.44	6.83	69.53	18.88	8.73	70.77
T_8 -	PE Calotropis @ 10 % + PW on 40 DAS	16.97	4.88	69.07	18.42	5.09	70.33
T_9 -	PE Calotropis @ 10 % + EPoECalotropis @ 10 %	19.33	7.68	8.35	20.90	10.47	6.34
T_{10} -	Pendi. @ 1.0 kg ha^{-1} + HW on 40 DAS	74.73	65.94	87.92	89.35	74.73	93.07
T_{11} -	Pendi. @ 1.0 kg ha^{-1} + PW on 40 DAS	74.33	65.65	87.66	89.37	74.44	92.86
T_{12} -	HW on 20 and 40 DAS	0.29	68.73	88.25	0.91	77.84	93.35
T_{13} -	PW on 20 and 40 DAS	0.22	68.40	87.32	0.70	77.67	92.55
T_{14} -	Unweeded control	-	-	-	-	-	-

Table.4: Nutrient removal by weed at 60 DAS as influenced by weed management practices in cotton

Treatments	**N, P, K removal by weeds at 60 DAS (kg ha^{-1})**					
	2012			**2013**		
	N	**P**	**K**	**N**	**P**	**K**
PE Calotropis @ 30 % + HW on 40 DAS	10.75	5.17	12.63	9.87	3.71	10.73
PE Calotropis @ 30 % + PW on 40 DAS	10.87	5.32	12.71	9.95	3.78	10.99
PE Calotropis @ 30 % +EPoECalotropis @ 30 %	16.81	6.89	19.69	14.59	5.75	16.09
PE Calotropis @ 20 % + HW on 40 DAS	12.34	6.83	15.13	11.59	4.66	12.32
PE Calotropis @ 20 % + PW on 40 DAS	12.82	6.91	15.34	11.69	4.75	12.56
PE Calotropis @ 20 % +EPoECalotropis @ 20 %	16.99	6.96	19.78	14.72	5.86	16.25
PE Calotropis @ 10 % + HW on 40 DAS	13.15	6.13	15.45	12.11	4.76	12.75
PE Calotropis @ 10 % + PW on 40 DAS	13.27	6.22	15.59	12.38	4.84	12.87
PE Calotropis @ 10 % +EPoECalotropis @ 10 %	17.34	7.13	19.83	15.01	5.91	16.54
Pendi. @ 1.0 kg ha^{-1} + HW on 40 DAS	7.22	3.88	10.89	7.15	2.71	8.09
Pendi. @ 1.0 kg ha^{-1} + PW on 40 DAS	7.29	3.96	10.96	7.32	2.80	8.15
HW on 20 and 40 DAS	7.12	3.71	10.74	6.94	2.58	7.96
PW on 20 and 40 DAS	7.35	4.09	11.14	7.46	2.89	8.32
Unweeded control	17.86	7.34	21.06	15.47	6.12	17.13
S. Ed	**0.56**	**0.25**	**0.72**	**0.50**	**0.20**	**0.57**
CD (P = 0.05)	**1.12**	**0.49**	**1.43**	**1.01**	**0.39**	**1.13**

Table.5: Effect of weed management practices on monopodial branches, yield attributes and yield of cotton in 2012

Treatments	**Growth attribute**	**Yield attributes and yield of cotton**			
	Monopodial branches $plant^{-1}$ (Nos.)	**Sympodial branches $plant^{-1}$ (Nos.)**	**Bolls $plant^{-1}$ (Nos.)**	**Boll weight (g $boll^{-1}$)**	**Seed cotton yield (kg ha^{-1})**
T_1 - PE Calotropis @ 30 % + HW on 40 DAS	1.67	14.37	21.61	3.68	1884
T_2 - PE Calotropis @ 30 % + PW on 40 DAS	1.67	14.31	21.33	3.68	1850
T_3 - PE Calotropis @ 30 % + EPoECalotropis @ 30 %	1.33	8.99	12.01	3.16	1408
T_4 - PE Calotropis @ 20 % + HW on 40 DAS	1.67	14.24	18.96	3.56	1638
T_5 - PE Calotropis @ 20 % + PW on 40 DAS	1.67	14.19	18.89	3.56	1603
T_6 - PE Calotropis @ 20 % + EPoECalotropis @	1.33	8.76	11.95	3.09	1385

20 %					
T_7 - PE Calotropis @ 10 % + HW on 40 DAS	1.67	13.34	18.62	3.47	1589
T_8 - PE Calotropis @ 10 % + PW on 40 DAS	1.67	13.25	18.56	3.47	1572
T_9 - PE Calotropis @ 10 % + EPoECalotropis @ 10 %	1.33	8.65	11.78	2.96	1374
T_{10} - Pendi. @ 1.0 kg ha^{-1} + HW on 40 DAS	1.67	19.11	23.42	3.71	2123
T_{11} - Pendi. @ 1.0 kg ha^{-1} + PW on 40 DAS	1.67	18.96	23.18	3.71	2087
T_{12} - HW on 20 and 40 DAS	1.67	19.36	24.50	3.72	2185
T_{13} - PW on 20 and 40 DAS	1.67	18.23	22.92	3.69	2045
T_{14} - Unweeded control	1.00	8.41	11.60	2.87	1356
S. Ed	0.40	0.63	0.82	0.15	80
CD (P = 0.05)	NS	1.25	1.63	0.30	159

Table.6: Effect of weed management practices on monopodial branches, yield attributes and yield of cotton in 2013

Treatments	**Growth attribute**	**Yield attributes and yield of cotton**			
	Monopodial branches plant^{-1} (Nos.)	**Sympodial branches plant^{-1} (Nos.)**	**Bolls plant^{-1} (Nos.)**	**Boll weight (g boll^{-1})**	**Seed cotton yield (kg ha^{-1})**
PE Calotropis @ 30 % + HW on 40 DAS	1.67	18.96	20.12	3.70	2010
PE Calotropis @ 30 % + PW on 40 DAS	1.67	18.91	20.01	3.69	1998
PE Calotropis @ 30 % + EPoECalotropis @ 30 %	1.33	10.57	14.21	3.00	1582
PE Calotropis @ 20 % + HW on 40 DAS	1.67	18.75	17.43	3.67	1823
PE Calotropis @ 20 % + PW on 40 DAS	1.67	18.68	17.13	3.67	1811
PE Calotropis @ 20 % + EPoECalotropis @ 20 %	1.33	10.49	13.55	3.00	1560
PE Calotropis @ 10 % + HW on 40 DAS	1.67	17.86	16.75	3.65	1782
PE Calotropis @ 10 % + PW on 40 DAS	1.67	17.79	19.64	3.63	1759
PE Calotropis @ 10 % + EPoECalotropis @ 10 %	1.33	10.41	12.99	2.98	1541
Pendi. @ 1.0 kg ha^{-1} + HW on 40 DAS	1.67	21.47	26.18	3.86	2232
Pendi. @ 1.0 kg ha^{-1} + PW on 40 DAS	1.67	21.33	25.82	3.81	2196
HW on 20 and 40 DAS	2.00	21.53	26.30	3.91	2293
PW on 20 and 40 DAS	2.00	20.45	24.76	3.75	2174
Unweeded control	1.00	10.37	12.90	2.96	1517
S. Ed	**0.39**	**0.62**	**0.88**	**0.16**	**86**
CD (P = 0.05)	**NS**	**1.24**	**1.77**	**0.31**	**172**

Table.7: Economics of different weed management practices in cotton during 2012

Treatments	**2012**			
	Total cost of cultivation (Rs ha^{-1})	**Gross income (Rs ha^{-1})**	**Net income (Rs ha^{-1})**	**B:C ratio**
PE Calotropis @ 30 % + HW on 40 DAS	49811	75360	24549	1.48
PE Calotropis @ 30 % + PW on 40 DAS	48466	74000	24534	1.50
PE Calotropis @ 30 % +EPoECalotropis @ 30 %	46388	56320	8932	1.19
PE Calotropis @ 20 % + HW on 40 DAS	49811	65520	14709	1.29
PE Calotropis @ 20 % + PW on 40 DAS	48466	64120	14654	1.30
PE Calotropis @ 20 % +EPoECalotropis @ 20 %	46388	55400	8012	1.17
PE Calotropis @ 10 % + HW on 40 DAS	49811	63560	12749	1.25
PE Calotropis @ 10 % + PW on 40 DAS	48466	62880	13414	1.27
PE Calotropis @ 10 % +EPoECalotropis @ 10 %	46388	54960	7572	1.16

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Pendi. @ 1.0 kg ha^{-1} + HW on 40 DAS	47296	84920	37624	1.80
Pendi. @ 1.0 kg ha^{-1} + PW on 40 DAS	45951	83480	37529	1.82
HW on 20 and 40 DAS	50049	87400	37351	1.75
PW on 20 and 40 DAS	46544	81800	35256	1.76
Unweeded control	41084	54240	13156	1.32

Table.8: Economics of different weed management practices in cotton during 2013

Treatments	**2013**			
	Total cost of cultivation (Rs ha^{-1})	**Gross income (Rs ha^{-1})**	**Net income (Rs ha^{-1})**	**B:C ratio**
PE Calotropis @ 30 % + HW on 40 DAS	56235	80400	23065	1.40
PE Calotropis @ 30 % + PW on 40 DAS	54530	79920	24290	1.44
PE Calotropis @ 30 % +EPoECalotropis @ 30 %	52308	63280	9872	1.18
PE Calotropis @ 20 % + HW on 40 DAS	56235	72920	15585	1.27
PE Calotropis @ 20 % + PW on 40 DAS	54530	72440	16810	1.30
PE Calotropis @ 20 % +EPoECalotropis @ 20 %	52308	62400	8992	1.17
PE Calotropis @ 10 % + HW on 40 DAS	56235	71280	13945	1.24
PE Calotropis @ 10 % + PW on 40 DAS	54530	70360	14730	1.26
PE Calotropis @ 10 % +EPoECalotropis @ 10 %	52308	61640	8232	1.15
Pendi. @ 1.0 kg ha^{-1} + HW on 40 DAS	53650	89280	35630	1.66
Pendi. @ 1.0 kg ha^{-1} + PW on 40 DAS	51945	87840	35895	1.69
HW on 20 and 40 DAS	56697	91720	35023	1.62
PW on 20 and 40 DAS	52352	86960	34608	1.66
Unweeded control	46412	60680	14268	1.31

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Regulation of Seed Germination and the Role of Aquaporins under Abiotic Stress

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***Abstract*—** *Aquaporins play a major role in governing the movement of water between neighboring cells during seed germination and are major players in response to abiotic stress conditions that affect water availability. Seeds of pea (Pisum sativum L. cv. Arkel) were used for studying cell growth, expression and function of aquaporins during seed imbibition, radicle emergence and growth.Water channel functioning checked by inhibitory test with mercuric chloride showed closed water channels prior to growth initiation. Addition of mercury scavenging agents dithiothreitol and β-mercaptoethanol along with the $HgCl_2$ overcame the observed inhibitory effects in terms of moisture content. The presence of aquaporin inhibitors ($HgCl_2$ and $ZnCl_2$) and NaCl reduced seedling growth. Here we studied expression of a plasma membrane intrinsic protein (PsPIP1;2) and a tonoplast intrinsic protein (PsTIP1;1) by using the semi quantitative RT-PCR in the germinated seedlings exposed to different abiotic stresses. Treatment with NaCl, $HgCl_2$ and $ZnCl_2$ differentially regulated gene expression in radicle, cotyledon and plumule. NaCl and Hg, upregulated expression of PsPIP1;2 and PsTIP1;1 in radicle and expression of PsTIP1;1 was significantly upregulated in radicle and suppressed in cotyledon by Zn. A possible role for aquaporins in germinating seeds and seedling response to abiotic stresses is discussed.*

***Keywords*—Seed germination, Aquaporins, Pisum sativum L., Heavy metals, dithiothreitol (DTT), β-mercaptoethanol (ME).**

I. INTRODUCTION

Seed germination determines successful crop production and is usually the most critical stage in seedling establishment (Almansouri et al. 2001; Bhattacharjee 2008). The germination of seeds requires great adeptness since the process is very complex, a seed from its stillness is woken up to its active state (Dow & Schwintzer 1999). Imbibition of water marks the onset of germination of seeds and triggers arrested metabolic activities which culminates in the emission of a radicle and finalization of germination (Nanogaki et al. 2010). Water moves through the plant tissue via three different pathways: the apoplastic pathway through cell walls and intercellular spaces, the symplastic path from cell to cell either through cytoplasm and plasmodesmata, the transcellular path traversing through cell membranes. The movement of water via the transcellular path involves aquaporins, which are water selective channels (Preston et al. 1992; Agre et al. 1998).

Plant aquaporins fall into five subfamilies: the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the nodulin26-like intrinsic proteins (NIPs), the small basic intrinsic proteins (SIPs), and the uncategorized X intrinsic proteins (XIPs) (Maurel et al. 2015). Aquaporins are concentrated in zones of cell division and enlargement, play a major role in governing the movement of water between neighboring cells during seed germination (Tyerman et al. 2002; Jain et al. 2008). Recent studies have implicated the importance of aquaporins in seed imbibition and subsequent germination (Schuurmans et al. 2003; Liu et al. 2007; Liu et al. 2013; Cardoso et al. 2015). The function of seed aquaporins may be related to the water imbibition and activation of the metabolic system in the seed, which results in higher germination (Liu et al. 2007).

Seed germination occurs only when conditions become favorable. Seeds in their germinating stage are highly sensitive and germination is arrested by various heavy metals like mercury, cadmium and zinc etc. (Du et al. 2004; Chugh & Sawhney 1996; Rout & Das 2009). Mercury is widely believed to block aquaporins by binding to a cysteine residue located in close proximity to the aqueous pore of the protein (Daniels et al. 1994; Agre et al. 1998). Due to its aquaporins blocking activity (Przedpelska-Wasowicz & Wierzbicka 2011), mercurial compounds have been used to assess the contributions of aquaporins in water transport at various growth stages of plant. A reversibility of mercury effects by reducing agents like dithiothreitol (DTT) and β-mercaptoethanol (ME), have also been used in conjuction with $HgCl_2$in current studies that seek to evaluate the activity of aquaporins (Przedpelska-Wasowicz & Wierzbicka 2011; Obroucheva et al. 2012; Liu et al. 2014).

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Several studies have shown that abiotic stresses such as water deficit, salinity and heavy metals differentially regulate expression of aquaporins in various plant organs (Sakurai et al. 2005; Tyerman et al. 2002; Suga et al. 2001; Alexandersson et al. 2005; Jang et al. 2004). In Arabidopsis, the expression of MIP genes in different organs in response to cold, salt, drought and ABA treatment has been determined by using a semi-quantitative slot blot analysis and quantitative real-time RT-PCR (Alexandersson et al. 2005;Weig et al. 1997). Most of the studies showed that several aquaporins were predominantly expressed in one organ and that many were markedly up or downregulated under the different stress conditions. Analysis of 33 rice MIP gene expression at different growth stages and in different plant organs also showed that gene expression varied with plant organ and growth stage (Sakurai et al. 2005). Heavy metals such as Cd, Cu and Hg are known to modulate gene expression and function of MIPs in various plant species. For example, in *Pisum sativum* reduction of root hydraulic conductivity (Lpr) by $HgCl_2$ treatment was accompanied by an increase in the expression of *PsPIP2;1*, suggesting that the increase in *PsPIP2;1* might compensate for the AQPs blocked by Hg (Beaudette et al. 2007), whereas in *Populus deltoides* roots subjected to copper stress genes encoding plasmalemma (PIP) and tonoplast (TIP) AQPs were downregulated under Cu application (Guerra et al. 2009).

The present study was undertaken to assess the role of heavy metals (Hg, Zn), salinity (NaCl) known aquaporin inhibitors, along with several chemical agents known to reverse the inhibitory effects of mercuric chloride in regulating the water relations via aquaporins during pea seed germination and investigated the expression and function of plasma membrane and tonoplast aquaporins in seedling tissues during seed imbibition, radicle emergence and growth.

II. MATERIALS AND METHODS

1. Effect of $HgCl_2$ on seed germination

Seeds of pea(*Pisum sativum* L. cv.Arkel) were procured from Manipur Seeds Corporation, Manipur. Theyhad no visible signs of injury and were uniform in size and weight.The seeds were placed in petri dishes (90 mm in diameter) lined with two layers of Whatman no.1 filter paper, moistened with 3 ml of distilled water and $HgCl_2$ solutions at concentrations of 100, 300, 500 and 1000 µM. The petri dishes were incubated at 25 ± 2^0C in dark for 72 h, and daily evaluations of germination were performed.

2. Effect of $HgCl_2$, ME and DTT on activity of aquaporins

To study the involvement of aquaporins on seed germination, known aquaporin inhibitor*$HgCl_2$*and mercury scavenging agents (ME and DTT) were tested during germination as per the method of Jain et al. (2008). The seeds were placed in petridishes lined with two layers of Whatman no.1 filter paper, moistened either with 3 ml of the test solution i.e., $HgCl_2$ (0, 100, 300, or 500 µM) or in combination with ME (250 µM) or DTT (500 µM). The petri dishes were incubated at 25 ± 2^0C in dark for 72 h. Five seedlings were used to calculate the percent moisture. The fresh and dry weights of seedlings were recorded, and the percentage moisture content was calculated as {(W_1-W_2)/W_1} x 100, where W_1 and W_2 represent fresh and dry weights of five seedlings, respectively. All the experiments were repeated thrice.

3. Effect of $HgCl_2$, $ZnCl_2$ and NaCl on seedling growth

In order to verify the significance of aquaporins in germinating pea seeds $HgCl_2$, $ZnCl_2$and NaCl was administered in germinating pea seeds. The germinated seeds were treated with different concentrations of $HgCl_2$(100, 300, 500 and 1000 µM), $ZnCl_2$(10, 25, 50 and 100 µM) and NaCl (100, 300, 500 and 1000 mM). Growth of the seedlings was determined by measuring the length of the radicle and plumule. Measurements were done inthree replicates using five plants and expressed as cm.The data were recorded after every 24 h upto 72 h.

4. Expression study of aquaporins

To study the effect of salt (NaCl) and heavy metals (Hg and Zn) on gene expression, germinated seeds were treated with NaCl (100 mM), $HgCl_2$(100 and 300µM),and $ZnCl_2$ (50 and 100µM). After 24 h and 48 h, radicle, plumule and cotyledon samples were taken from each treatment for RNA extraction.100 mg of the tissue samples were homogenized using liquid nitrogen and the total RNA was isolated using Total RNA purification kit (Nucleopore's) following the manufacturer's instructions. The RNA isolated were quantified spectrophotometrically at 260/280 nm. Out of total RNA isolated from the seed tissues, approximately 1000 ng was reverse transcribed using RevertAid™ First Strand cDNA Synthesis Kit #K1622 following the manufacturer's instruction. The cDNA thus obtained was kept at -20^0C till further use.The gene specific primers were designed from the nucleotide sequences of *PsPIP1;2* and *PsTIP1;1* (Gene bank accession number: AJ548795.1 and AJ243309.1 respectively). The gene specific forward primer 5'-TGATGCAGGTTCTTGGTG-3', reverse primer 5'-CGTGCTGGGTTGATACCA-3' were used for

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amplifying *PsPIP1;2*, and forward primer 5'-TGGCTGAGTTCATCTCCA-3', reverse primer 5'-CACTCCAACTCCTGCGGA-3' were used for amplifying *PsTIP1;1*.The PCR conditions were in house validated and reconfirmed for each aquaporin gene. Each reaction system contained 2.5 µl of 10x PCR buffer with $MgCl_2$, 1.0 µl of 10 pmole/µl each primer, 0.5 µl Taq DNA polymerase (5 U/µl), 2 µl cDNA and the volume was made up to 25 µl with deionized water.The thermal cycle used for *PsPIP1;2* was as follows: 96^0C for 2 min; 30 cycles of 96^0C for 30 s, 56^0C for 1min, 72^0C 30 s, and a final 72^0C for 5 min. The PCR reaction conditions for amplifying *PsTIP1;1*were the same as those for *PsPIP1;2* gene, but the annealing temperature was changed into 56^0C.The amplicons so generated were resolved on 1.2% agarose (Sigma-Aldrich,) gel electrophoresis. Then the gel was examined in Gel Doc (KODAK) and the photographs were taken.

III. RESULTS

1. Effect of $HgCl_2$ concentration on seed germination

Initial experiments were carried out to determine the effect of various concentrations of $HgCl_2$(0, 100, 300, 500 and 1000 µM) on seed germination. The percentage seed germination decreased gradually up to 1000 µM (Fig. 1). The seeds soaked in 100 µM $HgCl_2$ showed similar trend in germination as the seeds soaked in water and germination percentage were not significantly different from the control. However, 1000 µM $HgCl_2$ caused drastic reduction in germinationpercentage of the seeds.

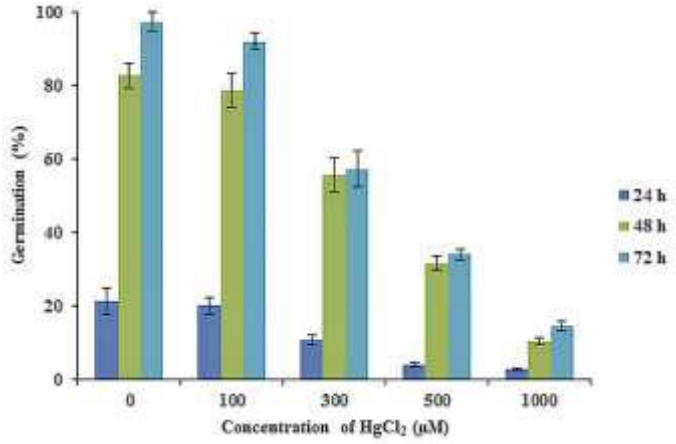

Fig. 1: Effect of different concentrations of $HgCl_2$ upon germination percentage of pea seeds. Values are means ± S.E. based on three independent experimental data.

2. Effect of mercury- scavenging agents on activity of aquaporins

It is well known that the inhibitory effect of $HgCl_2$ on aquaporins can be reversed by mercury-scavenging agents such as DTT and ME. The moisture content of the seedlings wasmeasured after treatment with distilled water, 250 µM ME and 500µM DTT. Their presence had no inhibitory effect on seedling moisture content (Fig. 2). Application of these agents individually with 100, 300 or 500µM of $HgCl_2$ overcame the inhibitory effect of $HgCl_2$in terms of moisture content. The seedling moisture content was significantly lower than the control with 500 µM of$HgCl_2$. This inhibitory effect of $HgCl_2$ was reversed by using DTT or ME (Fig. 2).

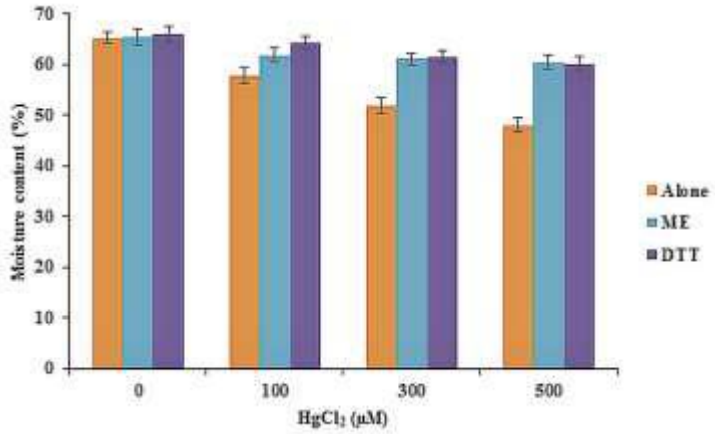

Fig. 2: Moisture content of pea seedlings germinated in the presence of different concentrations of $HgCl_2$ (0, 100, 300, or 500 µM) either alone or in combination with reversal agents ME (250 µM) or DTT (500 µM). Values are means ± S.E. based on three independent experimental data.

3. Effect of $HgCl_2$, $ZnCl_2$ and NaCl on seedling growth

Radicle and plumule length were measured to illustrate the impact of $HgCl_2$, $ZnCl_2$ and NaCl upon the growth of pea seedlings. Different concentrations of $HgCl_2$ (100, 300, 500 and 1000 µM) showed marked impact upon the measured growth parameters in pea seedlings. Both radicle and plumule length significantly inhibited at high concentration (1000 µM) as compared with the control (Fig. 3). Seed germination in $ZnCl_2$ also had reduced radicle and plumule length at a concentration of 100 µM compared to the control (Fig. 4). In the present study, radicle and plumule length decreased progressively with increasing NaCl concentration (Fig. 5).

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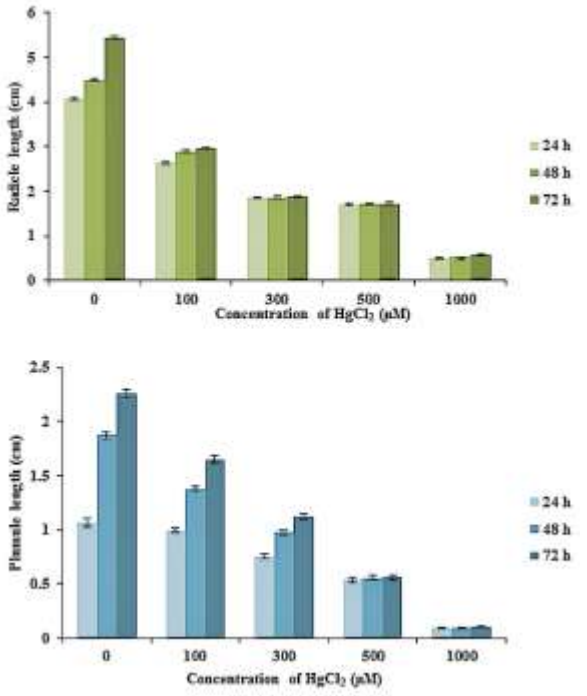

Fig. 3: Effect of different concentrations of $HgCl_2$ upon radicle length, plumule length of pea seedlings. Values are means ± S.E. based on three independent experimental data.

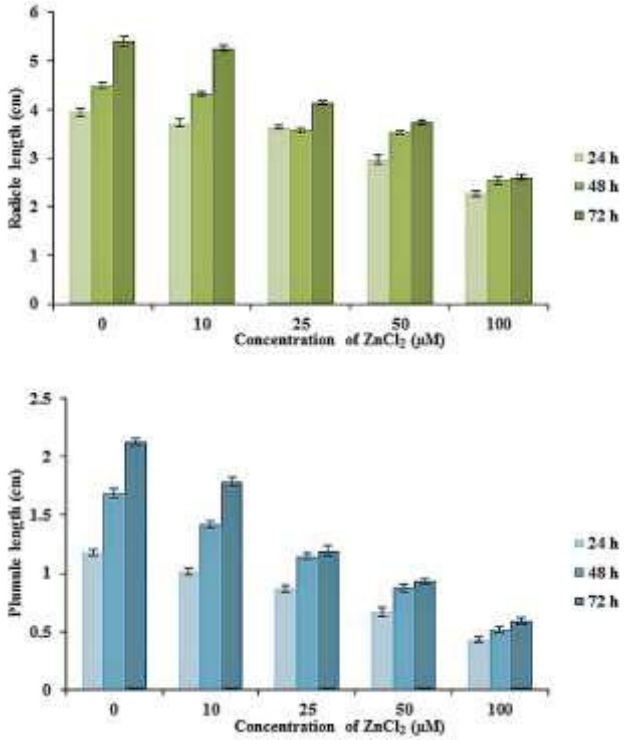

Fig. 4: Effect of different concentrations of $ZnCl_2$ upon radicle length, plumule length of pea seedlings. Values are means ± S.E. based on three independent experimental data.

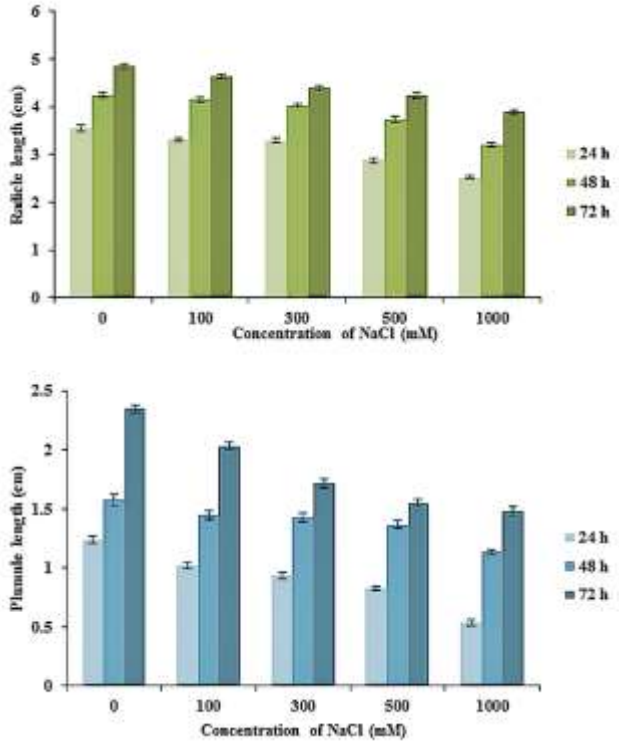

Fig. 5: Effect of different concentrations of NaCl upon radicle length, plumule length of pea seedlings. Values are means ± S.E. based on three independent experimental data.

4. Differential Expression of PsPIP1;2 and PsTIP1;1 during germination under stress conditions

The expression pattern of *PsPIP1;2 and PsTIP1;1* in pea seedling tissues under salt and heavy metal treatment was examined by semi-quantitative RT-PCR. The results showed that the transcripts of *PsPIP1;2* and *PsTIP1;1* were responsive to salt stress in radicle. Transcript level of *PsPIP1;2* accumulated 24 h after treatment of salt, then decreased as the same level as the control 48 h later. Transcript level of *PsTIP1;1* downregulated 24 h after treatment of salt and further decreased as the same level as the control 48 h after treatment (Fig. 6). Since heavy metals are known to block water movement by inhibiting the activity of aquaporins, we studied the effects of Hg and Zn on *PsPIP1;2* and *PsTIP1;1* expression. In radicle, Hg upregulated expression of *PsPIP1;2* and *PsTIP1;1* after 24 h and 48 h of treatment (Fig. 6).

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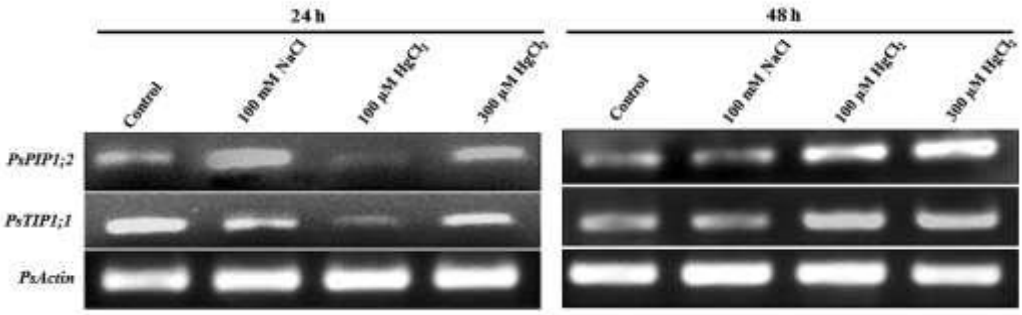

Fig. 6: Tissue Expression pattern of PsPIP1;2 and PsTIP1;1 in radicle of pea seedlings after 24 h and 48 h of treatment with 100 mM NaCl, 100 µM $HgCl_2$and 300 µM $HgCl_2$;PsActin primers were used as internal control.

Expression of *PsTIP1;1* was studied under different concentrations of Zn stress after 24 h in cotyledon, plumule and radicle.Zn downregulated expression of *PsTIP1;1*in cotyledon whereas upregulated in radicle. Expression of *PsTIP1;1* was not affected in plumule (Fig. 7).

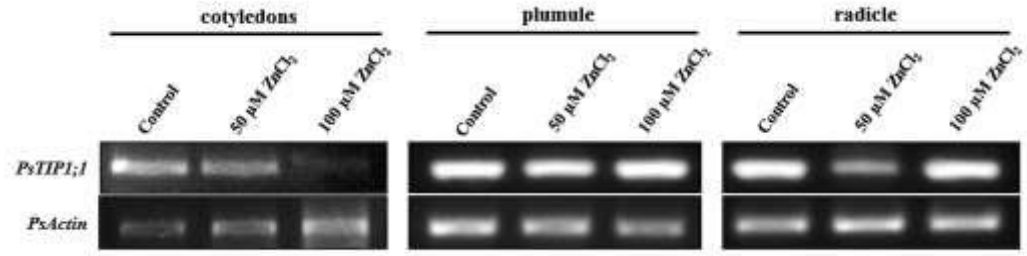

Fig.7: Tissue Expression pattern of PsTIP1;1 under $ZnCl_2$ stress in pea seedlings. PsTIP1;1 expression in cotyledons, plumule and radicle after 24 h treatment with 50 and 100 µM $ZnCl_2$;PsActin primers were used as internal control.

IV. DISCUSSION

Mercury at different concentrations affect seed germination differently.Higher concentrations of mercury cause death of the seed embryo by exerting oxidative stress and damaging cellular components. Mercury at low concentrations affectsseed germination by changing the hydration pattern (Munzuroglu & Geckil 2002; Jain et al. 2008).In previous studies mercurials reduced the speed of seed imbibition and seed germination in pea and Arabidopsis, respectively (Veselova et al. 2003; Vander Willigen et al. 2006). Since mercury blocks aquaporin activity and aquaporins play major role in transmembrane transport of water (Aroca et al. 2012), high concentration of $HgCl_2$ might have suppressed aquaporin activity and subsequent reduction in hydration and germination.

In this study, addition of DTT and ME to $HgCl_2$ treated seeds reduced the inhibition of imbibition. Similar results were obtained by Jain et al. (2008) in which addition of DTT and ME overcame the inhibitory effect of $HgCl_2$on tomato seed germination and moisture content. Veselova and Veselovsky (2006) reported that DTT restored reduced rate of water uptake by mercury-containing compound in pea.

Excessive accumulation of heavy metals in the soil environment due to rapid industrialization, urbanization and intensive agriculture adversely affects the germination of seeds, plant growth, alters the level of biomolecules in the cells and interferes with the activities of many key enzymes related to normal metabolic and developmental processes (Parmar & Chanda 2005; Jayakumaret al. 2008; Ogundiran2007). Diminution in plant growth is one of the clearest symptoms induced by heavy metals.Reduced root and shoot length in response to heavy metal has been reported by a number of investigators (Nag et al. 1989; Zhenguo et al. 1998; Tomulescuet al. 2004; Zhanget al. 2009; John etal. 2009).The visible symptoms of mercuric chloride toxicity were decrease in germination percentage, decrease in radicle length and plumule length. Root growth inhibition and lateral roots development are symptoms of mercury toxicity which can be attributed to the inhibition of mitosis, reduced synthesis of cell wall components and changes in photosynthetic activity. Similar observations with $HgCl_2$ treatment to *Arachis hyposea* seeds were

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noticed by Abraham and Damodaran (2012).To further substantiate the results, $ZnCl_2$, another inhibitor of aquaporins, was used. $ZnCl_2$ inhibits water transport in a permanent manner as it reacts with sulfhydryl groups of a cysteine in the vicinity of the conserved NPA motif, blocking the constriction region of the channel (Niemietz & Tyerman, 2002). According to Flowers et al.(2010) salinity (as NaCl) reduces seed germination and growth of pea seedlings. There is reduction in both seedling growth and root growth. It may be due to the fact that the root cannot balance the nutrient uptake due to osmosis. The osmotic effect takes place during salt stress which affects seed germination (Welbaum et al. 1990) which in turn slows down the water uptake by the plant. Neuman (1995) also observed that salinity inhibits root growth rapidly and hence the capacity of water uptake. Similar results were observed by Demir and Arif (2003) in safflower. This osmotic effect in the plant cell cause negative pressure in the pore of water channel affecting hydraulic conductivity (Ye et al. 2004). The hydraulic conductivity when lower also lowers the aquaporins activity. The reduction in root hydraulic conductivity is also correlated with a dynamic change in the post-translational modifications such as phosphorylation and amidation that affect aquaporin function(Khan et al. 2015). Aquaporin is also altered by ROS which leads to channel closure through a direct oxidative mechanism (Kourie 1998), and induces internalization of PIPs and reduces hydraulic conductivity through cell signaling mechanisms (Boursiac et al. 2008a, Boursiac et al. 2008b).

Since abiotic stresses such as salt and heavy metals induce osmotic stress to plants and disturb plant water balance, we tried to investigate the molecular mechanisms involved in maintaining osmotic homeostasis by studying gene expression and water transport activity of one PIP and one TIP isoforms in pea. Transcripts of *PsPIP1;2 and PsTIP1;1* studied were detected in both radicle and plumule at different levels. Our results showed that transcript levels responded differently to 100 mM NaCl treatment depending on the type of gene. In radicle, salt stress significantly upregulated transcript of *PsPIP1;2*during the initial 24 h of treatment but reached to same level as of unstressed after 48 h of treatment. The transient induction of *PsPIP1;2* by salt stress might confer the membrane permeability to water transport in water-deficient condition (Yamada et al. 1997). Our study is in accordance with a real-time PCR analysis of *PIP* gene expression (Jang et al. 2004), which revealed in salt-treated roots an increase in abundance more pronounced for PIP genes. Salt stress downregulated the expression of*PsTIP1;1*after 24 and 48 h of treatment. At the gene level, expression of TIPs and PIPs was co-ordinately reduced. This could mean that TIPs also contribute to control transcellular water transport in radicle. Mercury, having inhibitory effect on water transport activity (Carvajal et al. 1996; Maggio & Joly 1995) stimulated gene expression as in the current study and as reported earlier in pea(Beaudette et al. 2007). Mercury enhancing transcript accumulation of *PsPIP1;2* and *PsTIP1;1*, suggests that seedlings tend to overcome water shortage by compensating the water channels blocked by Hg.

Being the aquaporins of the tonoplast, TIPs are thought to permit a more rapid transcellular water flow by increasing the effective cross-section of the cytoplasm, and to facilitate osmotic adjustment between cytosol and vacuole (Barrieu et al. 1998). Previously Schuurmans et al. (2003) found *PsTIP1;1*and/or its close homologues and their abundant expression in cotyledons of developing and germinating pea seeds, and in roots and shoots of seedlingsand concluded that TIP1 members play a role in the rehydration of the dry seed during imbibition and subsequent germination. In our case, expression of *PsTIP1;1* was significantly high in plumule and radicle which wasconsistent with the expression pattern of TIP1s in *Arabidopsis* (Alexandersson et al. 2005). The expression of *PsTIP1;1* upregulated by Zn in radicle and suppressed in cotyledon after 24 h of treatment and was not affected in plumule. High expression of *PsTIP1;1* might contribute to transcellular water transport in germinating seeds and facilitate water supply to expanding tissues.

V. CONCLUSION

Based on the results of our study, higher concentration of mercuric chloride suppressed aquaporin activity and subsequent reduction in hydration and germination of pea seeds.Addition of mercury scavenging agents along with $HgCl_2$ overcame the inhibitory effects. It concludes a putative role for aquaporins in controlling pea seed germination, by possibly acting in the initial phases of germination. The presence of aquaporin inhibitors ($HgCl_2$ and $ZnCl_2$) and NaCl reduced seedling growth and differentially regulated expression of plasma membrane intrinsic protein (*PsPIP1;2*) and tonoplast intrinsic protein (*PsTIP1;1*) in different parts of the seedlings, suggesting that the isoforms have a distinct role under these stress conditions.

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Study of Mobile Phone Gratification Sought and Obtained by Aquaculture Farmers as Strategy for Advisory Services in Nigeria

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Abstract— *Mobile phone is strategic in the current effort to improve advisory services delivery and effectiveness of information sharing toenhance aquaculture entrepreneurship for food security, and wealth creation in the country. This prompted the study of mobile phone gratification sought and obtained among table size aquaculture fish food producers through the application of Uses and Gratification Theory. In pursuit of the set objectives, primary data was generated from 100 respondents in Niger State, Nigeria which was analysed with descriptive and inferential statistic tools. Personal profile revealed dominance of aquapreneur by people in middle age categories with mean age of 42 years and 4.5year of experience. Respondents top gratifications sought from mobile phone usage were to be accessible, connected, job accomplishment and socialization whereas obtained gratifications in enterprise were to support adoption of technologies, timely information, linkage to customers, quick response, and access to inputs. It was revealed that respondents had positive antecedent to mobile phone services subscription relating to caller tone, music, news alert, sports, and health. Socio-economic variables that correlate with gratification sought and obtained were marital status, religion, and education at 0.05 level. In view of the finding on responsible usage of mobile phone in aquaculture enterprise, more investment is required develop mobile phone applications and services. To sustain and improve on the benefits derived, respondents need capacity building to acquire more knowledge and skills to effectively participate in advisory services.*

Keywords— Mobile phone, aquaculture, gratification, innovative platform, Nigeria.

I. INTRODUCTION

Mobile phone technology is driving contemporary information society and behaviour which is transforming economies and livelihood activities at global, regional, national and individual levels. Report of GSM Association (2015a) revealed that as at 2014, global mobile industry achieved 3.6 billion subscribers, 50% penetration, 7.3 billion SIM connections, 2.4 billion mobile internet users, 2.6 billion smartphones adopters, 39% of 3/4G connections and contributed $3.0 trillion to gross domestic product (GDP). In sub-Saharan Africa, it is good news with 367 million subscribers representing 41% penetration rate, 680 million SIM connections representing 77% penetration rate, 24% broadband connection, 160million smartphone users and contributed $100 billion to the region's economy in 2015 (GSMA, 2015b). In the case of Nigeria as at June, 2016, there exist 213,113,202million connected GSM lines of which the active lines were 149,179,083 (70%) whereas internet users stood at 86million (http://www.ncc.gov.ng/).

Above evidence points to positive impact of mobile phone technologies in diverse business situations in many countries. Ogbeide and Ele, (2015) posits that it provides different opportunities to transfer information and knowledge among stakeholders in the agribusiness value chain. Therefore, deployment of mobile technologies which exist in infrastructure, services, and applications is capable of accelerating access to quality and effective information to agricultural community in the region. An innovation mix of mobile phone with radio programs (call-in or SMS) facilitates interaction, feedback and most cost-effective solution.

The World Bank (2006) acknowledged that information is a factor of production as enterprise do better with

information and communication. By implication, information is an input required by actors in agricultural value chain to take informed decision. As such, evolution of mobile phone has increased farmers' investment in information as an input through ownership, subscription and access to applications and services. Aker (2010) highlighted mobile phone functions and benefits in agricultural extension services whereas Gakuru *et al.* (2009) shared evidences of mobile phone in advisory services in sixteen African countries. On productivity, Bayes (2001) established higher returns of using mobile services for the poor compared to the non-poor. Mobile phones have been found to help improve the productivity of individuals and organizations within resource-constrained environments due to increased efficiency, effectiveness, and reach (Qiang, *et al.,* 2011; Hudson, 2006). In Nigeria, Ogbeide and Ele (2015) established that crop and livestock farmers that use mobile phone to access market and financial information significantly increased productivity .In capture and aquaculture fishery, Aphunu and Atoma (2011), Ifejika (2013) and Jiriko et. al (2015) established mobile phone ownership and usage to access fisheries information. Also, Ifejika (2015) empirically found that mobile phone ownership facilitates storage and evaluation of aquaculture information before usage.

In aquaculture production, mobile phone applications are needed for fish species identification, fingerlings counting, fish sex detection, water quality parameters measurement in pond, fish disease and adulterated fish feed. Likewise, mobile phone advisory services are needed to overcome extension deficiencies in manpower, knowledge, cover distance and effectiveness. Hecht (2006) hinted that aquaculture in sub-saharan Africa is contributing less than 1% to world aquaculture production. In 2015, fish production in Africa amounted to 11 million tons with 85% coming from catch fishing and 15% from fish farming which accounted for 7% of the world production (Ntagungira, 2016). However, outrageous fish importation bill of US$2.006billion spent by west African countries is a big threat to aquaculture development in the sub region.

In Nigeria, aquaculture performance is encouraging and exemplary in the sub region. It is interesting to note that Nigeria's aquaculture growth which picked up in 2000 coincided with the introduction of mobile phone in the same year. According to Federal Ministry of Agriculture and Rural Development (FMARD) (2012),aquaculture has attained double digit growth of 20% in domestic fish production from 5.50% to 24.75% between 2003 and 2011. Other positive attributes of aquaculture are; highly profitable venture of which the cost of production was ₦571, 231.79, the total revenue of ₦5, 853, 625.64 and the net income was ₦5, 282, 393.85 as found in Kaduna State (Kudi etal 2008); generates monthly income of ₦26, 553.40 which is higher than national minimum wage of ₦19, 000= US$126.67 (Oluwemimo and Damilola, 2013); fish farming activity reduces poverty by 34.2% as found in Adamawa State (Ndamu 2016); catfish fingerling producers attest that patronage was encouraging and demand for fingerlings is more than supply in Borno State (Olanrewaju et al, 2010); attractive to economic active age group including women and youths (Ifejika et al, 2015; Ndamu, 2016; Kudi et al, 2008). Others are transformation of a rural community is the case of "MonaiFish Farming Village" in Borgu council, Niger State, wealth creation in the value chain for hatchery operators, table size growers, fish feed millers and producers, input dealers, pond construction, fish smoking, security guards and attendants, hiring of ponds, transporters, fish marketing, and increase value of land.

Muir (2005) predicted that aquaculture should develop rapidly to increase by over 260% which translates to an annual average of more than 8.3% by 2020. Aquaculture in the region with less than 5% of the suitable land area being used, deserve attention and investment in mobile phone technologies services and applications for information delivery. Meanwhile, huge gap exists in literature on studies of mobile phone dedicated to aquaculture as well as on the application of Uses and Gratification Theory (UGT). Communication experts provided a new dimension in the study of mobile phone through UGT which provides insight on "why and how". Ruggiero (2000) wrote that as new mobile technologies present people with more and more media choices, motivation and satisfaction become even more crucial components of audience analysis. UGT belongs to social functionalism and psychological communication perspective school of thought (Luo, 2002). UGT is useful in understanding of gratifications sought and obtained as well as helpful in clarifying activity and activeness of media audiences. "Activity" refers to what the media consumer does whereas "activeness" refers to the audience's freedom and autonomy. In this direction, Quan-Haase and Young (2010) and Ruggiero (2000) posits that new media like mobile phone possess at least three attributes not commonly associated with traditional media: interactivity, demassification, and asynchroneity. Above attributes are critical in emerging mobile phone platforms in extension advisory services. Present study seeks to break the jinx and unfold why fish farmers acquire mobile phone and benefits derived from its usage in fish farming activity. Above arguments informed the decision to carry out investigation on aquaculture farmers' gratification sought and obtained from mobile

phone and its consequences on advisory services as strategy for aqua-business in the region.

Therefore, the study seeks to provide answers to the following research questions:

1. What are the gratifications fish farmers' sought and obtained from mobile phone?
2. What is their mobile phone communication behaviour?
3. What are their antecedents' to mobile phone services subscription?
4. What is their personal profile?

II. HYPOTHESES TESTING FOR THE STUDY

H_0. Null hypothesis: There is no significant relationship between gratifications sought and obtained from mobile with selected personal profiles of subjects.

H_1. Alternative hypothesis: Significant relationship exists between gratifications sought and obtained from mobile with selected personal profiles in the States.

III. AREA OF STUDY

In Nigeria, Niger State is located in the north central geo-political zone and in guinea savannah belt which lies on latitude 80º to 11º:30' North and Longitude 03º 30' to 07º 40' East (Niger State Planning Commission, 2011). It has twenty-five administrative councils and population figure of 3,950,249 million in 2006 census. About 85% of the land mass is arable whereas three hydroelectric power dams boostcapture fishing livelihoods. Based on survey of fish farms in the country, Niger State was categorized as low aquaculture zone due to low number of operators (AIFP Project, 2004). Between then and now, evidences of aquaculture intensification and scaling up are visible in Minna, Bida and New-Bussa environs. In the State, aquaculture is attracting investment, growth in number and practices which is quite significant and contributing to job creation, income and food security in the value chain.

IV. METHODOLOGY

Secondary information collected from Fisheries Subject Matter Specialist in the State Agricultural Development Programme (ADP), Research Institution, Fish Farmers groups in Minna, New Bussa and Bida as well as physical observations put the number of fish farmers in the State at close to 1080 which is the population for the study. While the study sample sizewas contact fish farmers with ADP and NIFFR found to be 250. Respondents' were randomly selected 110 active contact fish farmers' in the last six months representing 44% of the sample size. After data collection, ten respondents were discarded due to error from enumerators hence the valid respondents were 100. Primary data was generated from respondents in the three ADP zones namely Minna, Bida and Kontagora in the month of April to July, 2013. Instrument for data collection was semi-structured questionnaire which was face validated by experts in agricultural extension and fisheries subject matter specialist. Reliability was measured with Cronbach Alpha values and alpha value of 0.70 coefficients was obtained which confirmed instrument as reliable. Primary data was collected through face to face interview by trained enumerators fluent in local and English languages. Variables were measured at nominal, ordinal and interval levels and scored accordingly. Dasgupta (1989) procedure was adopted to categorise respondents into high (50% <) and low (50% >) from the index score. Generated data was analysed with descriptive and inferential tools of frequency, mean, percentage, standard deviation and Pearson Product Moment of Correlation (PPMC) which is presented in tables, figures and charts.

V. RESULT AND DISCUSSIONS

Figure 1 show respondents' antecedent behaviour to adoption of subscribed mobile phone services which discloses familiarity with mobile phone services. Mean adoption score revealed low antecedent behaviour by majority (57%) to mobile phone services compared to highantecedent behaviour (43%). Respondents' antecedents suggest high awareness but low adoption of services. Subscribed mobile phone services among respondents with high adoption above the mean score (> 27) were on caller tone (32%), news alert (20%), music (19%) while low adoption behaviour below the mean score (< 27) was observed in joke, health, love, and sport services. Probably, low adoption of subscribed services among fish farmers can be traced to high charges ranging from ₦50 to ₦100, reckless deduction of credit by service providers, unappealing andirrelevant messages. These factors might be responsible for the negative reaction of respondents to stop usage by 0.71% and trial only without adoption by 12.28%. Respondents antecedents predict possibility of participation in mediated aquaculture mobile phone advisory services. However, discontinuance behaviour is a caution that respondents will pull out if subscribed services don't meet expectations to satisfy information need in terms of quality, efficiency and effectiveness.

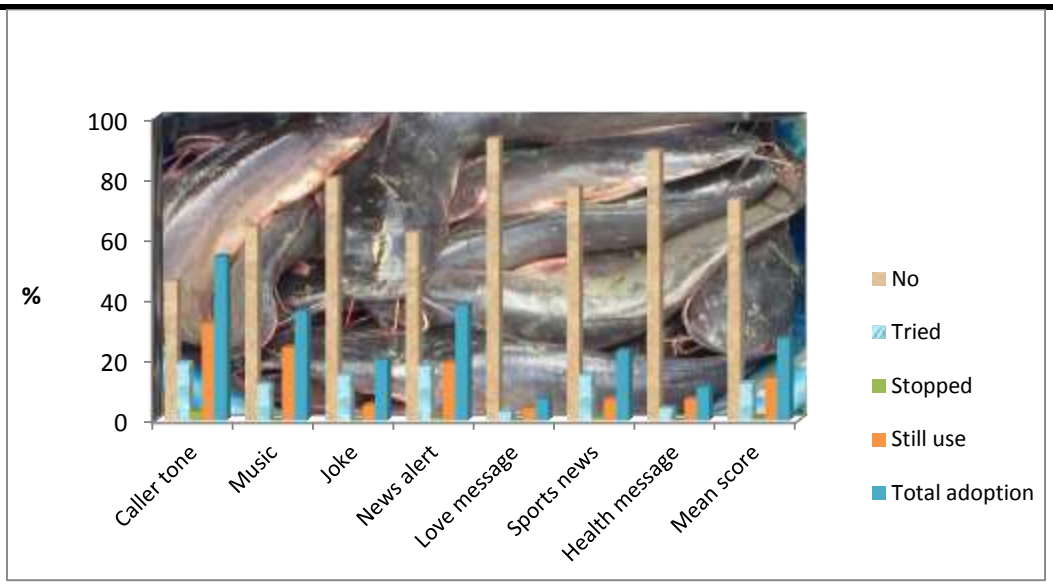

Fig.1: Antecent behaviour to paid mobile phone services adoption

Source: Field data (2013)

Displayed in Table 1 is result of gratifications sought by aquaculture farmers to acquire mobile phone. Total mean score confirmed that higher proportion of subjects (57%) sought high gratification against smaller proportion (43%) to acquire mobile phones. Sought gratifications with high expectations (> = 39.9) among subjects was found to be mobile and accessible (96%), connect to people (81%), job accomplishment (68%) and socialization (45%) whereas low motive was observed on time management (32%), security and boost image (30%) respectively. The result confirmed that aquaculture farmers were highly motivated to acquire mobile phone to satisfy social and job related task among others. Wei (2001), Wei and Lo (2006) studies corroborated the finding on high gratification sought for usage of mobile phone on mobility, intimacy, job and sociability. Response on job gratification by 68% justify investment in mobile phone acquisition and subscription to services as an input with return on investment.

Table.1: Respondents gratification sought to acquire mobile phone (%)

Sought variables	No	Low	High
Socialization	12	43	45
Job Accomplishment	6	26	68
Connected to people	4	15	81
Time management	46	22	32
Mobile & Accessible	2	2	96
Security	40	30	30
Boost image	31	39	30
Categorization			
Mean		39.9 (> = high), 0.365 (< = low)	
Standard deviation		4.2	
High		57	
Low		43	

Source: Field data (2013)

Table 2 provides information on job gratifications obtained from using mobile phone in aquaculture practice. Mean score (78.2%) attests that majority (71%) obtained high benefits on aquaculture work compared few (29%) that derived low benefit. As seen, mobile phone facilitated the following benefits: adoption of

technologies (99%), timely information delivery & link to customers (94%) respectively, market information (92%), quick response/intervention (90%) among others satisfactions in the table below. The findings on high job gratificationsobtained in the usage mobile phone in economic activity agreed with Puro (2002) and Hooper and Zhou(2007). Positive contribution of mobile phone to accomplishment of work task justify investment in subscription to servicesand applications. It further attests to mobile phone delivery of quality information in terms of relevance, credibility and effectiveness to support decision making process among aquaculture farmers.

Table.2: Job related gratifications obtained in aquaculture practice

Benefits obtained	**No**	**Yes**
Timely information	6	94
Take informed decision	21	79
Adopt new technologies	1	99
Increase fish yield	16	84
Link customers	16	94
Market information	8	92
Better sells & profit	16	84
Improve access to inputs	19	81
Quick response & intervention	10	90
Reduce risk	17	83
Categorization		
Mean	78.2	
Low	29	
High	71	

Source: Field data (2013)

As shown in Table 4, respondents mean age (42yeras) was slightly above the young group category indicating dominance of people in the early and middle adulthood (52%) than the youth and young categories (44%) in fish farming. Ifejika *et al* (2015) and Karem *et al* (2009) agreed on the activeness of the two age categories as contending force in aquaculture. On gender, men (82%) dominated aquaculture practice over the women (18%) in the study area. Previous findings consistent with the result on men dominating aquaculture enterprise were Ifejika *et al* (2015) and Karem *et al* (2009). However, women involvement in fish farming enterprise is gradually rising unlike ten years ago in the country. Probably, investment in mobile phone services and applications in aquaculture might provide the needed leverage to attract more women. Result on religion indicates that it is not a barrier to the practice of catfish fish farming in the States by either Muslim (59%) or Christians (41%). Rather religious should be an asset for platform formation as network to promote knowledge sharing and solve challenges in aquaculture. On marital status, majority were married (75%) and few singles (21%) among the young people. Corroborating the fact on high involvement of family members over singles in aquaculture enterprises in the country were Ifejika *et al* (2007), Nwosu and Onyeneke, (2013). Fish farming is supporting family farming enterprises which provides income, food and job security as well as labour, information and entry point. Majority (50%) had between 4-6years, 34% had between 1-3years which signify that 95% entered the business in less than ten years.Experience is a critical factor to success of aquaculture practice as most operators depend on past knowledge to evaluate information before usage. In view of this result, fish farmers' opinions should be respected and consulted in aquaculture mobile phone advisory services. Response on education strongly confirmed that fish farming is an elite agricultural enterprise compared to crop and livestock. Large proportions of respondents (81%) hold higher degrees of National Diploma (33%), BSc (35%) and MSc (16%). In agreement with the result on engagement of graduates into fish family were Karem *et al* (2009) who found 82% as graduates in Ogun State and Okunola *et al* (2011) found 63% in Ondo State. This development is a good omen for aquaculture as they will be flexible in thinking and innovative towards mobile phone driven advisory services. Response on English language communication skill, over 90% affirmed to have competency to read, write and speak English language very well. This is not surprising as over 80% of them were graduates, hence, dissemination of aquaculture information in English language will not be a challenge to subscribers. As such respondents, can make use of verbal and nonverbal communication tools to send and receive information with mobile phone.

Table.4: Personal profile of respondents

Age	**%**	**Years of experience**	**%**
20-30 Youth	23	1 to 3	34
31-40 (Young)	21	4 to 6	50
41-50 (Early adulthood)	28	7 to 9	11
51-60 (Middle adulthood)	24	10 to 12	3
< 61 (Late adulthood0	4	13 to 15	2
		Mean 4.52	
Mean	42	**Education**	
Religion		No school	2
Christianity	41	Primary	0
Muslim	59	JSS	0
Traditional	0	SSS	14
Marital status		NCE/ND	33
Single	21	BSc	35
Married	75	MSc	16
Widow	4	**Gender**	
Divorced	0	Men	82
		Women	18

Language skill	***No***	***Partial***	***Very well***
Speak Good English	2	7	91
Read Good English	2	7	91
Write Good English	2	6	92

Source: Field data (2013)

Table 5 shows result of personal variables that correlates with gratification sought and obtained**.** Personal characteristicsthat positively correlated with mobile phone gratification sought were religion (p=.029) and marital status (p=.000) whereas gratification obtained were positively correlated with education (p=.017).Collaborating the finding was Ofuoku *et al.*, (2007) on the positive contribution of education to usage of mobile phone among poultry farmers in Delta State. By implication, education influences thinking and reasoning of farmers towards adoption of innovation. This is an indication that respondents need capacity building to build knowledge and skills to derive more benefits from mobile phone usage. Also, training will stimulate positive attitude and behaviour necessary for participation and investment in mobile phone services initiative in aquaculture value chain.

Table.5: Correlation analysis between gratification sought and obtained with selected personal characteristics

Independent Variables	**Correlation coefficient (r)**	**p-value (r^2)**	**Remarks**
Age	-.151^{sought}	.133	Negative &weak NS
	.083^{obtain}	.412	Positive &weak NS
Gender	-.152^{sought}	.132	Negative &weak NS
	-.059^{obtain}	.562	Negative &weak NS
Marital status	-. 218 ^{Sought}	.029	Negative & positive s*
	-.015^{obtain}	.88	Negative & weak NS
Religion	-. 417 ^{sought}	.000	Negative & stable*
	-.023^{obtain}	.821	Negative & weak NS
Education	.239 ^{obtained}	.017	Positive & strong S*

Significant; *= 0.05 levels; **=0.001 levels

VI. CONCLUSION AND RECOMMENDATIONS

Outcome of the study was useful in understanding the gratifications fish farmers sought and obtained byacquiring mobile phone and their antecedent behaviour to mobile services.Respondents are deriving high gratification from mobile phone to support their aquaculture enterprise. Education and accomplishment of family responsibilities are influencing the responsible usage of mobile phone in fish farming activities. As such, aquaculture operators the study area are ready to invest and subscribe to mobile phone services platform that will deliver useful information. Government and private services providers are encouraged to consider setting up mobile phone platform for aquaculture farmers. Also, investment is highly needed in mobile phone applications that will help reduce drudgery, support adoption and market information access.

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Bush meat sold on the markets in Kisangani: analysis addressed to the right on species conservation in the Democratic Republic of the Congo

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Abstract— *In order to identify the game species sold on the central market of Kisangani and to check up the respectability of the regulation of hunting by the Congolese population, we collected data from January to August 2009 and from December 2014 to May 2015. The results indicate that 29,525 game carcasses marketed, belong to 8 orders, 13 families and at least 27 species. On the central market of Kisangani, Artiodactyla (40.06%) are the most sold followed by Primates (37.79%). The family Bovidae (37.98%) is the most represented followed by Cercopithecidae (37.61%). Based on counting carcasses, Cercopithecus sp (35.35%), followed by Cephalophus monticola (22.96%) are the most sold. The Low n° 82-002 which regulates hunting is not observed in Kisangani, as well as the ministerial decree n° 14/003 of 11 February 2014 relating to the conservation of nature. In fact, the regular hunting period is not observed. In addition, Loxodonta africana, Manis gigantea, Okapia johnstoni, and Pan troglodytes which are totally protected, Cephalophus sylvicultor, Potamochoerus porcus, and Syncerus caffer nanus which are partially protected are exploited. Therefore, it is essential to implement mechanisms for integrated management of wild fauna which respect the Congolese legislation and international conventions.*

Keywords— bush meat, law, conservation, Kisangani.

I. INTRODUCTION

In DRC, hunting is a main activity after agriculture for the majority of the Congolese population living in rural areas. Bush meat provides them with animal protein. In Kisangani, breeding is less practiced and occasionally it concerns poultry farm and small livestock. Kisangani is one of the Congolese towns with higher demographic pressure on forest resources, with approximately 1,186,479 inhabitants in 2009 and 1,602,144 inhabitants in 2015 [1]. Urbanization has increased a strong demand for bush meat because breeding of small livestock, poultry and fishing alone cannot cover the needs in of animal protein. Indeed, hunting for subsistence or for commercial purposes, constitutes a threat to the whole ecosystem biodiversity of the Congo Basin forests [2].

Hunting has always provided rural population with bush meat protein. The low density of the population ensured the sustainability of hunting, a better protection and traditional management of natural resources. Today, this balance is disturbed as the population from the forest areas is growing as well as the development of the bush meat markets in DRC. In addition, because the legislation is not respected and/or unsuitable, the chain of bush meat is dominated by the informal sector. However, it participates as other activities to create a great number of jobs and the circulation of wealth throughout the extent of the country, from urban centers to the rural areas [3]. Bush meat is not a resource in free access. Each village controls a part of the forests intended for hunting, limits are determined on the basis of the density of the population [3].

Due to the crisis of governance in DRC as in Kisangani, the chain of bush meat is facing the management crisis. This bush meat management crisis is contributing to the extinction of some species with consequences for a large number of rural populations to loss their main source of animal protein and of their means of survival.

Therefore, the main goal of this study consists to identify animal species sold as game on the central market of Kisangani, to address the Congolese law 82-002 of 28

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May 1982 which regulates hunting in relation with sustainable management of bush meat in the region of Kisangani.

II. MATERIAL AND METHODS

The study was carried out on the central market of Kisangani, the capital city of the province of Tshopo. Kisangani has six communes which are Kabondo, Kisangani, Lubunga, Makiso, Mangobo, and Tshopo. Kisangani totalizes 1,602,144 inhabitants consisting of riparian, farmers and hunters [1]. The central market is located in the commune Makiso. Kisangani is crossed by the Tshopo River and the Congo River which facilitate the transport of goods, whaleboat, paddle-canoe or motorized-canoe [4].

The study is supported by data collected from January to August 2009 [5], from December 2014 to May 2015 [4], from December 2014 to May 2015 [6]. The data collected on bush meat in this period were analyzed regarding the legal tools.

III. RESULTS

Organization of the sector

From the hunters and/or the trappers in villages to the consumers in the city, the marketing chain seems to present very complex way as Babuchet and Ioveva [7] have recognized in the south of Cameroon.

Bush meat trade is part of the informal economy which escapes all control systems in the region of Kisangani. The official services concerned by the conservation of nature in their responsibility do not have reliable data. The owner of bush meat fixes the prices according to his immediate needs and financial means of the purchasers. The sector faces higher fiscal pressure (taxes, fees).

The institutional reform to enshrine biodiversity by the Constitution of 18 February 2006 remains unfinished. In the practice, sharing benefits among the central administration, the provinces and the territorial entities is unclearly defined. Conflicts of competence are observed throughout different administration levels which do not foster local development. The confusion of competences among the services of the National Economy to check the price and the services of the environment with a normative mission in the application of the legislation on the environment and the protection of wildlife.

Concerning the organization of the sector, it should be noted that in DRC, just as in other African countries of the Congo River basin, bush meat trade is organized around a long series of intermediaries and it is very rare that hunters themselves sell their catches on markets in the city [8].This complex chain indicates how the suppliers, the hunters, the sellers of the game interact in the organization of the hunting [9].

Species collected as bush meat

Proportion of animals sold as game on markets in Kisangani (data compared with the tables I, II and III of the Law nº 82-002 of 28 May 1982 on the regulation of hunting)

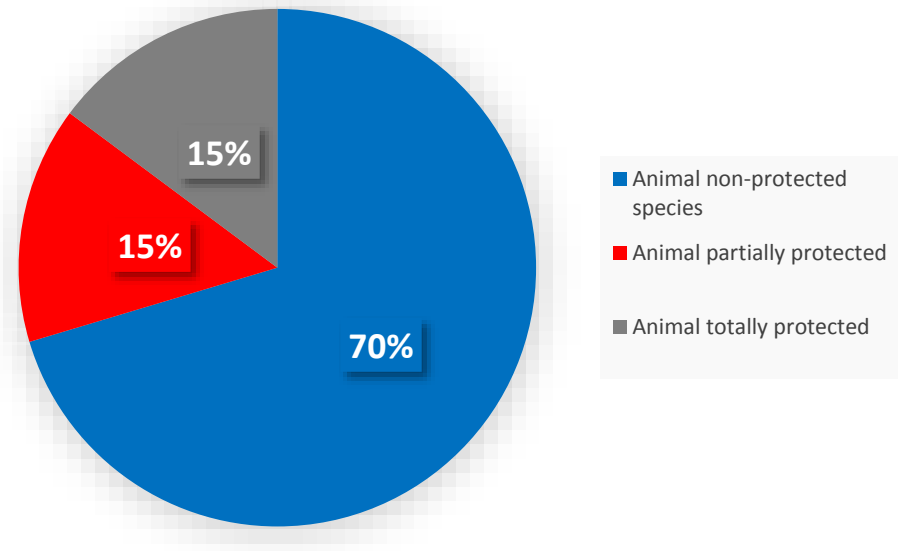

Fig. 1: proportion of species by animal partially protected, totally or not protected sold to markets of Kisangani

In DRC, the Law nº 82-002 of 28 May 1982 which regulates hunting distinguishes 3 categories of animals: the animals that are fully protected, animals partially protected and non-protected animals. The fig. (1) informs that on markets in Kisangani, all 3 categories of animals are sold: non-protected animal species come in head (70%) followed animals fully protected (15%) and animals partially protected (15%).

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Techniques used by hunters and/or trappers in villages

Table.1: The capture techniques used.

Hunting techniques	Part of the sale (%)	Part of the consumation (%)
Firearm : artisanal caliber 12 & caliber 12 Baikal	58.4	19.9
War weapon :SMG, Mi-Mag & AK 47	1.4	0
Traps built with creeper, nylon, metal cables and net	40.2	80.1
Total	100	100

Source: [9]

The artisanal caliber 12 and the industrial caliber 12 Baikal are actively used for commercial hunting (58.4%) than for consumption (19.9%). Traps built with nylon and metal cables enhance the effectiveness of trapping because of their resistance than the traditional traps made with creepers found in forests. Such traps contribute with 40.2% of game for commercial hunting against 80.1% for household consumption.

Marketing of the bush meat

In DRC, the Law nº 82-002 of 28 May 1982 which regulates hunting recognizes hunting for home consumption (including animal species partially protected, and non-protected animal species), and animal species fully protected [10]. In DRC, totally protected species are those which are also listed by the International Convention on the Species of Flora and Fauna threatened of extinction (CITES) and the ministerial decree nº 056/CAB/MIN/AFF-ECNPF/01/2000 of 28 March 2000 on the regulation of the international trade of endangered species of flora and fauna threatened of extinction. The trade of living wild animals protected cannot be done without the approval of the Central Organ of CITES Management which delivers on payment of an special license. Partially protected species and non-protected species are those officially authorized for local consumption. The ministerial decree n° 056 has established the rules, and conditions of hunting, detention, trading and transporting such species (article 1). In fact, in DRC, all wild animals qualified as "the non-protected species" and their by-products for a commercial purpose might be killed as game only after obtaining an approval license issued by the Secretary General having hunting under his responsibility, by payment of a license of export or import. Article 81 of the Law nº 82-002 of 28 May 1982 stipulates that the import or export of any protected species or any part non-perishable of one of these species is legally feasible only if the activity is covered by a legitimate certificate issued by the Department with the Hunting under his responsibility. Similarly CITES determines the import and export of animals as listed in third annex, to the prior grant and presentation respectively of a permit to import and export [10].

Table.2: Estimated number of carcasses of animals found on the markets.

Order	**Family**	**Genus / Species**	**Protection status**	**Total of carcasses**	**%**
Artiodactyla (40.06%)	Bovidae (37.98%)	Cephalophus monticola (Thumberg, 1789)	ANP	6778	22.96
		Cephalophus sp (C. H. Smith, 1827)	ANP	1840	6.23
		Cephalophus sylvicultor (Afzelius, 1815)	APP	33	0.11
		Cephalophus dorsalis (Gray, 1846)	ANP	2526	8.56
		Tragelaphus spekei	ANP	83	0.28
		Syncerus caffer nanus (Sparman, 1779)	APP	36	0.12
	Suidae (1.23%)	Patamochoerus porcus (Linnaeus, 1758)	APP	362	1.23
	Tragulidae (0.85%)	Hyemoschus aquaticus (Ogilby, 1841)	ANP	250	0.85

	Giraffidae (0.00%)	Okapia johnstoni (P. L. Sclater, 1901)	ATP	1	0.00
Primates (37.79%)	Cercopithecidae (37,61%)	Cercopithecus ascanius (Audebert, 1799)	ANP	506	1.71
		Cercopithecus sp (Linneaus, 1758)	ANP	10438	35.35
		Papio anubis (Lesson, 1827)	ANP	63	0.21
		Cercopithecus hamlyni (Pocock, 1907)	ANP	1	0.00
		Cercopithecus mitis (Wolf, 1822)	ANP	27	0.09
		Cercopithecus lhoesti (p. Sclater, 1899)	ANP	68	0.23
	Hominidae (0.18%)	Pan troglodytes (Blumenbach, 1775)	ATP	53	0.18
Rodentia (10.77%)	Cricetidae (8.95%)	Cricetomys emini (Wroughton, 1910)	ANP	2643	8.95
	Hystricidae (1.82%)	Atherurus africanus (Gray, 1842)	ANP	537	1.82
Chiroptera (8.99%)		Eidolon helvum (Kerr, 1792)	ANP	2655	8.99
Pholidota (0.15%)	Manidae (0.15%)	Manis gigantea (Illiger, 1815)	ATP	20	0.07
		Manis tetradactyla (Linneaus, 1766)	ANP	6	0.02
		Manis sp	ANP	18	0.06
Carnivora (0.05%)	Viverridae (0.05%)	Bdeogale nigripes (Pucheran, 1855)	ATP	14	0.05
Proboscidea (0.01%)	Elephantidae (0.01%)	Loxodonta africana (Blumenbach, 1797)	ANP	2	0.01
Crocodilian (0.00%)	Crocodilidae (0.00%)	Crocodylus sp	ANP	1	0.00
		Epomops franqueti (Tomes, 1836)	ANP	150	0.51
		Hypsignathus monstruosus H. (Allen, 1861)	APP	414	1.40
		Total	///////	29525	100

Source: [4], [5] and [6]

Key: ATP: Animal totally protected; APP: Animal partially protected; ANP: Animal non-protected species.

The table 2 shows that 29,525 carcasses of game have been inventoried on markets in Kisangani. They represent 8 orders, 13 families and at least 27 species. Artiodactyla are the most sold (40.06%), followed successively by Primates (37.79%), Rodentia (10.77%), Chiroptera (8.99%), Pholidota (0.15%), Carnivora (0.05%), Proboscidea (0.01%) and of Crocodilian (0.00%).

The family *Bovidae* (37.98%) which occupies the first place, is respectively followed by the families *Cercopithecidae* (37.61%), *Cricetidae* (8.95%), *Hystricidae* (1.82%), *Manidae* (0.15%), *Viverridae* (0.05 %), *Elephantidae* (0.01%) and *Crocodilidae* (0.00%). The genus *Cercopithecus* (35.35%; 10,438 carcasses) occupies the head of the ranking followed by *Cephalophus monticola* (22.96%: 6,778 carcasses) and *Crocodylus* (1 carcass).

The table indicates also all the three categories of animal are hunted as games: *Pan troglodytes*, *Okapia johnstoni*, *Loxodonta africana*, *Cephalophus sylvicultor*, *Manis gigantea*, *M. tetradactyla*, *Crocodylus* sp are totally protected species; *Tragelaphus spekei*, *Cercopithecus hamlyni* are partially protected, and all the others are the non-protected species authorized for local consumption.

IV. DISCUSSION AND CONCLUSION

Three categories of animals (totally protected species, partially protected species, and non-protected species) are hunted as games and sold on markets in Kisangani based

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on legal instruments, both national and international which regulate the chain of bush meat. Clearly, these legal instruments are not observed in Kisangani. Fargeot [11] revealed that the Artiodactyla, and in particular the duikers suffer from the strongest levy of trade. Observation from FAO [12] revealed also that today more sophisticated techniques and non-selective are used by professional hunters and amplify game destruction.

In DRC, hunting regulation is not respected either by official authorities and local hunters in villages. The techniques and procedures prohibited by the Law n° 82 are always used to hunt. In effect, the Law n° 82 in reference with article 21 prohibits the use of the following devices for hunting:

1. the automatic weapon firing in bursts, the projectiles containing explosives, the guns TUE-Fauves and rifles fixed;
2. the luminous device or equipped with dazzling lights or any device illuminating;
3. the collars and the laces and metal threads of netting;
4. the poisons and other toxic products;
5. the circular light wrap;
6. the weapon manufactured illegally;
7. the weapon and ammunitions of war component or having dialed the arming regulatory framework of FARDC, gendarmerie, military or police affairs;
8. the striped weapon with a caliber of less than 6.5 mm if hunting concerns animals other than birds, rodents, small monkeys and small carnivores that are not protected;
9. the smooth weapon of some caliber as it is or the striped weapon with a caliber of less than 9 mm for hunting large game.

On the other side, the Law n° 82, in reference with article 23, except by derogation of the hunting department authority, it is prohibited to import, to hold, to expose for sale, to transfer or to receive in any title and to transport or to peddle traps or device prohibited. The ministerial decree nº 014/CAB/MIN/ENV/2004 of 29 April 2004 concerning measures of execution of the Law n°82 also prohibits the use of hunting-spears as well as pits.

Indeed, in Kisangani, the exploitation of the game is not respected in regard to the relevant species concerned. The first decree of 21 April 1937 during colonial era on the preservation of wildlife, the regime of hunting and fishing constitutes the basic of all the texts elaborated later for the management of forests and the hunting process [10]. This decree was revised on 15 January 1957, and later amended by the Legislative decree nº 52-273 of 24 June 1958, the Decree of 27 June 1960, and the Law n° 82. Today, this Law is facing a growing crisis due to the failure of good policy coordination of the laws, production and the implementation measures, of suitable governance based on convergence between the different complementary sectors involved in the promotion of the right of hunting in DRC.

The promulgation of the Congolese Forest Code n° 011/2002 on 29 August 2002, the ministerial decree n° 056 of 28 March 2000 on the regulation of the International Trade of species of Fauna and Flora in danger of extinction (CITES) of 6 March 2001 laying down the sampling periods of the parrot-gray in DRC, the adoption of the ministerial decree n°014/CAB/MIN/ENV/2004 of 29 April 2004 related to the application measures of the Law n°82-002 of May 28 1982, all reinforce the new framework of the Congolese legislation in the field of preservation of the wildlife, and all have played an important role that led to the adoption of the decree n°14/003 relating to the conservation of the nature of 11 February 2014. However, there is not an approach effective jurisprudence on the part of the Congolese legislator.

The Law n° 82 is based on the principle of exploitation of wildlife rather than on its protection. Better to say, it is subject to an ambiguous interpretation concerning the local community authority and the parties in the presence. The consequence is a bad exploitation the fauna resources which is leading to the loss of the flagship species in DRC.

Hunting is positioned on the list of informal activities and in fact, it is beyond doubt out of the control of the official authorities as on markets in Kisangani all the three categories of animals are hunt for meat, e.g. the totally protected species (*Okapia johnstoni*, *Pan troglodytes*, *Loxodonta africana*, *Manis gigantea*, etc.), the partially protected species (*Syncerus caffer nanus*, *Potamochoerus porcus*, etc.), and the non-protected species. Probably, this list is not exhaustive as usually the smoked-carcasses represent at least 95% of the stock sold [4, 5, 6]. All of them have not been identified to the rank of species to be compared with the list in the three annexes of the Law nº 82. The Congolese Law n° 82-002 to regulate hunting is not respected regarding the games listed as "non-protected species" in annex III. In reality, these species are placed under the regime which regulates the opening period and closing period for hunting.

Fargeot [2] reported that the legislation to be applied and the attitudes of hunters are generally confused, and the entire chain of bush meat is completely managed as informal activity. These attitudes indicate that the hunting pressure on games is uncontrolled.

To conclude, due to the higher exploitation of bush meat based on the whole different laws on nature conservation in DRC, the sustainability of the species is threatened. Our study recommends quickly to respect the Congolese legislation and international conventions governing the conservation of nature and to implement mechanisms for

integrated management of wildlife, in order to better durable conservation of biodiversity for present and future generations.

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Microwave-Assisted Alkali Delignification Coupled with Non-Ionic Surfactant Effect on the Fermentable Sugar Yield from Agricultural Residues of Cassava

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***Abstract*—** *Cassava stem, leaves and peel are agricultural residues generated as waste biomass during the cultivation and processing of cassava. The potential of these biomasses as feedstock for ethanol production depends on the effective deconstruction via pretreatment and saccharification. The effect of alkaline hydrogen peroxide (AHP) treatment on microwave (MW)-irradiated or steam-exposed aqueous slurry was compared with MW-irradiation (300 W) of alkali slurry in delignifying the biomass and degrading the polysaccharides. Cellulose was degraded to a higher extent than hemicellulose in the AHP treatments. The steam-exposed and AHP pretreated residues on saccharification with Cellic (Cellulase complex) alone or Cellic along with Tween 20 resulted in high conversion of carbohydrate to reducing sugars (RS) in leaves (64-70%) and peel (74-78%), with slightly lower conversion in stem. MW-irradiation of alkali slurry (5 min.) followed by Tween 20 supplemented saccharification was a better strategy degrading cellulose and hemicellulose to very high extent. Tween 20 supplementation was beneficial in enhancing the RS release from the biomasses even when Cellic dosage was halved. Ultrastructural studies indicated the disappearance of starch granules from stem and peel samples after MW-irradiation and saccharification, while fragmented cellulose fibers were visible in leaf samples. The study showed that MW-assisted alkali pretreatment followed by saccharification with Cellic in presence of Tween 20 was very effective in releasing maximum sugars from these biomasses.*

***Keywords*—Cassava processing residues, Composition, Microwave-alkali pretreatment, Saccharification, Tween 20, Ultrasructure.**

I. INTRODUCTION

Lignocellulosic biomass (LCB) comprising wood, agricultural residues and dedicated grasses such as switchgrass, Bermuda grass or *Miscanthus* sp. is considered as the most advantageous feedstock for biofuel production, owing to the cheap and abundant availability [1]. Nevertheless, the cost effective production is still a major challenge due to the highly recalcitrant nature, and other technological barriers such as high enzyme costs, low conversion rate, generation of saccharification/fermentation inhibitors etc. [2-4]. Lignin contributes to the high recalcitrance, protecting the cellulose and hemicellulose from degradation. Besides its unproductive binding to cellulase making only low quantity of enzyme for hydrolysis, lignin also creates a barrier to the free entry of enzymes for high degree of hydrolysis [3, 5]. Parameters such as cellulose crystallinity, accessible surface area, degree of cellulose polymerization and acetylation of hemicellulose etc. have been reported as major bottlenecks in the successful enzymatic cleavage of polysaccharides to fermentable sugars [1]. Pretreatment aims at breaking down the lignin-hemicellulose matrix and reducing cellulose crystallinity so that it becomes susceptible to enzymatic hydrolysis [6, 7]. It should result in high yield of fermentable sugars after saccharification, reduce operating costs and restrict the formation of fermentation/saccharification inhibitors [6]. Although several pretreatment techniques have been developed during the past three decades such as mechanical, physico-chemical, chemical, biological and organosolv treatments, most of them require severe processing conditions, costly chemicals or sophisticated set up besides low efficiency [4, 6, 7]. Further, the type of pretreatment varies with biomass depending on its composition and hence necessitates optimization for each biomass [8].

Alkaline hydrogen peroxide (AHP) is reported to delignify agricultural biomass and increase its saccharification efficiency and the main effect is solubilisation and separation of lignin from the hemicellulose matrix [9].Very high fermentable sugar yields have been reported from AHP-pretreated wheat straw, rice hulls and barley straw [10-12]. Alkaline hydrogen peroxide pretreatment has also been suggested

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as a promising approach for enhancing the saccharification of rice straw, sugarcane bagasse, corn stover etc. [13-15]. There has been a recent upsurge in interest in the use of microwave irradiation as a feasible option for the pretreatment of biomass and several reviews have appeared on this topic [16-18]. Microwaves interact with both polar molecules and ions in LCBs and produce thermal and non-thermal effects, which help breakdown the biomass in a shorter time compared to conventional heating [19, 20]. Microwave (MW)-assisted alkali pretreatment has been attempted by many researchers as an effective tool to delignify the biomass [21, 22]. Hydrogen peroxide has been reported as an activator of MW irradiation as it degrades easily to water and oxygen [23].

Cassava (*Manihot esculenta* Crantz) is a tropical root crop cultivated in approximately 102 countries of the world for its starchy tubers capable of providing energy [24]. During the harvesting of the crop for human consumption or industrial processing of the roots for starch production, three types of wastes are generated such as peels, leaves and stems (constituting approximately 44% of the total plant biomass) [25]. We had earlier reported the relative content of polysaccharides such as cellulose, hemicellulose and starch in these biomasses and found that the peel contained *ca.* 30% starch, besides 14% cellulose and 23% hemicellulose, while the stem contained 15% starch, 23% cellulose and 28% hemicellulose. Leaves had the least content of starch (2.4%) besides 17% cellulose and 27% hemicellulose [25]. As starch contributes substantially towards the fermentable sugar yield in these residues especially the peel and stem, they require alternative pretreatment and saccharification approaches. Steam pretreatment of moist samples or MW-assisted dilute sulfuric acid pretreatment were earlier reported from our laboratory as effective techniques to enhance the fermentable sugar yield from cassava peel, but not optimal for the other two residues during saccharification with Accellerase [25]. Subsequent studies using another cellulolytic complex, Cellic CTec 2 also showed that very high yield of sugars was possible from peel. Nevertheless, optimum hydrolysis of polysaccharides could not be achieved for the other two biomasses [26].

Non-ionic surfactants such as Tween 20 (polyethylene glycol sorbitan monolaurate) and Tween 80 (polyethylene glycol sorbitan monooleate) have been reported to prevent the non-productive binding of lignin to cellulases [27]. Various effects have been reported for surfactants including alteration in the structure [28, 29], stabilization of enzymes thereby preventing their denaturation [29], positive interaction between substrate and enzymes etc. [27]. Surfactants with high hydrophilic-lipophilic balance (HLB) such as Tween 20 (HLB 16.7) have been reported to be more effective in extracting hydrophobic lignin degradation products into the soluble phase [30]. Hence, the aim of the present study was to compare the fermentable sugar yield from agricultural residues of cassava in three pretreated systems such as AHP pretreatment on steam-exposed or MW-irradiated biomass slurry as well as MW-assisted alkali pretreatment. The effect of supplementing Tween 20 at the saccharification stage in steam-exposed AHP and MW-assisted alkali pretreatments in enhancing the sugar yield was also studied. The extent of delignification in the various treatments, compositional changes in cellulose and hemicellulose and the ultrastructural alterations brought about in the effective combinations were also studied.

II. MATERIALS AND METHODS

2.1 Samples

Stems and leaves were collected from fully mature and healthy cassava plants (variety: Sree Jaya) grown at the Institute farm. Leaves along with the stalk were separated from the stems and allowed to wilt in the shade for 18 h to facilitate the elimination of cyanoglucosides to the maximum. Stems were chopped to small pieces (*ca.* 5.0 cm long) and both wilted leaves and stems were dried in the sun for 36–48 h. Dry stems and leaves were powdered in a hammer mill to particle size of *ca.* 2-3 mm and the powder was used without further sieving (unscreened) for the study. Peels (skin ± rind) were manually separated from the roots, chopped into pieces of *ca.* 2-3 cm length and dried in the sun for 36-48 h. Dry peels were powdered in a hammer mill to particles of similar size as before and stored in airtight bottles until use.

2.2 Enzyme Source

Cellic® CTec 2, an improved cellulase enzyme cocktail from M/s Novozymes, Bagsvaerd, Denmark, containing beta-glucosidase as well as xylanase, with reportedly high tolerance to product inhibition was used for saccharification [31]. The optimum temperature and pH of Cellic were standardized in our laboratory on these biomasses and were found to be 50 °C and 5.5 respectively [26].

2.3 Pretreatment

2.3.1. Experiment 1: Alkaline hydrogen peroxide (H_2O_2) pretreatment of steam-exposed/MW-irradiated biomass

MW-irradiated slurry

Ten percent (w/v) slurry of the dry biomass powders were prepared in distilled water and exposed to MW irradiation at 300 W for 20 min. in a general purpose Microwave Oven (M/s Samsung, Thailand). The equipment had MW radiation at power levels ranging from 100 W to 900 W. The flasks after exposure were treated with 2.5 ml and 5.0

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ml 35% H_2O_2 and pH adjusted to 11.5 with 4M NaOH and volume raised to 100 ml. The microwave power regime was chosen based on our earlier studies [25, 26]. The flasks were then incubated either for 4 h at 50 ºC or 24 h at room temperature (30±1 ºC) in a thermostatic water bath (M/s Julabo SW22).

Steam-exposed biomass slurry

The powdered samples (10.0 g each of dry powder of stems, leaves and peels) were moistened with distilled water to raise their moisture contents (MC) to *ca*. 40 % and exposed to steam in a Vegetable Steamer for 30 min. at 100 ºC as per the techniques standardized earlier [25]. The steam treated (ST30) slurry (equivalent to 10% w/v original dry biomass) was treated with 2.5 ml 35% H_2O_2 and the pH was raised to 11.5 using 4M sodium hydroxide (NaOH) and volume raised to 100 ml (Set 1). Another set of flasks containing the same quantity of each biomass along with 5.0 ml H_2O_2 was pH adjusted to 11.5 and volume raised to 100 ml (Set 2). Two flasks from each set (for each biomass) were incubated for 4 h at 50 ºC in a thermostatic water bath while another two sets were incubated at room temperature for 24 h in the water bath.

Compositional analysis

The samples after the respective pretreatments were first squeezed through a muslin cloth and then through Whatman No. 1 filter paper and the residues were dried in an Oven at 60 ºC for 18 h. The dry weight of the pretreated residues was assessed and they were further analysed for the compositional changes such as cellulose, hemicellulose and lignin as per the methods reported earlier [25].

Cellulose content was determined in the residue using acetic-nitric reagent by the method of Updegroff [32]. Hemicellulose content was determined as the difference of Neutral detergent fibre (NDF) and acid detergent fibre (ADF). The ADF and NDF were analyzed as described by Goering and Vansoest [33]. The ash content of the biomasses was determined by the standard procedure [34] and the lignin content of pretreated biomass was calculated as the difference of the sum of cellulose and ash from the acid detergent fiber [25].

Enzyme saccharification

Based on the compositional analysis, the best pretreatment with regard to lignin reduction was found to be steam pretreatment and hence this alone was carried over to further enzymatic saccharification studies. In the case of stem and leaf samples, 5.0 ml H_2O_2 for 24 h at RT group gave the pretreated biomass with low lignin content and hence these were selected. Nevertheless, for peel samples, 2.5 ml H_2O_2 for 24 h at RT group had the lowest lignin retention and hence this was used.

Steam pretreatment was done as described above and the slurry from each biomass was adjusted to pH 5.5 and equilibrated at 50 ºC for 10 min. Twenty milligrams of sodium azide were added to each flask to prevent microbial contamination during incubation. Cellic equivalent to 500 mg enzyme protein was added to each sample and incubated at 50 ºC for 120 h (full dose enzyme set, T1).

In another set of flasks, Cellic was added along with 250 mg Tween 20 (T2) to find out the enhancing effect on sugar yield due to the prevention of non-productive binding of lignin to cellulases by the non-ionic surfactant.

In a third set of flasks, half the dose of Cellic (250 mg enzyme protein) was added along with 250 mg Tween 20 (T3) to find out whether the enzyme dosage could be reduced in the presence of Tween 20.

All the flasks were incubated for 120 h after which the reducing sugars released in the supernatant fraction was assayed by Nelson-Somogyi method [35] and the composition of the enzyme saccharified residues was further determined to evaluate the extent of retention of cellulose, hemicellulose and lignin.

2.3.2. Experiment 2: MW-assisted alkali pretreatment of biomass and enzymatic saccharification

This experiment was conducted to find out whether alkali pretreatment alone under MW irradiation was better than initial exposure of aqueous slurry to MW and further pretreatment with H_2O_2 and alkali (Experiment 1). Preliminary trials showed that there was extensive swelling and volume reduction when alkali slurry was exposed to MW for 20 min. and as free movement of water was necessary for effective MW irradiation, the pretreatment time was reduced to 5 min. at 300 W MW power. Biomass slurry (10% w/v) in 3% NaOH was prepared for each sample and the alkaline slurry was exposed to MW irradiation at 300 W for 5 min.

The slurry was then enzymatically saccharified as described earlier using full dose of Cellic (T1), full dose with Tween 20 (T2) or half dose of Cellic with Tween 20 (T3). The composition of the saccharifed residue as well as the RS content in the supernatant was studied.

2.4 Ultrastructural Studies

The changes in the reorientation/structural alterations of cellulose, hemicellulose and lignin due to MW-assisted alkali pretreatment/enzymatic saccharification (adjudged as the best from the RS values) were studied using Scanning Electron Microscopy (SEM; HITACHI Scanning Electron Microscope Model S- 2400). The dry residues after pretreatment as well as after enzymatic

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saccharification were subjected to SEM. The samples were applied on the double side carbon pasted on an aluminium stub. A thin gold-platinum coating (10-15 nm thick) was applied for 3 min. using E-1010 Ion Sputter Unit under 15 kV and 10 Pa vacuum and discharge current of 10 mA and the SEM photographs were visualized at 1500x magnification.

2.5 Statistical Analysis

The various biochemical constituents in the pretreated/saccharified residue were expressed as percentage of the original biomass, based on the water insoluble residue weight obtained from each pretreatment. Two replicates were kept for each experiment and duplicate analyses were performed on each replicate. The data were subjected to Analysis of Variance (ANOVA) for statistical testing of the mean values and was followed by least significant difference (LSD) for pair-wise comparison of mean values by using the statistical package, SAS 9.3 [36].

III. RESULTS AND DISCUSSION

3.1 Alkaline hydrogen peroxide (H_2O_2) pretreatment of steam-exposed/MW-irradiated biomass

3.1.1 Structural polysaccharide changes in the AHP pretreated agricultural residues from cassava

Compositional alterations in the structural polysaccharides (cellulose and hemicellulose) consequent to alkaline hydrogen peroxide (AHP) pretreatment of microwave (MW)-irradiated and steam-exposed biomass (stem, leaves and peels of cassava) are given in **TABLE** 1. AHP pretreatment at higher temperature (50 ºC) for 4 h had a significant effect in lowering the cellulose content of MW-irradiated stem and peel at both levels (2.5 and 5.0% v/v) of H_2O_2, while in the case of leaves, significant effect was noticed only for 5.0 % level. Both room temperature (RT) exposure for 24 h and high temperature (HT) exposure for 4 h to AHP (5.0% v/v) were not significantly different for stem. Steam-exposed samples when exposed to AHP pretreatment brought about highly significant cellulose removal from cassava peel compared to the other two biomasses (**TABLE** 1) and maximum reduction was observed for T6 (2.5% AHP for 24 h at RT). There was no significant change in hemicellulose content in peel samples subjected to AHP pretreatment after MW-irradiation of aqueous slurry for 20 min. Nevertheless, in the case of stem and leaf samples, significant reduction in hemicellulose was observed for most MW-irradiation treatments. On the contrary, hemicellulose content was significantly reduced for peel samples only when the steam-exposed samples were subjected to AHP treatment (**TABLE** 1). In the case of stem, HT (50 ºC) facilitated removal of HC from steam-exposed AHP pretreated samples.

*Table.1: Structural polysaccharide changes in pretreated biomass subjected to AHP treatment**.*

Treatments	H_2O_2 levels (% v/v)	Cellulose content (g/100 g original biomass on dry basis)			Hemicellulose content (g/100g original biomass on dry basis)		
		Stem	Leaves	Peel	Stem	Leaves	Peel
Initial (without any treatment)**		22.8^{a}	17.3^{a}	14.17^{a}	28.8^{a}	27.6^{a}	23.4^{a}
MW-irradiated samples							
T1	2.50	16.90^{c}	16.67^{a}	10.82^{b}	27.10^{ab}	25.33^{b}	23.30^{a}
T2		18.26^{b}	17.35^{a}	12.19^{ab}	26.00^{b}	25.63^{b}	22.63^{a}
T3	5.00	18.00^{b}	15.73^{b}	11.84^{b}	26.50^{b}	24.33^{c}	23.13^{a}
T4		19.08^{b}	16.71^{a}	13.13^{a}	25.30^{c}	25.53^{b}	22.17^{a}
Steam-exposed samples							
T5	2.50	19.77^{b}	15.35^{b}	10.69^{c}	27.00^{b}	27.40^{a}	19.20^{b}
T6		20.91^{ab}	16.85^{a}	9.13^{d}	28.50^{a}	25.37^{b}	18.50^{b}
T7	5.00	22.40^{a}	17.47^{a}	10.22^{c}	26.80^{b}	27.20^{a}	19.20^{b}
T8		21.98^{a}	16.65^{a}	11.66^{b}	28.10^{a}	26.83^{a}	17.20^{c}

* Treatments T1, T3, T5 and T7 were exposed to AHP for 4 h at 50 °C; T2, T4, T6 and T8 were exposed to AHP for 24 h at room temperature (30±1 ºC); ** Ref. [26]; statistical comparison was made within each column and values with different superscripts are significant at $p < 0.05$.

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Alkaline peroxide pretreatment is known to decrystallise cellulose [9]. Banerjee et al. [37] reported that AHP pretreatment was beneficial in enhancing lignocelluloses deconstruction at low temperature and atmospheric pressure and had less environmental effect as well.

3.1.2 Lignin changes

Highest delignification occurred when steam-exposed stem samples were subjected to AHP treatment using 5.0% v/v H_2O_2 followed by MW-irradiation and AHP (5.0% v/v H_2O_2) treatment (Fig. 1).Nevertheless, lignin changes in cassava leaves subjected to various treatments (T1- T7) were insignificant and significant reduction was noticed only in T8 (5.0% v/v for 24 h at RT). In the case of peel, delignification was the highest for steam-exposed sample subjected to AHP (2.5% v/v H_2O_2 for 24 h at RT), which was not significantly different from the MW-irradiated sample (T2). It was found in the present study that steam-exposed samples subjected to AHP treatment had better delignification than MW-irradiated samples. Singh et al. [38] reported that MW-assisted H_2O_2 treatment of rice straw disrupted the ester linkages between carbohydrates and lignin and helped in efficient delignification. Prolonged reaction time at 30 °C for 24 h was beneficial than 50 °C in eliminating lignin from steam-exposed and AHP pretreated agricultural residues (Fig. 1).

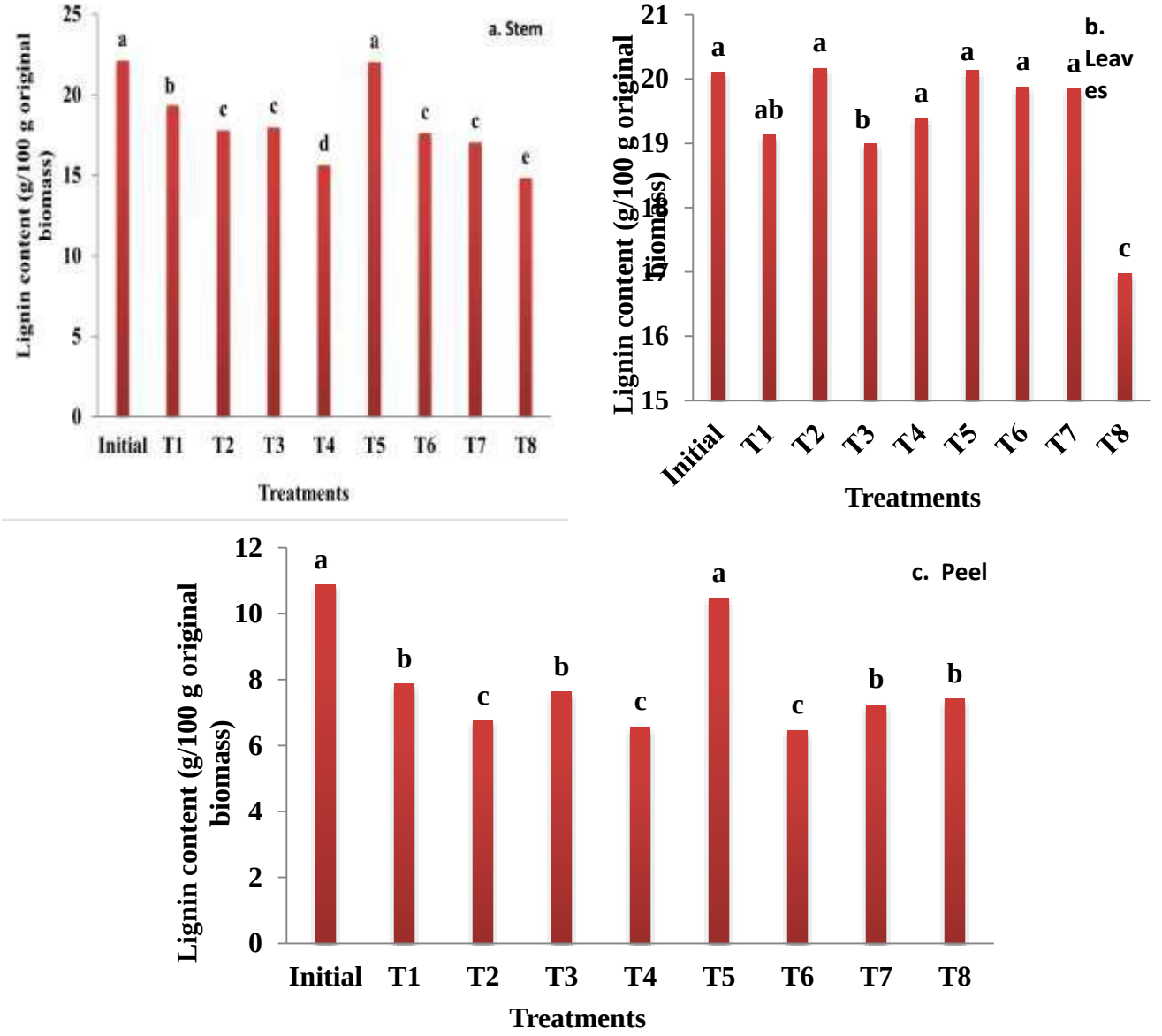

Fig.1 (a-c): Lignin changes in MW-irradiated or steam-exposed biomass subjected to AHP treatment; bars with different alphabets are significant at p <0.05.

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3.1.3 Enzymatic saccharification

Structural polysaccharides in residue after saccharification

Based on delignification, the steam-exposed biomasses subjected to AHP treatment alone were used further for saccharification. The AHP levels found optimum for cassava stem and leaves were 5.0% v/v and 2.5% v/v for cassava peel and the reaction time was 24 h at room temperature. The effect of Tween 20 supplementation along with full dose of Cellic CTec 2 (T2) or half dose of enzyme (T3) was studied to understand whether enzyme saving was possible in Tween 20 supplemented system. Significant hydrolysis of cellulose occurred after enzymatic saccharification in stem and leaves when Cellic-based system was supplemented with Tween 20. Nevertheless, when Cellic dosage was reduced to half (250 mg enzyme protein/10g biomass), there was significant reduction in cellulose hydrolysis in these two biomasses (**TABLE** 2).

However, in the case of peel, neither Tween-20 supplementation nor Cellic dosage reduction had any significant effect in cellulose hydrolysis and *ca.* 80-86% cellulose hydrolysis was observed after 120 h saccharification in T1-T3. Hemicellulose was hydrolysed to the extent of 45%, 60% and 63% respectively from stem, leaves and peel, when saccharified with Cellic (500 mg enzyme protein/10g biomass). There was no significant variation in hemicellulose hydrolysis in cassava leaves, when Tween 20 was supplemented or Cellic dosage was halved, indicating that the effect of Tween 20 was more on enhancing cellulose hydrolysis. Tween 20 supplementation was found to negatively impact hemicellulose hydrolysis in cassava stem and only 25% hydrolysis occurred. In the case of leaves and peel, significant alterations were not noticed due to Tween supplementation. Saha and Cotta [10] also reported that alkaline peroxide pretreated wheat straw could be converted to fermentable sugars with excellent yield. The commercial enzyme preparation Cellic® CTec 2 was reported to contain very high beta-glucosidase activity (*ca.* 34,495 ± 2,935 nkat/g) [39]. The hemicellulase co-activities of cellulase enzyme complex might be remaining unaffected even after Tween 20 supplementation. Karagoz et al. [40] studied the advantage of AHP pretreatment for enhancing bioethanol production from rapeseed straw and found that 5% (v/v) H_2O_2 at 50 ºC for 1 h was the optimal pretreatment condition. They found that as high as 94% of cellulose was digested by enzymes during this pretreatment and saccharification. One of the greatest advantages of AHP pretreatment was that the pretreatment and enzymatic hydrolysis could be performed in the same reactor [41]. They used a lower concentration of H_2O_2 (1.25%) than the present study and reported that 75% glucose and 71% xylose were released from AHP pretreated corn stover after 48 h enzymatic saccharification.

Table.2: Structural polysaccharide changes after saccharification (120 h) of steam-exposed and AHP pretreated biomass with or without surfactant supplementation.*

Enzyme treatments	**Cellulose content (g/100g original biomass)**			**Hemicellulose content (g/100g original biomass)**		
	Stem	**Leaves**	**Peel**	**Stem**	**Leaves**	**Peel**
Cellic alone (T1)	7.90^{b} (65.35)**	5.39^{a} (68.84)	2.04^{a} (85.60)	15.78^{c} (45.21)	10.9^{a} (60.51)	8.61^{b} (63.21)
Cellic+ Tween 20 (T2)	7.03^{b} (69.17)	3.40^{c} (80.35)	2.00^{a} (85.89)	21.48^{b} (25.42)	10.00^{a} (63.77)	8.33^{b} (64.40)
Cellic (half dose) + Tween 20 (T3)	9.30^{a} (59.21)	4.39^{b} (74.62)	2.83^{a} (80.03)	23.80^{a} (17.36)	9.56^{a} (65.36)	11.40^{a} (51.28)

* Statistical comparison was made within each column and values with different superscripts are significant at $p < 0.05$;
** indicates the percentage decrease from the original value in the respective biomasses.

Residual lignin

Residual lignin after enzymatic saccharification (expressed as % of the original biomass) indicated significant reduction in levels in the three biomasses (Fig. 2). Except in the case of leaves, saccharified with full dose of Cellic (T4), there were no significant differences in the lignin content among the various enzyme treatments with or without Tween supplementation. Deconstruction of lignocelluloses coupled with the hydrolysis of cellulose and hemicellulose along with the starch present in stem and peel might have resulted in further release and solubilisation of lignin hydrolytic products into the supernatant. Gould [9] reported the use of alkaline H_2O_2 (pH 11.5) in delignification of LCBs and the same pH was adopted in the present study as well. Lignin degradation releases soluble phenolics into the supernatant liquid which are reported to be inhibitory to cellulases [42, 43]. Surfactants such as Tween and polyethylene glycol have been reported to reduce the levels of lignin hydrolytic products and enhance

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saccharification [27, 44]. Previous studies showed that Tween 20 was highly effective in removing up to 80% phenolics from the prehydrolysates from lignocellulo-starch biomasses, by possible complex formation with them [44].

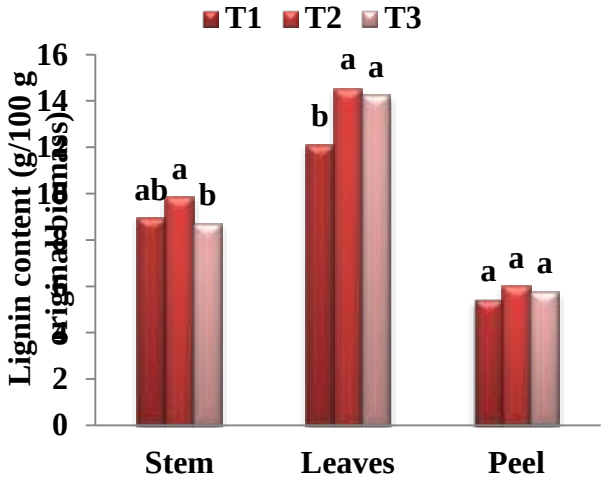

Fig.2: Lignin changes after saccharification (120 h) of steam exposed and AHP pretreated biomass with or without surfactant supplementation; bars with different alphabets are significant at p<0.05

Reducing sugar content and Overall Conversion Efficiency (OCE)

The reducing sugar content in the saccharified mash as given in **TABLE** 3 indicates that the highest RS release was obtained for cassava peels, which was evidently due to its high total carbohydrate content (71.77 % on dry basis) [25] . Highest RS release was obtained from Tween 20 supplemented system with full dose of Cellic for peel and stem, although there was no difference between full and half dose in the case of leaves. The OCE expressed as percentage conversion of total carbohydrate to RS was also the highest for cassava peel. A higher conversion of carbohydrate to RS was observed for leaves compared to stem and the effect of Tween 20 in enhancing the RS yield was evident for the three biomasses (**TABLE** 3**)**. Divya Nair et al. [45] found that Tween 20 supplementation during saccharifcation of steam or dilute acid pretreated cassava starch factory residue significantly enhanced the RS and ethanol yield from it. The beneficial effect of surfactants such as Tween 20 in preventing the non-productive binding of lignin onto cellulase by forming complex with lignin and resulting enhanced saccharification yield has been reported by several researchers [30, 45, 46]. Previous studies indicated that the use of Cellic alone during saccharification of steam-exposed (30 min.) slurry from cassava stem or peel released *ca.* 38 and 73 g/L RS after 120 h saccharification, while *ca.* 27 g/L was only released from the leaves [26]. This showed that AHP treatment of steam-exposed slurry was superior to simple steam pretreatment in the case of leaves only. Saha and Cotta [11] reported that the addition of Tween 20 @ 4.3 g/L in AHP treated rice hull hydrolysate was not effective in enhancing the saccharification yield, while Kaar and Holtzapple [29] reported the additive effect of Tween 20. The low OCE obtained for leaves and stem indicated the need to improve the saccharification yield by modifying the pretreatment strategies. Hence the effect of microwave-assisted alkali pretreatment followed by enzymatic saccharification was attempted.

Table.3: Reducing sugar content (g/L) and Overall Conversion Efficiency (%) after saccharification of steam-exposed and AHP pretreated biomass with or without surfactant supplementation

Enzyme treatments	**Reducing sugar content (g/L of saccharified mash)**			**Overall Conversion Efficiency (%)**		
	Stem	**Leaves**	**Peel**	**Stem**	**Leaves**	**Peel**
Cellic alone (T1)	35.31^{b}	31.74^{b}	53.43^{b}	51.43^{b}	64.21^{b}	74.43^{b}
Cellic+ Tween 20 (T2)	37.74^{a}	34.96^{a}	56.20^{a}	54.96^{a}	70.72^{a}	78.29^{a}
Cellic (half dose) + Tween 20 (T3)	31.56^{c}	34.37^{a}	48.99^{c}	45.96^{c}	69.53^{c}	68.24^{c}

* Statistical comparison was made within each column and values with different superscripts are significant at p < 0.05.

3.2. Microwave-assisted alkali pretreatment and saccharification

3.2.1 Structural carbohydrates and lignin in saccharified residues

There was significantly higher degradation of cellulose, hemicellulose and lignin in MW-assisted alkali pretreated biomass saccharified using Cellic compared to steam-exposed and AHP preteatment followed by saccharification (**TABLE** 1 *vs.* **TABLE** 4). The percentage reduction in cellulose ranged from 90-93% when Cellic alone was used which increased to 91-95% when Tween 20 was also supplemented. Nevertheless,

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when Cellic dosage was reduced to half, there was significantly lower hydrolysis. A similar trend was observed in the case of hemicellulose also, although the hydrolysis was less than cellulose. Cellulose and hemicellulose hydrolysis could be significantly improved from both stem and leaves when MW-assisted alkali pretreaed residue was saccharified (**TABLE** 4). Tween 20 supplementation helped to enhance the breakdown of both cellulose and hemicellulose. Even when the Cellic dosage was halved, there was 84-88% hydrolysis of cellulose and 77-85% hydrolysis of hemicellulose from the various residues, whereas the extent of hydrolysis under similar conditions of saccharification in steam-exposed AHP pretreatment was only 59-80% for cellulose and 17-65% for hemicellulose (**TABLE** 4 vs. **TABLE** 2). This indicated that MW-assisted alkali pretreatment was superior to steam-exposure followed by AHP treatment of the selected biomasses. Singh et al. [47] observed that alkali concentration, MW-irradiation time and substrate concentration were important for the optimum pretreatment efficiency of rice straw and reported the optimum values as 2.75%, 22.5 min and 30g/L respectively. However, much lower irradiation time (5 min.) and higher substrate concentration (100 g/L) was used in the present study. Zhu et al. [48] also reported higher fermentable sugar yield from MW (700 W)-assisted alkali (1% NaOH) pretreatment for 25 min followed by enzymatic saccharification of wheat straw, compared to conventional alkali treatment. Nevertheless, as compared to the reported results for wheat or rice straw, very high hydrolysis of both cellulose and hemicellulose was observed in the present study for the biomasses when 3% alkali slurry was subjected to MW-irradiation at 300 W for 5 min. Partial hydrolysis of hemicellulose during pretreatment is reported to result in high levels of xylooligomers in the pretreated liquor, which are strongly inhibitory to cellulase [49]. The very high hydrolysis of cellulose and hemicellulose obtained indicated that the possibility of such inhibition was negligible in the treated biomasses. Zhu et al. [50] obtained 12 times more sugar yield from *Miscanthus* sp. subjected to MW (300 W)-assisted alkali (0.2M NaOH) for approximately 10 min. compared to conventional alkali or dilute acid pretreatment. Budarin et al. [51] reported that 180 ºC was the crucial turning point in the MW degradation of cellulose and this temperature is achieved in MW oven at 300 W and the same was used in the present study as well.

Table.4: Structural polysaccharide changes after saccharification (120 h) of MW- assisted alkali pretreated biomass with or without surfactant supplementation.*

Enzyme treatments	**Cellulose content (g/100g original biomass)**			**Hemicellulose content (g/100g original biomass)**		
	Stem	**Leaves**	**Peel**	**Stem**	**Leaves**	**Peel**
Cellic alone (T1)	2.37[a] (89.61)**	1.40[b] (91.91)	1.02[a] (92.80)	6.89[a] (76.08)	3.92[a] (85.80)	3.85[a] (83.55)
Cellic+ Tween 20 (T2)	2.05[a] (91.01)	1.21[b] (93.01)	0.64[a] (95.48)	4.80[b] (83.33)	3.67[a] (86.70)	3.34[a] (85.73)
Cellic (half dose) + Tween 20 (T3)	2.73[a] (88.03)	2.73[a] (84.22)	1.69[a] (88.07)	6.73[a] (76.63)	4.08[a] (85.22)	3.98[a] (82.99)

* statistical comparison was made within each column and values with different superscripts are significant at $p < 0.05$;
** indicates the percentage decrease from the original value in the respective biomasses.

Residual lignin in the three biomasses subjected to MW-assisted alkali pretreatment followed by enzymatic saccharification (120 h) showed that Tween 20 supplementation facilitated the removal of more lignin from the biomass possibly through complex formation with it (Fig. 3). While lignin was removed to a greater extent from MW-assisted alkali treated and saccharified cassava leaf and peel samples compared to AHP treatment (Fig. 2), higher retention was observed in the respective stem samples treated with either Cellic alone or with half dose of Cellic and Tween 20. High removal of lignin from MW-assisted alkali pretreated biomass has been reported by several researchers [47, 48, 52]. The ester linkages between lignin and hemicellulose are broken down during MW-irradiation which then facilitates rapid hydrolysis of polysaccharides during saccharification.

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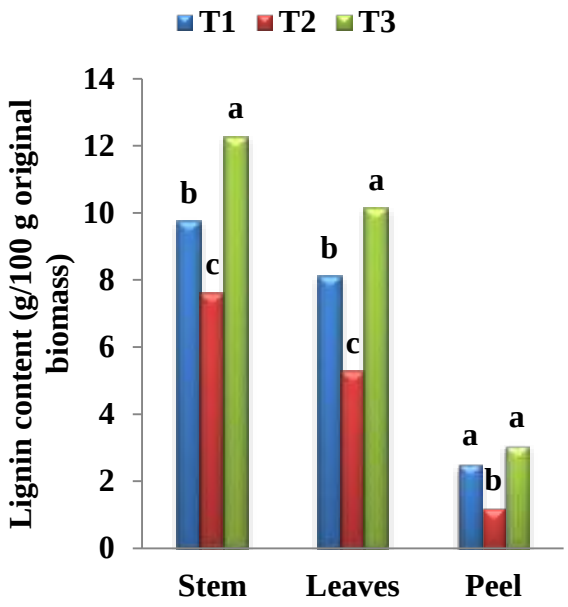

Fig.3: Lignin changes after saccharification (120 h) of MW-assisted alkali pretreated biomass with or without surfactant supplementation; bars with different alphabets are significant at p<0.05

3.2.2 Reducing sugars and Overall Conversion Efficiency

The saccharified mash from MW-assisted alkali pretreated biomass had significantly higher RS content compared to steam-exposed and AHP pretreated biomass (**TABLE** 5 and **TABLE** 3). There was evidently higher release of RS from Tween 20 supplemented system with full dose of Cellic (**TABLE** 5). Accordingly the OCE was also high for the biomass residues with as high as 82-94% of the potential carbohydrates getting hydrolyzed after 120 h saccharification. Hu and Wen [22] also reported 90% conversion of carbohydrates to sugars when MW-assisted alkali (1% NaOH) pretreated switchgrass was saccharified. Singh et al. [47] observed that MW-assisted alkali pretreated rice hull had low cellulose crystallinity which facilitated its effective hydrolysis during the saccharification step. Lignin hydrolytic products especially low molecular weight phenols have been reported to be inhibitory to cellulases [43] and the effect of Tween 20 in preventing the non-productive binding of cellulases to lignin has been reported [27, 46]. The very high OCE obtained in the present study in Tween supplemented system is evidently due to lignin channeling effect. Haven and Jorgensen [39] found that as high as 65% the β-glucosidase activity in Cellic CTec 2 was adsorbed onto lignin from pretreated wheat straw and such adsorption could be reduced by supplementing the system with bovine serum albumin or polyethylene glycol.

Table.5: Reducing sugar content (g/L) and Overall Conversion Efficiency (%) after saccharification of MW-assisted alkali pretreated biomass with or without surfactant supplementation.*

Enzyme treatment	Reducing sugar content (g/L of saccharified mash)			Overall Conversion Efficiency (%)		
	Stem	Leaves	Peel	Stem	Leaves	Peel
Cellic alone (T1)	54.12^{b}	41.60^{c}	56.25^{b}	78.82^{b}	84.16^{c}	78.36^{b}
Cellic+ Tween 20 (T2)	57.79^{a}	46.23^{a}	59.55^{a}	84.17^{a}	93.52^{a}	82.95^{a}
Cellic (half dose) + Tween 20 (T3)	53.76^{b}	44.12^{b}	54.97^{b}	78.30^{b}	89.25^{b}	76.57^{c}

* Statistical comparison was made within each column and values with different superscripts are significant at $p < 0.05$

3.3 Ultrastructural studies

The ultrastructural changes brought about in the three biomass residues due to MW-assisted alkali pretreatment as well as after enzymatic saccharification were compared with the native (untreated) biomass using Scanning electron microscopy. It was found that the native samples of stem and peel had many starch granules with a preponderance of them in peel. Fiber particles could also be seen in the stem samples, which might have got fragmented during the powdering operation. However, intact and rigid cellulose fibrous structure was visible in the cassava leaf (Fig. 4 a, d and g). During MW-assisted alkali pretreatment, most of the starch granules disappeared from the stem and peel samples and in the case of stem, broken or defibrillated structures could be seen (Fig. 4 b). The surface morphology of the peel samples indicated gelatinized starch coating over fibrous particles due to the preferential hydrolysis of hemicellulose (Fig. 4 h). Nevertheless, in the case of leaf samples, lot of fragmented cellulose fibres were seen, indicating the efficacy of MW-assisted alkali pretreatment in deconstructing the cellulose. Zhu et al. [50] also reported large scale separation of fibers due to lignin removal by the MW-assisted alkali pretreatment. Thin and striated surface morphology was reported in MW-assisted alkali pretreated switchgrass [22]. Microwave

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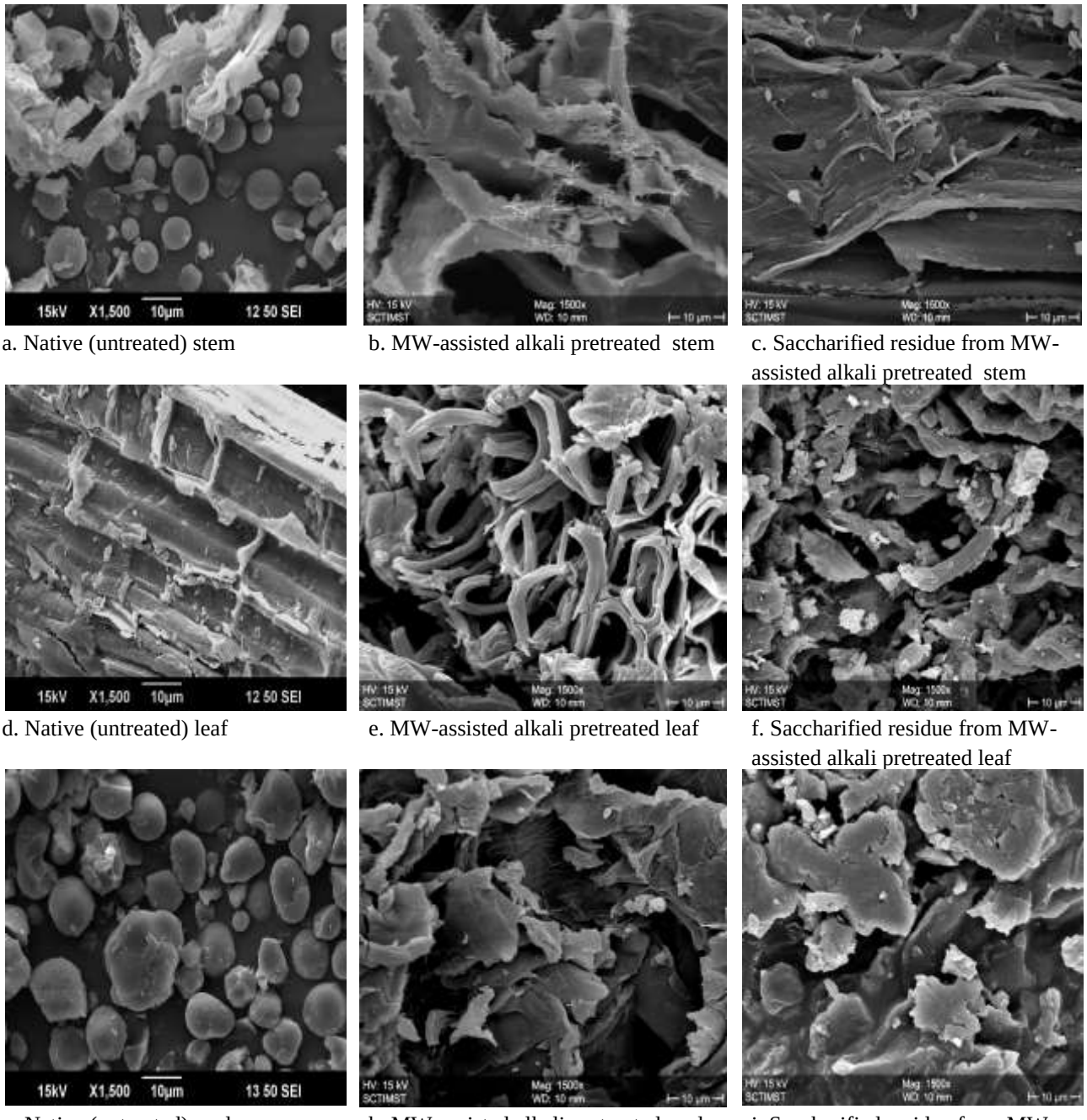
irradiation has been reported to create hot spots within the lignocelluloses matrix leading to an explosive effect on the recalcitrant structure, facilitating its faster disruption than in conventional heating [22].

The saccharified residue from the three biomasses presented a different surface morphology from the pretreated residue. In the case of stem, although starch granules disappeared totally, fragmented cellulose sheaths were visible indicating that the hydrolysis was incomplete (Fig. 4 c). It was found that *ca.* 84% of potential carbohydrates were only hydrolysed from cassava stem (Table 5). Highly fragmented fiber particles were seen in leaf samples (Fig. 4 f). Although intact starch granules were not seen in saccharified residue from peel, coating of starch over fibrous particles were visible (Fig. 4 i), which stressed the need to incorporate a starch degrading enzyme also into the hydrolytic enzyme cocktail for complete saccharification.



a. Native (untreated) stem
b. MW-assisted alkali pretreated stem
c. Saccharified residue from MW-assisted alkali pretreated stem
d. Native (untreated) leaf
e. MW-assisted alkali pretreated leaf
f. Saccharified residue from MW-assisted alkali pretreated leaf
g. Native (untreated) peel
h. MW-assisted alkali pretreated peel
i. Saccharified residue from MW-assisted alkali pretreated peel

Fig. 4 (a-i): SEM photographs (x 1500) of native (untreated) vs. MW-assisted alkali pretreated and enzyme saccharified cassava stem, leaf and peel.

Pooja and Padmaja [26] reported the presence of alpha-amylase existing as a co-activity along with cellulase and β-glucosidase in Cellic CTec 2. However, the levels may not be sufficient to bring about complete hydrolysis of starch. Previous studies on the ultrastructure of steam (30 min.) and MW (300 W)- irradiated (20 min.) samples of stem, leaves and peels showed the highly intact structure of stem with unbroken sheaths of fiber and starch-coated surface morphology for leaves and peel [26]. However, compared to those pretreatments, MW-assisted alkali pretreatment was found to efficiently deconstruct the biomasses leading to higher extent of saccharification.

IV. CONCLUSION

The efficacy of alkaline hydrogen peroxide treatment of steam-exposed/MW-irradiated aqueous slurries of cassava stem, leaves and peel was compared with MW-assisted alkali pretreatment in enhancing the biomass deconstruction and saccharification. While cellulose was degraded to a higher extent than hemicellulose in the saccharified residue from AHP pretreatment, both were effectively hydrolyzed in the MW-irradiated alkali slurry subjected to saccharification. Tween 20 supplementation coupled with Cellic enabled higher release of reducing sugars from the three biomasses, due to higher removal of lignin from the MW-irradiated alkali slurry. Disappearance of starch granules consequent to saccharification was evident in the ultrastructural pictures of stem and peel. Very high conversion of potential carbohydrates to reducing sugars in Tween 20 supplemented system from stem (84%). leaves (93.5%) and peel (83%) indicated that MW-assisted alkali pretreatment could effectively deconstruct the polysaccharides enhancing their hydrolysis and releasing maximum quantity of fermentable sugars.

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Maize Hybrids Yield as Affected by Inter and Intra Row Spacing

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Abstract**—** *To study the effect of different, inter and intra-row on some new maize hybrids under on yield and its components. Two field experiments were carried out during summer seasons of 2014 and 2015. The results showed that highest ear length, ear diameter, grains weight/ear, shelling percentage, 100-grain weight and grain yield/fed. S.C 2055 hybrid was recorded the greatest value number of rows/ear. S.C 2066 hybrids recorded the highest number grains/row, the lowest ear length, ear diameter, grains weight/ear, shelling percentage and 100-grain weight. Sown maize plants in width rows (70 cm) produced the highest number of ear/plant, number of rows/ear and number grains/row and ear length, ear diameter, grains weight/ear, shelling percentage and 100-grain weight. Sown maize plants in hills 30 cm apart produced the greatest numbers of ears/plant and thick ears, highest grains weight/ear, shelling percentage and 100- grain weight. However, sown maize plants at hill spacing of 25 cm apart produced tallest ears. It could be concluded that sown S.C. 3084 hybrid at 60 cm row width and hill spacing of 20 cm apart maximized maize productivity under the environmental conditions of Dakahlia Governorate, Egypt.*

Keywords**— Row spacing, hill spacing maize yield, yield components.***

I. INTRODUCTION

Maize (*Zea mays* L.) is considered as a one of the most important strategic cereal food crops in Egypt and the world. Recently, is mixed with wheat flour for making bread to reduce the gap between production and consumption of wheat. There is agonizes from the shortage of cereal production such maize. To intensification grain corn production per unit area of maize in Delta soils in Egypt, it must be resolute the appropriate maize hybrids at both row and hill spacing to exploit its productivity. Maize hybrids may be dissimilar in agronomic characters due to row width, hill spacing and plant population density that affect production per unit area. Maize hybrids differed with different row spacing, plant population and hill spacing. Maize hybrids differences on agronomic characters and grain yield. In this respect, [1, 2] summarized that for obtaining a higher maize yield and net income, maize hybrids had different responses to agronomic characters and grain yield. [3, 4] showed a significant difference between maize hybrids in plant height, No. of ear/plant, barren %, LAI, No. of kernels/row, grain weight/ear and grain yield/plant. [5] initiate that hybrid 30Y87 was early in maturity, produced more No. of grain row/cob, less No. of grains /row and less cob length than the hybrid 31R88 similarly 1000-grain weight, grain yield and straw yield of hybrid 30Y87 was significantly greater than the hybrid 31R88. [6] noticed that hybrid SiPAA-444 surpassed hybrid Ts-13 for grain yield. [7] found that S.C. 128 produced the highest value when planting in ridges 80 cm apart 22 cm between hills and one plant hill. [8] set up that hybrid 90-22-13 was superior to other varieties investigated. [9,10] concluded that maize hybrid S.C. 10 with 429 Kg N/ha, recorded the tallest cob. Also, hybrid S.C. 10 gave the maximum 1000-kernel weight and grain yield. [11,12] showed that maize hybrid significantly differed in final grain yield and some yield components as cob yield and number of grains/cob. [13] indicated that maize hybrids DKC6589 and Mobeen had the highest and lowest grain yield among studies hybrids. Higher grain yield in DKC6589 was due to the higher number of grains /ear and 100-grain weight. [14] found that number of ears per m^{-2} of SC 320 hybrid was significantly higher than SC 301 hybrid, but number of grains/ear and 1000-grain weight in SC 320 hybrid was significantly lower than SC301 hybrid. [15,16] showed that the harvests performed after physiological maturity decreased the real grain productivity, especially for the hyper-early hybrids.

Row width plays a great effect on the maize plant population. In this respect, [17,18] designated that increasing distance between rows from 60 to 70 and 80 cm lead to a significant increase in growth character, grain and its components due to better interception and utilization of solar radiation and the increase in photosynthetic processes. [2,19] showed that increasing

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ridge spacing significantly recorded No. of days to 50% tassling and silking, plant and ear heights were in some direction, planting on the 80 cm ridge was associated with a significant increase in ear length No. of kernels/row, 1000 kernel weight and grain yield. [2,20] point out that planting maize in ridges 80 or 90 cm apart produced the highest values of all studied characters. Planting maize in ridges 70 cm apart gave the lowest values of these characters. Recently, [21] reported that maize plants sown in line having (60 cm) row to row distance had highest plant, heavier 1000 grains weight and highest grain yield.

Growth and grain yield of maize is more affected by variations in hill spacing than other members of the grass family. Hill spacing affected of agronomic, flowering characteristics, and grain yield. Many investigators studied the effect of plant density of maize as a spacing between hills, in this regard, [1,2] described that highest grain yield and harvest index obtained at 10 plants/m^2. The highest No. of the grain/ear, stem diameter and cob length were recorded at 8 plants/m^2, while the highest values of plant height were recorded at 12 plants/m^2. [22,23] establish that grain yield increased in the narrow rows due to limited intra-row plant competition for light, nutrients and water. Population above the optimum has resulted in lodging that has caused a reduction in maize production. [1,7] showed that increase in intra-row spacing from 20 to 25cm significantly increased No. of row /cob, cob diameter, 100-grain weight and grain yield. [9] reported that highest grain yields for some hybrid was obtained at plant denser of 8 plants /m^2 reached their maximum grain yield and increased density in the grain yield and its components. Therefore, the best option to achieve the highest grain yield. [24] showed that the 70 x 30 and 60 x 40 cm spacing gave higher values of the morphological parameters than 80 x 20 cm. With regard to yield, 80 x 20 cm gave the highest average cob weight and 1000-grain weight. With respect to the interaction between maize hybrids and row width will present in this respect, [21] described that Hybrid-3025 sown in ridges having a 60 cm row to distance produce more grain yield as compared to Azam variety. Concerning the interaction among maize hybrids and hill spacing, in this respect, [25,26] concluded that maize hybrids react differently to various plant population densities. The interaction between the spaces between the hills and maize hybrids was significant for ear length and grain yield. Regarding to the interaction between row width and hill spacing, in this respect, [27] decided that this interaction had a significant for number of ear/plant, grain yield/plant and per faddan. They added that planting maize on 80 cm rows of plant densities of 25-30 thousand plants/fed (17-20 cm between hills) maximized grain yield. Concerning to the interaction among maize hybrids, row width and hill spacing, in this respect, [1,28] described that the highest grain yield due to increased plant population and reduced row spacing, depended mainly on different factors, like the hybrid type in use. Therefore, the present investigation was objective to study inter- intra-row spacing and plant population density on the growth, yield and yield components of some single cross maize hybrids.

II. MATERIALS AND METHOD

2.1. Research time and location:

The current investigation was carried out in the extension field at Mahelt Engaq Village, Sherbin Center, Dakahlia District during summer growing seasons of 2014 and 2015 to study the effect of inter and intra-row spacing on plant growth, yield, and yield components of some maize hybrids. Two separate field trials were conducted during each year of 2014 and 2015 summer seasons. One trail for each row spacing (RS), *i.e*. 60 and 70 cm between ridges. The experimental design used in each trail was split-plot design with four replications. The main plots were assigned for maize hybrids i.e. (SC) 3084, (SC) 3062, (SC) 2055 and (SC) 2066 and hill spacing were randomly distributed in the sub-plots i.e. 15, 20, 25 and 30 cm hill spacing apart. Each plot consisted of five ridges, 4.5 m long and the ridge width was differed according to the treatment. The combined analysis was done over the two row pacing experiments. Eight plant population densities and its distribution were the combination of four hybrids and four plant spacing. The outer two ridges (1^{st} and 5^{th}) were considered as borders. Grain yield and yield components were determined from the remaining two ridges. The previous crop was wheat in both years. Planting date was done on June 16 in the 2014 season, and June 6 in the 2015 season. Calcium superphosphate 15.5% P_2O_5 at the rate of 480 kg/ha was applied before planting. Three grains were hand planted in each hill, then thinning to one plant per hill was done before the first irrigation. Hoeing twice was done for controlling weeds before the first and second irrigations. Nitrogen fertilizer in the form of urea (46.0 %N) at the rate of 288 kg/ha was applied in two equal doses before the first and the second irrigations, respectively. Recommended agricultural practice in the region was applied. These distributed of eight plant population densities was presented in Table 1.

2.2. Studied Characters:

At harvest (after 120 days from planting) random samples of guarded ten plants were taken at random from each sub - plot to determine the yield components. Number of ears/plant was calculated as the mean number of ears of ten plants. Ear length (cm) was measured as the means of ten ears length. Ear diameter (cm) was measured by using

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a Vernier Caliper as the means of ten ears randomly. Number of rows/ear was counted as the average of the number of rows of ten ears randomly. Number of grains/row was counted as the means of a number of grains in each row of ten ears randomly. Ear grains weight (g) was obtained by averages weight of ten ear grains in grams. Shelling percentage (%) was determined by dividing the weight of ten ears shelled grains by their weight and multiplied by 100. 100-grain weight (g) was taken from clear grains and determined as the mean weight of four random samples of 100 grains of each plot and adjusted to 15.5 % moisture content. Grain yield/ha was determined by the weight of grains per kilograms adjusted to 15.5 % moisture content of each plot, then converted to t/ha.

2.3. Experimental analysis:

All obtained data were statistically analyzed according to the technique of analysis of variance (ANOVA) for the split – plot design to each experiment (row spacing), then combined analysis was done between row spacing trails as published by [29] by using "MSTAT-C" computer software package. A Least significant of the difference (LSD) method was used to test the differences between treatment means at the 5 % level of probability as described by [30].

III. RESULTS

3.1. Effect of row width:

Regarding to the effect of row width (60 and 70 cm between ridges) number of ear/plant, ear length, ear diameter, number of rows/ear, number of grains/row, ear grains weight, shelling percentage, 100-grain weight and grain yield/ha, the results in Tables 2 and 3 clearly showed a significant difference in both seasons due to row width. Sown maize plants in width rows (70 cm) produced the highest number of ear/plant, number of rows/ear and number grains/row and ear length, ear diameter, grains weight/ear, shelling percentage and 100-grain weight. Sown maize plants on narrow row width (60 cm) produced the highest values of grain yield/ha. This may be due to increases in photosynthesis due to increase light penetration through maize canopies.

3. 2. Performance of maize hybrids:

A significant difference among four yellow maize hybrids *i.e.*SC 3084, SC 3062, SC 2055 and SC 2066 on number of ear/plant, ear length, ear diameter, number of rows/ear, number of grains/row, ear grains weight, shelling percentage, 100-grain weight and grain yield/ha in both seasons as shown in Tables 2 and 3. The results showed that highest ear length, ear diameter, grains weight/ear, shelling percentage, 100-grain weight and grain yield/ha. S.C 2055 hybrid was recorded the greatest value number of rows/ear. However, S.C 2066 hybrids recorded the highest number grains/row, the lowest ear length, ear diameter, grains weight/ear, shelling percentage and 100-grain weight. S.C 3062 hybrid was recorded the lowest values of grain yield in both seasons. While, S.C 3084 hybrids recorded the lowest number of ear/plant and number grains/row.

3.3. Effect of hill spacing:

Concerning to the effect of hill spacing (15, 20, 25 and 30cm hill spacing apart) on number of ear/plant, ear length, ear diameter, number of rows/ear, number of grains/row, ear grains weight, shelling percentage, 100-grain weight and grain yield/ha, the results in Tables 3 and 2 clearly indicated that hill spacing significantly affected these traits in both seasons. Sown maize plants in hills 30 cm apart produced the greatest numbers of ears/plant and thick ears, highest grains weight/ear, shelling percentage and 100- grain weight. However, sown maize plants at hill spacing of 25 cm apart produced tallest ears. On the other side, sown maize plants at 15 cm apart produced the greatest number of rows/ear, the number grains/row and highest grain yield/ha.

3.4. Interaction effects:

Results in Tables 2 and 3 indicated that there was no significant interaction between maize hybrids and row width on number of ear/plant, ear length, ear diameter, number of rows, number of grain/rows and grain weight/ear. However, the effective interaction between maize hybrids and row width on the 100 grain weight and grain yield/ha significant effected on these traits in both seasons. The interaction between maize hybrids and row width on ear diameter the highest weights of 100 grain weight and grain yield/ha were produced from sown S.C.3084 at 70 and 60 cm, respectively as shown in Figs. 1 and 2. Results in Tables 2 and 3 indicated that there was no significant the interaction between maize hybrids and hill spacing of number of ear/plant, ear length, ear diameter, number of rows, number of grains/rows and grain weight/ear. However, the statistical analysis showed a significant interaction between maize hybrids and hill spacing on the 100 grain weight and grain yield t/ha. The results showed that highest interaction of 100 grain weight from S.C. 3084 at 30cm apart as graphically shown in Fig. 3. Highest grain yield/ha from sown S.C. 3084 in 20 cm hill spacing as illustrated in Fig. 4. Results in Tables 2 and 3 indicated that there was insignificant of the interaction between row width and hill spacing of number of ear/plant, ear length, ear diameter, number of rows, number of grains/rows and grain weight/ear. The results showed that highest interaction between row width and hill spacing on 100 grain weight was obtained from sown at 70 cm row width and 30 cm hill spacing as

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shown in Fig. 5. Highest grain yield/ha was produced from sown at 70 cm row width and 20 cm hill spacing as illustrated in Fig. 6. Concerning the third interaction among three studied factors, *i.e.* maize hybrids, row width and hill spacing, in significantly affected on all studied characters in both seasons.

IV. DISCUSSION

The results revealed a significant difference in both seasons due to row width. The increases in those yield components contributed to the higher productivity presented by narrowing sown maize. Therefore, the larger availability of solar radiation probably allowed plants to set more grains per ear and to produce heavier grains. These results in good accordance with those reported by [2,17,20,21,27]. The difference among four yellow maize hybrids *i.e.* SC 3084, SC 3062, SC 2055 and SC 2066 on number of ear/plant, ear length, ear diameter, number of rows/ear, number of grains/row, ear grains weight, shelling percentage, 100-grain weight and grain yield/ha**.** The differences in yield and yield components due maize hybrids may be due to the genetic factors. These results in good agreement with those reported by [2,4,11,12,13,14,16,27,]. Hill spacing significantly affected number of ear/plant, ear length, ear diameter, number of rows/ear, number of grains/row, ear grains weight, shelling percentage, 100-grain weight and grain yield/ha. The increases in grain yield when plants were sown at lowest hill spacing (15 cm) may be due to increase in number of rows/ear and number of grains/ear. These results in good agreement with those reported [4,26]. This may be due to more approach uniformity by sown at 15 cm hill spacing. Therefore, the higher yields obtained with the use of narrow spacing cannot be attributed to a different pattern of leaf area development or a larger leaf surface area to intercept solar radiation. A similar conclusion was reported by those reported by [1,24]. The effective interaction between maize hybrids and row width on the 100 grain weight and grain yield/ha significant effected on these traits in both seasons. There were varietal differences in response to intra-row spacing. Grain yield is the product of crop dry matter accumulation and the proportion of the dry matter allocated to the grain and harvest index in corn declines when plant density increases above the critical plant density. Highest grain yield/ha from sown S.C. 3084 at narrow row width (60 Cm) in 20 cm hill spacing i.e. 59.999 plants/ha reduced competition between, which will be more approached to uniformity which helps sun radiation penetration within plants then increase net photosynthesis, consequently increase grain yield per unite area.

V. CONCLUSION

It could be concluded that sown S.C. 3084 hybrid at 60 cm row width and hill spacing of 20 cm apart maximized maize productivity under the environmental conditions of Dakahlia Governorate, Egypt.

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Table.1: Different plant population densities due to row width and hill spacing.

Row width	Hill spacing	Plant populations densities
60 cm	15 cm	111.999 Plant/ha
60 cm	20 cm	84.000 Plant/ha
60 cm	25 cm	67.200 Plant/ha
60 cm	30 cm	59.999 Plant/ha
70 cm	15 cm	96.000 Plant/ha
70 cm	20 cm	72.000 Plant/ha
70 cm	25 cm	57.600 Plant/ha
70 cm	30 cm	48.000 Plant/ha

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Table.2: Number of ear/plant, number of plants at harvest, ear length, ear diameter and number of rows/ear as affected by maize hybrids, row width and hill spacing as well as their interactions during 2014 and 2015 seasons.

Characters / Treatments	Number of ear/plant		Ear length (Cm)		Ear diameter (Cm)		Number of rows/ear	
	2014	2015	2014	2015	2014	2015	2014	2015
A- Row width:								
60 cm	2.14	2.14	24.89	24.79	4.21	4.17	15.81	15.81
70 cm	2.17	2.25	25.07	24.89	4.27	4.25	16.20	15.87
F. Test	NS	*	*	NS	NS	*	*	NS
B- Maize Hybrids:								
SC 3084	2.12	2.06	26.18	26.48	4.50	4.50	15.53	15.56
SC 3062	2.00	2.15	23.62	22.90	4.30	4.26	15.37	15.37
SC 2055	2.25	2.56	25.09	24.89	4.05	4.04	16.68	16.31
SC 2066	2.25	2.00	25.03	25.09	4.10	4.04	16.43	16.12
F. Test	*	*	*	*	*	*	*	*
LSD at 5 %	0.22	0.18	0.54	0.36	0.11	0.08	0.57	0.40
C- Hill spacing:								
15 cm apart	1.90	2.06	24.23	24.32	4.22	4.23	16.00	16.12
20 cm apart	2.31	2.09	25.18	24.85	4.21	4.16	16.03	15.81
25 cm apart	2.21	2.28	25.29	25.12	4.22	4.19	16.06	15.75
30 cm apart	2.18	2.34	25.21	25.05	4.30	4.26	15.93	15.68
F. Test	*	*	*	*	*	*	NS	*
LSD at 5 %	0.21	0.19	0.33	0.30	0.06	0.06	-	0.34
D- Interactions F-Test:								
A × B	NS	NS	*	NS	*	NS	NS	NS
A × C	NS	NS	NS	NS	*	NS	NS	NS
B × C	NS	NS	NS	*	NS	*	NS	*
A × B × C	NS	NS	NS	*	NS	*	NS	NS

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Table.3: Number grains/row, grains weight/ear, shelling, 100-grain weight and grain yield/fed as affected by maize hybrids, row width and hill spacing as well as their interactions during 2014 and 2015 seasons.

Characters / Treatments	Number of ear/plant		Ear length (Cm)		Ear diameter (Cm)		Number of rows/ear	
	2014	2015	2014	2015	2014	2015	2014	2015
A- Row width:								
60 cm	50.37	50.12	294.2	288.8	87.98	85.11	40.07	39.76
70 cm	50.62	49.89	295.0	293.4	88.13	87.97	41.42	41.17
F. test	NS	NS	NS	NS	*	*	*	*
B- Maize Hybrids:								
SC 3084	51.31	51.31	322.0	321.5	88.73	87.94	45.59	45.62
SC 3062	47.09	47.00	290.1	281.0	87.76	87.65	43.34	42.81
SC 2055	51.71	51.31	284.2	281.8	88.17	83.22	36.40	35.93
SC 2066	51.87	50.40	282.1	280.0	87.55	87.35	37.65	37.50
F. test	*	*	*	*	*	*	*	*
LSD at 5 %	0.76	0.68	7.9	9.1	0.42	0.47	1.32	1.17
C- Hill spacing:								
15 cm apart	50.71	50.56	289.8	280.0	87.64	86.01	39.87	38.90
20 cm apart	50.62	49.71	291.2	287.9	88.06	83.83	40.46	40.31
25 cm apart	50.34	49.68	291.2	291.7	88.03	87.88	40.59	40.15
30 cm apart	50.31	50.06	306.2	304.8	88.48	88.43	42.06	42.50
F. Test	NS	*	*	*	*	*	*	*
LSD at 5 %	-	0.51	5.4	6.8	0.39	0.58	1.05	0.95
D- Interactions F-Test:								
A × B	NS	NS	*	NS	NS	*	*	*
B × C	*	NS	NS	*	NS	*	*	*
B × C	NS	*	NS	*	NS	*	*	*
A × B × C	*	NS	*	NS	*	NS	NS	*

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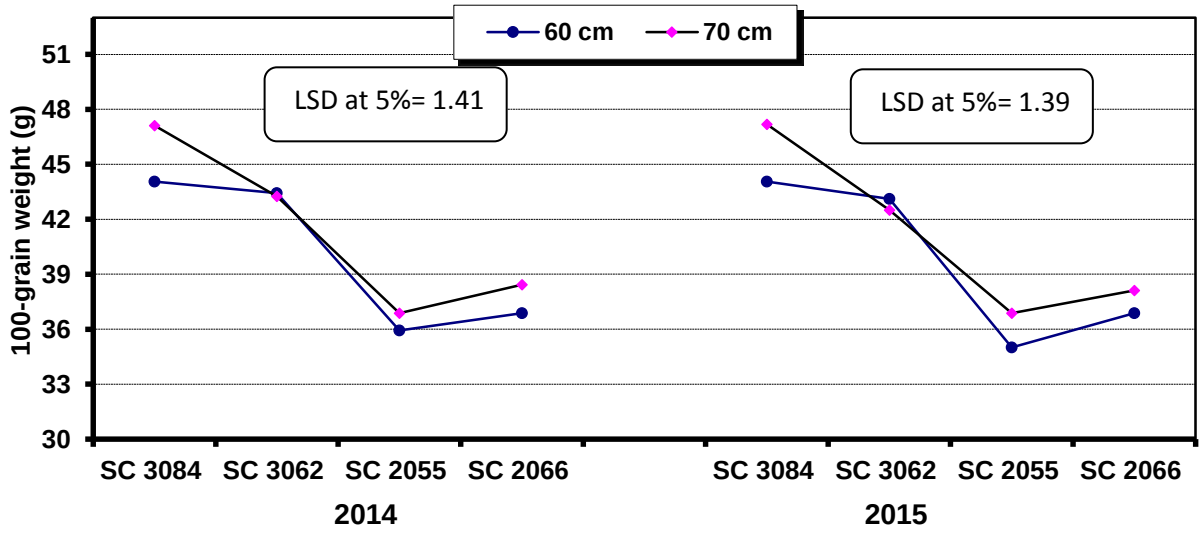

Fig.1: 100-grain weight (g) as affected by the interaction between maize hybrids and row width during 2014 and 2015 seasons.

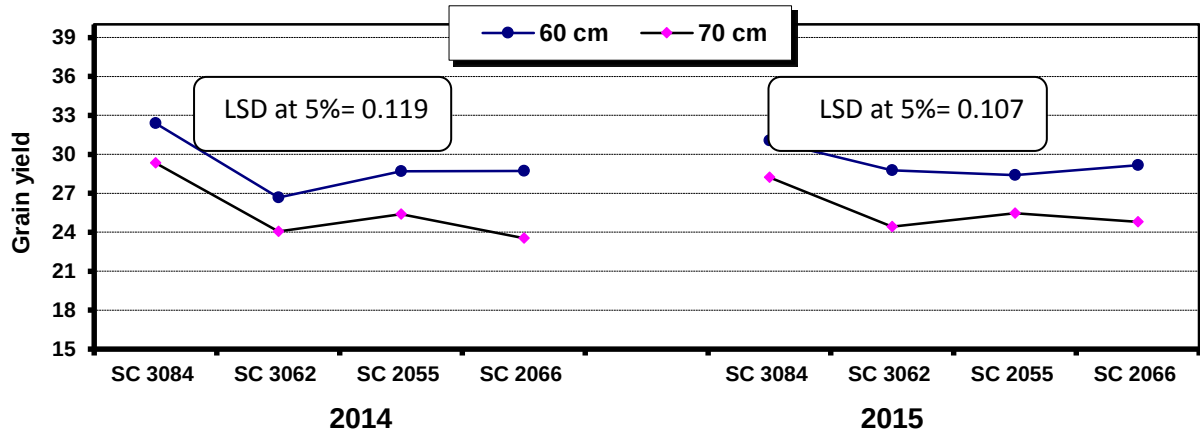

Fig. 2: Grain yield/ha as affected by the interaction between maize hybrids and row width during 2014 and 2015 seasons.

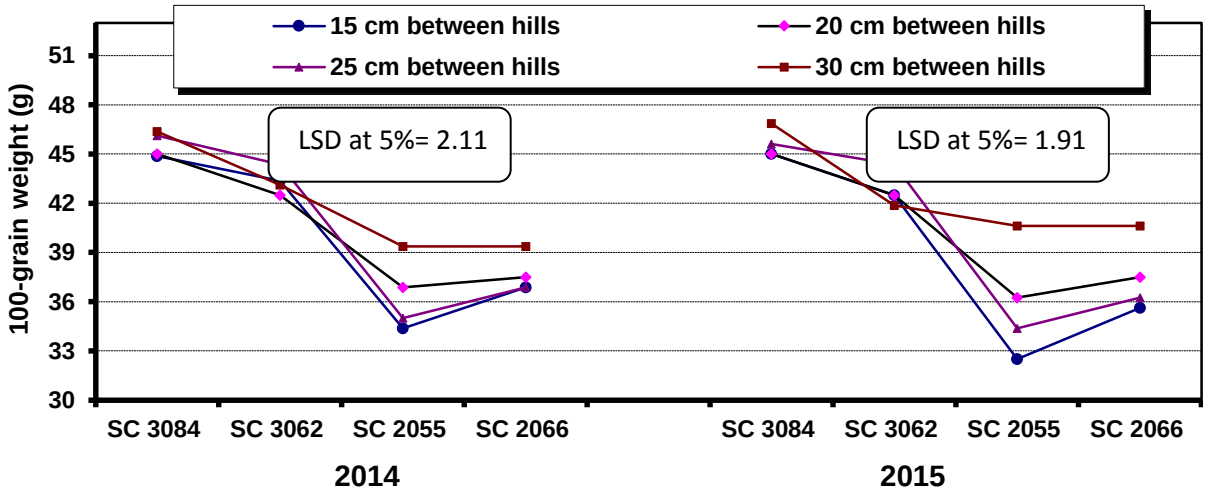

Fig.3: 100-grain weight (g) as affected by the interaction between maize hybrids and hill spacing during 2014 and 2015 seasons.

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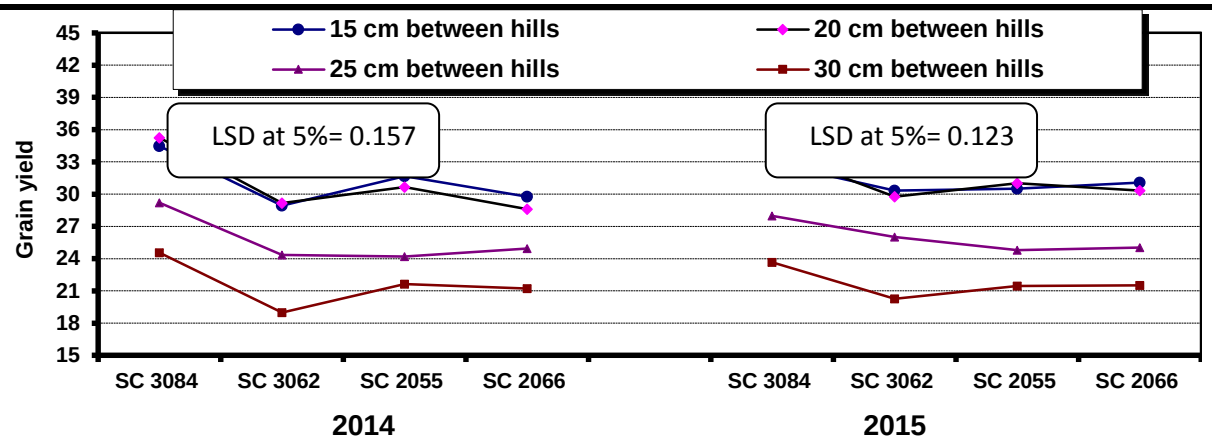

Fig.4: Grain yield/ha as affected by the interaction between maize hybrids and hill spacing during 2014 and 2015 seasons.

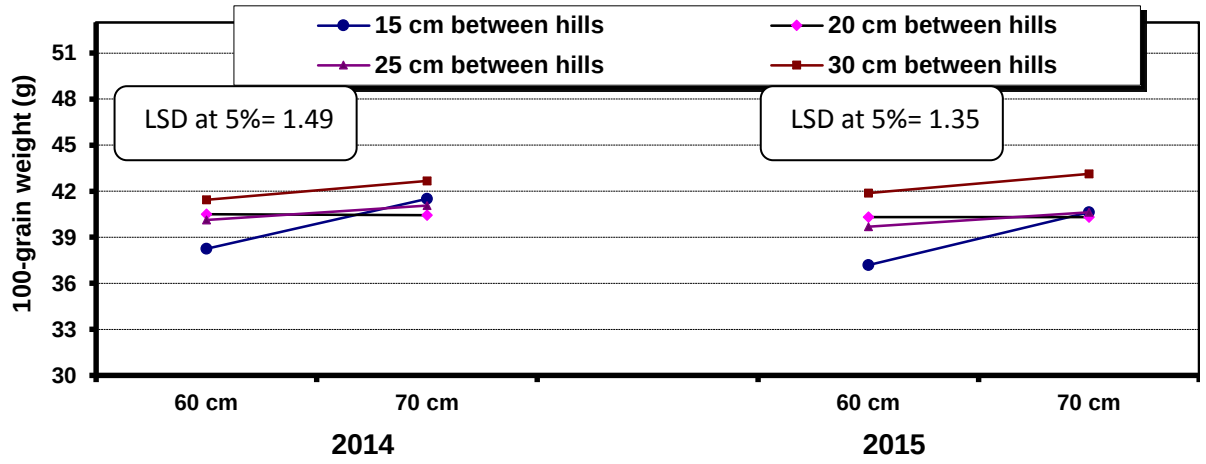

Fig. 5: 100-grain weight (g) as affected by the interaction between row width and hill spacing during 2014 and 2015 seasons.

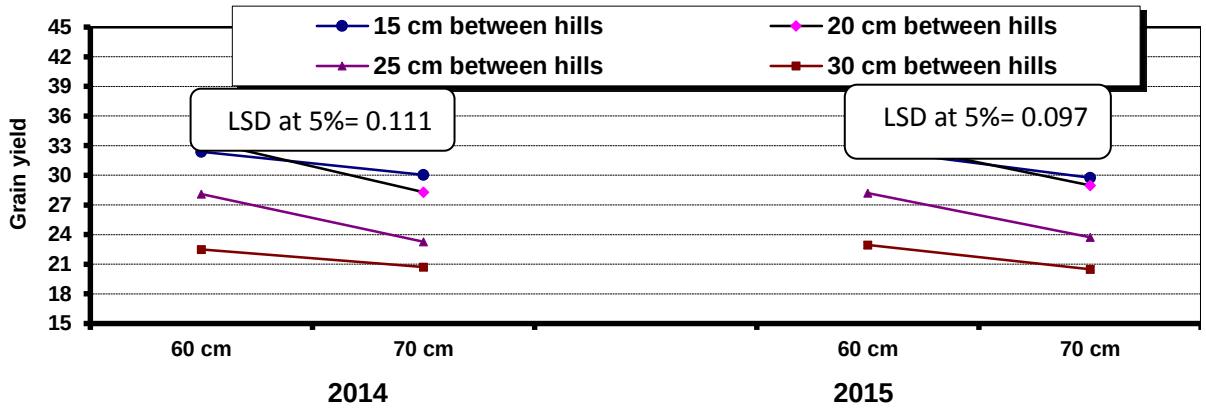

Fig.6: Grain yield/ha as affected by the interaction between row width and hill spacing during 2014 and 2015 seasons.

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Performance of canola (*Brassica napus* l.) genotypes under drought stress

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***Abstract*— *Drought is a wide spread problem seriously influencing rapeseed (Brassica napus L.) production, mostly in dryland regions. To investigate the effects of water deficit on some canola (Brassica napus L.) genotypes. Four drought treatments i.e. 4800m^3/ha, 3840m^3/ha, 2880 m^3/ha and 1920 m^3/ha on yield and yield components of six canola genotypes i.e. Serw 4, Serw 10, Pactol, Line 51. Two field experiments were conducted during 2014/2015 and 2015/2016seasons. Results revealed that irrigation using 3840 m^3/ha at four times came in the second rank for all studied parameters It increased above aforementioned traits using 1920 m^3/ha as two times by 9.4, 26.2, 40.5, 45.6, 46.0,54.4, 20.5, 25.8 and 58.3%, respectively comparing by irrigation using 1920 m^3/ha in two times as average of both seasons. Whereas, sown Serw 4 cultivar surpassed Serw 10 cultivar in plant height, No. of branches/plant, No. of silica/plant, seed weight/plant, seed, oil and protein yield/ha by 3.0, 21.8, 30, 21.6, 33.9, 26.7 and 37.9%, respectively as average in both seasons. It could be recommended that irrigation five times by 4800 m^3/ha of Serw 4 cultivar significantly maximized seed, oil protein yield/ha.*

Keywords*— *Brassica napus L., genotypes, drought treatments, seed and oil yield.

I. INTRODUCTION

Increasing plant productivity is one of the main targets of the Ministry of Agriculture in Egypt. This could be achieved through the suitable agricultural practices, i.e. using promising cultivars under different irrigation water regimes. Canola cultivation in Egypt may deliver an opportunity to overcome the shortage of edible oil production in Egypt. Drought tolerance consists of ability of crop for growth and production under water deficit conditions. A long term drought stress effects on plant metabolic reactions associates with, plant growth stage, water storage capacity of the soil and physiological aspects of plant. Canola is one of the most important oil crops in the world [1].The agricultural use of water in the world is more than 85% of total water use, moderate to severe intermittent or terminal drought is a common occurrence, and dry most crops cannot be grown without supplemental irrigation [2]. Water deficits in plants may lead to physiological disorders, such as a reduction in photosynthesis and transpiration [3]. Under drought stress in plant growth is affected by a number of morph-physiological disorders that cause reduction in nutrient uptake and impaired active transport of photosynthesis [4]. It has been observed that seed yield can be hampered, even by short period of soil moisture stress during reproductive stages [5]. Shortage of good quality water limits the production of agricultural crops to varying degree throughout the world, particularly in arid and semi-arid regions [6].The canola cultivars showed a variable response to drought stress and variation mainly depended on the cultivar, growth stage and the plant's ability to tolerate drought stress [7]. Research on drought tolerance in rapeseed is limited and mostly based on a few genotypes [8]. Water deficit during reproductive growth was more than that during vegetative growth of canola [9]. Oil yield was affected by water stress and it was dramatically decreased. Highest seed yield was obtained from GKH1103 cultivars under the conditions of full irrigation. The reproductive growth stage was found to be more sensitive to spells of drought stress than other growth stages [10]. The generated information suggested that managing water supply at reproductive stage to reduce yield losses in canola under the environments with low moisture availability [11]. Therefore, the objectives of this investigation were aimed to explore the educating growth and productivity of canola by using different cultivars at various irrigation water regimes under the reclaimed soils.

II. MATERIALS AND METHODS

2.1. *Research time and location*:

Two field experiments were conducted out at the experimental Station Farm of El-Serw Agricultural Research Station of the Agricultural Research Center, during the two successive winter seasons of 2014/2015

and 2015/2016 to study the performance of canola genotypes to irrigation treatments under newly reclaimed saline soil conditions. Two experiments were designed with a strip plot design in a RCBD with four replications. Each experiment included sixty treatments comprising, four canola genotypes and four irrigation treatments. The horizontal-plots were included the following four irrigation treatments, i.e.1-Irrigation five times (I_1)by 400 m^3 for each (4800m^3/ha).2-Irrigation four times (I_2)by 400 m^3 for each (3840m^3/ha).3-Irrigation three times (I_3)by 400 m^3 for each (2880 m^3/ha).4-Irrigation two times (I_4)by 400 m^3 for each (1920 m^3/ha).The vertical-plots were included the four canola genotypes i.e.1-Serw 4: Egyptian cultivar was produced via anther culture as mid early flowering.2-Pactol: A mid flowering, French cultivar introduced to Egypt by Agriculture Research Center, ARE.3-Serw 10: Local line mid flowering, was produced by Field Crop Institute, Agriculture Research Center, ARE.4-Line 51: Local line late flowering, was produced by Field Crop Institute, Agriculture Research Center, ARE.A plastic strip, sheet between horizontal stripes was made to insulate between the experimental units. Seeds of the studied cultivars were obtained from Oil Research Section, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt. Each experimental unit included five ridges 60 m apart and 3.5 m long occupying an area of 10.5 m^2. The soil in the preceding crop was sunflower in both seasons. The soil of experimental site was characterized as saline loamy clay soil, PH was 7.8 and 7.7, E.C. dS/m^{-1} was 4.6 and 4.8, Organic matter was 1.28 and 1.31%, available nitrogen was 14.9 and 17.8 ppm and available phosphorus was 41.8 and 39.6 ppm, which mechanical and chemical properties according to [12,13].

2.2. ***Agricultural practices:***

The experimental field was well prepared through two ploughings, compaction, division and then divided into the experimental units with dimensions as previously mentioned. Calcium super phosphate (15.5 % P_2O_5) was applied during soil preparation (after ploughing and before division) at the rate of 476 kg/ha. Potassium sulfate (48 % K_2O) at the rate of 178 kg/ha was applied during soil preparation. Nitrogen fertilizer in the form of ammonium nitrate (33.5 % N) was applied at the rate of 108 kg/ha was added in two equal portions before the first and second irrigation. Seed was sown in hills 15 cm apart on 20th and 25th of November for both seasons. The common agricultural practices for growing canola, according to the recommendations of the Ministry of Agriculture were followed, except the factors under study.

2.3. ***Studied Characters:***

At harvesting, the middle row was harvested randomly from each plot to estimate the following characters: 1-Number of days to 50% flowering (days):Number of days from sowing to 50% flowers/plot.2- Plant height (cm): It measured from the soil surface to the top of the main stem.3- Number of branches/plant: Its determined from average of five plants.4- Number of silica/plant: It was measured by counting the number of silica/plant from average of five plants.5- Seed weight/plant: It was estimated by weight seed of five plants.6-Oil Percentage: Oil content was determined according to[14]. 7-Crude protein percentage: Total nitrogen was estimated by the improved Kjeldahl method according to [14], modified by distilling the ammonia into saturated boric solution and titration in standard acid. The crude protein percentage was calculated by multiplying the total nitrogen values in canola flour by 5.75. 8-Seed yield/ha: It was calculated by weighting of two ridges and air dried, the seed at 15 % moisture were weighted and converted to kg/ha. 9- Oil yield kg/ha: multiplied with seed yield/ha to obtain protein and oil yields in kg/ha.10-Crude protein yield/ha: It calculated by multiplying the crude protein percentage then multiplied with seed yield/ha to obtain protein and oil yields in kg/ha.

2.4. ***Experimental analysis:***

All obtained data were statistically analyzed according to the technique of analysis of variance (ANOVA) for the strip - plot design as published by[15]by using MSTAT statistical package (MSTAT-C with MGRAPH version 2.10, Crop and Soil Sciences Department, Michigan State University, USA). Least Significant Difference (LSD) method was used to test the differences between treatment means at the 5 % level of probability as described by[16].

III. RESULTS AND DISCUSSION

3.1. ***Drought treatment effects:***

Results accessible in Tables 1 and 2 clearly designated that irrigation five times by 400 m^3 for each, *i.e.* 4800 m^3/ha, 960m^3/ha for each significantly affected No. of days from sowing to 50% flowering, plant height, No. of branches/plant, No. of silica/plant, seed weight/plant, oil and protein percentage, seed, oil protein yield/ha in both 2014/2015 and 2015/2016 seasons. Irrigation using 3840 m^3/ha at four times,960 m^3/ha for each came in the second rank for all studied parameters It increased above aforementioned traits using 1920 m^3/ha as two times960 m^3/ha by 9.4, 26.2, 40.5, 45.6, 46.0,54.4, 20.5, 25.8 and 58.3%, respectively comparing by irrigation using1920 m^3/ha in two times as average of both seasons. The results showed that increases in seed yield/ha due to irrigation five times using 4800 m^3/ha960 m^3/ha may be due to increases in yield attributes such as number of branches, silica and seed/plant as shown in Table (1). Regarding to increases in both oil and protein yields/ha due to irrigation five times using 4800 m^3/ha,960 m^3/ha, it's the fact that

these increases due to increases in seed yield/ha and both oil and protein percentages as shown in Table (2).Results revealed that reducing irrigation to two times by 400 m^3 for each, *i.e.* 1920 m^3/ha recorded the lowest values of No. of days from sowing to 50% flowering, plant height, number of branches, silica and seed/plant, oil and protein percentage, seed, oil protein yield/ha in both 2014/2015 and 2015/2016 seasons. Physiological growth indices were reduced under drought stress. This condition can be the most important environmental factor for the increase of total dry matter of control of irrigation [17]. A long term drought stress effects on plant metabolic reactions associates with, plant growth stage, water storage capacity of the soil and physiological aspects of plant. Canola is one of the most important oil crops in the world[1].The agricultural use of water in the world is more than 85% of total water use, moderate to severe intermittent or terminal drought is a common occurrence, and dry most crops cannot be grown without supplemental irrigation [2].Regularly, water deficit stress has detrimental effects on many processes in plants, which include reducing photosynthesis, accumulation of dry matter, stomatal exchanges, and protein synthesis that affects their growth stages[18,19].Grain yield showed high sensitivity to water deficit, proving that irrigation can definitely benefit crop grain yield [20]. Generally, plants respond to water deficit stress through developmental, biochemical and physiological changes and the type of the observed response depends on several factors such as stress intensity (SI), stress duration and genotype [21]. The stresses imposed at a later stage of development, reduce sink size, shorten the duration of seed filling and decrease the opportunity of crop to recover. Irrigation had more influence on seeds per pod than other yield components and water deficit influenced flowering to maturity stages more than other growth stages [5]. Water stressed conditions, those of rapeseed cultivars which were able to maintain their relative water content at high levels had a higher seed yield. Since water stress during seed development did effect on the sink size (seeds per plant), decreased source capacity led to reduction of seed weight [22].A similar result was reported by[3,4,5,6,23].

3.2. *Canola genotypes performance:*

Regarding to canola genotypes performance, the results existing in Tables 1 and 2 clearly showed that studied canola genotypes significantly differed in No. of days from sowing to 50% flowering, plant height, number of branches, silica and seed/plant, oil and protein percentage, seed, oil protein yield/ha in both 2014/2015 and 2015/2016 seasons. Sown Serw 4 cultivar surpassed studied canola genotypes in all above aforementioned traits followed by sown Line 51 and Serw 10 cultivar came in the last rank in both seasons. The results clearly showed that sown Serw 4 cultivar surpassed Serw 10 cultivar in plant height, No. of branches/plant, No. of silica/plant, seed weight/plant, seed, oil and protein yield/ha by 3.0, 21.8, 30, 21.6, 33.9, 26.7 and 37.9%, respectively as average in both seasons. The results displayed that Serw 4 cultivar recorded highest values in seed yield/ha may be due to increases in yield attributes such as number of branches, silica and seed/plant as shown in Table (1). Whereas, Serw 4 surpassed studied genotypes in both oil and protein yields/ha due to increases in seed yield/ha and both oil and protein percentages as shown in Table (2). Fido cultivar surpassed Tower in all traits under study which gave seed yield/fed by 12.05% as an average of both seasons [24]. Cultivators the L210 selected as the best cultivar for the normal condition and the L73 is the best cultivars in stress was started from the stem elongation stage and stress was started from flowering stage, also, the cultivar L183 is the best cultivars in stage of stress was started with pod formation [25]. Karaj3 and Talaye cultivars showed the highest seed yield in normal and stress conditions, respectively [26]. The canola cultivars showed a variable response to drought stress and variation mainly depended on the cultivar, growth stage and the plant's ability to tolerate drought stress [7]. Research on drought tolerance in rapeseed is limited and mostly based on a few genotypes [8]. The effect of water deficit during reproductive growth was more than that during vegetative growth of canola [9].The least reduction of seed yield in water deficit conditions has produced in Zarfam cultivar. Also, this cultivar had lower decreasing of oil yield in stress conditions and it has the best adaptation in water deficit conditions. These results may be due to the reduction of photosynthesis and chlorophyll content [27]. Oil yield was affected by water stress and it was dramatically decreased. Highest seed yield was obtained from GKH1103 cultivars under the conditions of full irrigation. The reproductive growth stage was found to be more sensitive to spells of drought stress than other growth stages [10]. The generated information suggested that managing water supply at reproductive stage to reduce yield losses in canola under the environments with low moisture availability [11].

3.3. *Interaction between drought treatments and studied genotype effects:*

Concerning to the interaction between drought treatments and studied canola genotypes, the results accessible in Tables 1 and 2 clearly indicated that this interaction insignificantly affected No. of days from sowing to 50% flowering, plant height, number of branches, silica and seed/plant, oil and protein percentage in both 2014/2015 and 2015/2016 seasons. Results graphically illustrated in Fig 1, 2, 3, 4, 5 and 6

showed that irrigation five times by 400 m^3 for each, *i.e.* 4800 m^3/ha of Serw 4 cultivar significantly increased seed, oil protein yield/ha in both 2014/2015 and 2015/2016 seasons. However, Serw 10 cultivar when irrigated with two times by 960 m^3/ha for each, *i.e.* 1920 m^3/ha recorded the lowest values of above aforementioned traits in both seasons. Water stress significantly limits plant growth and crop yield. Hence, the efficient management of soil moisture and the study of metabolic changes which occur in response to drought stress are important for agriculture. Cultivars differed significantly with respect to seed yield. Zarfam and Elvice cultivars under stress condition had the lowest seed yields. They suggested that, Zarfam and Elvice cultivars would be important for breeding programs designed for water-stress environments and in identifying drought-tolerant lines under arid and semi-arid conditions [28].The high oil yield and thousand grain weight were achieved by Okapi cultivar under control irrigation, highest grain yield and silique number per plant were obtained by Licord cultivar under control irrigation and highest grain number per silique was achieved by Zarfam cultivar under control irrigation and high drought tolerance index was exhibited by Licord cultivar [17].Reason of the grain yield reduction in different cultivars can be due to the level of used stress and its effect on some yield components such as pod per plant, seed per pods and the weight of thousand seeds [27]. The interaction between water deficit stress and type of cultivars affected yield, grain per pod, pod per plant and length pod. 'Hyola 308' and 'Sarigol' showed highest and lowest yields under stress conditions [29].

IV. CONCLUSION

It could be recommended that irrigation five times by 4800 m^3/ha,960 m^3/ha of Serw 4 cultivar significantly maximized seed, oil and protein yield/ha.

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Table.1: Mean of No. of days to 50% flowering, plant height, No. of branches/plant, No. of silica/plant and seed weight/plant as affected by irrigation treatments of some canola genotypes during 2014/2015 and 2015/2016 seasons.

Treatments	**No. of days to 50% flowering**		**Plant height (cm)**		**No. of branches/plant**		**No. of silica/plant**		**Seed weight/plant (g)**	
	2014/2015	*2015/2016*	*2014/2015*	*2015/2016*	*2014/2015*	*2015/2016*	*2014/2015*	*2015/2016*	*2014/2015*	*2015/2016*
A. Irrigation treatments:										
I_1:4800m^3/ha.	*86.8*	*93.7*	*175.2*	*178.0*	*11.2*	*12.5*	*993.8*	*1120.2*	*83.0*	*92.8*
I_2:3840m^3/ha.	*84.1*	*90.3*	*163.7*	*173.1*	*10.1*	*11.3*	*922.2*	*1050.3*	*74.0*	*82.0*
I_3:2880 m^3/ha.	*81.6*	*87.8*	*151.8*	*162.5*	*9.1*	*10.3*	*741.2*	*876.9*	*55.5*	*62.3*
$I_4$1920 m^3/ha.	*79.8*	*83.7*	*138.1*	*146.8*	*6.7*	*7.4*	*514.3*	*633.9*	*45.3*	*49.6*
F-test	*	*	*	*	*	*	*	*	*	*
L.S.D. 5%	*1.5*	*1.0*	*1.9*	*3.0*	*0.8*	*1.5*	*10.2*	*9.0*	*2.3*	*3.4*
B. Canola genotypes:										
Serw 4	*84.1*	*86.2*	*156.5*	*166.5*	*10.8*	*12.1*	*958.2*	*1163.9*	*77.5*	*82.1*
Pactol	*83.2*	*89.5*	*157.1*	*161.2*	*9.2*	*10.0*	*726.8*	*800.6*	*62.4*	*69.5*
Serw 10	*79.8*	*89.5*	*152.8*	*160.5*	*8.8*	*9.1*	*716.9*	*768.3*	*60.0*	*65.1*
Line 51	*85.1*	*90.3*	*163.0*	*172.1*	*9.4*	*10.3*	*869.6*	*948.5*	*64.9*	*70.0*
F-test	*	*	*	*	*	*	*	*	*	*
L.S.D. 5%	*1.9*	*1.8*	*1.7*	*2.9*	*0.9*	*1.4*	*8.1*	*12.8*	*1.4*	*3.0*
Interaction AXB										
F-test	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*	*N.S.*

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Table.2: Mean of seed yield t/ha, oil and protein percentage and oil and protein yield kg/ha as affected by irrigation treatments of some canola genotypes during 2014/2015 and 2015/2016 seasons.

Treatments	Seed yield t/ha		Oil%		Protein%		Oil yield kg/ha		Protein yield kg/ha	
	2014/2015	2015/2016	2014/2015	2015/2016	2014/2015	2015/2016	2014/2015	2015/2016	2014/2015	2015/2016
A. Irrigation treatments:										
I_1:4800m^3/ha.	2.329	2.551	43.1	43.5	37.9	37.9	1003.9	1110.8	882.7	964.1
I_2:3840m^3/ha.	2.062	2.115	42.5	42.8	37.6	37.6	876.0	905.0	774.9	799.2
I_3:2880 m^3/ha.	1.561	1.650	41.6	42.0	33.5	33.1	649.2	691.9	530.4	550.5
$I_4$1920 m^3/ha.	1.082	1.132	39.2	39.6	28.2	28.0	431.3	448.5	306.0	318.0
F-test	*	*	*	*	*	*	*	*	*	*
L.S.D. 5%	0.018	0.027	0.7	0.6	0.7	0.7	11.7	15.3	10.6	13.4
B. Canola genotypes:										
Serw 4	2.051	2.248	41.6	41.7	35.0	35.1	709.4	946.5	718.3	814.1
Pactol	1.685	1.854	41.6	41.8	34.2	34.1	700.5	785.7	576.0	655.2
Serw 10	1.381	1.456	42.2	42.9	33.5	33.1	582.5	629.7	462.5	488.8
Line 51	1.726	1.892	41.1	41.4	34.3	34.4	612.9	796.8	593.8	673.4
F-test	*	*	*	*	*	*	*	*	*	*
L.S.D. 5%	0.017	0.023	0.7	0.6	0.9	0.8	11.2	18.7	10.6	17.2
Interaction AXB										
F-test	*	*	N.S.	N.S.	N.S.	N.S.	*	*	*	*

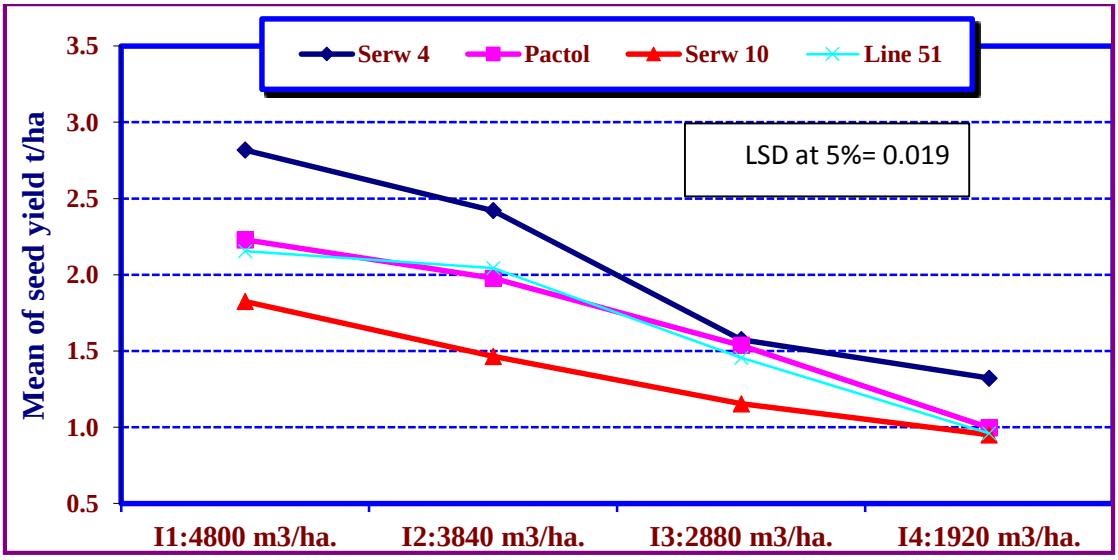

Fig..1: Mean of seed yield t/haas affected by irrigation treatments and canola genotypes during 2014/2015 seasons.

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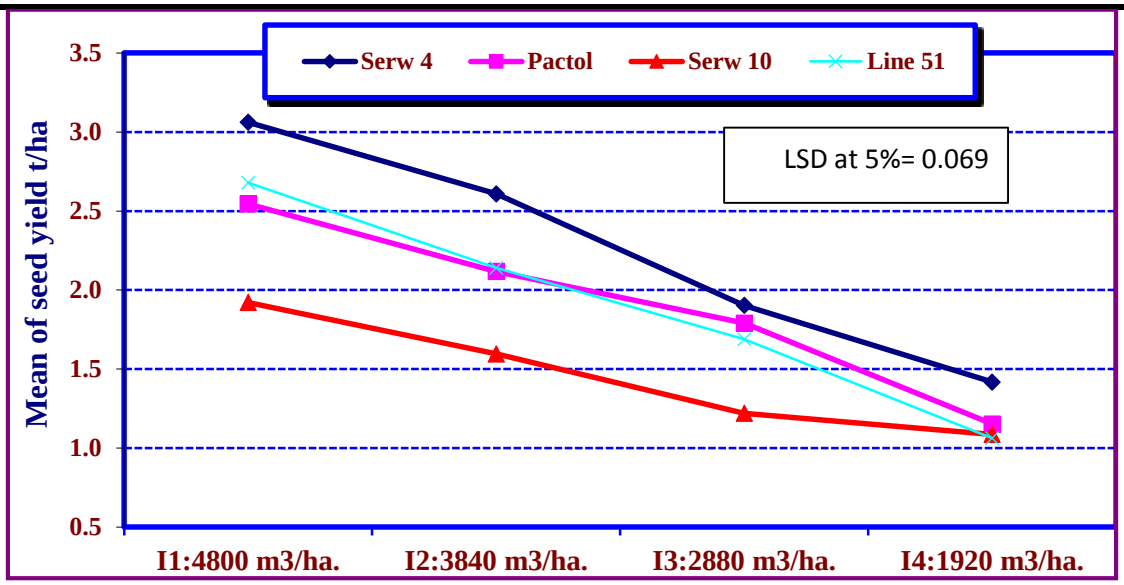

Fig. 2: Mean of seed yield t/haas affected by irrigation treatments and canola genotypes during 2015/2016 seasons.

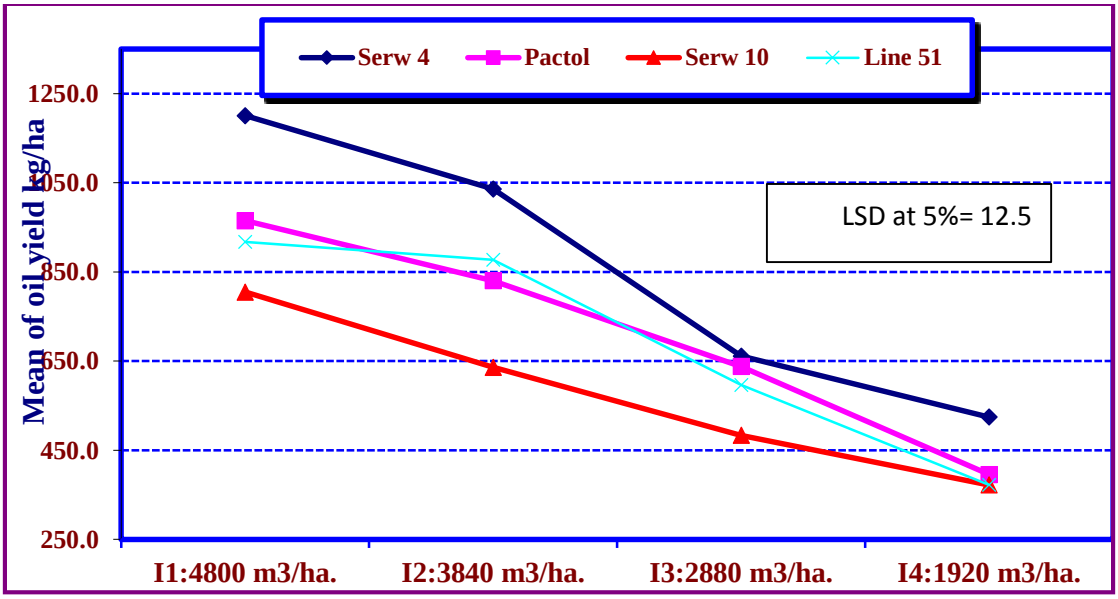

Fig. 3: Mean of oil yield kg/haas affected by irrigation treatments and canola genotypes during 2014/2015 seasons.

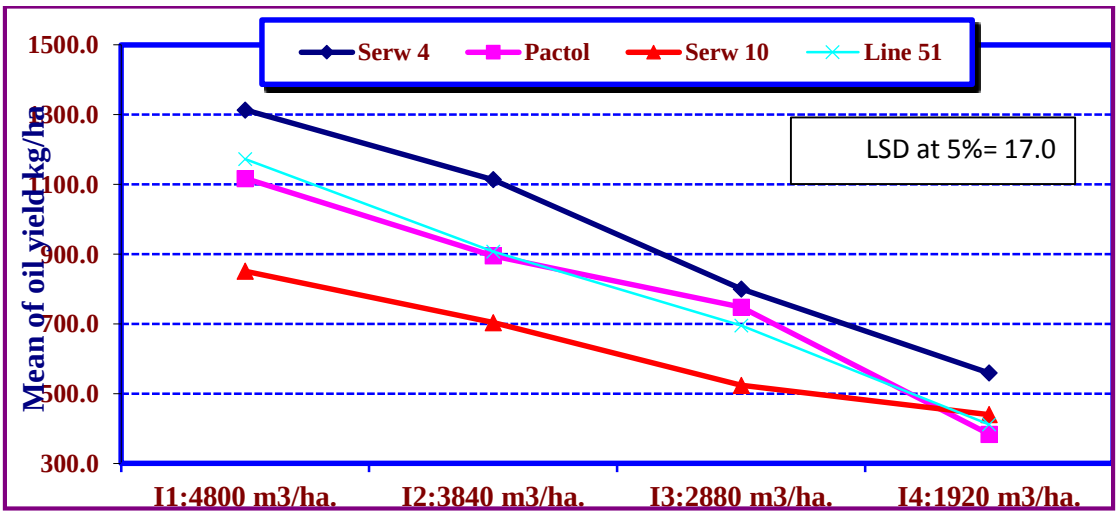

Fig. 4: Mean of oil yield kg/haas affected by irrigation treatments and canola genotypes during 2015/2016 seasons

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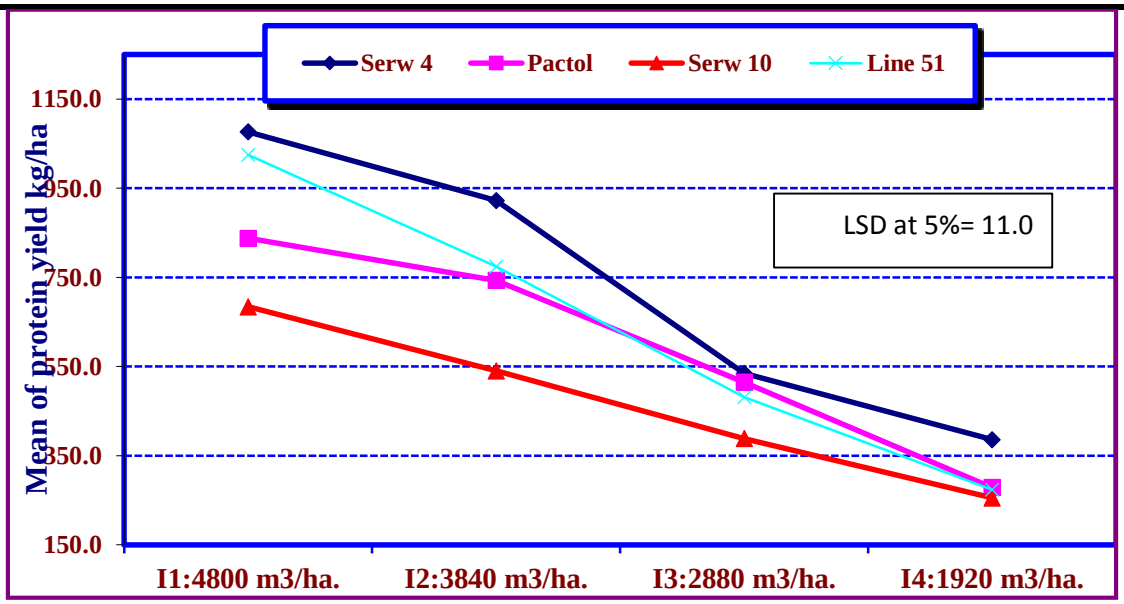

Fig. 5: Mean of protein yield kg/haas affected by irrigation treatments and canola genotypes during 2014/2015 seasons.

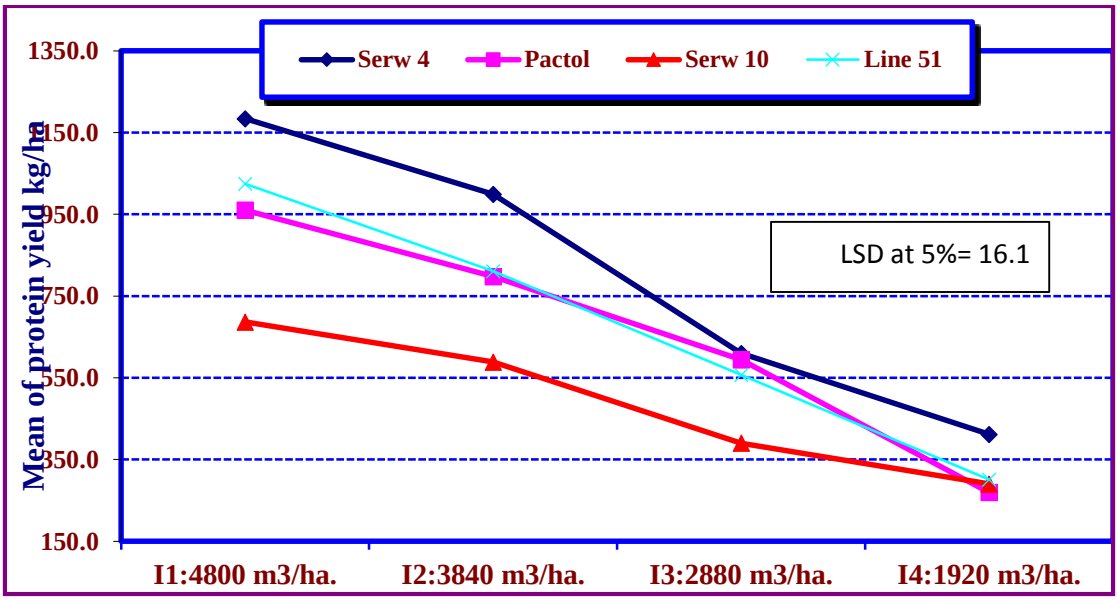

Fig. 6: Mean of protein yield kg/haas affected by irrigation treatments and canola genotypes during 2015/2016 seasons.

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Micro-propagation of *Alstroemeria Hybrida* Cv. Pluto

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***Abstract*—** *The experiment entitled micropropagation of Alstroemeria hybrida cv. Pluto was conducted to standardize protocol for aseptic establishment, callus induction, proliferation, and rooting from rhizome tips, rhizome sections, shoot tips, shoot nodal segments and inflorescence buds. Highest culture asepsis of 79.20 per cent at 2 weeks of culture and 68.08 per cent at 4 weeks of culture was recorded in rhizome tips following sterilization treatment with Carbendazim 200 ppm for 30 minutes + $HgCl_2$ (0.1 %) dip for 10 minutes and final treatment with ethyl alcohol (70 %) for 1 minute. Rhizome tips and rhizome section explants survived sterilant treatment better than other explants. MS-liquid medium supplemented with BAP + IBA: 1.5 + 0.2 mg l^{-1} proved best for culture establishment (89.42 %) in case of rhizome tips and (56.13 %) in case of rhizome sections. MS-solid medium with plant growth regulator combinations BAP + IBA: 1.0 + 0.2 mg l^{-1} fortified with activated charcoal resulted in an establishment of (78.25 %) in rhizome tips and (40.24 %) in case of rhizome sections. Callus induction was highest in MS-solid medium fortified with BAP + NAA: 0.5 + 4.5 mg l^{-1}. Rhizome tips cultured on MS-medium BAP + IBA + GA_3 + Activated charcoal: 2.0 + 0.4 + 0.5 + 1000 mg l^{-1} gave highest proliferation (88.85 %) along with highest number of erect shoots (5.75) , number of new rhizome buds (3.75), rhizome fresh weight/shoot complex (6.05), and multiplication index (2.76). Highest Rooting (54.81 %) along with lowest days to appearance of root (10.87), highest number of roots (3.12) and highest root length (16.42 mm) was recorded in MS-liquid medium fortified with NAA 1.5 mg l^{-1}.*

Abbreviations used— AC; Activated charcoal, BAP; 6-Benzyl amino purine, BA; 6-Benzyladenine, 2, 4-D; 2, 4dichloro-phenoxyacetic acid,GA_3; Gibberelic acid, IAA; Indole-3-acetic acid, IBA; Indole-3-butyric acid, MS; Murashige and Skoog's (1962) medium, NAA; Naphthalene acetic acid and µm; Micro molar.

***Keywords*— Tissue culture, micropropagation, Alstroemeria, growth regulators.**

I. INTRODUCTION

Alstroemeria also known as Inca lily, lily of Incas or Peruvian lily, is a rhizomatous monocot belonging to the family *Alstroemeriaceae*. *Alstroemeria* hybrids are very popular cut flowers occupying position within top ten cut flowers of the world due to diversity of colours, low energy requirement and longer vase life. *Alstroemeria* are also grown as bedding or potted plants (Bridgen, 1997) and has a premium potential as a cut flower crop in temperate and sub temperate areas of the country especially of Himachal Pradesh, Jammu and Kashmir, Uttar Pradesh, Uttaranchal, West Bengal and Nillgiri hills of Tamil Nadu. The increase in production area and the introduction of new hybrids by breeding have necessitated development of efficient methods for cloning, since modern *Alstroemeria* hybrids are often sterile (triploids) and can only be propagated vegetatively. *Alstroemeria* are conventionally propagated vegetatively by rhizome division but it is inefficient, time consuming, requires large area for maintaining stock plants and contributes to the spread of viral diseases. Globally, the demand for clean healthy plant materials for agriculture, horticulture, forestry and ornamental industries is in excess of 16 trillion units per year, which equals US$ 4 trillion. For ornamentals, it is estimated that the global sale of cut flowers and pot plants is US$ 90 and 60 billion, respectively. The production of ornamentals by commercial micropropagation was in 1986, 130 million plants globally. Over one billion ornamentals are produced yearly through micropropagation (Prakash, 2009).

Alstroemeria plants consist of a sympodial fleshy, multi-stemmed rhizome from which shoots and fibrous roots arise. Some of the fibrous roots latter become thickened storage roots as the plants develop. The storage roots are called *'Radices Medullosae'*. The shoots can be either reproductive or vegetative depending upon the environmental conditions. *Alstroemeria* shoots produce a whorled cymose inflorescence. Each cyme is sympodially branched with up to five florets that open one after another. The sexual parts of the flowers are dichogamous. The

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stamens open first and shed pollen before the stigma becomes receptive. The flowers come in a variety of colours. Post-harvest life is terminated by petal drop or yellowing of the leaves or both.

There are several commercial cultivars of *Alstroemeria* available. Van Scheepen (1991) gave descriptions of species and the common ones include *A. aurea*, *A. brasiliensi*, *A. caryophyllaea*, *A. chiliensis*, *A. ligtu*, *A. pelegrina*, *A. psittacina* etc. Among the cultivated *Alstroemeria*, *Ligtu* hybrids (LH) have originated from a natural crossing between *A. ligtu* and *A. ligtu spp. Simsii* (Robinson, 1963). They are widely used for cut flowers in Japan. On the other hand *A. pelegrina* var. *rosea* (PR) is dwarf and has large flowers. The interspecific hybrids between LH and PR have also been produced (Ishikawa *et al.*, 1997).

Alstroemeria is a relatively recent introduction into the world's floriculture scene and has become a major cut flower. It is also used as a potted flowering plant for the home decoration and as an herbaceous landscape plant in the mild climates. Plants have routinely survived in the Netherlands and in Maryland (Zone-6) in the United States. Cultivars are patented and growers must sign agreements. Division is not legal unless authorized.

In India *Alstroemeria* is a recent introduction. The crop was introduced in 2001 by Ministry of Agriculture, GOI, under Food and Agriculture Organization programme at three Model Floriculture Centers in India-Ooty (Tamil Nadu), Chial (Himachal Pradesh) and Srinagar (Jammu & Kashmir). The crop was introduced in SKUAST-Kashmir, Shalimar in 2005-06 under ICAR sponsored Horticulture Mini Mission-I. Initial results in the poly house and open conditions were promising.

Attempts have been made by few workers to multiply Alstroemeria *in vitro* through rhizome tips (Hussey *et al*., 1979; Gabryszewska and Hempel, 1985; Lin and Monette, 1987; Chiari and Bridgen, 2000) but contamination was the major bottleneck to start the cultures. Alstroemeria can be started with apical shoot tips but there is also a strong dominance effect suppressing the growth of axillary buds (Bond and Alderson, 1993b).

Several components and combinations of compounds interfere with the mechanism of apical dominance, thereby releasing buds from their inhibited state like Tri-iodobenzoic acid, an anti-auxin (Niedergang and Skoog, 1956; Rubery, 1987) and Thidiazuron (TDZ), having a cytokinin like mode of action (Mok *et al*., 1982).

In vitro multiplication of elite plant genotype is the widely used commercial application of plant biotechnology and offers immense opportunities to multiply more number of disease free planting material in a shortest possible time (Lin *et al*., 2000).

Alstroemeria is generally propagated by rhizome division of the three year old plants. However, multiplication rate is low. Propagation through seed is not commonly practiced due to variability in hybrids and long and difficult germination. Legal planting material imported from foreign breeder companies is still very costly ranging from Rs. 500-600/plant. Cultivars introduced in late nineties in India are currently out of the patent regime. However, planting material is not easily available due to constraints imposed on multiplication rate under conventional propagation methods of rhizome division. This has been the single major stumbling block that has prevented *Alstroemeria* from becoming widely adopted by cut flower growers in Kashmir and Himachal Pradesh. There is a need to establish *in vitro* propagation protocols for the cultivars already available with research institutes and Government departments. This shall not only ensure availability of quality planting material for growers but also open up avenues for cultivar improvement through *in vitro* mutagenesis. Keeping in view the importance of *Alstroemeria* as a lower energy requirement cut flower crop for growers in Kashmir the current study was conducted with the following objectives:

i). Standardization of disinfection protocol for aseptic establishment.
ii). Identification of suitable explant for establishing *in vitro* cultures in *Alstroemeria*
iii). Standardization of media and growth regulator regimes for callus induction, callus proliferation and organogenesis if any.
iv). Development of protocol for multiplication and rooting of *Alstroemeria in vitro*.

II. REVIEW AT A GLANCE

Results obtained by different workers on *in vitro* propagation of *Alstroemeria* as well as other related crops with similar problems have been reviewed under the following sub-heads.

2.1 Explant
2.2 Surface sterilization of explant
2.3 Establishment and proliferation of cultures
2.4 Callus induction and regeneration
2.5 Rooting

2.1 Explant

2.1.1 Rhizome

Rhizomes of pale orange-coloured *Alstroemeria cv*. 'Caralis' were used as the source of explants by Amir *et al*. (2012). Pumisutapon *et al.* (2009) prepared four types of explants - an intact rhizome with two intact shoots

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(+R+2S), an intact rhizome with two decapitated shoots (+R-2S), a decapitated rhizome with two intact shoots (-R+2S), and a decapitated rhizome with two decapitated shoots (-R-2S), *in vitro* to study the apical dominance in *Alstroemeria*. Yousef *et al*. (2007) compared the regeneration ability of plantlets using *in vitro* and *in vivo* grown rhizome buds as explants that were cultured on MS basal medium with 3 different compositions of growth regulators (1, 0.2 mg l^{-1} NAA with 1 mg l^{-1} BA and 0.2 mg l^{-1} IAA with 1 mg l^{-1} BA) and the cultures were incubated in 18 + or -1 degrees C at 16 h photoperiod.

Elliott *et al*. (1993) established *Alstroemeria cv*. Parigro Pink rhizome tip explants. Gabryszewska (1995) successfully cultured rhizome apical and axillary tips cultured on Murashige and Skoog medium with BA at 2 mg l^{-1} and NAA at 0.5 mg l^{-1}.Hakkaart *et al.* (1988) used a technique for elimination of *Alstroemeria* mosaic virus from infected *Alstroemeria* cultivars in which meristems were excised from rhizome tips and placed on a medium containing indole-3-butyric acid at a cultivar dependent concentration. Pierik *et al.* (1988), used terminal and lateral tips from fleshy rhizomes that were isolated *in vitro* and induced to form new rhizomes.

Gabryszewska and Hempel (1985), Han *et al*. (1994), Chiari and Bridgen (1996, 2000) cultured rhizome pieces of Alstroemeria for *in vitro* plantlet regeneration. Lin and Monette (1987) regenerated plantlets from rhizome tips cultured on solid and liquid media based on Murashige and Skoog salt formulation. The quality of the cultures was superior when intact rather than longitudinally sliced rhizome tips were used as explants, and when a temperature of 8° rather than 22°C was used at the initiation stage.

Fig. 1 and Fig. 2. Rhizome explant of Alstroemeria hybrida Cv. Pluto used in this study.

2.1.2 Leaf

Khaleghi and Azadi (2011) selected vegetative explants (node, internode, and leaf) and employed embryogenic calluses for gene transformation in *Alstroemeria* and studied the embryogenic callus induction in *Alstroemeria* cv. Fuego. Lin *et al.* (1997), Jang *et al*. (1999), Lin *et al*. (2000) reported that leaves of Alstroemeria can be used as an explant to enhance the multiplication efficiency. Lin *et al*. (1998) induced direct shoot regeneration from leaf explants of *Alstroemeria* clone VV2406, a selection from a tetraploid breeding line. Explants contained a leaf blade and a small portion of stem node, which were cut from erect shoots of *in vitro*-multiplicated plantlets. Shoot regeneration capacity of the excised leaf explants was significantly related to the position of the explant on the stem. The youngest explant which was located closest to the shoot apex gave the highest response.

2.1.3 Inflorescence stem

Different types of explants have been employed to establish cultures. Ziv *et al.* (1973) used young actively growing tissue explants from *Alstroemeria* inflorescence stem taken at a distance of 1-2 mm below the apex that proved capable of regenerating buds and roots from which small plantlets could be established.

2.1.4 Somatic Embryos

Schaik *et al*. (1996) studied the plant regeneration ability of callus obtained from zygotic embryos of diploid *Alstroemeria* inodora and a tetraploid cultivar. The best explants for somatic embryogenesis were immature zygotic embryos in half-ovules when the endosperm was soft and white. Hutchinson *et al*. (1994) obtained callus when mature zygotic embryos were cultured on MS medium supplemented with 20 µM kinetin and 10 or 20 µM NAA. Callus that was transferred to MS medium supplemented with 20 µM kinetin and 20 µM NAA for long term culture, maintained a regeneration capacity of 40 per cent over an 8 month period. Gonzalez and Alderson (1992) cultured excised somatic *Alstroemeria cv.* Butterfly embryos on solid MS medium alone, or on MS medium supplemented with 0.1 mg BA l^{-1} + 10% (v/v) coconut water or 0.1 mg

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GA_3 l^{-1}. Cultures were incubated at 25 or 15°C and the number of embryoids which developed into single shoot plantlets was generally higher at 15 than 25°C. After 4 weeks of culture, the greatest percentage of cultures with single shoot plantlets (25%) was obtained from the medium supplemented with GA_3 and cultured at 15°C, but the greatest percentage of cultures with callus (75%) was obtained from the same medium at 25°C. Gonzalez and Alderson (1990) obtained a callus from mature embryos of cv. Butterfly on MS medium supplemented with either 2 or 4 mg picloram l^{-1} or 4 mg 2, 4-D l^{-1} combined with benzyladenine (BA) or kinetin (0-4 mg l^{-1}). Shoots regenerated and torpedo-like structures (somatic embryos) formed when callus was transferred to regeneration media containing BA plus picloram or 2, 4-D.

2.1.5 Root, stem segments, shoot tips, rhizome buds

Gonzalez and Alderson (1995) attempted Callus induction in *Alstroemeria* using explants from roots, stem segments, shoot tips and rhizome buds of *cv.* Carmen and mature embryos of *cv.* Butterfly which were cultured on MS basal medium supplemented with various concentrations of 2,4-D, picloram, NAA, kinetin, BA and GA_3. The best results were obtained with mature embryos and after 18 days in culture, callus was produced on 40, 34 and 32 per cent of embryos cultured on MS medium supplemented with 4 mg 2, 4-D l^{-1}, 2 mg picloram l^{-1} and 4 mg picloram l^{-1}, respectively.

2.1.6 Leaf, stem, rhizome, inflorescence apices

Pedraza-Santos *et al*. (2006) developed a protocol for the *in vitro* regeneration of *Alstroemeria cv.* 'Yellow King', using several explant sources (leaf, stem apices, rhizomes and immature inflorescence apices) and various temperature and light/dark regimes, hormone and salt concentrations and several hormone concentrations for shoot multiplication and rooting and found that only the young floral apices produced adventitious shoots by direct organogenesis. The highest shoot induction rate (10.4 shoots per explant) was obtained by incubation in the dark for 15 days at 8 °C followed by 15 days at 25 °C and a 16-h/8-h light/dark regime, on a Murashige and Skoog (1962) liquid medium at 50 per cent of the salt concentration, supplemented with 2.5 mg l^{-1} Kinetin, 1.5 mg l^{-1} BA and 1.0 mg l^{-1} NAA, using a piece of filter paper to support the explant. The highest shoot multiplication rate (9 shoots per explant) was obtained on a liquid MS medium at full strength supplemented only with BA at 1.0 mg l^{-1}.

2.1.7 Shoots

Fujita *et al*. (2010) observed high rhizome-formation ability but frequent contamination with soil microorganisms *in vitro* when apical meristem explant taken from underground shoots of 3-4 cm length that had sprouted from the rhizome. Apical meristem cut from vegetative shoots of 50-100 cm length had hardly any rhizome-formation ability, and these were not suitable as explants for micro propagation.

2.1.8 Leaf, Node and Internode

Seyyedyousefi *et al.* (2013) used segments of nodes and internodes of *Alstroemeria cv*. Fuego as explant that were cultured in MS basal medium with different concentrations of BAP (0.0 and 0.5 mg l^{-1}) and NAA (0.0, 1.0 and 2.0 mg l^{-1}) to produce callus.

Khaleghi and Azadi (2008) studied the plant regeneration ability of callus in the ornamental monocot *Alstroemeria cv*. Fuego. High frequency (23%) of compact callus induction was obtained on a Schenk and Hildebrandt (SH) medium supplemented with 2 mg l^{-1} picloram from nodal segments excised from plants grown in the greenhouse and also employed embryogenic calluses for gene transformation in *Alstroemeria* and studied the embryogenic callus induction in *Alstroemeria* cv. Fuego. The vegetative explants (node, internode, leaf) along with various concentrations of auxins (picloram, NAA, IAA, 2, 4-D) either with or without BAP were taken into experimentation. The nodal explants provided the highest embryogenic calluses. The internodal explants resulted in a lower embryogenic callus production than the nodal ones. The leaf explants did not prove suitable for callus induction. The highest induction rate of embryogenic calluses was obtained for ½ MS medium supplemented with 2 mg l^{-1} of NAA. As for regeneration, callus was transferred to regeneration medium supplemented with 2 mg l^{-1} of BAP (Khaleghi and Azadi, 2011).

Kim *et al*. (2006) obtained high frequencies of compact embryogenic callus (CEC) induction (~40%) and friable embryogenic callus (FEC) induction (~15%) in *Alstroemeria* from nodes with axil tissue cultured first on a Murashige and Skoog (MS) medium supplemented with 10 µM thidiazuron and 0.5 µM indole-3-butyric acid and after that on a Schenk and Hildebrandt (SH) medium supplemented with 9.1 µM 2, 4-dichlorophenoxy acetic acid and 2.2 µM benzylaminopurine (BA). Both types of callus were maintained on modified MS medium supplemented with 20.8 µM picloram. Regenerated plants were established in the greenhouse and flowered normally.

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2.1.9 Other Related Crops

Arimura *et al.* (2000) took basal portion of ginger as an explant and cultured on MS medium containing different concentration of NAA. De Him and De Paez (1998) used shoot tips of ginger for *in vitro* multiplication. Sharma and Singh (1995) advocated that shoot tips of ginger can be regenerated into plantlets. Devi and Nayar (1993) used shoot tips as explants excised from one and three months old suckers. Similarly, Malamug *et al.* (1991) while working with *ginger cv*. Kintoki used 1-2 mm shoot tip explants.

Hosoki and Sagawa (1997) took the rhizome buds of ginger and cultured on modified MS medium. Nathan *et al*. (1993) used axillary and terminal buds of Heliconia Psittacorum as a source of explant. Tissue blocks of approximately 1 cm size containing either the apical bud or axillary buds were excised from shoots and after surface sterilization further trimmed to approximately 3 mm size before culturing. In ginger (*Zingiber officinale* Rosa.) the newly emerging buds are the favourite explant for initiating the cultures. Meristem of size 0.1-0.5 mm were excised from pale yellow sprouted buds of ginger for establishment of cultures (Bhagyalakshmi and Singh, 1988). Nel (1985) established cultures with shoot-tips of ginger. Shetty *et al.* (1982) cultured single sprouting buds of Turmeric on modified MS medium containing sucrose (40 g l^{-1}) and kinetin (0.2-0.5 mgl^{-1}).

2.2 Surface sterilization of explant

Seyyedyousefi *et al*. (2013) also used fragments of stem containing node and internode and washed thoroughly under running tap water for 20 minutes and disinfected with 1.5 per cent NaOCl aqueous solution for 15 minutes. Sathyagowri and Seran (2011) also reported reasonable level of culture asepsis and survival with Carbendazim (0.3%) + Doxycycline (0.2%) for 10 minutes followed by 70% ethanol for 1 minute in ginger rhizome expants with buds. Jyothi *et al*. (2008) reported use of 1.0 per cent mercuric chloride for 8 minutes in ginger a plant with a underground rhizome similar in architecture as *Alstroemeria.*

Pedersen and Brandt (1992) developed a procedure for the disinfection of *Alstroemeria* rhizome tips based on scale leaves immersed for 1-10 min in 3 per cent Korsolin or 1 per cent NaOCl. Dekker's *et al*. (1991) used 1 per cent potassium hypochlorite for 20 minutes for the disinfection of axillary buds obtained from rhizomes (*Zingiber officinale, Curcuma amada, C. domestica*), aerial stem nodes (*Costus* spp.) and bulbils (*Alpinia parpurata*) arising from inflorescence.

Pierik (1987) identified four source of infection during *in vitro* propagation of *Alstroemeria* viz. the plant (internal as well as external), the nutrient medium (insufficiently sterilized), the air and the aseptic work. The rhizome tip explant regenerated profusely but contamination was found to be a major bottleneck. Since rhizome tips grow below the surface of soil and it was quite difficult to disinfect and also reported that prior to sterilization. *Alstroemeria* rhizomes are to be washed under tap water to remove soil etc. Later rhizome segments are to be surface sterilized initially in 70 per cent ethanol for 2-3 seconds followed by 20min dip in 1.5 NaOCl (with few drops of Tween-20) and rinsed thrice in sterile tap water (Pierik *et al*., 1988).

Lin and Monette (1987) obtained best results with the treatment of rhizome tips of *Alstroemeria cv.* 'Alsaan' with 0.6 per cent sodium hypochlorite plus 0.1 per cent Tween-20 for 20 minutes with continuous stirring followed by rinsing 3 times in sterile distilled water. Hakkart and Versluijs (1985) initially rinsed the rhizomes of *Alstroemeria cv's* 'Rosario', 'Toledo' and 'Jubilee' with tap water and later dipped for few seconds in 70 per cent alcohol. Final sterilization was done in a laminar flow cabinet with 5 per cent Ca-hypochlorite followed by rinsing 3 times with sterile water.

2.3 Establishment and proliferation of cultures

Pumisutapon *et al*. (2011) studied apical dominance in *Alstroemeria* and used rhizome as the standard explant with a tip and two vertically growing shoots from which the larger part had been excised leaving ca. 1 cm stem. The axillary buds that resumed growth were located at this 1-cm stem just above the rhizome. They were released by removal of the rhizome tip and the shoot tips. Replacement of excised tips by lanolin with indole-3-butyric acid (IBA) restored apical dominance. The auxin transport inhibitors 2, 3, 5-triiodobenzoic acid (TIBA) and N-1-napthylphthalamic acid (NPA) reduced apical dominance. 6-Benzylaminopurine (BAP) enhanced axillary bud outgrowth but the highest concentrations (>9 µM) caused fasciation.

Hutchinson *et al.* (2010) observed the effect of Thidiazuron, NAA, and BAP on *in vitro* propagation of *Alstroemeria aurantiaca cv.* Rustica from shoot tip explants. Successful micro-propagation of *Alstroemeria* in liquid medium using slow release of medium components was done by Klerk and Brugge (2010) in which *Alstroemeria* rhizomes were micro-propagated on semi-solid medium and in liquid medium. In liquid medium, growth was much enhanced (ca. 70%).

Fujita *et al*. (2010) observed high rhizome-formation ability but frequent contamination with soil microorganisms *in vitro* when apical meristems explant taken from

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underground shoots of 3-4 cm length that had sprouted from the rhizome and also the apical meristem cut from vegetative shoots of 50-100 cm length had hardly any rhizome-formation ability, and these were not suitable as explants for micropropagation. Apical meristems of floral shoots that sprouted in the field were cultured on MS medium containing 0.01 mM BAP, 0.001 mM NAA, and 3% sucrose and a large number of rhizome buds were propagated by subculturing on a medium containing 6% sucrose, 0.01 mM BAP, and 0.001 mM NAA. Rhizome buds were subcultured in a culture vessel with ½ N-MS medium containing 9% sucrose, 0.01 mM BAP, and 0.001 mM NAA.

Pumisutapon *et al.* (2009) prepared four types of explants- an intact rhizome with two intact shoots (+R+2S), an intact rhizome with two decapitated shoots (+R-2S), a decapitated rhizome with two intact shoots (-R+2S), and a decapitated rhizome with two decapitated shoots (-R-2S), *in vitro* to study the apical dominance in *Alstroemeria*. Khaleghi *et al.* (2008) in his experiment, used lateral and terminal buds of rhizomes (4-6 mm) that were cultured on solidified MS medium containing 30 g l^{-1} agar supplemented with different concentrations of BAP and NAA after surface disinfection and sub cultured every three weeks. The greatest number of shoots was obtained from the medium supplemented with 1.5 mg BAP l^{-1} and 0.2 mg NAA l^{-1}.

Yousef *et al*. (2007) compared the regeneration ability of plantlets using *in vitro* and *in vivo* grown rhizome buds as explants. *In vitro* and *in vivo* grown rhizome buds were cultured on MS basal medium with 3 different compositions of growth regulators (1, 0.2 mg l^{-1} NAA with 1 mg l^{-1} BA and 0.2 mg l^{-1} IAA with 1 mg l^{-1} BA) and the cultures were incubated in 18 + or -1 ^{0}C at 16 h photoperiod. Four subcultures of explants were done on the same fresh media with 3 weeks intervals. Pedraza-Santos *et al*. (2006) developed a protocol for the *in vitro* regeneration of *Alstroemeria* cv. 'Yellow King', by testing for shoot induction, using several explant sources (leaf, stem apices, rhizomes and immature inflorescence apices), temperature and light/dark regimes, hormone and salt concentrations and tested several hormone concentrations for shoot multiplication and rooting and found that only the young floral apices produced adventitious shoots by direct organogenesis. The highest shoot induction rate (10.4 shoots per explant) was obtained by incubation in the dark for 15 days at 8 ^{o}C followed by 15 days at 25 ^{o}C and a 16-h/8-h light/dark regime, on a Murashige and Skoog (1962) liquid medium at 50 per cent of the salt concentration, supplemented with 2.5 mg l^{-1} Kinetin, 1.5 mg l^{-1} BA and 1.0 mg l^{-1} NAA, using a piece of filter paper to support the explant. The highest shoot multiplication rate (9 shoots per explant) was obtained on a liquid MS medium at full strength supplemented only with BA at 1.0 mg l^{-1}.

Chiari and Bridgen (2002) excised stem apical meristems, rhizome apical meristems and rhizome axillary meristems from *Alstroemeria* plants and were grown *in vitro* on modified Murashige and Skoog (MS) media containing different concentrations of Gibberelic acid and 6-benzylaminopurine [benzyladenine] (BA). Plantlets developed from stem apical meristems never regenerated a rhizome and eventually died but the highest regeneration rate (74.1%) of plantlets with a rhizome was observed when rhizome axillary meristems were grown on modified MS medium containing 8.9 µM of BA. Lin *et al.* (2000) micro propagated six tetraploid *Alstroemeria* clones by rhizome multiplication, and within a 3-week subculture interval, the average rhizome multiplication rate for all genotypes was 2.3. Chiari and Bridgen (2000) investigated the growth of *in vitro Alstroemeria* hybrids through morphological studies and produced rhizome halves that regenerates by cutting the rhizome with a horizontal or vertical longitudinal cut.

Jang *et al.* (1999) investigated the efficiency of shoot induction from leaf explants, and the subsequent development of shoots into complete plants with rhizomes and developed a good regeneration system applicable for micro propagation in *Alstroemeria* culture conditions. The youngest ex-plant located close to the shoot apex gave rise to the highest regeneration rate. Lin *et al*. (1998) induced direct shoot regeneration from leaf explants of *Alstroemeria* clone VV2406, a selection from a tetraploid breeding line. Explants contained a leaf blade and a small portion of stem node, which were cut from erect shoots of *in vitro* multiplicated plantlets. The youngest explant which was located closest to the shoot apex gave the highest response. A gradient response toward the shoot apex was observed in percentage of shoot regeneration and in the number of shoots/regenerating explant.

Lin *et al*. (1997) advocated a two-step protocol for the induction of shoots from leaf explants of *Alstroemeria*. Leaf explants with stem node tissue induced best results when cultured initially for 10 days on MS medium containing 10 µM Thidiazuron and 0.5 µM indole butyric acid followed by several subculturing on regeneration medium containing 2.2 µM BAP.

Podwyszynska *et al.* (1997) developed an effective micro propagation method for new Polish cultivars of *Alstroemeria cv*. Juanita, and *in vitro* experiments were conducted to improve the efficiency of multiplication and rooting stages. Rhizomes were cultured on media containing 1.5, 3 or 6 mg BAP [benzyladenine] l^{-1}. The

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greatest number of aerial shoots and shortest roots, but the poorest rhizome rooting ability, was observed at 6 mg BAP l^{-1}.

Gabryszewska (1995, 1996) successfully cultured rhizome apical and axillary tips cultured on Murashige and Skoog medium with BA at 2 mg l^{-1} and NAA at 0.5 mg l^{-1} and observed the presence of BA in the medium markedly increased the number of upright growing shoots and more shoots at 25°C than at 17°C. The highest number of lateral rhizomes was observed on a medium containing 60 or 80 g sucrose l^{-1} and BA. Presence of BA in the medium markedly influenced the formation of upright growing shoots; the tallest shoots were found in cultures on media containing 20 or 30 g sucrose l^{-1}. Low and high concentrations of sucrose inhibited the formation and elongation of upright growing shoots.

Han *et al.* (1994) cultured rhizome tips of *Alstroemeria hybrid cultivars* 'Othello', 'Lilac Glory', 'Cyprus' and Yellow prince' on MS medium supplemented with various growth regulators and obtained best results for rhizome multiplication on MS medium containing 1.0-2.0 mg BA + 0.3 mg l^{-1} IAA or 0.2 mg NAA l^{-1}. Apical rhizomes produced more branched rhizomes/explant than did lateral rhizomes. Further continuous lighting at 3000 lux resulted in greater shoots and rhizomes formation than did 16 hours lighting or culture in darkness.

Bond and Alderson (1993a) assessed the effects of mechanical and chemical methods of removing or reducing apical dominance on the multiplication of *Alstroemeria* cultivars Valiant, Parade and Eleanor grown *in vitro* and observed significantly enhanced rhizome multiplication on sub culturing rhizome explants without aerial shoots and rhizome apices, and rhizome explants divided into single internodes with or without aerial shoots, but sub culturing rhizome explants with only the aerial shoot or rhizome apices removed had no significant effect. Bond and Alderson (1993b) observed that for good multiplication the requirements for the culture environment were a temperature of 15°C, an irradiance of 5 W m^{-2} with a day length of 8 hours. Elliott *et al*. (1993) established *Alstroemeria cv*. Parigro Pink rhizome tip explants on modified MS medium with P added as KH_2PO_4 at 0, 0.01, 0.05, 0.25, 1.25 or 2.5 mM and cultures were transferred to fresh media every 4 weeks. Explants supplied with 1.25 or 2.5 mM P produced significantly more shoots and growing points, and greater FW of rhizomes and shoots, than those provided with lower P concentrations. Shoot tissue P concentrations > 7 mmol kg^{-1} FW were required for maximum *in vitro* growth of *Alstroemeria cv*. Parigro Pink.

Pierik *et al*. (1988) used terminal and lateral tips from fleshy rhizomes that were isolated *in vitro* and induced to form new rhizomes and studied the influence of temperature, light, rhizome portion, plant growth regulators, length of multiplication cycle, media and saccharose concentration on mean number and length of upright growing shoots as well as on rhizome multiplication rate (RMR). A temperature of 15 ^oC was ideal for increasing the number of elongated growing shoots whereas, 21 ^oC was optimal for increasing the length of shoots as well as significantly increased rhizome multiplication rate. Saccharose 3-4 per cent, BA @ 3-4 mg l^{-1}, and a multiplication cycle of 3 weeks given 5 times, was most optimal for rhizome multiplication. Gabryszewska and Hempel (1985) recommended the use of BA for tissue multiplication and used MS medium containing NaFeEDTA 40.3 mg l^{-1}, mesomositol 100 mg l^{-1}, thiamine 4 mg l^{-1} and different concentrations of plant growth regulators for initial establishment of cultures of Alstroemeria. Maximum shoot multiplication was obtained on MS medium supplemented with BA (8 mg l^{-1}).

Hakkart and Versluijs (1985) established cultures from meristem tips in *Alstroemeria cv.* 'Rosario' on MS medium containing 2-ip [isopentenyladenine] 0.01 mg l^{-1} and NAA 1 mg l^{-1} in addition to thiamine 0.4 mg l^{-1}, sucrose 3 % and Difeo Bacto agar 0.6 per cent. Ziv *et al*. (1973) established cultures of Alstroemeria from inflorescence segments equally on White's medium and Murashige and Skoog's medium. A higher ratio of auxin to cytokinin resulted in root regeneration, while the reverse ratio promoted bud differentiation. Plantlets were obtained from bud subculture on a low sucrose medium supplemented with IAA but without kinetin and also used young actively growing tissue explants from *Alstroemeria* inflorescence stem taken at a distance of 1-2 mm below the apex that proved capable of regenerating buds and roots from which small plantlets could be established.

Fig.3. Establishment of rhizome tip and rhizome section explant of Alstroemeria hybrida Cv. Pluto in liquid media (Fig. 3a and 3d), solid media (Fig.3b and 3e) and solid with activated charcoal media (Fig. 3c and 3f)

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Establishment of rhizome tips in liquid media

3a) BAP + IBA: 1.5 + 0.2 mg l^{-1}

Establishment of rhizome sections in liquid media

3d) BAP + IBA: 1.5 + 0.2 mg l^{-1}

Establishment of rhizome tips on transparent solid media

3b) BAP + IBA: 1.0 + 0.2 mg l^{-1}

Establishment of rhizome sections on transparent solid media

3e) BAP + IBA: 1.0 + 0.2 mg l^{-1}

Establishment of rhizome tips on activated charcoal media

3c) BAP + IBA + AC: 1.0 + 0.2 + 1000 mg l^{-1}

Establishment of rhizome sections on activated charcoal media

3f) BAP + IBA + AC: 1.0 + 0.2 + 1000 mg l^{-1}

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Sprouted buds of rhizome tips in liquid media

4a) BAP + IBA: 1.5 + 0.2 mg l^{-1}

Sprouted buds of rhizome sections in liquid media

4d) BAP + IBA: 1.5 + 0.2 mg l^{-1}

Sprouted buds of rhizome tips in transparent solid media

4b) BAP + IBA: 1.0 + 0.2 mg l^{-1}

Sprouted buds of rhizome sections in transparent solid media

4e) BAP + IBA: 1.0 + 0.2 mg l^{-1}

Sprouted buds of rhizome tips in activated charcoal media

4c) BAP + IBA + AC: 1.0 + 0.2 + 1000 mg l^{-1}

Sprouted buds of rhizome sections in activated charcoal media

4f) BAP + IBA + AC: 1.0 + 0.2 + 1000 mg l^{-1}

Fig.4: Sprouted buds of rhizome tip and rhizome section explant of Alstroemeria hybrida Cv. Pluto in liquid media (Fig. 4a and 4d), solid media (Fig.4b and 4e) and solid with activated charcoal media (Fig. 4c and 4f)

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Rhizome tip proliferation on transparent solid media

5a) BAP + IBA + GA_3: 1.0 + 0.2 + 0.5 mg l^{-1}

Erect shoots on transparent solid media

5d) BAP + IBA + GA_3: 1.0 + 0.2 +0.5 mg l^{-1}

Rhizome tip proliferation in media with activated charcoal

5b) BAP + IBA + GA_3 + AC: 2.0 + 0.4 + 0.5 + 1000 mg l^{-1}

Erect shoots in media with activated charcoal

5e) BAP + IBA + GA_3 + AC: 2.0 + 0.4 + 0.5 + 1000 mg l^{-1}

Rhizome tip proliferation in media with activated charcoal

5c) BAP + IBA + GA_3 + AC: 1.0 + 0.2 + 0.5 + 1000 mg l^{-1}

Erect shoots in media with activated charcoal

5f) BAP + IBA + GA_3 + AC: 1.0 + 0.2 + 0.5 + 1000 mg l^{-1}

Fig.5: Proliferation and erect shoots of rhizome tip explant of Alstroemeria hybrida Cv. Pluto in MS solid media (Fig. 5a and 5d), solid with activated charcoal media (Fig.5b, 5c and 5e, 5f)

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2.3.1 Other related crops

Archana *et al*. (2013) developed improved micro propagation protocol for *Zingiber moran* and *Z. zerumbet*, two wild species of the genus *Zingiber* and tested the effects of growth regulators, sugar concentrations, and nutrients on the rate of shoot initiation and multiplication and observed an increase in proliferation and multiplication that occurred in modified Murashige and Skoog (MS) medium supplemented with benzyladenine and kinetin and also observed that about 2 % sucrose and 0.7 % agar to be the optimum for shoot multiplication and regeneration. Arimura *et al*. (2000) obtained adventitious shoots in ginger on MS medium containing Kinetin 25 µM. Roots were induced on growth regulator free MS medium. Kim *et al*. (2000) induced callus in ginger on N6 medium supplemented with 2 mg l^{-1} NAA and further obtained plantlets regeneration from callus on MS medium supplemented with BA 1.2 mg l^{-1} .Sharma and Singh (1997) developed disease free clones (7.7 shoots/bud) of *Zingiber officinale* by cutting active buds on MS medium supplemented with Kinetin 2 mg l^{-1} and sucrose 20 g l^{-1}. Devi and Nayer (1993) obtained successful establishment and multiplication of shoot tips of banana on MS medium consisting inositol (5.5 µM), thiamine HCl (2.97 µM), BA (22 µM), sucrose (12 µM) and coconut water (15 %). Bhagyalakshmi and Singh (1988) induced shoots from meristems in ginger when cultured on MS medium containing sucrose 6%, coconut milk 20%, ascorbic acid 100ppm, glutamine 400 ppm, activated charcoal 250 ppm, BA 0.5 ppm, IBA 0.4 ppm and agar 0.8%. Meristems derived shoots exhibited consistent multiplication on this medium. Further, liquid media was less effective than solid medium for micro-propagation. Inden *et al*. (1988) cultured shoot tips of ginger on MS basal medium supplemented with 5 mg l^{-1} IBA, 0.5 mg l^{-1} NAA and obtained 4 shoots of 20 to 30 mm size from an explant within 6 weeks.

Illahi and Jabeen (1987) induced callus in ginger on ½ strength MS media containing different combination of growth regulators and obtained regeneration on MS medium containing 2,4-D. Sato *et al*. (1987) obtained satisfactory *in vitro* shoot formation in ginger on Gamborge B5 obtained on MS medium. Pillai and Kumar (1982) established and multiplied ginger cultures on Schenk and Hildebrandt medium and obtained 15 daughter shoots per original shoot in 3 months. In case of turmeric, Shetty *et al.* (1982) cultured buds on MS medium containing sucrose (40 g l^{-1}) and Kinetin (2.5 mg l^{-1}). After 1-2 subculturing callus produced numerous buds which later on developed into plantlets. Hosoki and sagawa (1977) successfully cultured buds of ginger on MS medium consisting MS major elements.

2.4 Callus induction and regeneration

Seyyedyousefi *et al.* (2013) evaluated the effect of explant type and plant growth regulators (NAA and BAP) on callus formation of *Alstroemeria cv*. Fuego and showed that the explants source and different concentrations of growth regulators influenced callus production. Segments of nodes and internodes were cultured in MS basal medium with different concentrations of BAP (0.0 and 0.5 mg l^{-1}) and NAA (0.0, 1.0 and 2.0 mg l^{-1}) to produce callus and observed that node was better explant than internode to produce callus best at 0.5 mg l^{-1} of BAP and 2.0 mg l^{-1} of NAA. Amir *et al.* (2012) evaluated the number of growth regulators as well as supplements to the MS-basal medium on the regeneration of *Alstroemeria* rhizome explants. In an experiment on vegetative explants (nodes, internodes and leaves) on *Alstroemeria cv.* 'Feugo', Khaleghi and Azadi (2011) used various concentrations of auxins (Picloram, NAA, IAA, 2, 4-D) with or without BAP and reported that nodal explants provided highest embryogenic calluses.

Khaleghi and Azadi (2008) obtained a high frequency (23%) of compact callus induction in *Alstroemeria cv*. Fuego on a Schenk and Hildebrandt (SH) medium supplemented with 2 mg l^{-1} picloram from nodal segments excised from plants grown in the greenhouse. After three months of culture, compact embryogenic calluses (CECs) were transferred to the modified Murashige and Skoog (MS) medium supplemented with 5 mg l^{-1} picloram for further proliferation of CECs. Kim *et al.* (2005) developed an efficient system for the regeneration of plants from protoplasts in *Alstroemeria.* Friable embryogenic callus (FEC) proved to be the best source for protoplast isolation and culture when compared with leaf tissue and compact embryogenic callus. Micro-calluses were formed after 4 week of culture. Ninety per cent of the micro-calluses developed into FEC after 12 week of culture on proliferation medium. FEC cultures produced somatic embryos on a regeneration medium and half of these somatic embryos developed shoots(Kim *et al.,* 2005) and also obtained high frequencies of compact embryogenic callus (CEC) induction (~40%) and friable embryogenic callus (FEC) induction (~15%) in *Alstroemeria* from nodes with axil tissue cultured first on a Murashige and Skoog (MS) medium supplemented with 10 µM thidiazuron and 0.5 µM indole-3-butyric acid and after that on a Schenk and Hildebrandt (SH) medium supplemented with 9.1 µM 2, 4-dichlorophenoxy acetic acid and 2.2 µM benzylaminopurine (BA). Both types of callus were maintained on modified

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MS medium supplemented with 20.8 µM picloram (Kim *et al*., 2006). and also concluded that the nodal explants can be successfully used as a source for transformation in combination with the MS medium containing 1 mg l^{-1} 2, 4-D, 0.25 mg l^{-1} BAP, 3% sucrose (w/v) and 0.75% (w/v) micro agar for the production of a high level of compact callus and somatic embryos (Kim *et al.*, 2001)

Akutsu and Sato (2002) developed an efficient procedure for plant regeneration from calluses of *Alstroemeria* by somatic embryogenesis. Suspension cells were maintained in Murashige and Skoog's (MS) medium supplemented with 1 mg l^{-1} picloram and then used for either solid or liquid culture. Friable embryogenic calluses formed in liquid half-strength MS medium supplemented with 0.5 mg l^{-1} naphthalene acetic acid (NAA) and 0.5 mg l^{-1} benzyl adenine (BA). The friable calluses developed pro-embryos after transfer to solidified half-strength MS medium without growth regulators and described an efficient procedure for transformation of calli of the monocotyledonous plant *Alstroemeria* by Agrobacterium rhizogenes. Inoculated calli were plated on medium that contained cefotaxime to eliminate bacteria. Four weeks later, transformed cells were selected on medium that contained 20 mg l^{-1} hygromycin. Plants derived from transformed calli were produced on half-strength MS medium supplemented with 0.1 mg l^{-1} GA_3 after about 5 months of culture (Akutsu and Sato, 2004).

Hutchinson *et al*. (1997) cultured Embryogenic callus induced from mature zygotic embryos on MS medium supplemented with 40 µM NAA and 20 µM kinetin, and used as inoculum for liquid cultures. When transferred to a semi-solid, half-strength MS medium supplemented with casein hydrolysate, cell aggregates successfully differentiated into plantlets which later grew to maturity under greenhouse conditions. Lin *et al*. (1997) developed a 2-step protocol for the induction of shoots from *Alstroemeria* (genotype VV024) leaf explants with stem node tissue attached were incubated on shoot induction medium for 10 days, and then transferred to regeneration medium. Shoots from the area adjacent to the region between the leaf base and node tissue regenerated within 3 weeks after transfer, without a callus phase. The best induction was obtained with Murashige and Skoog medium containing TDZ (10 µM) and IBA (0.5 µM). The regeneration medium contained 6-benzylaminopurine [benzyladenine] (2.2 µM) and after several subcultures of leaf explants with induced shoots, normal plantlets with rhizomes were formed.

Schaik *et al*. (1996) studied the plant regeneration ability of callus obtained from zygotic embryos of diploid *Alstroemeria* inodora and a tetraploid cultivar. The best explants for somatic embryogenesis were immature zygotic embryos in half-ovules when the endosperm was soft and white. Nodular embryogenic callus was induced on callus induction medium with a success rate of 54%. The best callus induction period was 10 weeks. Somatic embryos were formed after transfer of the callus to regeneration medium. These somatic embryos had the typical features of zygotic *Alstroemeria* embryos. Gonzalez and Alderson (1995) attempted callus induction in *Alstroemeria* using explants from roots, stem segments, shoot tips and rhizome buds of *cv.* Carmen and mature embryos of *cv.* Butterfly which were cultured on MS basal medium supplemented with various concentrations of 2,4-D, picloram, NAA, kinetin, BA and GA_3. The best results were obtained with mature embryos and after 18 days in culture, callus was produced on 40, 34 and 32% of embryos cultured on MS medium supplemented with 4 mg 2, 4-D l^{-1}, 2 mg picloram l^{-1} and 4 mg picloram l^{-1}, respectively. Callus fresh weight increased at a higher rate over the next 45 days in the medium supplemented with 2 mg picloram/litre than in the other 2 media.

Hutchinson *et al*. (1994) obtained callus when mature zygotic embryos were cultured on MS medium supplemented with 20 µM kinetin and 10 or 20 µM NAA. Callus that was transferred to MS medium supplemented with 20 µM kinetin and 20 µM NAA for long term culture, maintained a regeneration capacity of 40% over an 8 month period. Gonzalez and Alderson (1992) cultured excised somatic *Alstroemeria cv.* Butterfly embryos on solid MS medium alone, or on MS medium supplemented with 0.1 mg BA l^{-1} + 10% (v/v) coconut water or 0.1 mg GA_3 l^{-1}. Cultures were incubated at 25 or 15°C and the number of embryoids which developed into single shoot plantlets was generally higher at 15 than 25° C. After 4 weeks of culture, the greatest percentage of cultures with single shoot plantlets (25%) was obtained from the medium supplemented with GA_3 and cultured at 15°, but the greatest percentage of cultures with callus (75%) was obtained from the same medium at 25°C. Gonzalez and Alderson (1990) obtained callus from mature embryos of *cv*. Butterfly on MS medium supplemented with either 2 or 4 mg picloram l^{-1} or 4 mg 2,4-D l^{-1} combined with BA or kinetin (0-4 mg l^{-1}). Shoots regenerated and torpedo-like structures (somatic embryos) formed when callus was transferred to regeneration media containing BA plus picloram or 2, 4-D. Shoots were also produced by somatic embryos upon transfer to other media.

Fig. 6 and Fig.7 represents the callusing of leaf explant of Alstroemeria hybrida Cv. Pluto in MS solid media fortified

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with plant growth regulator combination BAP + NAA: 0.5 + 4.5 mg l^{-1}.

2.5 Rooting

Pedraza-Santos *et al*. (2006) developed a protocol for the *in vitro* rooting of shoots on a liquid MS medium, either with or without plant hormones using several explant sources (leaf, stem apices, rhizomes and immature inflorescence apices), temperature and light/dark regimes, hormone and salt concentrations and several hormone concentrations for rooting. Chiari and Bridgen (2000) produced plants with the split technique that rooted and flowered regularly, and were true-to-type. Kristiansen *et al*. (1999) observed a synergistic promoting effect of PPFD (Photosynthetic photon flux density) and sucrose on root formation. Root formation after transfer to rooting medium was affected by sucrose and PPFD during the multiplication phase. PPFD did not influence root formation after propagation on 7% sucrose, whereas on 3 or 5% sucrose root formation was gradually inhibited when PPFD was decreased below 17 µmol m^{-2} s^{-1}. The formation of thick roots was promoted by propagation in light but not influenced by sucrose concentration. Root formation on rooting medium was reduced by BA and promoted both by NAA and high levels of sucrose. Thick roots were only produced in the presence of NAA and not affected by sucrose treatment.

Podwyszynska *et al.* (1997) observed that the greatest number of aerial shoots and shortest roots, but the poorest rhizome rooting ability, at 6 mg BAP l^{-1} and used rhizome cultures of Polish *Alstroemeria X hybrida cv*. Juanita to enhance the effectiveness of a micro propagation method for new cultivars and selections. The effects of cytokinins (benzyl adenine, kinetin and 2iP), auxins (IAA, IBA and NAA) and growth retardants (paclobutrazole and flurprimidol), alone or in combination, were studied in relation to rhizome branching, aerial shoot production and rhizome rooting. Application of BA at low concentration with paclobutrazole (0.1-0.5 mg l^{-1}) or flurprimidol (0.01-0.1 mg l^{-1}) in the presence of 1 mg NAA/litre resulted in a high number of aerial shoots (5-6, but these were shorter) and higher rooting ability of the rhizomes. Growth retardants applied with NAA strongly stimulated root formation but suppressed their elongation (Podwyszynska *et al*., 1998).

Pedersen *et al*. (1996) investigated flower induction by testing 9 genotypes at four temperature regimes (5, 10, 15 or 20°C for 6 weeks) and three methods of propagation (by seeds, rhizome division or micro propagation) and for micro propagated plants, temperature treatments were carried out during root formation *in vitro*. Gonzalez and Alderson (1995) observed no root formation when experiments were attempted on callus induction in *Alstroemeria* using explants from roots, stem segments, shoot tips and rhizome buds of *cv.* Carmen and mature embryos of *cv.* Butterfly which were cultured on MS basal medium supplemented with various concentrations of 2,4-D, picloram, NAA, kinetin, BA and/or GA_3. Han *et al.* (1994) successfully cultured rhizome tips of the hybrid cultivars 'Othello', 'Lilac Glory', 'Cyprus' and 'Yellow Prince' on MS medium supplemented with various growth regulators. The most effective (for percentage rooting, number of roots and branched rhizomes/explant and root length) was IBA at 3.0 mg l^{-1}.

Hakkart and Versluijs (1988) reported that *Alstroemeria cv.* 'Rosario' rooted when *in vitro* regenerated shoots were cultured on MS medium containing IAA (1 mg l^{-1} or NAA 2 mg l^{-1}). Out of 32 cultures, 12 rooted cultures were obtained and observed that root formation was better on filter paper bridges in a liquid medium than on a solid medium. Subsequent transfer into soil was more successful with the plantlets rooted in liquid medium than that with those rooted on solid medium. Pierik *et al*. (1988) conducted *in vitro* rooting experiments in *Alstroemeria cv.* Toledo and

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reported that MS medium containing NAA 0.5 ppm in addition to saccharose 5 per cent was most ideal. Further rooting was promoted by a day length of 18h in comparison to 8h and an irradiance of 7 W/m^2. Initial dark treatments had a negative effect and optimal rooting occurred at 21 ^{0}C in comparison to 25^o and 27 ^{o}C.

Lin and Monette (1987) regenerated plantlets from rhizome tips cultured on solid and liquid media based on Murashige and Skoog salt formulation. The quality of the cultures was superior when intact rather than longitudinally sliced rhizome tips were used as explants, and when a temperature of 8° rather than 22°C was used at the initiation stage. More roots were produced on rhizome tips containing a rhizome apical meristem than on rhizome sections lacking such a meristem and 90 per cent of the rooted plantlets were successfully acclimatized and developed into true-to-type flowering plants. Gabryszewska and Hempel (1985) recommended the use of BA for tissue multiplication and NAA (1.0-16.0 mg l^{-1}) for rooting. Ziv *et al*. (1973) advocated a higher ratio of auxin to cytokinin for *in vitro* root regeneration in *Alstroemeria.*

Rooting of rhizome tip of *Alstroemeria* hybrida Cv. Pluto in MS liquid fortified with NAA 1.5 mg l^{-1} using sterilized guage as bridge (Fig. 8, 9, 10 and 11)

2.5.1 Other related crops

Archana *et al*. (2013) developed improved micro propagation protocol for *Zingiber moran* and *Z. zerumbet*, two wild species of the genus *Zingiber* and observed that Naphthalene acetic acid at 0.5 mg l^{-1} produced the best rooting response for both the species. Regenerated plantlets were acclimatized successfully and cytogenetic stability was confirmed by RAPD profiling and ploidy checks. Rout and Das (1997) obtained rooting in *in vitro* regenerated shoots on half strength MS supplemented with IBA or IAA and 2 per cent sucrose.

Babu *et al.*(1996) developed profuse callus in ginger on MS medium supplemented with 2,4-D 1mg l^{-1}alone or 2,4-D (0.5 mg l^{-1}) + BA (1 mg l^{-1}) and found that individual embryoids developed into plantlets with better rooting when NAA (1 mg l^{-1}) was added to culture medium. Huang (1995) regenerated *in vitro* plantlets with complete root system directly from shoot tips (20.2-0.9 mm in length) of ginger on MS medium containing BA 2.0 mg l^{-1} and NAA 0.6 mg l^{-1}. Dogra *et al.* (1994) rooted excised rhizome buds of elite lines of ginger on MS medium supplemented with BA 2.5 mg l^{-1} and NAA 0.5 mg l^{-1}. The maximum number of roots were formed on MS medium supplemented with

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NAA (1 ppm). Nathan *et al*. (1993) obtained *in vitro* rooting in *Heliconia psittacorum* on hormone free medium. A high (92 %) rooting was observed when MS medium was supplemented with thiamine HCl (0.5 ppm), myoinositol (100 ppm), sodium hydrogen phosphate (170 ppm), adenine sulphate (80 ppm), gelrite (2 g l^{-1}) and sucrose (3 %). Chang and Criley (1993) could induce roots in *in vitro* regenerated shoots of ornamentals pink ginger (*Alpinia purpurata*) within 4 weeks on agar solidified on half strength medium with 2 per cent sucrose.

Malamug *et al*. (1991) regenerated the shoots from callus in *ginger cv.* 'Kintoki' and concluded that the multiplication medium containing NAA (1 mg l^{-1}) and BA (5 mg l^{-1}) was optimal to produce satisfactory root system. Bhagyalakshmi and Singh (1988) found that prolonged culturing on multiplication medium resulted into root hair formation in case of ginger. However, unrooted shoots can be induced to root by sub culturing in the quarter strength MS medium supplemented with sucrose (3 5), ascorbic acid (100 mg l^{-1}), activated charcoal (100 mg l^{-1}) and agar 8 per cent. This medium did not require any growth regulator for root induction. Successful rooting of *in vitro* regenerated shoots of ginger was obtained within 2 months when the shoots were transferred to basal MS medium supplemented with BA 1 ppm (Hosoki and Sagawa, 1977). Pillai and kumar (1982) could induce root hair formation in ginger shoots by prolonged culturing on establishment/multiplication medium. However, to induce more root hair development, daughter plantlets were subcultured on blotting paper bridges in liquid medium.

III. CONCLUSION (Fig.12)

i. Rhizome tips taken during the vegetative growth are suitable explant for starting the culture in *Alstroemeria hybrida Cv.* Pluto.
ii. Maximum uncontaminated growing cultures were obtained with Carbendazim 200 ppm for 30 minutes followed by mercuric chloride (0.1%) treatment for 10 minutes and final treatment with ethyl alcohol (70%) dip for 1 minute.
iii. Maximum culture establishment was obtained in MS- liquid media fortified with plant growth regulator combination BAP + IBA: 1.5 + 0.2 mg l^{-1}.
iv. Proliferation of rhizome tip explants in media fortified with activated charcoal was significantly higher as against the other treatments. [BAP + IBA + GA3 + Activated charcoal: 2.0 + 0.4 + 0.5 + 1000 mg l^{-1}].
v. Highest callus induction per cent was obtained with plant growth regulator combination BAP + NAA: 0.5 + 4.5 mg l^{-1}.
vi. Best rooting was recorded in MS- liquid media fortified with NAA 1.5 mg l^{-1}.

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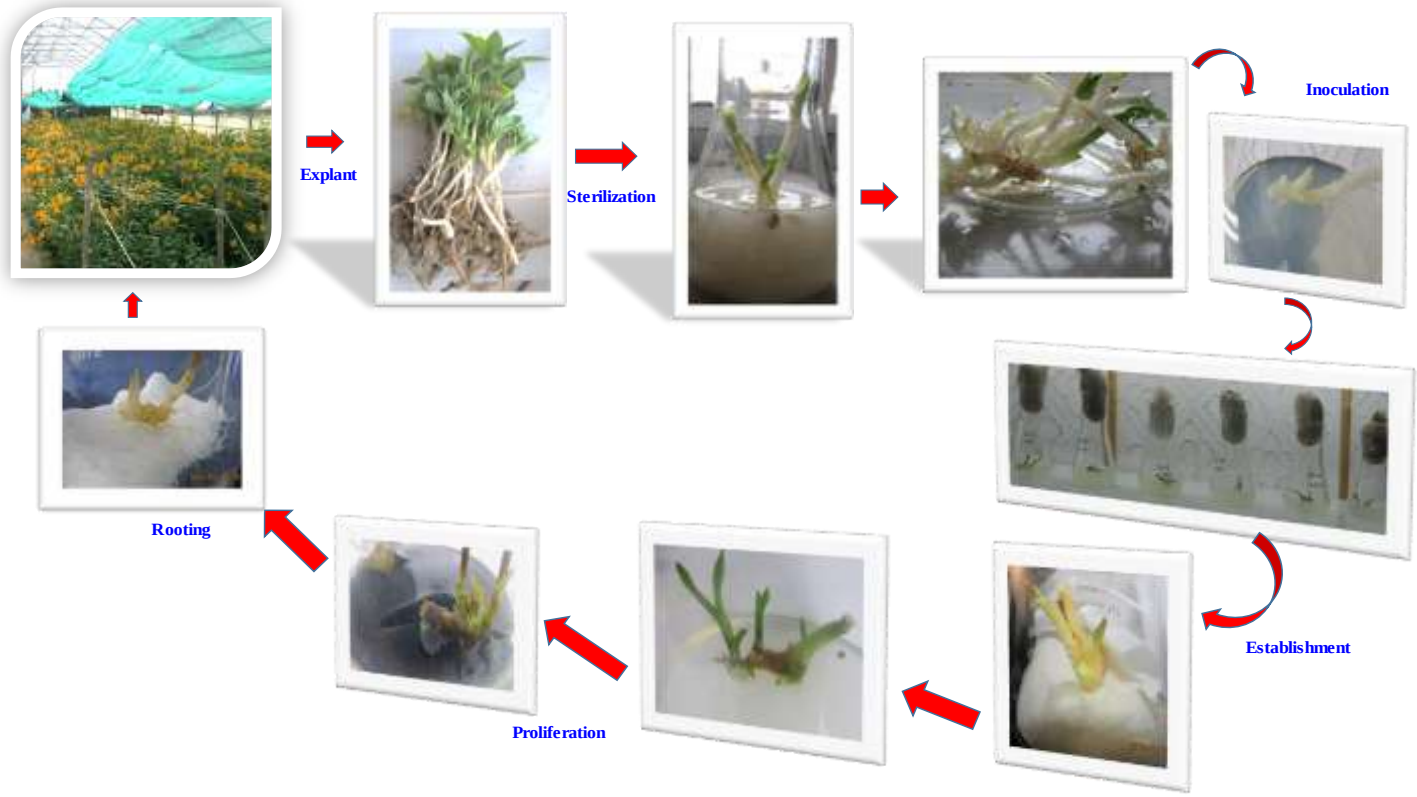

Fig.12: Flow chart representing micro-propagation of Alstroemeria hybrida cv. Pluto

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Cashew Tree Gum: A Scientific and Technological Review

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Abstract*— *Cashew gum can be obtained from the exudate released from the stem of the species "Anacardium occidentale", commonly called cashew tree, a tree typical of Brazil and subtropical countries. It is a heteropolysaccharide complex that after hydrolysis presents a high content of monosaccharides with a varied composition depending on its origin. Due to its biological origin, the ability to form gels and the fact that it has properties similar to synthetic polymers, it is a great option for the application in several sectors of industry. In the food industry it can be used as a thickener and a stabilizer for juices, an emulsifier in salad sauces, a stabilizer in the emulsions of meats such as sausage, and in food compositions containing chocolate. In the manufacture of pharmaceuticals it may be used as an agent for suspending, emulsifying, disintegrating, binding, gelling, tabletting drugs with release control and also as a mucoadhesive agent. In medicine, studies also indicate a phytotherapeutic potential in the reduction of blood pressure and even in some types of cancer.

Keywords*— *Anacardium occidentale, cashew gum, food industry, phytotherapy, polysaccharide.

I. INTRODUCTION OF THE SPECIES *ANACARDIUM OCCIDENTALE*

The *Anacardium* word is a Greek word meaning inverted heart, in reference to the format of the fruit. Cashew is a fruit of high nutritional value with high levels of vitamin C, minerals, Ca, Fe, Zn [1].

The fleshy part, edible in natura, is a pseudofruit, and the chestnut cashew, consisting of a shell, almond and pellicule, is the true fruit of the cashew tree. The shell contains the cashew nut shell liquid (CNSL), a phenolic compound which can be used as a source of phenols in the industry of plastic, paints, varnishes, in the manufacture of auto parts and others. The roasted almond without the shell can be consumed as food and it is rich in high quality oils [1].

Anacardium occidentale is the scientific name of the cashew tree, a tree native to Brazil known to the indigenous inhabitants of northeastern Brazil in 1500 who used their fruit, the cashew, as one of the most complete and important foods. The nuts were toasted over the fire to remove the CNSL (cashew nut shell liquid) and used as food. The cashew pulp was squeezed to release the juice, which in turn was fermented for the production of wine. The cashew trees were also used to establish the boundaries of the territories and to provide shade. The name of the fruit itself is due to the Indians since it comes from Tupi, "acaiu". When fruiting cashew, the tribes that descended from the interior had to fight the tribes of the coast in order to own the cashew plantations. These fights became known as "cashew wars" and are supposed to explain how the cashew tree (which is not very demanding in terms of availability of water and soil) has spread throughout the northeastern interior of dry and arid lands. The words "acaiu" (nut that is produced) and "yu" (yellow) probably formed the word “caju” (cashew in Portuguese) [2,3,4,5].

In the 16th century the cashew trees became very popular among settlers. The naturalist monk Thevet, on a visit to Brazil in 1558, was the first to draw a cashew by showing how Indians harvested fruits and squeezed the pulp into a pot. Later, in 1576, the book "History of the Province of Santa Cruz" by Pero de Magalhães Gandavo was published, where a scientific description of the geography, fauna and flora of the Brazilian coast was written. In this publication Gandavo described the cashew tree as having the size of an apple tree and pear tree, and the cashew nut looking like a bean, being as tasty as the almond [4,6]. The cashew trees were brought in the first half of the century by the Portuguese to Goa in India, where they easily adapted to the region and were planted for the production of wines, cognacs and liqueurs. From India they were taken to other Asian countries, then to Mozambique and Angola, and later to Nigeria, Kenya, the Philippines, and Ceylon so that by the end of the eighteenth century there were already cashew trees scattered throughout the Indian Ocean region [2, 3, 5, 7, 8]. Meanwhile, in Brazil, the cashew trees were being replaced by sugarcane plantations.

At the beginning of the 20th century, India dominated the trade in cashew nuts [2]. During World War II the cashew agroindustry began in Brazil, due to high external demand for cashew nut shell liquid (CNSL) used in the manufacture of high voltage cables. The United States was the main consumer in the world and the crop was explored in an extractive way, and chestnuts were used

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exclusively as raw material for the manufacture of CNSL [9].

In the 1960s, with tax incentives from the Northeast Development Superintendency (SUDENE), Brazil was the country with the largest planted area of cashew trees and an industry for processing the production of nuts, with emphasis on the state of Ceará [9]. In the 1970s, SUDENE once again financed the implementation of large plantations. In the 1980s, Embrapa's National Center for Tropical Agroindustry was implemented with the participation of the Agricultural Research Company of Ceará (EPACE), where large researches were carried out with several generations of clones of precocious dwarf cashews of expressive productivity and high genetic quality, which contributed significantly to the increase in cashew planted areas and cashew production not only in Ceará but all over Brazil [10].

Since its production is focused on countries considered as underdeveloped or developing, cashew is considered a product of great socio-economic importance worldwide. According to data from Companhia Nacional de Abastecimento (CONAB), Brazil produced, in 2015, about 102,000 tons of cashew nuts, where 12,957 tons were exported mainly to the Netherlands, Canada and the United States.

According to data from the Food and Agriculture Organization of the United Nations (FAO), Brazil was the eleventh largest producer of cashew nuts in 2013, with a production of 109,679 tons. Vietnam, Nigeria, India and Côte d'Ivoire were the largest producers with productions of 1,110,800; 950,000; 753,000 and 450,000 tons. The following figure shows the evolution of the world production of cashew nuts between the years 2000 and 2013.

It is possible to note that Nigeria, India and Côte d'Ivoire increased their production while Brazil has kept its production stagnated in recent years.

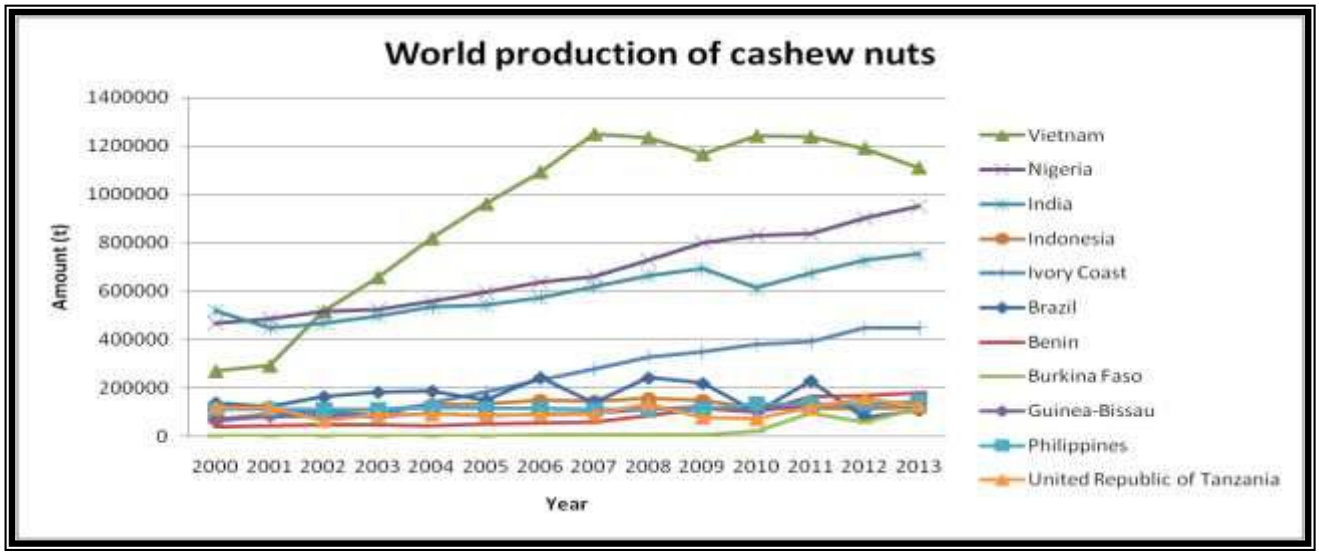

Fig. 1: World production of cashew nuts

II. CASHEW GUM

The term "gum" is generally used to characterize high molecular weight compounds capable of forming gels in the presence of suitable solvents and forming suspensions or solutions with high viscosity even at low concentrations. According to this definition, some hydrophobic substances such as high molecular weight hydrocarbons derived from petroleum, many resins and proteins, some synthetic polymers may also be referred to as gums. However, it is common to refer to polysaccharides of vegetable or microbial origin as gums, and they dissolve integrally or partially in hot or cold water producing viscous solutions or suspensions. Thus, these substances are also called hydrocolloids [11].

When the cashew stem undergoes any sort of environmental aggression, it releases an exudate, which is a resin that varies in color from yellow and brown, and after going through a purification process, it becomes a gum rich in polysaccharides [1]. The resin is produced in the epithelial cells located in the outer channels of the plant. The gum encapsulating cells pass through the cell wall into the channels, carrying the lysed cell material therein. When the flowable resin reaches the outer surface of the plant through a cut, the resin dries by evaporation [1].

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Table.1: Cashew gum compositions

Monosaccharide	Cashew gum composition in differents regions (%)					
	Brazil [15, 16]	Brazil [17]	Brazil [14]	India [18]	Papua [18]	Venezuela [19]
galactose	72 - 73	81.7	69.78	61	63	49
arabinose	4.6 - 5	1.9	11.84	14	16	31
mannose	0 - 1	-	0.97	2	1	4
xylose	-	-	1.29	2	-	1
rhammose	3.2 - 4	1.9	2.28	7	7	7
glucose	11 - 14	9.5	9.78	8	9	-
glucuronic acid	4.5 - 6.3	5	0.52	6.2	5.7	8

According to Tiomno (1946) [12]; Mothé & Rao (2000) [13], cashew gum resembles gum arabic and may be used in the pharmaceutical industry, in cosmetics, and adhesive industry. It can also be used in food industry as emulsifiers or stabilizers since it is not toxic nor presents odor or taste.

Thus, the gum can add more value to cashew agrobusiness, minimizing Brazil's imports of gum arabic and then exporting to other countries.

III. CASHEW GUM CHARACTERIZATION

According to Botelho (1999) [14], cashew gum is considered a heteropolysaccharide of molar mass (Mw) 1.5 x 10^4 and a polydispersity index of approximately 1.49 (values obtained by size-exclusion chromatography). It is usually composed of galactose, arabinose, glucose, rhamnose, mannose and glucuronic acid, whose concentrations vary depending on the source of the gum.

The following table 1 shows the composition of cashew gum in different countries.

It can be observed that the gum has large quantities of galactose in relation to other monosaccharides. The gum from Brazil has larger amounts of galactose and glucose compared to the gums from other countries. Xylose was observed in small amounts only in the gums in India, Venezuela and Brazil, studied by Botelho (1999) [14].

The gum of the cashew tree has connections (1 → 3) which guarantee a lower symmetry of molecule contributing to improve its solubility. These leads are interspersed by glycosidic linkages (1 → 6), which contribute to increase the solubility of the molecule [1].

Anderson & Bell (1975) [18] proposed a possible structural fragment of the cashew gum, presented in the figure below, where R is D-mannose, D-xylose, L-ramanose, L-arabinose and R" is D-glucose or glucuronic acid.

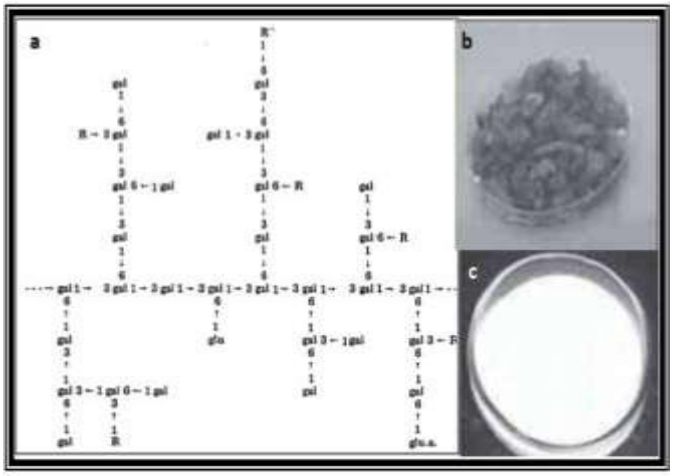

Fig.2: (a) Possible structural fragment of the cashew gum [18]; (B) Exudate of the cashew tree (c) Purified cashew tree gum [1]

IV. CASHEW GUM EXTRACTION AND PURIFICATION PROCESSES

Costa et al (1996) [20] studied a purifying method consisting of the steps of grinding, dissolution in water (4% in water) in an NaOH enviroment, filtration and precipitation with ethanol. The obtained precipitate is washed with ethanol and acetone and dried in aqueous medium, thus obtaining the isolated gum. Isolated gum suffers a first purification process which aims to remove the cations present in the gum. This first purification of the isolated gum consists of the steps of obtaining isolated gum in the dissolution step being carried out with water (4% in water) in a NaCl environment. To remove NaCl precipitate excess, a second purification method is carried out, which differs from the first only in the dissolution step, which is performed with 3% in water and NaCl.

Mothe & Freitas (2013) [21] performed the purification of cashew gum through grinding, solubilization, centrifugation, precipitation with alcohol and vacuum drying. The presence of total sugars was analyzed by the UV-Visible spectrophotometer method. The characterization on the purified sample of the gum was

performed by GC-MS, and the following proportion was found: 56.7% Galactose; Arabinose 10.6%; Glucose 19.7% and 13% Rhamnose. The uronic acid was not quantified.

In a publication made by Tiomno (1946) apud Mothe et al (2006) [1], a qualitative analysis of the ashes of the cashew gum was made, and the presence of potassium, silicon, magnesium, calcium, aluminum and iron was found, as well as traces of sodium and manganese. Mothe et al, 2002 [22] after performing X-ray fluorescence analysis of cashew gum ashes, found that the calcium content was lower than 1%, and also found traces of manganese, potassium and iron.

The rheological behavior of aqueous dispersions of cashew gum and gum arabic was studied by Mothe & RAO (1999) [23] and it was concluded that both gums show a pseudoplastic, non-Newtonian behavior in concentrations of 4 to 50%. This result confirmed the study by Zaccaria & RAHMAN (1996) [24] who had observed the same behavior for the cashew gum.

A comparison of thermal behavior of gum arabic and cashew gum was performed by Mothe & RAO (2000) [13]. Thermogravimetric curves (TG) of cashew gum were performed at different concentrations (w / w) at temperatures of 0 to 800 ° C in nitrogen atmosphere. Two stages of decomposition were observed, the main one at 252º C. In this study, it was found that cashew gum and gum arabic have similar thermal behavior, so in terms of thermal stability, the gum arabic can be replaced by the cashew gum in various applications.

V. STUDIES OF CASHEW GUM BLENDS

Table 2: Cashew gum blends with other polymers

Blends of Cashew gum with other polymers		
Polymers	Purpose	References
Acrylamide	Graft copolymerisation of acrylamide onto cashew gum	Silva et al, 2007 [25]
Alginate (ALG)	Floating bead as a matrix for larvicide release	Paula et al, 2012 [26]
Alginate (ALG)	Nanoparticles for essential oil encapsulation	Oliveira et al, 2014 [27]
Carboxymethylcellulose (CMC)	Formulations as protective coatings on intact and cut red guavas	Forato et al, 2015 [28]
Cassava starch Carnauba waxe	Influence of cassava starch and carnauba waxe on physical properties of cashew tree gum based films	Rodrigues et al, 2014 [29]
Chitosan	Synthesis and thermal stability of chitosan/carboxymethyl cashew gum polyelectrolyte complex	Maciel et al, 2005 [30]
Chitosan	Chitosan/cashew gum nanogels for essential oil encapsulation	Abreu et al, 2012 [31]
Chitosan	Development and characterization of hydrogels of policaju and chitosan	Soares et al, 2014 [32]
Chitosan	Assess effect on the swelling and BSA release from CHI/CMCG MIC	Magalhães et al, 2009 [33]
Chitosan	Polysaccharide-based nanoparticles formation by polyeletrolyte complexation of carboxymethylated cashew gum and chitosan	Silva et al, 2010 [34]
Metallic phthalocyanines Polyallylamine hydrochloride	Nanobiomedical devices	Araújo et al, 2012 [35]
Polyaniline	Multilayer films electrodes for dopamine determination	Barros et al, 2012 [36]
Polyvinyl alcohol (PVA)	Bioactive film for wound dressing aplication	Moreira et al, 2015 [37]
Polyvinyl alcohol (PVA)	Stimuli-responsive and bioactive film	Silva et al, 2016 [38]
Polyvinyl alcohol (PVA)	Film for fungal growth inhibition	Silva et al, 2012 [39]

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An important application of cashew gum is in the formulation of blends or mixtures. The gum may be associated with other polymers giving good filmogenic features which provide new industrial applications for the same. Table 2 below presents a list of published works associating cashew gum with other polymers such as Acrylamide [25], Alginate [26, 27], Carboxymethylcellulose [28], Cassava starch and Carnaubawaxe [29], Chitosan [30, 31, 32, 33, 34]; Metallicphthalocyanines, polyallylamine, hydrochloride [35].

Studies of cashew rubber blends with polyvinyl alcohol (PVA) have presented several applications in the formulation of films. Moreira et al (2015) [37] and Silva et al (2016) [38] produced bioactive films for use in dressings. Silva et al (2012) [39] studied a bioactive film inhibitor of fungal growth.

VI. CASHEW GUM APPLICATION

By having a biological origin, the ability to form gels and because it has properties similar to synthetic polymers, gums are an excellent choice for the use in several sectors of industry [40]. Depending on the size and molecular orientation, particle size, temperature, concentration, ionic and hydrogen bonds, the functional properties of the gums are affected.

In the food industry, it is possible to apply the cashew hum as a thickener and stabilizer for juices, as an emulsifier in sauces [22] and emulsions of meats such as sausage [41]. Recently, Mothe & Lannes (2015) [42] developed chocolate formulations containing cashew gum in candy bars, studying their thermal, rheological and sensorial properties.

In the pharmaceutical industry it may be an agent for suspending, emulsifying, disintegrating, binding, tabletting drugs with release control and also as a mucoadhesive and gelling agent.

Mothé & Silva, 2005 [44] studied the use of cashew gum in the reduction of blood pressure in spontaneously hypertensive rats. Blood pressure reduction was observed in up to 20% of the rats that were fed with cashew gum. There was also a 4% decrease in the ratio of left ventricular mass and heart mass of the rats treated with the gum. This indicates that cashew gum may have contributed as a promoter of cardiac cells, retarding their hypertrophy.

In the area of neoplasia, the effect of cashew gum was studied in mice with induced sarcoma 180, where antitumor activity was observed after the use of the gum, as well as a significant reduction of tumors [45].

Anti-diarrhea properties of cashew gum in rodents were also observed by Araújo (2015) [46].

Another application of cashew gum was in the flotation of minerals, where its use was studied as a depressor agent in the flotation process of the tailings of marble and granite industries. The benefit of this application is the fact that cashew gum is of organic origin and therefore it is non-polluting, unlike the generally used depressants, of inorganic origin [47].

VII. TECHNOLOGICAL MONITORING OF CASHEW GUM

In order to get an overview of technological and scientific development related to application of cashew gum, a technological prospection was carried out, with a database of patents and articles.

The prospection of patent applications was carried out in the period between 1976 and September 2016, a total of 40 years, and the databases of the Institute of Industrial Property (INPI), United States Patent and Trademark Office (USPTO) and World Intellectual Organization (WIPO) were used. The following terms were used as keywords: Anacardium occidentale and cashew gum. The following table 3 shows the number of patents found per database and application.

Table.3: Number of patents found per research base

Application	Reserch source			
	INPI until set 2016	USPTO (1976 to set 2016)	WIPO (1978 to set 2016)	TOTAL
Personal hygiene & Cosmetics	2	0	4	6
Flotation of calcareous minerals	1	0	0	1
Hydrogels manufacture	1	0	0	1
Pharmaceuticals	3	0	13	16
Food	5	0	22	27
Plastics industry	1	0	1	2
Production process of cashew gum	2	0	3	5
DNA sequencing	0	3	2	5
Effluent and water treatment	1	0	0	1
TOTAL	**16**	**3**	**45**	**64**

It is noteworthy that 13 patent applications found in the INPI database were also found in the WIPO database and 2 patent applications found in the USPTO were also found in the WIPO database.

The most found patent applications have applications in the pharmaceutical and food industries, which shows the importance of the cashew agribusiness in these industry segments.

Although literature has a large number of cashew gum applications, only 5 patent applications on this subject were found in the INPI database, 2 of the production process or isolation of gum, one on its use as a depressant in calcareous mineral flotation, another as a flocculant for water and wastewater treatment and another on the production of superabsorbent hydrogels.

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For the technological prospection of articles, the Web of science database was used with the keywords "cashew gum". The search period was from 1900 to September 2016, a total of 116 years. 71 records of publications were found.

Figure 3 below shows the progress per year of publications found.

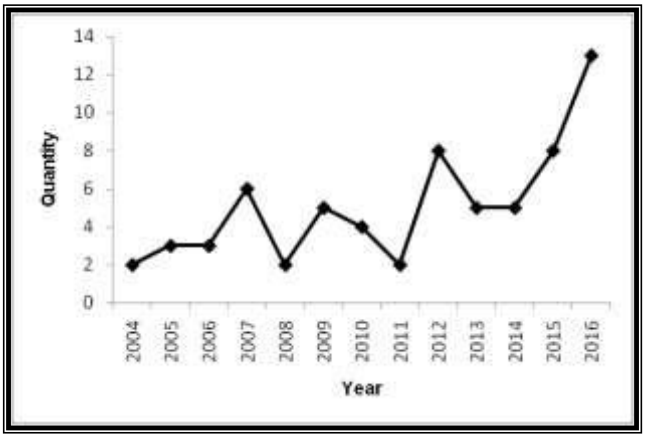

Fig.3: Evolution of publications on cashew tree gum (using data from the Web of Science)

It is possible to observe a significant increase of publications on cashew gum in recent years. In 2016, up to September, 13 publications were found.

The next graph shows the percentage of publications found, by country.

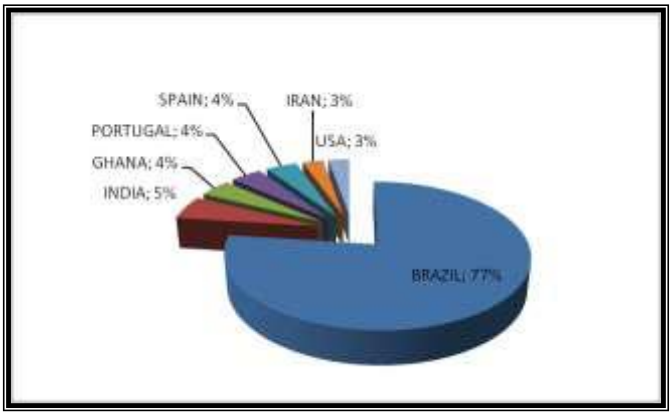

Fig.4: Publications on cashew tree gum by country (Own elaboration from Web of Science data)

It is possible to observe that Brazil is the country that has published the most about gum cashew, with 77% of publications found. India, Ghana, Portugal, Spain, Iran and the United States made publications, but in small quantities, varying from 3 to 5%.

An analysis of publications was also performed by field of study, which is presented in figure 5 below.

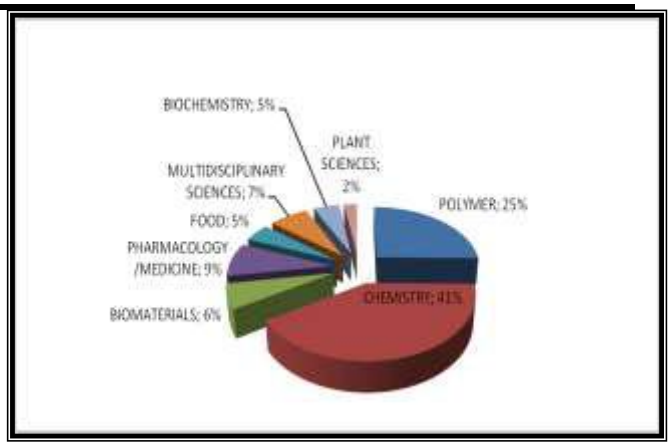

Fig.5: Publications on cashew tree gum by field of study

Most publications found, 41%, referred to the chemistry field in general. The polymer field is second with 25% of the publications, then the fields of pharmacology / medicine, with 9% of the publications.

VIII. CONCLUSION

In cashew agribusiness, there is still interest in the production of nuts and the pseudo fruit (cashew), besides of the cashew nut shell liquid (CNSL). The exudate has been considered as a solid residue, that is, with great potentiality. The perspective of the production and availability of cashew gum in Brazil, associated with recent publications on its properties and performance, such as lowering blood pressure and antitumor properties against sarcoma 180, makes this polysaccharide an excellent choice of raw material to be explored or used by the pharmaceutical industry and especially by the food industry, in the development of functional foods.

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Bio Gas Generation from Biodegradable Kitchen Waste

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***Abstract*—** *Generation of Solid wastes in general and biodegradable waste in particular is increasing at house hold level over the last two decades. Per capita generation of the waste has been increasing steadily due to population growth and changing socio-economic characteristics and cultural habits and varies from 250g to 600g. Any material which can be decomposable by the action of microorganisms in a short period of time is called biodegradable Mostly food waste; vegetable peels and fruit pulp are biodegradable. These materials readily mix with the soil by the action of bacteria. During decomposition, these materials release carbon dioxide, methane, ammonia and hydrogen sulphide into the environment thereby contributes to air pollution and odour pollution. The gases that are released during the decay of biodegradable wastes can be captured for the economic utility and as well as to save the environment. An attempt is being made in this technical research paper to demonstrate the possibilities energy recovery from biodegradable kitchen waste that is collected from residential societies which can be utilized for the benefits of the society. Kitchen and food waste collected from a high end residential community of 300 families in Mumbai city suburbs is analyzed for the quantification of bio gas. Bio gas is captured through a fabricated anaerobic digester. Experimentation and results are discussed. The results are encouraging.*

Keywords*— *Anaerobic digester, Biodegradable Kitchen and Food Waste; Bio gas from Food Waste.

I. INTRODUCTION

Biodegradable waste is the waste that can be decomposed and will be broken down into carbon dioxide, water, methane or simple organic molecules by the action of micro-organisms in reasonably less time. These wastes are generated out of human and industrial economic activity and the sources could be the residential areas, commercial areas and industrial areas. Characteristics of these wastes vary depending upon its source of generation. The composition the waste varies from urban to rural and also from community to community due to their socio-economic –cultural and living conditions. Normally biodegradable wastes are food and kitchen waste, manure, agricultural and forestry waste, paper waste, and textiles. For the disposal wastes different disposal methods available and there is a need to select eco-friendly options to save the environment and natural resources.

Solid waste generated from the residential communities and also out of food processing, commercial and agricultural activity contain significant organic matter which are of biodegradable nature. The per capita waste generation rate in India has increased from 0.44 kg/day in 2001 to 0.6 kg/day in 2011. The reasons for the increase in solid wastes are by changing lifestyles and increased purchasing power of urban Indians. There are 53 cities generate 86,000 TPD (31.5 million tons per year) of MSW at a per capita waste generation rate of 500 grams/day (Annepu, 2012[1]). Non availability of data and also inconsistency in available data on solid wastes are the major hurdle while studying the solid waste management process among Indian cites. At present more than 32% of country's population is living urban areas and wastes generated in 366 Indian cities contribute 70% of solid waste from India's urban population. Estimations made by different agencies and institutions reported that the total MSW generated in urban India at 68.8 million tons per year (TPY) or 188,500 tons per day (TPD). The data collected indicate a 50% increase in MSW generated within a decade since 2001. In a "business as usual scenario", urban India will generate 160.5 million TPY (440,000 TPD) by 2041, in the next decade, urban India will generate a total of 920 million tons of municipal solid waste that needs to be properly managed in order to avoid further deterioration of public health, air, water and land resources, and the quality of life in Indian cities[2]. Also from the studies conducted and also analysis of solid waste samples collected from different cities across the country in India reveal that

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composition of urban Municipal Solid Waste (MSW) in India constitute 51% organics, 17.5% recyclables (paper, plastic, metal, and glass) and 31 % of inert materials. The moisture content of urban MSW is 47% and the average calorific value is 7.3 MJ/kg (1745 kcal/kg).

Aim: The aim of the study is to convert biodegradable kitchen waste in to bio-gas.

- To convert biodegradable kitchen waste into biogas.

Objectives:

- To produce a renewable bio energy from kitchen waste.
- To reduce air pollution.
- To find ecofriendly disposal methods
- To generate revenue from the waste that is generation of wealth from the waste.
- To reduce global warming.

II. REVIEW OF LITERATURE

Biogas refers to a gas made from anaerobic digestion of kitchen waste. Methane is a clean gas which generates energy and one of the main constituent of cooking gas. Abundant kitchen waste (biomass) in terms vegetable peelings, kitchen waste, food waste are abundantly available from the each and every house of Indian communities. These kitchen waste biomass mass can be a source for Methane production where combination of waste treatment and energy production would be an advantage. In this connection many researches carried studies and investigations for the generation of bio gas the Methane from biodegradable waste.

Dr. Anand Karve (President Appropriate Rural Technology of India, pune ARTI[3],[4] developed a compact biogas system that uses starchy or sugary feedstock material and the analysis shows that this new system is 800 times more efficient than conventional biogas plants.

Hilkiah Igoni[5] (2008) studied the Effect of Total Solids concentration of Municipal Solid Waste on the Biogas Produced in an Anaerobic Continuous Digester. The total solids (TS) concentration of the waste influences the pH, temperature and effectiveness of the microorganisms in the decomposition process. They investigated various concentrations of the TS of MSW in an anaerobic continuously stirred tank reactor (CSTR) and the corresponding. Shalini sing, sushil kumar, M.C. Jain, Dinesh kumar (2000), [6] carried studies on cattle dung and their residues insemination with kitchen waste and found that the increased biogas production using microbial stimulants. Ranjeet Singh, S. K. et.al [7]collected inocula from four different sources such as Jajmau tannery waste treatment plant (ITW), Jajmau municipal waste treatment (IMW), Unnao distillery (IDW) and batch reactor and carried studies for the generation of waste. Jong Won Kang et al (2010) [8]studied the On-site Removal of H2S from Biogas Produced by Food Waste using an Aerobic Sludge Bio filter for Steam Reforming Processing. They show that a bio filter containing immobilized aerobic sludge was successfully adapted for the removal of H2S and CO2 from the biogas produced using food waste. Abhishek et.al (2015)[9] carried research on production of bio-gas from the food and degradable waste. Leta Deressa etal (2015)[10] analysed the production of bio gas from fruit and vegetable waste mixed with cow dung in anaerobic digester Sudha.G.et.al, 2012[11] published a paper titled "Production of biogas from different fruit pulp". Anaerobic digestion process was adopted to convert the biodegradable fruit pulp into biogas. It was observed that the more the Chemical Oxygen Demand (COD) in fruit pulp, the more is the biogas. COD value is directly proportional to biogas production.

III. RELEVANCE

Kitchen waste is organic material having the high calorific value and nutritive value to microbes, that's why efficiency of methane production can be increased by several order of magnitude, using higher efficiency and size of reactor. The bio gas used can be used for cooking and lighting purposes. And also on large scale generation the cost of biogas production is reduced. Also in most of cities and places, residential communities, hostels of university campuses, agricultural market yards, fruits and vegetable market huge amounts of bio degradable wastes are generated and are being disposed on to the landfill or discarded in open dumping yards. The traditional methods of bio degradable wastes results in public health hazards and diseses like malaria, cholera, typhoid. Inadequate management of wastes like uncontrolled dumping bears several adverse consequences: It not only leads to polluting surface and groundwater through leachate and further promotes the breeding of flies , mosquitoes, rats and other disease bearing vectors. Also, it emits unpleasant odour & methane which is a major greenhouse gas contributing to global warming. Hence forth the kitchen waste can be used as a raw material to produce biogas. The produced biogas can be used as a cooking gas in the kitchen. This concept is energy from waste.

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IV. BENEFITS OF BIO GAS TECHNOLOGY

- Production of energy.
- Transformation of organic wastes to very high quality fertilizer.
- Improvement of hygienic conditions through reduction of pathogens.
- Environmental advantages through protection of soil, water, air etc.
- Micro-economic benefits by energy and fertilizer substitutes.
- Macro-economic benefits through decentralizes energy generation and environmental protection.

V. MATERIALS AND METHODS

The bio degradable waste materials used for the demonstrative study are vegetable peelings, fruit peelings, food waste collected from the residential housing societies. In Siddartha Nagar, Kandivili East at Radha Residence CHS there are about 300 families with a population of about more than 1200 people are living. From the house hold survey and from the society office registers it has been revealed that on an average 400 kg of exclusive organic waste is collected from house to house. The kitchen waste has been used for the demonstration of the experiment.

For the demonstration, the technology used by ARTI – Appropriate Rural Technology of India, Pune (2003) has been used. At ARTI developed a compact biogas plant which uses waste food rather than any cow dung as feedstock, to supply biogas for cooking. The plant is sufficiently compact to be used by urban households. The design and development of this simple in this the bio gas plan uses waste grain flour, spoilt grain, overripe or misshapen fruit, nonedible seeds, and fruits.

Yet powerful technology for the people, has won ARTI the Ashden Award for sustainable Energy 2006 in the Food Security category. Dr. AnandKarve (ARTI) developed a compact biogas system that uses starchy or sugary feedstock and rhizomes, green leaves, kitchen waste, leftover food, etc. Just 2 kg of such feedstock produces about 500 g of methane, and the reaction is completed with 24 hours.

For the generation of the bio-gas an anaerobic digester is fabricated to convert biodegradable kitchen waste (BKW) into biogas. The digester can be a hard plastic tank or iron tank. The volume of the tank is designed based on the quantity of BKW produced per day. Cow dung or animal dung is added initially to introduce anaerobic bacteria into the digester. A Non-return valve (NRV) is fitted to both inlet and outlet pipes of the digester.

VI. DESCRIPTION OF THE PARTS OF THE PROCESS

Slurry preparation tank: The kitchen waste is made into slurry with 45-50% solids. Initially 10% cow dung is required. At a later stage it can be reduced to 5%. The process is a batch process. Each batch can be retained for 4 days in the digester.

Digester A digester is a huge vessel where chemical or biological reactions are carried out. Anaerobic digestion is a series of processes in which microorganisms break down biodegradable material in the absence of oxygen. It is used for industrial or domestic purposes to manage waste and/or to release energy. The digestion process begins with bacterial hydrolysis of the input materials to break down insoluble organic polymers, such as carbohydrates, and make them available for other bacteria. Acidogenic bacteria then convert the sugars and amino acids into carbon dioxide, hydrogen, ammonia, and organic acids. Acetogenic bacteria then convert these resulting organic acids into acetic acid, along with additional ammonia, hydrogen, and carbon dioxide. Finally, methanogens convert these products to methane and carbon dioxide. The digester is made up of fiber material like a plastic tank or steel tank.

Valves: A valve is a device that regulates, directs or controls the flow of a fluid (gases, liquids, fluidized solids, or slurries) by opening, closing, or partially obstructing various passageways. In an open valve, fluid flows in a direction from higher pressure to lower pressure. The ball screw valve is made by plastic material.

Gas Purifier The gas produced from digester consists of carbon dioxide, hydrogen sulphide and methane. The removal of both H2S and CO2 can be done by passing it through water. This simple process is used to produce a pure methane gas.

Pressure Gauge Many techniques have been developed for the measurement of pressure and vacuum. Instruments used to measure pressure are called pressure gauges or vacuum gauges. The pressure gauge is a device used to measure the pressure inside the digester. The pipe lines are made up of PVC (polyvinyl chloride). It's used to flow the waste crushed food into the digester and also to flow the gas from digester to gas purifier. The anaerobic digester develop in the pilot study is shown in the line diagram in figure-1 below.

Process flow sheet

1. Slurry preparation tank
2. Anaerobic digester
3. Methane gas collection tank
4. Sludge collection tank.

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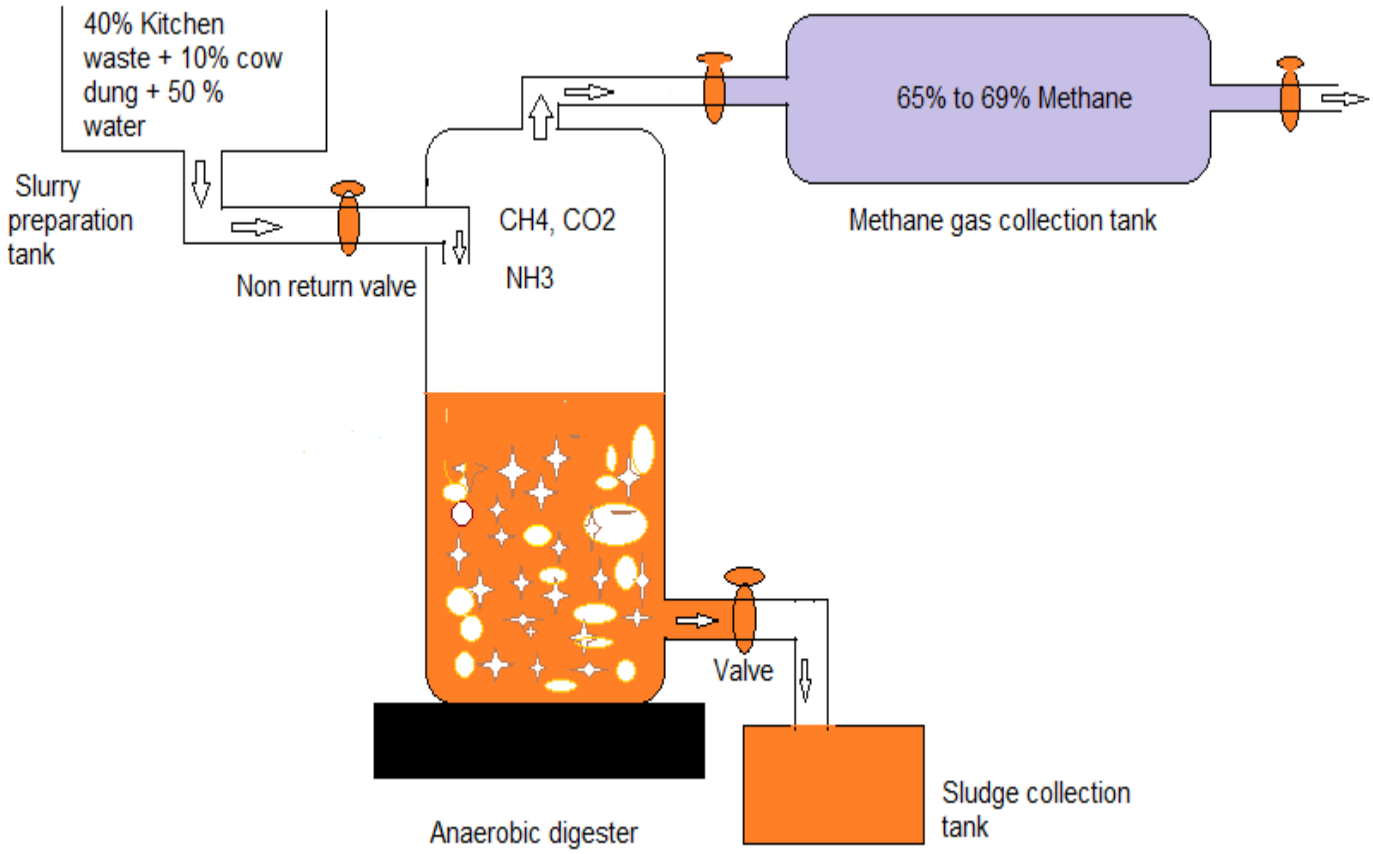

Fig.1: Anaerobic Digester Experimentation unit model

VII. EXPERIMENTAL PROCEDURE

The fresh kitchen waste consisting of fruit pulp, vegetable peeling and food waste is mixed with cow dung and water to prepare slurry. The ratios are 40% kitchen waste plus 10% cow dung and 50% water. The slurry was pumped into an anaerobic digester tank. The tank was thermally insulated and was provided with a low speed agitator that worked continuously; under mesosphilic conditions (38^0C).This slurry was transferred into the anaerobic digestion tank and retained nearly for 96 hours. After 96 hours of maturation time due to micro bacterial actions and anaerobic digestion in the absence of oxygen different gases such as Methane, Ammonia, Carbon dioxide and sulphur dioxide are generated. Methane gas was collected in a separate gas collection tank. The sludge from the anaerobic digester is collected in a sludge collection tank, which can be used as a bio-fertilizer.

VIII. DESCRIPTION OF METHOD

The biodegradable kitchen waste (BKW) is made into slurry by adding suitable, quantity of water. The slurry is introduced into the anaerobic digester through the inlet pipe. Nearly 10-kilogramme cow dung slurry is passed into the digester. The microorganisms decompose BKW into Carbon dioxide and methane. The methane gas is collected in a storage tank through the outlet pipe.

BKW + Cow dung + Water → Methane + Carbon dioxide + ammonia

IX. RESULTS AND ANALYSIS

The percentage of methane varies from 65% to 69% and carbon dioxide varies from 15% to 25%. The remaining % is ammonia and hydrogen sulphide. The percentage of methane can be increased by decreasing the carbon dioxide percentage in the anaerobic digester.

Waste characteristics

Waste is collected from a sample of ten housing units in a residential community of 300 residential fats. The waste are collected for a period of seven days and the average collection per day per house is reported in the table below. The housing community selected for the study is Radha Residency, Siddarth Nagar, Borivili (E) situated in Mumbai western suburbs.

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Table.1: Analysis of Bio degradable waste and food waste is collected from Radha Residency Community of 300 Residential Flats

House no	No of persons in Housing unit -Flat	Wet Waste Kg/dayFlat	Dry Waste Kg/day /Flat	Total Waste kg/day/flat	Per Week wet waste Collection from the Sample housing units in kg/day/flat	From the Housing Society(300Flats) in Kg /week
1	4	0.8	1	1.8	5.6	168
2	4	1	1.2	2.2	7	210
3	5	1.5	1.4	2.9	10.5	315
4	4	0.88	1.2	2.08	6.16	184.8
5	5	1.25	1.4	2.65	8.75	262.5
6	6	1.44	1.47	2.91	10.08	302.4
7	4	0.92	1.06	1.98	6.44	193.2
8	5	1.2	1.3	2.5	8.4	252
9	3	0.66	0.735	1.395	4.62	138.6
10	3	0.63	0.84	1.47	4.41	132.3

From the table of analysis it is seen that on an average about 2 tons of biodegradable waste is collected from the residential community of 300 flats. Bio degradable kitchen waste mix is analyzed in the laboratory and the results of the mix are furnished below.

Table.2: Characteristics of kitchen waste

Sl.No	**Description**	**g/Kg**
1	Total Solids	250
2	Organic Total Solids	280
3	aNo3-N	<0.11
4	aNHp4-N	<0.2

Results and Discussions:

At residential community, Radha Residency co-operative housing society where in about three hundred families are staying and generating about two tons of BKW per week and is disposed on to open land dumping following the conventional methods of disposal . On pilot study experimentation it is revealed the BKW can be used in this project can be used to generate biogas. For demonstration purposes a fixed drum type model is used in the pilot study. From the lab scale experiment 75:25 Ratio of food waste and cow dung will provide more efficient gas. From this experiment it is recorded that it is able to produce around 45 00ml of biogas daily in a 8 liter reactor (digester).

X. CONCLUSIONS

The gap between demand and supply for energy sources can be reduced by converting Bio degradable kitchen waste into a biogas. It is a source of renewable green energy. The biogas can be used as a cooking gas and also can be used in turbine to generate electricity. The left over sludge can be packed and used as a manure and compost for agriculture forming. There is need, further to investigate the cost economics and utility returns to establish the plant and running the unit for 365 day a year at residential community level.

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Characterization of Selected Honey in South-East Nigeria: Theoretical Translation

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Abstract— *With the vast honey bee species producing honey for international export and consumption in Nigeria, there is need for theoretical translation of quality assessment and characterization of honey for human consumption. The physicochemical and mineral contents of some selected honey in the five South east geopolitical states of Nigeria was performed for above mentioned application. The results were evaluated with 3D plot to identify the statistical significance of the parameters analyzed. The levels of glucose and fructose were accepted by codex alimentation standard and rejected samples B, C, and G. A correlation of similar botanical origin was demonstrated in sample B, C and G and similarly observed in their moisture content been > 21%. The pH and electrical conductivity showed no significant variation. The codex hydroxyl methyl furfural standard identified samples B, E and L to be "aged honey" or falsified honey in circulation. The 3D plot showed the significant variation of hydroxyl methyl furfural content of samples. A hypothesis was observed when the samples and previously analyzed Nigerian samples were compared; metal concentration levels of Group 1 elements > Group 2 > Transition metals in Nigerian honey and formed an identification trend.*

Keywords—Honey, bees, honeybees characterization, consumption.

I. INTRODUCTION

For centuries, honey has been known for its potent activity against antibiotic resistant bacteria and as a potential source of antimicrobial compounds [1, 2]. As previously reported, the typical constituents of honey by dry matter is sugar, with small amounts of about 22 additional complex sugars and other constituents like pigments, acids and minerals that bring about unique honey types. Subsequently, honeybees are related to the identity and quality of their honey, however, there are not many studies that establish the stability of chemical compounds present in honey and ensuring that honey consumption are devoid of toxic compounds for human health [3, 4].

A study conducted on stability of Nigerian honey showed that if natural harvested honeys are produced hygienically and stored, that its stability can be up to 2 years [5], while the geographical and botanical origin of the flora, type and activity of the bee can also affect the quality and honey composition [6]. Nevertheless, the beehive type does not affect their physicochemical properties [7]. Thus promoting honey with information on its characterization [8] has opened avenues for local products (exports) to meet international standards [9, 10]. Hence, it created the need for legislation on honey quality given the vast bee species in existence [11, 12]. Studies on the proximate analysis and mineral contents of honey for different countries, have confirmed the presence of trace metals such as Fe, Al, Mn, Mg, Na, K, Cu and Ca [13, 14, 15]. These exist in some form of natural combination which can be used as nutritional supplement for humans [16]. Yet, despite the compositional and nutritional value of honey bees, comparative relationship data between the nutritional components and biochemical composition of the vast honey species are limited in Nigeria [17].More over studies have shown that certain commercial honey has higher levels of some parameters which suggested some kind of adulteration [18] or poor hygiene and handling [19]. Hence, this research work will study statistical application and theoretical translation on characterization of selected honey in south-east Nigeria of some selected honey in the five South east geopolitical states of Nigeria to establish their quality for human consumption.

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II. MATERIALS AND METHOD

Collection of samples

The samples were purchased from 12 pre-named locations in Abia State, Imo State, Enugu State, Ebonyi State and Anambra State of Nigeria. They samples collected were tagged (A, B, C, D, E, F, G, H, I, J, K, L) for easy identification. The collected samples were placed inside plastic bottles of 250ml each and tightly sealed and tagged. Afterwards the sample bottle was labeled with sample volume, date, location, time of collection and stored at 4 OC prior to analysis. Honey sample was obtained from National Root Crop and Research Institute Umudike in

Nigeria (NRCRI) known for honey and bee keeping production and research..

Physicochemical analysis

The physicochemical characteristics of honey are a routine laboratory analysis. It provides quantitative results and allows for approximate estimation of the presence of honey blend. The study was carefully carried out using recently reported approach [20]. The moisture content was determined using a digital refractometer calibrated with double distilled water. Ash content was measured with reported AOAC method in previous study [20]. Hanna Instrument (HI 98127) was used to determine pH of the prepared solution while Hanna Instrument (HI 98311) was used for electrical conductivity of the prepared solution. Milli-Q water was used for preparing honey solution for Hanna Instruments. For quality assurance, we ran each experiment 4 times and reported only the average of the quadruple values.

To determine the Hydroxymethylfurfural (HMF); 5g of honey was dissolved in 25mL of distilled water. The absorbance was measured at 284 and 336 nm against a filtered solution treated with NaHSO3. The HMF value were then determined using the following the equation below [10, 12]. Where D = dilution factor and W = sample weight in grams:

HMF (mg/kg of honey) = (Abs_{284}-Abs_{336}) X 149.7 X 5 X D/W.

Metal assay

The metal contents were determined using commonly reported standard AOAC method [6]. This was carried out by measuring 5ml of 10% HCl solution and added to the ash and warmed in a water bath to dissolve. 5ml of 10% Trioxonitrate (v) acid was further added and warming continued until total dissolution in the water bath. A stirring rod was used to transfer the solution into a funnel then to a clean dry 50ml standard volumetric flask. The "ashed" solution was analyzed for Ca, Mg, Fe, K, Zn, Pb and Cd by direct aspiration via atomic absorption spectrophotometer (AAS).

Determination of sugars

1.0g of honey sample was weighed into a graduated 100ml cylinder. 10ml of distilled water was added to the sample and stirred with a long glass rod. 13ml 52% perchloric acid reagent was further added and stirred for 20 minutes. The content was filtered into 25ml graduated flask and diluted to full mark with distilled water.10ml of the sample extract was diluted to 100ml with distilled water, and then 1ml of the dilute filtrate was transferred into a test tube. Blanks were also prepared using double distilled water. The standards of the different sugars (glucose, fructose and sucrose) were prepared using 1ml of each. 5ml of freshly prepared anthrone reagent was added rapidly and the tubes closed to allow for mixing of the contents. They solution was then placed in water-bath for 12 minutes and allowed to cool at room temperature to read the absorbance at 630nm against the blanks [21]. Results were calculated on the following formulae:

% glucose, fructose or sucrose = (25 X H) / (S X W)

Where H = Absorbance of dilute honey sample

S = Absorbance of dilute standard

W = Weight of sample used

III. RESULTS AND DISCUSSION

Physicochemical and sugar analyses

The result for determination of composite sugars (fructose, glucose and sucrose) is as shown in Table 1. Observation showed that the monosaccharide's (glucose and fructose) and the disaccharide (sucrose) in the all 12 samples make up 70% composition of each honey sample respectively. Sample A and E had the highest fructose content, sample C and E showed the highest glucose value while samples B C and G gave the highest sucrose content. They result confirmed good storage conditions for the samples that had sugar concentrations above 70% except samples B, H, J, and L. They conditions are often reflected in the stability of the honey products. Thus, the formation of undesirable products like 2-acetylfuran, Isomaltol and hydroxy-2-methyl-5-6-didropiran-4-one that can cause change in color, odor and taste of honey are prevented. [3].

Observation shows that the concentration of glucose, fructose and sucrose varies, as well as the ratio between them. This useful indicator [3] showed the classification of the honey samples based on geographical origin and botanical origin as using their ratios. Samples A and E are of same botanical origin, Samples D, F and H are of same botanical origin, samples B, C, and G are of similar geographical origin while samples I, J, K and L are same geographical origin.

On the other hand, the results indicated that glucose and fructose are the main composition of the honey samples and corresponds to Codex Alimentations commission international standard [22]. The higher levels of sucrose in samples B, C and G could be an indication of adulteration or early harvesting as seen in Table 1. Thus these formed a correlation with the botanical origin based classification of samples using their ratios. However, in samples A, D, F and H, the glucose content were below 5 %. This showed the natural feeding of the honey bees and high quality of the honey produced from the farms [20]. The results were further compared with previous published results (sugars in honey) for honey samples in Nigeria [5, 18, 23, 24]. The results showed no significant difference from previous studies.

The codex alimentarius committee on sugars recommended moisture content should not exceed 20g 100g-1 [22]. The samples analyzed were all within 16% to

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20.7% except samples B, C and G greater than 21%. However, research has shown that moisture content in honey samples lower than 20% elongates the shelf life of honey [20], while factors of temperature and relative humidity in the geographical origin affects honey moisture content. Therefore all samples showed no significant difference with samples in the south east region of Nigeria [16, 17] and were within 17% to 21%. While samples from the North were lower in moisture content about 15% to 18% when compared to previous results in Nigeria [4, 6]. There was no significance difference between the samples in pH and electrical conductivity while the ash content showed variation

The pH values of the twelve honey samples were measured and the obtained results confirmed that, all tested samples were acidic (pH 3.0–4.2) as seen in Table 2 and within the standard limit of Codex Alimentatarius value of pH 3.40–6.10 [22] except sample D and F. However they were all close to previously reported values in Nigeria [5, 6, 16, and 21].

The higher the ash content, the higher the electrical conductivity. This showed a linear relationship with the both ash content and electrical conductivity and similarly had been observed by research [20]. All samples were below the standard electrical conductivity limit of 0.8mS/cm recommended by codex alimentatarius [22]. The Ash content of 6 samples (B, C, E, G, J & L) were within the acceptable range (0.6–1.2 g/100 g), while samples (A, D, F, H, I & K) were not accepted by codex range Codex Alimentations. This disparity could be an indication of the honey colonies fed with sugar syrup that had lower ash [20]. The codex HMF standard [22] for honey is recommended at maximum 40mgkg-1for processed honey and 80mgkg-1 for declared tropical origins. Thus all honey samples met the standard but samples B, E and L were not accepted by codex alimentations. This identified samples (A, D, F, H) to be fresh honey samples, samples (C, G, I, J) were not fresh while sample (B, E and L) were either "aged honey" or falsified honey. This can be obtained by adding invert syrup since 5-HMF can be produced to the inversion of sucrose by heated sugars in the presence of an acid. [3]

Metal Assay

Studies have shown that the mineral content in honey ranges from 0.04% in light honeys to 0.2% in dark honeys. In addition, the chemical components reflect the type of soil from which both the plant and nectar were obtained [3].From Table 1, the mineral concentration of calcium was the highest in all the 12 samples followed by magnesium with similar concentration levels. The concentrations of lead and cadmium were all below the jointly proposed acceptable levels of 25µg kg-1 for Pb^{2+} and 7µg kg-1 for Cd^{2+} by world health organization (WHO) and the Food and Agricultural organization (FAO) [25]. The remaining elements $Fe^{2+,}$ K^{+}, $Zn^{2+,}$ were all below 0.7mg/l. These reflected the botanical origin of the studied honey samples and formed a correlation that south east Nigerian honey are similar to Hungarian Linden honey based on mineral content [26].The samples D, E and F obtained from Owerri urban in Imo-state were compared with previous results from Owerri urban [18]. A correlation was seen with Pb^{2+} been very negligible, Fe^{2+} in very small concentration, while K^{+}, Mg^{2+} and Ca^{2+} were of higher concentration levels [18]. Thus our result was in agreement with the study that reported samples in Owerri as having the least occurrence of metals. Furthermore, obtained information (data) in all the honey samples of Nigeria as analyzed by previous researchers showed the following hypothesis; the metal concentration levels of Group 1 elements > Group 2 > Transition metals in Nigerian honey [6, 16, 17, 18,] and may form an identification. (though we only analyzed K^{+} in group 1)

3D Plot

The results from the 3D plot can be seen below in fig1. The variations of HMF concentration could be observed at Anambra, Imo and Abia in fig 1A. In Addition, pH, electrical conductivity, ash content and, moisture content were uniformly distributed within the 12 sampling points. The fig 1B depicts the concentrations of heavy metals in the 12 sampling locations. Magnesium and calcium concentrations can be clearly identified as having concentrations greater than 1.0 mg/l but less than 2.50 mg/l each. The concentration of potassium can also be seen with variations in all the sample locations e.g., it had the lowest points at Ebonyi state with 0.07 mg/l and 0.06 mg/l and shown as a flat bar respectively.

IV. CONCLUSION

Samples A and E had the highest fructose content, sample C and E showed the highest glucose value while samples B C and G gave the highest sucrose content. The results also confirmed that the honey samples can be classified based on geographical origin and botanical origin. All honey samples were moderately acidic while the moisture content was highest in B, C and G and L. codex alimentations identified samples (A, D, F, H) to be fresh honey samples, samples (C, G, I, J) were not fresh while sample (B, E and L) were either "aged honey" or falsified honey. The mineral content of the were within world health organization (WHO)/(FAO) recommendations. The following identification trend was observed to exist; the metal concentration levels of Group 1 elements > Group 2 > Transition metals in Nigerian honey.

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Table.1: Physicochemical parameters of honey from 5 south-eastern states in Nigeria

CODE	LOCATION	STATE	Moisture content (%)	pH	Electrical Conductivity (mS/cm)	Ash content (g/100g)	HMF content (mg/kg)	Fructose (%)	Glucose (%)	Sucrose (%)
A	NRCRI Umudike	*Abia*	19.5	4.2	0.469	0.189	12.3	40.20	39.30	4.20
B	Ariara Market;	*Abia*	21.2	3.5	1.341	0.690	97.0	21.30	24.00	20.80
C	Okpara Square Market	*Abia*	22.1	4.0	1.131	0.570	42.0	19.35	39.40	18.75
D	Emekuku Convent	*Imo*	16.7	3.3	0.206	0.038	13.1	37.60	38.00	2.40
E	EkeUkwu Market	*Imo*	20.3	4.1	1.490	0.776	98.0	40.75	40.70	6.05
F	FUTO	*Imo*	19.3	3.0	0.406	0.153	12.9	31.60	35.97	3.60
G	Ogbete Market	*Enugu*	20.7	4.3	1.212	0.616	47.0	20.15	37.40	15.50
H	Oba Market;	*Enugu*	20.4	4.2	0.715	0.330	29.6	26.35	39.18	1.80
I	Ohaozara:	*Ebonyi*	17.6	3.9	0.704	0.324	30.3	24.35	39.20	8.80
J	Abakaliki Market;	*Ebonyi*	19.5	4.2	1.284	0.657	52.0	22.30	26.70	13.18
K	Marvis Mall;	*Anambra*	19.4	4.0	1.144	0.577	36.2	29.34	36.30	10.20
L	Relief Market	*Anambra*	21.7	3.9	1.203	0.611	121.0	29.70	26.75	12.30

Table.2: Mineral composition of honey samples from 5 south-eastern states in Nigeria

CODE	LOCATION	STATE	Ca^{2+} (mg/l)	Mg^{2+} (mg/l)	Fe^{2+} (mg/l)	K^{+} (mg/l)	Zn^{2+} (mg/l)	Pb^{2+} (mg/l)	Cd^{2+} (mg/l)
A	NRCRI Umudike	*Abia*	2.29	1.07	0.06	0.44	0.05	Nil	Nil
B	Ariara Market;	*Abia*	1.47	1.15	0.07	0.58	0.20	0.01	Nil
C	Okpara Square Market	*Abia*	1.54	1.00	0.05	0.53	0.08	Nil	Nil
D	Emekuku Convent	*Imo*	2.17	0.94	0.05	0.41	0.04	Nil	Nil
E	EkeUkwu Market	*Imo*	1.42	0.97	0.03	0.58	0.10	0.02	Nil
F	FUTO	*Imo*	2.04	1.05	0.04	0.50	0.07	Nil	Nil
G	Ogbete Market	*Enugu*	1.35	1.09	0.03	0.65	0.11	0.01	Nil
H	Oba Market;	*Enugu*	1.67	1.02	0.04	0.50	0.07	Nil	Nil
I	IshieluOhaozara:	*Ebonyi*	1.69	1.03	0.02	0.07	0.13	Nil	Nil
J	Abakaliki Market;	*Ebonyi*	1.66	1.02	0.06	0.06	0.09	Nil	Nil
K	Marvis Mall Awka;	*Anambra*	1.58	1.20	0.06	0.42	0.08	Nil	Nil
L	Relief Market	*Anambra*	1.30	1.09	0.03	0.63	0.12	0.01	Nil

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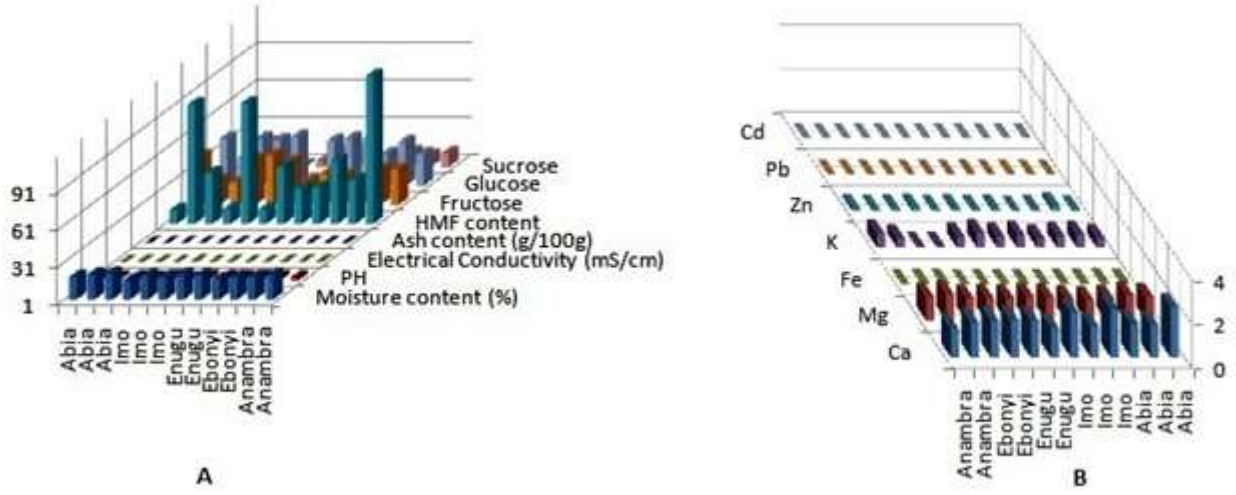

Fig.1: (1A): The physicochemical parameters of honey from 12 sample points; 1(B): The heavy metal concentration of the honey samples

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Elemental Concentrations of Aerosols in the City of Gaborone

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Abstract— *This paper presents aerosol studies carried out in Gaborone, the capital city of Botswana. The Gaborone aerosol is varied consisting of elements from Si to Au. Traffic contribution to the aerosol of Botswana is clearly visible as illustrated by strong positive bromine and lead correlation. The use of unleaded petrol could be the cause of the decrease of ambient lead (Pb) and bromine (Br) concentrations when the present measurements are compared to previous measurements. The elements present in the aerosol of Gaborone range from silicon to lead.*

Keywords— Particulate matter, Correlation, Regression, Ambient.

I. INTRODUCTION

The health effects of urban aerosols and particularly those derived from vehicles are the focus of attention in many countries. Particulate matter (PM) with aerodynamic diameter ≤ 10 μm (PM_{10}), especially the fine particle fraction of $PM_{10,}$ i.e. $PM_{2.5}$ (particulate matter with aerodynamic diameter ≤ 2.5 μm) was found to associate with urban health problems such as increase in daily mortality (e.g. Dockery and Pope, 1994) and asthma (e.g. Anderson et al., 1992). This has been reflected in an increase in the amount of routine monitoring of atmospheric particles (Clarke et al., 1999).

Continues monitoring of airborne particulates is of great importance since a data base on concentration and its fluctuations at different representative sites of a given urban area is of particular relevance to any future planning towards the improvement and control of air quality.

In Botswana air pollution surveillance is carried out by the Department of waste management and pollution control pursuant to the Atmospheric Pollution (Prevention) Act of 1971 (Air Pollution Control, 1995). Most air pollution stations throughout the country are designed to measure sulphur dioxide (SO_2) and total suspended particulates. Thus, only a few aerosol characteristics have been measured so far. In some parts of Botswana one can notice the effects on vegetation due to emissions from mineral smelting industries. This could be a result of inefficient methods of production although the industries are few compared to those in industrialised countries.

Similar studies were conducted for about ten years at University of Botswana, Gaborone, where continuous monitoring of concentration of aerosols and polluting gases were carried out by using particle counters and gas analysers. The investigation from this studies have been described in several papers such as Jayaratne and Verma,2001; Verma and Thomas,2007; Verma and John,2009 and Verma et.al 2010.

This paper presents results of a study of urban aerosol particle composition the capital city of Botswana (Gaborone). This involved aerosol sampling with a dichotomous virtual impactor as well as sampling of black carbon and sulphur dioxide. The results have been compared to a previous study carried out in 1997 in the same city (Selin Lindgren et al., 1998).

II. EXPERIMENTAL

Sampling took place in the capital city of Botswana (Gaborone (22.71°S, 25.9°E)) with a population of 250 000. The sampling site lies in a residential area located ≈500 meters on the western side from the city centre. The measurement campaign extended from the 10th to 30th of August, 1999. The sampling duration was 12 hrs, between 8:00 am and 20:00 pm local time.

Aerosol particles were sampled with a dichotomous virtual impactor (Anderson model 245) operating at a total flow rate of 16.7 l/min. In the impactor used in 1999 the cut-point between coarse and fine particles was 2.5 μm and the upper cut-off for coarse particles was 10 μm, while the lower and upper cut-off for the impactor used in the 1997 measurements were 3.5 μm and 18 μm respectively. Teflon membrane filters, manufactured by millipore (SA240PR100), were used. These filters have an aerial density of 0.9 mg cm^{-2}, a diameter of 37 mm and a pore size of 2.0 μm. The filter material had been evaluated before

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sampling with regard to its blank values, and was shown to be very clean.

All impactor samples were analysed by multielement energy dispersive X-ray fluorescence (EDXRF) technique. The characteristic radiation from the sample is detected by a Si (Li) detector (active area 80 mm^2, FWHM at 5.9 KeV of 173 eV). The X-ray tube was operated at a voltage of 55 kV and a current of 25 mA. The live time of each spectrum was 1000 s. For a detailed description of the spectrometer see papers by e.g. Standzenieks and Selin [1979] and Selin et al. [1991]. The detection limits for the spectrometer used are shown in Table 1.

Black carbon (BC) was sampled with a device consisting of a pump, a flow meter, a brass filter holder and a tube that ends with a funnel with the wide end facing downwards. The sampling rate was 16 l/min. The filters used were of glass fibre material (GF 10) (Schleicher&Schnell, ref.no 370393) in close agreement with the recommended type for the reflectometer (ESM Environmental Monitoring, 1998). The areal density of the filter is 6.7mg cm^{-2}. The filters were subsequently analysed with a light reflectance technique.

Sulphur dioxide was sampled with a self-contained high sensitivity pulsed fluorescence analyser Model 43S from Thermo Environmental Inc. operating at a flow rate of 0.5 l/min (Thermo Environmental Instruments Inc., 1996). Ten minutes averages were logged in a logger (Campbell CR10). The logger also recorded supporting weather variables like wind speed and direction. Table 2 presents a summary of the instruments used in this study.

Table.1: Detection limits for quantification of elements in airborne particles analysed with EDXRF technique from a measurement using Teflon filters.

Element	Detection limit [a] (ng/cm^2)	Minimum airborne concentration[b] (ng/m^3)
Si	1400	720
S	90	46
Cl	40	21
K	15	7.7
Ca	8.0	4.1
Ti	5.1	2.6
V	2.8	1.4
Cr	2.1	1.1
Mn	2.5	1.3
Fe	1.3	0.67
Ni	1.2	0.62
Cu	0.95	0.49
Zn	0.89	0.46
Br	0.62	0.32
Rb	0.54	0.28
Sr	0.63	0.32
Pb	0.92	0.47

[a]*Detection limits are calculated using the three times square root of background (3σ). The time of spectrum acquisition considered is 1000 s.*

[b]*The minimum airborne elemental concentrations are calculated with respect to the 12 hours sampling time used in this study.*

Table.2: Instruments used to measure atmospheric variables in the present work.

INSTRUMENT	MODEL
Dichotomous Impactor	Anderson
SO_2 Analyser	Model 43S from Thermo Environmental Inc.
Logger	Campbell CR 10
Wind Speed and direction	Wind monitor-AQ
Black Carbon sampler	Laboratory modified with a brass holder

III. RESULTS AND DISCUSSION

3.1. Element concentrations

The levels of measured elemental concentrations in the urban aerosols of Gaborone are presented in Table 3 for the fine and coarse particle fractions, respectively. The data from a previous measurement by Selin Lindgren et. al. (1998) are presented for comparison.

As can be noted from Table 3 the values from the 1999 measurements are of the same order of magnitude as those from 1997. The soil derived elements from the Gaborone 1999 measurements are slightly higher that the ones from Gaborone 1997. This could be explained by the fact that the measurements in 1999 were taken at 1.5 meters above ground level while the 1997 measurements were taken on a rooftop 15 meters above ground level. It may be noted that the Pb and Br levels for Gaborone 1999 are lower than

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those of 1997. There has been a shift in Botswana since 1997, from using leaded to unleaded gasoline, which can explain the decrease in the Pb and Br levels. Secondly the Gaborone 1997 site is closer to the university car parking lot which can also have an influence on the Pb and Br levels. The observation of decreasing Pb and Br levels demonstrates that the increasing use of unleaded petrol during the last years, is leading, as desired, to diminished lead ambient concentrations. Note, that the sum of coarse and fine Pb and Br for Gaborone 1999 is still smaller than the fine particle fraction alone for Gaborone 1997, and hence the decreasing concentrations can not be attributable to the difference in impactor cut-offs.

The concentration of Lead, one of the pollutants present in the atmosphere, with many related health effects is investigated. The investigation is to determine the effects of the implemented restriction on usage of leaded petrol in Botswana(Botswana Govt. 1 April 2006).The monitoring of lead present in environment of Gaborone was conducted , and it was found over a period of two months the concentration declined in 84 days was seen to be about 24% (Verma et al 2010)

It is thought that this effect is due to the transition of the usage of unleaded petrol in Botswana which began from 1 April 2006. We should bear in mind that the lead particle emitted from the leaded petrol before the transition date could remain in the atmosphere for several months. The measurements (Hana et al., 1983) show that 25 % of the vehicular emission, lead, is in the form of large, coarse particles which settle on the road-side and the rest are of a fine size which remain floating for a considerable time due to their high residence.

It is interesting to note that the concentrations of SO_2 and BC in Gaborone 1999 compares to those of cities in industrialised countries. Cu and Ni concentrations are low in Gaborone when compared to other places where Cu and Ni are mined (Chimidza and Moloi, 2000).

Table.3: Means, medians and Interquartile ranges (IQR) for this study (Gab99) and a previous study carried out in 1997 (Gab97). Elements in both fine (f) and coarse (c) particles are presented along with BC and SO_2. Units are ng m^{-3} for the element concentrations and $\mu g\ m^{-3}$ for BC and SO_2.

Element	Gab99			Gab97		Vemadata (2009)
	Mean	Median	IQR	Mean	Median	IQR
Si(c)	6700	7000	2100	4400	4600	1800
Si(f)	720	630	720	BDL	BDL	BDL
S(f)	1400	1200	750	1200	1100	340
Cl(c)	200	190	110	110	87	120
Cl(f)	BDL	BDL	BDL	100	45	89
K(c)	1100	1100	370	590	560	170
K(f)	310	300	86	650	630	210
Ca(c)	900	940	350	760	780	430
Ca(f)	100	99	50	160	130	110
Ti(c)	280	280	110	190	190	76
Ti(f)	34	32	21	41	39	34
Cr(c)	6.1	4.8	3.0	BDL	BDL	BDL
Cr(f)	1.2	0.9	1.0	BDL	BDL	BDL
Mn(c)	52	50	20	53	51	19
Mn(f)	7.8	7.1	3.4	14	13	13
Fe(c)	2600	2600	970	2400	2300	960
Fe(f)	350	320	210	600	460	540
Ni(c)	2.5	2.7	1.0	BDL	BDL	BDL
Ni(f)	0.7	0.6	0.3	BDL	BDL	BDL
Cu(c)	6.4	6.5	3.2	10	8.0	6.0
Cu(f)	2.1	1.7	1.4	7.0	6.0	5.0
Zn(c)	14	12	7.0	21	19	13

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Zn(f)	6.2	5.4	4.0	28	22	18
Br(c)	11	11	7.1	15	16	15
Br(f)	55	54	38	120	100	130
Rb(c)	10	11	4.6	12	11	4.0
Rb(f)	1.4	1.1	0.9	3.0	3.0	4.0
Sr(c)	4.1	4.3	1.8	6.0	6.0	2.0
Sr(f)	0.4	0.3	0.3	2.0	1.0	2.0
Pb(c)	20	20	12	38	39	33
Pb(f)	79	79	64	230	210	250
BC	1.3	1.3	0.3	nm	nm	nm
SO_2	54	55	27	nm	nm	nm

BDL: data was below detection limit.
nm: data was not measured.
Gab99:Gaborone 1999 measurements, Gab97: Gaborone 1997 measurements.
For Gab99 fine particles (f), d_a<2.5 μm and coarse particles (c), 2.5 μm < d_a <10 μm
For Gab97 fine particles (f), d_a<3.5 μm and coarse particles (c), 3.5 μm < d_a <18 μm

3.2. *Elemental ratios of some fine particle elements*

Fig. 1 represents the regression line of fine particle Br against fine particle Pb in Gaborone aerosols. As can be noted there is a strong correlation (R^2 =0.99). This could indicate internally mixed Pb and Br originating from the same source (automobile exhausts). The corresponding slope of the regression line is 0.7 and thus is higher than the ratio of 0.45 obtained in the 1997 measurements ('Shell South Africa' personal communication). An explanation could be that the petrol additive used in Gaborone 1999 has a different Br-Pb ratio from the one used in Gaborone 1997.

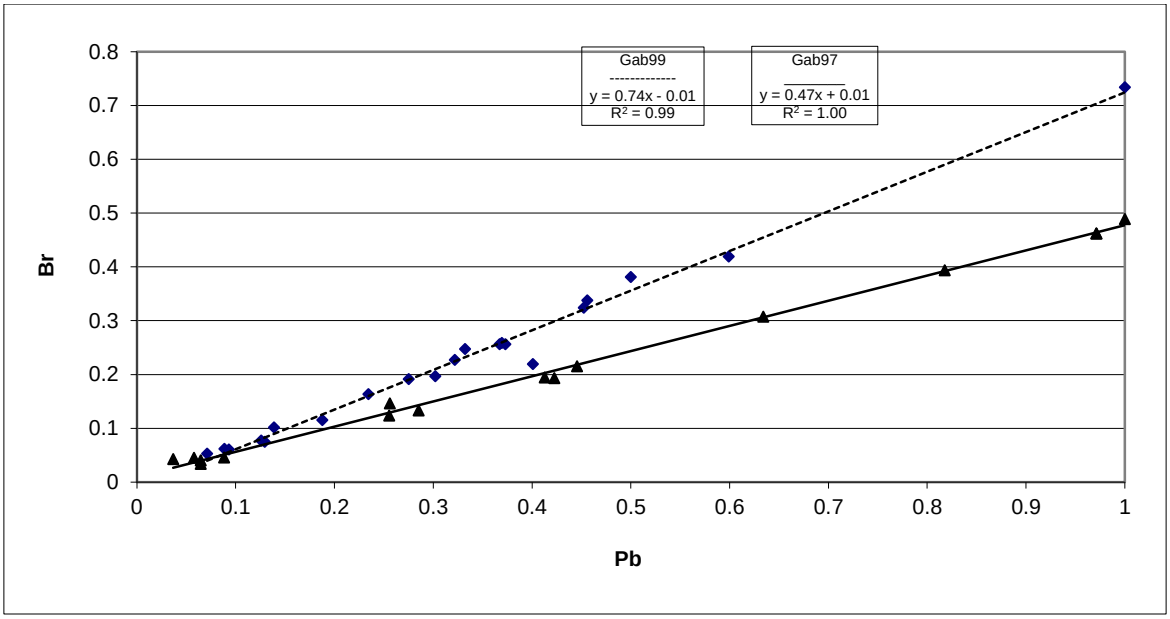

Fig.1: Regression line of normalised fine particle Br against normalised fine particle Pb for Gaborone 1999 and Gaborone 1997. The plotted values for both Br and Pb are normalised to the maximum value of Pb in both cases. (For Gaborone 1997, $[Br]_{max}$=290 ng/m^3, $[Pb]_{max}$=600 ng/m^3 ; For Gaborone 1999, $[Br]_{max}$=180 ng/m^3, $[Pb]_{max}$=240 ng/m^3).

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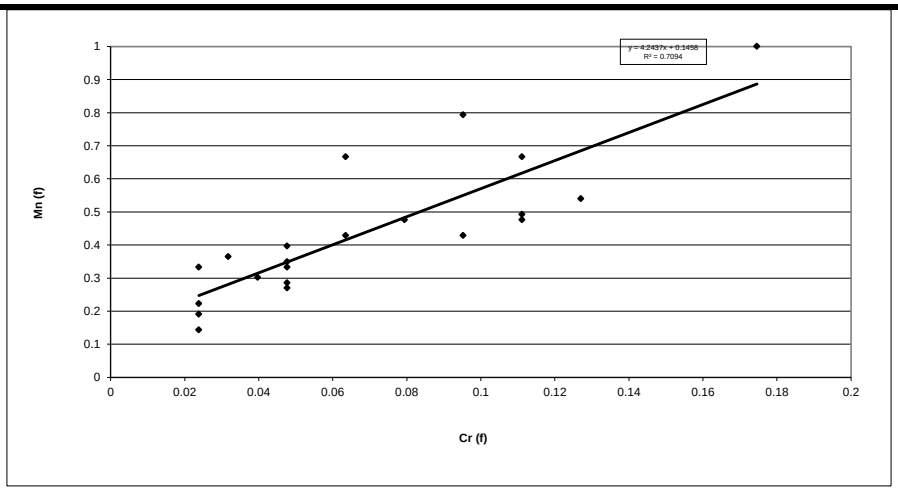

Fig.2: Regression line of normalised fine particle Mn versus normalised fine particle Cr for Gaborone 1999. The values are normalised to the maximum value of Mn. ($[Mn]_{max}$= 17 ng/m^3, $[Cr]_{max}$=3.1 ng/m^3)

Many of the anthropogenic fine particle elements have positive correlation. As an example Fig. 2 illustrates that the two elements Mn and Cr could have a common source or come from the same source region. The two elements Mn and Cr are very harmful to the environment and they have been found to be enhanced near an oil refinery (Mikula, 1997). Both are also effluent elements in the manufacture of stainless steel. Some Cr compounds are known to be carcinogenic to humans (Edme et al., 1997).

The study conducted at University of Botswana (Verma and John 2009) has also investigated elemental analysis of the elements present in the environment of Gaborone. Their findings revealed that elements detected in the atmospheric particles of Gaborone were Al, Si, Fe, K, Ca, Mg, Zn, Na, Cu, Pb, Ti, Ni, Pt, Au and Cr. The frequently occurring elements were silicon, aluminium, iron, copper and lead. The most frequently occurring element was silicon and least frequently occurring element was platinum. The major percentage of elements present in the atmosphere of Gaborone was nickel, copper, lead and gold.

IV. CONCLUSION

Comparison of results from Gaborone 1999 and Gaborone 1997 indicates that lead concentrations have significantly decreased during the last years. This demonstrates that the increasing use of unleaded petrol during the last years, is leading, as desired, to diminished Pb ambient concentrations.

Regression analysis has pointed to a few sources, which contribute to the Gaborone aerosol. The results indicate relatively high levels of local air pollution originating from both the natural and anthropogenic sources. As an example it can be mentioned that Pb and Br show a very high correlation coefficient indicating that they come from the same source which in this case is traffic.

The Gaborone aerosol consists of a wide range of aerosols both natural and anthropogenic. It varies from silicon to lead.

Although Botswana is a developing country some localised aerosol concentration like SO_2 and BC compared to those of industrialised countries.

The results from this study compared with similar studies done by Verma et. al.(2009) and(2010)., It is desirable that measurements with longer sampling times be undertaken in the future in order to further elucidate some of the observed phenomena. Longer time sampling measurements will give results with more statistical significance.

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Biogas Production Potential of Food Waste

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Abstract*—At present our country is facing various problems, among that energy crisis has become more serious in next coming years. Both energy crisis and pollution problems could be controlled by adopting an alternative method of biogas production form waste products. Food waste is the best alternative for biogas production in a community level biogas plant. Hence in the present study, an attempt has been made to study the rate of biogas production in a lab scale biogas digester model for the efficient conversion of the food waste (starch –rich materials) generated from PRIST University Campus. The biogas production depends on the maximum biogas yield, the concentration of volatile solids of the input, the density of the effluent, the density of the biogas and the reaction rate constant, which are all substrate - or process - specific. The experiments were carried out for 40 days and the rate of gas production was measured by water displacement method. The pH value of the cow dung and food waste was initially measured and adjusted to nearer to neutral and gradually increased to acidic and again it got stabilised to the neutral pH which favoured the production of biogas. The percentage of total solids was 69.86, 93.56 and 25.67 for cow dung, food waste and digested slurry respectively. The percentage of volatile solids was 52.5, 86.3 and 18.9 for cow dung, food waste and digested slurry respectively. The percentage of volatile fatty acid was 285, 356 and 365 for cow dung, food waste and digested slurry respectively. Observations on daily basis were made on the constituent of biogas, pH, volume and rate of biogas production. The rate of biogas production continuously increased as days progressed and there was maximum yield in biogas after 20 days. Thus continuous feeding helps in daily biogas production and can be used at a small as well as larger scale to manage the organic waste and energy production for various applications.*

Keywords*— *Anaerobic digestion Continuous fermenter, Digester, Food waste, Slurry.

I. INTRODUCTION

The prime challenge for the country is to provide the minimum energy services to allow the rural people to achieve decent standard of living. The biogas plant is a boon to the Indian farmers. The two main products of the biogas plants are enriched compost manure and methane, where as the compost manure helps to meet the fertilizer requirements of the farmers in a more economical and efficient manner that boost ups the agricultural production [1].

Due to industrial revolution and population explosion there has been an increase in the energy demand. To fulfil this energy demand non-renewable energy resource such as fossil fuels are the key energy generators. These non-renewable energy resources are limited and have various environmental impacts. This has resulted in researching heavily on alternatives forms of renewable sources of energy such as biogas production from biomass.

Biogas can be produced by anaerobic digestion from nearly all kind of biological feedstock types. They are from the primary agricultural sectors and from various organic waste streams. The largest resource is represented by animal manure and slurries from cattle and pig production units as well as from poultry, fish etc. In India million tones of animal manure are produced every year. When untreated or poorly managed, animal manure becomes a major source of air and water pollution. The animal production sector is responsible for 18 % of the overall green house gas emissions, measured in CO_2 [2]. It is smokeless, hygienic and more convenient to use than other solid fuels [3].

Biogas is used for cooking and lighting purposes and in larger plants, as motive power for driving small engines. It is produced when bacteria degrade organic matter in the absence of air. Biogas contains around 55-65 % of methane, 30-40% of carbon dioxide. The calorific value of biogas is appreciably high (around 4700 kcal or 20 MJ at around 55 % methane content). The gas can effectively be utilized for generation of power through a biogas based power-generation system after dewatering and cleaning of the gas. In addition, the slurry produced in the process provides valuable organic manure for farming and sustaining the soil fertility.

The bio-gas produced from food waste and decomposable organic material consisting of methane and a little amount of carbon dioxide is an alternative fuel for cooking gas (LPG). Also, the waste materials can be disposed off efficiently without any odour or flies and the digested

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slurry from the bio-gas unit can be used as organic manure in the garden.

Therefore, this work was carried out to explore the potential of biogas production from co-digestion of cow dung with food waste, along with various organic wastes. Hence, in this study an effort was made to study the cumulative biogas generation during digestion and the relationship of other parameters such as pH, EC, Total Solids (%), Total Dissolved Solids (ppt/l), Volatile Solids (%), Volatile fatty acids (VFA %), Total Khejdhal nitrogen (TKN %), Total organic carbon (TOC) and percentage of methane.

II. MATERIALS AND METHODS

2.1. Components of the Bio-gas Plant

The major components of the bio-gas plant consists of a digester tank, an inlet for feeding the food waste, a gas holder tank, an outlet for the digested slurry, a gas delivery system for taking out and utilizing the produced gas.

The Biogas plant consists of a digester tank, where the organic material was stored and the microorganisms work on them and release gas. The gas thus produced was collected in a tank known as gas collector. In a floating type model, this tank is floating in the slurry and moves up-and-down based on the amount of gas stored in it. It was fitted with a guide pipe that helps the gas collector tank to move up-and-down inside the digester tank. The waste material was fed through feed pipe inside the digester tank. The fully digested slurry drained out through the outlet pipe. This was collected, diluted and used as fertilizer for plants. A gas pipe line from the Gas collector tank helps in utilizing the gas for cooking and lighting.

2.2. Sample Collection and Processing

Cow dung sample were collected from the animal farm house at Vallam, Thanjavur and they were homogenized using through mixing. Food waste was brought from boy's hostel and canteen of PRIST University in a plastic container. Fresh feed material (food waste) was collected every three days and was stored at 4ºC. The preparation included homogenization in a kitchen blender, diluting with water (1:1 ratio).

All the samples brought to the laboratory and further processed for experimental analysis. Cow dung and food waste in combinations were used as a substrate to find out the efficient conversion of biogas.

Fig.1: Collection of food waste from PRIST University Hostel

2.3. Preparation of Inoculum

The inoculum was prepared from just one day old cow dung. 25 kg of cow dung were mixed with 25 litre of water. The samples were taken from the homogenized slurry for further physico - chemical analysis. About 25 kg of inoculums were fed into the digester through inlet chamber. The purpose of inoculums is to make a culture of the microorganisms so that when fresh food waste is added to it, the biogas production is enhanced.

2.4. Feeding of waste into the digester tank

After the acclitimization period, the gas formation was noted after 5 days of the inoculation of cow dung slurry. At this stage, the digester is ready for feeding the waste material. About 100 kg of waste material was fed to the digester (food waste from canteen/hostel) through inlet pipe fitted to the bottom of the digester. The dilution was kept less than 1:1 because food waste had already enough water content in it. The preparation of slurry was made by homogenization in a kitchen blender, diluting with water. The samples were taken from the homogenized slurry for further analysis.

2.5. Physico – chemical analysis of Cow dung and food waste

The physico- chemical analyses of the substrates were carried out and are shown in Table 1 and 2. pH, EC, salinity, Total solids (TS) and Total Dissolved Solids (TDS) were measured by potentiometeric method. TOC [4] and TKN were determined according to standard procedures as outlined in APHA [5]. Volatile solids

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(VS), Volatile fatty acids (VFA), were analyzed for fresh substrates and then for the digested slurry according to method of [6].

The bio gas composition was also analyzed. The biogas was collected and measured in a graduated beaker by means of water displacement method. The amount of gas produced is equal to the amount of water displaced in the beaker.

III. RESULTS AND DISCUSSION

3.1. Components of the Bio-gas Plant

The major components of the bio-gas plant consists of

- A digester tank, an inlet for feeding the food waste
- A gas holder tank, an outlet for the digested slurry
- A gas delivery system for taking out and utilizing the produced gas.

The volume of the digester tank constructed for lab scale was 200 litres capacity of PVC material. The volume of the gas collector tank was 150 litres capacity this acts a reservoir of gas collection. The produced biogas was collected in a gas collector tank and is shown in fig.2.

Fig.2: Lab scale model biogas digester and measurement of the biogas using water displacement method

3.2. Analysis of samples

The results of the analysis of physicochemical parameters of the fresh substrates and the digested slurries are shown Table 1 and 2. The pH ranged from 5.89 to 6.8 for the substrates used for the study. The pH was adjusted to neutral (7.0 to 7.2) by adding NaOH to enhance and accelerate the production of biogas in the digester.

Anaerobic digestion depends on several different parameters for an optimum performance. Different groups of microorganisms are involved in the methane production, and suitable conditions have to be established to keep all the microorganisms in balance. Some of these parameters are: pH, temperature, mixing, substrate, C/N ratio, and hydraulic retention time (HRT). Digestion is a slow process and it takes at a minimum of three weeks for the microorganisms to adapt to a new condition when there is a change in substrate or temperature [7].

pH is essentially a measure of the acidity and alkalinity of a solution before feeding to a digester. A pH value of 7 is regarded as neutral, less than 7 as acidic and more than 7 as alkaline. In the present study, the values of pH were ranging from 6.8 and 5.89 for the cow dung and food waste (before digestion process) respectively. The results of the present study correlated with previous workers which favours biogas generation. A symbiotic relationship is necessary between the hydrogen-producing acetogenic microorganisms and the hydrogen-consuming methanogens. Furthermore, a neutral pH is favorable for biogas production, since most of the methanogens grow at the pH range of 6.7 – 7.5.

Augenstein *et al*. [8] suggested that during anaerobic fermentation, micro-organisms require a natural or mildly alkaline environment for efficient gas production. An optimum biogas production is achieved when the pH value of input mixture in the digester is between 6.25 and 7.50 [9,10]. The pH value in a biogas digester is also a function of the retention time. In the initial period of fermentation, as large amounts of organic acids are produced by acid forming bacteria, the pH value inside the digester can decrease below 5. This inhibits or even stops the digestion or fermentation process. Methanogenic bacteria is very sensitive to pH value and do not thrive below a value of 6.5. Later, as the digestion process continues, concentration of NH_4 increases due to the digestion of N_2, which can increase the pH value to above 8. When the CH_4 production level is stabilised, the pH range remains between 7.2 and 8.2. According to studies in China, during the period when ambient temperature varies between 22 and 26°C, it takes approximately 6 days for pH value to acquire a stable value SPOBD [11]. Similarly, during the period when ambient temperature ranges between 18 and 20°C, it takes approximately 14-18 days for pH value to attain a stable value [12].

The C/N ratio of fresh substrates was 19.07, 24.81 for cow dung and food waste respectively. It achieved a ratio of 20.37 after the digestion process completed.

In the present study optimum C/N ratio was obtained, which well correlates with study of Deublein and Steinhauser, [7]. The carbon and nitrogen ratio should be around 16:1 – 25:1. Too much increase or decrease in the carbon/nitrogen ratio affects biogas production. The concentration of solids in the digester should vary between 7 % and 9 %. Particle size is not an important factor compared to other parameters such as pH and temperature. However, the size of the particles used

affects the degradation and ultimately the biogas production rate [7,12,13].

The relationship between the amount of carbon and nitrogen present in organic materials is expressed by the carbon/nitrogen (C/N) ratio. A suitable C/N ratio plays an important role for the proper proliferation of the bacteria for the degradation process [14].

Biochemical parameters such as total solids (%), volatile solids (%) and volatile fatty acid (VFA) content of the cow dung admixtures with food waste (before feeding) and slurries (after digestion) were analysed and shown in Table 1 and 2.

Fresh cattle waste consists of approximately 20 % total solid (TS) and 80 % water. TS, in turn, consist of 70 % volatile solids and 30 % fixed solid. For optimum gas yield through anaerobic fermentation, normally, 8-10 % TS in feed is required TERI [15]. This is achieved by making slurry of fresh cattle dung in water in the ratio of 1:1.

The biochemical composition of the substrates mixture during the digestion period showed that there was a gradual decrease in biochemical characteristics such as total solids, volatile solids from the 0^{th} to 40^{th} day. Volatile fatty acid content is represented in the fig. 3. It was increased in the initial stage of digestion and gradually increased up to the 6^{th} day of digestion process and again trending towards decreasing nature up to 14^{th} day. There was a sharp raise in the value in the 16^{th} day and there was a variation in the value thereafter. The decrease of VFA in the middle of digestion process indicated the biogas production yield was better in the reactor. (Fig.3). The organic nitrogen content was subsequently increased during the digestion process and considerable solid removal has been achieved in all the substrates mixtures, which was supported by the reduced rate of total organic carbon (Table 1 & 2).

3.3. Quantitative Analysis of Biogas yield

Cumulative yields of biogas (expressed in litres) from the admixtures of cow dung and food waste are presented in Table 2. The typical set up for the measurement of gas is shown in fig.2 and the rate of gas production at ambient temperature of food waste (FW) admixtures with cow dung (CD) at different hydraulic retention time is presented in fig.4. There was a gradual increase in the production of bio gas as the number days increased i.e. retention time. There was sharp increase in the peak from 26^{th} day to 35^{th} day and there was a decline in the gas production towards the end of the digestion period. The yield of biogas obtained during the study period amounted to about more than 90 percent of the total gas. The CH_4 is to CO_2 ratio was 65: 33 during the end of the digestion process. In the first 20 days of operation, the content of bio gas in the reactor was 65 %, and after 30 days of operation the biogas content was 70 %. The temperature recorded during this process was 35º C to 38º C. The rate of bio gas production was dependent on the temperature and when there was variation in temperature, bio gas production rate was significantly varied.

The major parameters affecting methanogenic reactions in a digester are the C/N ratio, temperature, pH value, presence of volatile substance, biological oxygen demand (BOD), chemical oxygen demand (COD) [16,17]. The rate of biogas production depends on the ambient temperature of a particular region also; it decreases considerably if the ambient temperature falls below 15°C or if it exceeds 45°C. In the present study the ambient temperature of 38°C was recorded which enhances the biogas production rate. Optimum pH, C/N ratio and volatile solids of the present study results favoured the production of biogas in the reactor.

Table.1: Physicochemical parameters in fresh slurry

S. No.	Name of the waste	pH	EC	Salinity (mg/l)	TS (%)	TDS (mg/l)	VS (%)	VFA (mg/l)	Nitrogen (%)	TOC (%)	C/N ratio
1.	Cow dung	6.8	4.6	4.26	69.67	5.32	52.5	285	2.8	53.4	19.07
2.	Food waste	5.9	13.8	12.56	93.56	8.12	86.3	356	1.6	39.7	24.81

Table.2: Physicochemical parameters in digested slurry

S.No	Name of waste	pH	EC	Salinity (mg/l)	TS (%)	TDS (mg/l)	VS (%)	VFA (mg/l)	Nitrogen (%)	TOC (%)	C/N ratio	CH_4 : CO_2
1.	Cow dung + Food waste slurry	4.32	8.6	10.56	25.67	5.95	18.9	365	1.9	36.8	20.37	65:33

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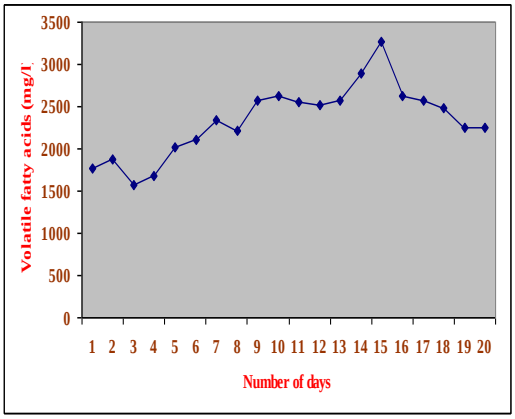

Fig.3: Showing the VFA content of food waste vs number of days

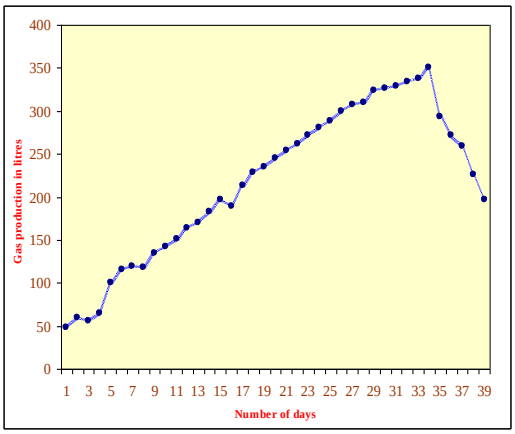

Fig.4: Showing the gas production rate of food waste vs number of days

IV. CONCLUSION

Since food waste is easily biodegradable and is having high volatile solids, it can be potentially used as a feed stock for biogas production. Use of inoculums can significantly reduce the lag phase of bacteria in food waste and hence biogas generation is continuous. Thus continuous feeding helps in daily biogas production and can be used at a small as well as larger scale to manage the organic waste and also produce the energy which can be used for the domestic purpose like cooking, lighting etc.

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Comparative Assessment of the Effect of Ripening Stage on the Vitamin C Contents of Selected Fruits Grown within Nsukka Axis of Enugu State

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Abstract— *Studies were carried out on the quantitative determination of vitamin C in fresh fruits (orange, cashew, pawpaw, lemon, grape) at different ripening stages using iodometric titration method. The study revealed that the vitamin C contents in the fruits at different ripening stages decreased in the following order: half-ripe < ripe < unripe. Orange was found to have the highest vitamin C content of 77.96 ± 0.44mg/100g while lemon has the least content of the vitamin with a value of 11.83 ± 0.10mg/100g.*

Keywords— Vitamin C, Ripening effect, Cashew, Orange, Lemon, PawPaw and Grape.

I. INTRODUCTION

Fruit is a part of flowering plant derived from specific tissues of the flower, with one or more ovaries and in some cases accessory tissues (Mauseth, 2003). Fruits are the means by which plant disseminate seeds. Many plants bear edibles fruits. Humans and animals have become dependent on fruits as a source of food and hence accounts for a substantial fractions of the world's agricultural output (Lewis, 2002). Example of fruits include; mango, apple, cashew, orange, grape, water melon, lemon and pineapple e.t.c.

Cashew (*Anarcadium occidentale*) is a tropical evergreen tree that produces the cashew nut and cashew apple. It can grow as high as 14m and has proved more profitable with earlier maturity and higher yields. The cashew apple is a light reddish to yellow fruit whose pulp can be processed into a sweet, astringent fruit drink or distilled into liquor (Vaughae, 2005). Grape fruit (*Citrux paradix*) is a sub-tropical fruits tree known for its sour to semi-sweet fruit.Grape fruit is a hybrid originating in barbados as an accidental cross between two introduced sweet species, sweet orange and pomelo. The evergreen grape fruit trees usually grow to 5 – 6m tall. The fruit has antioxidant properties (Feller *et al*., 2000) Lemon (*Citrux limon*) is a species of small ever green tree in the flowering plant family rutaecea, native to Asia. The tree is ellipsoidal yellow fruit and is used for culinary and non-culinary purposes throughout the world (Morton, 1981). Paw paw tree (*Cariaca papaya*) is native to the tropics is the Americas. It is a small, sparsely branched tree usually with a single stem growth, 5 to 10m tall with spirally averages confined to the top of the fruit. The ripe fruit of the papaya is usually eaten raw without skin or seed. It has both culinary and medicinal uses (Titayi *et al*., 2008). Orange (*Citrus sinensis*) is a tropical ever green tree from the flowering plant family of rutaecea. The tree is ellipsodal yellow fruits that serves nutritional requirements of man (Nicolosi *et al*., 2000).

Most plants and animals have the ability to synthesize vitamin C, the only mammals that are unable to synthesize vitamin C are premates including man and guinea pigs. Therefore, humans depend on exogenous source of the vitamin which includes fruits and vegetable as well as food supplements (Okiei *et al*., 2009). Vitamin C is the most important vitamin for human nutrition that is supplied by fruits and vegetables. It is a valuable food component because of its antioxidant and therapeutic properties (Okiei *et al*., 2009). Ascorbic acid also known as vitamin C, when pure is white crystalline water soluble vitamin found especially in citrus fruits, vegetables and organ meats. The amount of ascorbic acid in plant varies greatly, depending on such factors such as variety, weather and maturity (Rahman *et al*., 2007).

Vitamin C is a very important water soluble nutrients, critical for sustaining cellular function. It promotes health in a variety of ways, such as preventing scurvy, promoting bone structure and strength, reducing inflammation and aiding the cardiovascular system through anti-oxidation and improving endothelial cell function (Wannamethee *et al*., 2006; Naidu, 2003). Vitamin C is crucial for maintaining a healthy body and is involved in over 300 biological processes. It is necessary for the manufacture of collagen (Melissa and Ock, 2016).

The only way humans uptake ascorbic acid is via food. The estimated average requirement and recommended dietary allowance of vitamin C is between 100 – 120mg per day (Levine *et al*., 1999). However, findings suggests that people's need for vitamins and other nutrients vary

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markedly and that to maintain good health, many people need amounts of nutrients much greater than the recommended doses (Klezczewska, 2000).

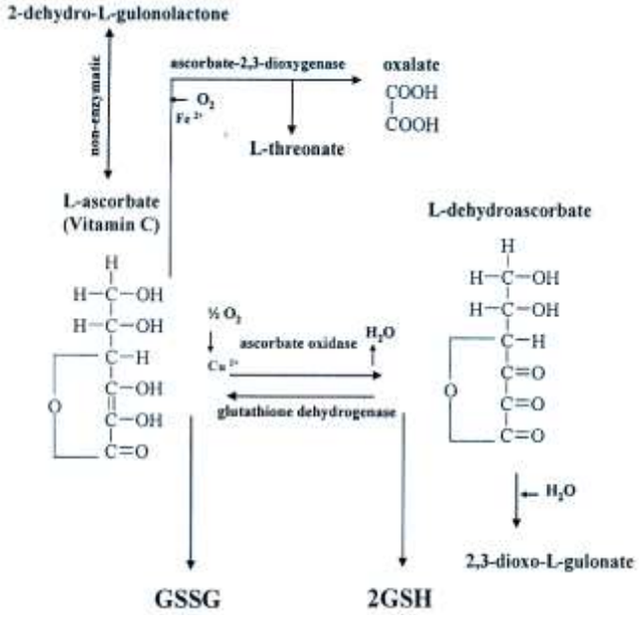

Fig.1: Scheme of a biological function of ascorbic acid (GSH – reduced glutathione, GSSG – oxidized glutathione)(Klezczewska, 2000).

Because of the important role played by vitamin C in the wellbeing of man and animals as a very important nutrient requirement, studies were carried out to assess the effects of ripening stage on the vitamin C contents of selected edible fruits grown within the Nsukka axis of Enugu State.

II. MATERIALS AND METHODS

Sample Collection and Preparation

Twenty five samples (ripe, half-ripe and unripe) were obtained from markets and farms within the studied environment. The fruit samples were; orange, lemon, grape, cashew and paw-paw. Fruits sample were blended with a blender and filtered using a muslin cloth and made up to 100ml with distilled water respectively.

Preparation of reagents and estimation of ascorbic acid

1% starch indicator solution was prepared by dissolving 0.50g soluble starch with 50ml of distilled water and the solution constantly stirred until it dissolves completely (Harris, 2000).

Iodine solution was prepared by weighing 5.00g of potassium iodide (KI) and 0.262g of potassium iodate into a 300ml beaker and dissolving with 200ml distilled water. 30ml of 3M concentration of H_2SO_4 was added into the beaker solution was made up to mark with distilled water (Harris, 2000).

Vitamin C standard solution was prepared by dissolving 0.250g of ascorbic acid in a 100ml conical flask with distilled water. The solution was transferred into 250ml volumetric flask and made up to mark with distilled water.

Titration of iodine solution with vitamin C standard solution was done by pipetting 25ml into a 125ml Erlenmeyer flask. 10 drops of 1% starch solution was added and then titrated against iodine solution until blue-black colour was observed. Each sample was run three times to obtain triplicates measurements and ascorbic contents were evaluated accordingly (Harris, 2000).

III. RESULTS AND DISCUSSION

Table.1: below showed the vitamin contents in Table 1 (mg/100g) for the fruit sample analyzed.

Fruits	Average concentration of vitamin C (mg/100g)		
	Ripe	Half ripe	Unripe
Grape	30.13 ± 0.96	39.08 ±0.11	20.78 ± 0.15
Lemon	11.83 ± 0.10	20.12 ± 0.60	16.45 ± 0.20
Pawpaw	61.09 ± 0.62	68.69 ± 0.19	50.14 ± 1.93
Orange	64.85 ± 1.05	77.96 ± 0.44	48.85 ± 0.37
Cashew	34.61 ± 0.74	42.82 ± 0.33	29.05 ± 0.18

The results of vitamin C contents (mg/100g) obtained from the study are indicated in Table 1. The mean values of the vitamin C contents decreased in the following order: orange >paw paw>cashew >grape >lemon. Orange was observed to have the highest vitamin C concentration in all the fruits analyzed while lemon has the least concentration. The results obtained is in agreement with literature findings that the major source of vitamin C are citrus fruits, especially sweet oranges (Okiei *et al*., 2009). The variation in vitamin C contains of the analyzed fruits could be due to factors such as variety, weather and maturity (Rahman *et al*., 2007).

Grape

The contents of vitamin C in grape as indicated in Table 1 were in the order: half ripe > ripe > unripe. This implies that half ripe grape has the highest mean vitamin C content of 39.08 ± 0.11mg/100g while ripe grape has the least concentration of 20.78 ± 0.15mg/100g.

Lemon

The vitamin contents in lemon decreased in the following order: half ripe > unripe > ripe with mean values of 20.12 ± 0.60mg/100g, 16.45 ± 0.20mg/100g and 11.83 ± 0.10mg/100g respectively.

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Pawpaw

The mean concentration of vitamin C in pawpaw indicated in Table I increased in the following order: unripe < ripe < half ripe with values of 50.14 ± 1.93mg/100g, 61.09 ± 0.62mg/100g and 68.69 ± 0.19mg/100g respectively.

Orange

The study showed that the mean contents of vitamin C in the orange samples decreased in the following order: half ripe > ripe > unripe with values of 77.96 ± 0.44mg/100g, 64.85 ± 1.05mg/100g and 48.85mg/100g respectively.

Cashew

The result of the vitamin C contents in cashew as indicated in Table I increased in the following order: unripe < ripe < half ripe with values of 29.05 ± 0.18mg/100g, 34.61 ± 0.74mg/100g and 42.85 ± 0.33mg/100g respectively. The results of this study was in agreement with values reported by (Mahdavi *et al*., 2010) for the vitamin C contents of orange and lemon fruits in grown local districts of Pakistan. The results of the study showed that fruits at half ripening stage indicated the highest concentration of vitamin C. It has been reported that ascorbic acid contents in fruits decreases upon ripening, temperature increase and time which were attributed to degradation caused by heat and oxidation (Muhammed *et al*., 2014).

IV. CONCLUSION

The results obtained in this study showed that fruits are very essential source of vitamin C to man and animals. The study further indicated that fruits at half-ripe stage contains the highest vitamin C concentration hence daily consumption of fruits (especially orange and paw paw) at that maturity stage will ensure that people's needs for the vitamin is met on daily basis.

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Species diversity and distribution of ruderal flora on landfills in Maradi city, Niger

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Abstract*—Waste management continues to be a critical environmental issue in cities. It impacts on the well being of the population, the environment and the biodiversity. In the city of Maradi, in Niger, more interest is given to the problem in order to understand the whole waste management system. It is in this context that this study is carried out to investigate on the role of ruderal flora on the municipal solid wastes dumpsites and landfill sites in Maradi city. The specific objectives are to determine the floristic diversity and distribution of ruderal flora on the municipal solid waste disposal sites, and to identify potential species that can play an important role in the phytoremediation of these sites. In total, 65 species belonging to 52 genera and 24 families were recorded. These species can be categorised into two groups containing anthropic and nitrophilic species according to the ascending Hierarchical Classification (AHC) at 25% similarity. Characteristic species of the first group G1 are Amaranthus viridis and Cucurbita pepo, and Datura innoxia and Cucumis melo for the second group G2. Other ruderal species, namely Amaranthus spinosus L., Amaranthus viridis L., Celosia trygina L., Datura innoxia Mill., and an introduced woody species, Cuphea hyssopifolia Kunth., found are not included in the Maradi city list of species. Datura innoxia, Amaranthus viridis and Amaranthus spinosus are species known to tolerate different degrees of pollution and their ecology should be further study to better understand how they can be used for phytoremediation on this kind of sites.*

Keywords*—*Landfills, flora, species, diversity, distribution, Maradi.

I. INTRODUCTION

Cities in developing countries are subject, on one hand, to a high demographic expansion and on the other, to a massive and fast rural urban migration (Seidl and Mouchel, 2003). Effects of globalization have led population of several large West African cities to an increase in their consumption resulting to an exorbitant increase of household waste (Oxfam-Québec, 2007). Waste production, particularly the solid ones, in urban areas is growing at an unprecedented rate and takes large proportions in those developing countries where their disposal has become an issue of growing concern and paramount (Redjal, 2015).

However, the garbage accumulation and local population exposure create discomforts and affect the population health (Maiti *et al*., 2004; Ouedraogo, 2010).

Consequently, there is a proliferation of disease transmitters and deterioration of the urban environment quality (Tchaou, 2011). Furthermore, dumpsites are characterized by the daily presence of household waste.

The wastes, in many cases, contain matters from animal, vegetal (food rest, organic debris ...) and mineral sources. Thus, they contribute highly to the soil enrichment of these sites especially in organic matter and nutrients.

Also, these sites offer very specific ecological conditions on one hand by the accumulation of organic matter and, secondly, by the existence of a certain contamination by heavy metals. Indeed, several heavy metals buried during waste storage have been identified in urban landfills and dumps, namely, Aluminium (Al), Lead (Pb), Cadmium Cd), Zinc (Zn), Copper (Cu), Iron (Fe), Arsenic (As), etc. (Jourdan *et al.*, 2005; Kimani, 2007; Beyene and Banerjee, 2011; Tankari *et al.*, 2013; Abdourahamane *et al*., 2015).

The presence of these organic matters, inorganic and metal pollutants often alters biodiversity in the area. It leads to the modification of ecological factors that often results in dynamics of plant association and floristic composition of the environment (Falcon, 2012). Also, the transfer of metals, particularly lead, zinc and copper, to aquifers or to plants can lead to harmful effects on people through the food chain because of their toxicity (Fifi, 2009; Jourdan *et al*., 2005; Tankari, 2011). It is well known that hyper-accumulating and accumulating ruderal

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plants accumulate metals, whatever the concentration rate in soil (Leteinturier & Malaisse, 1999). For example, according to Prasad (2001), *Amaranthus spinosus* and *Amaranthus spinosus* accumulate cadmium in their root, stem and leaves while Abou-Shanab *et al.* (2007) reported that *Cynodon dactylon* accumulate lead, cooper and zinc in their root and shoot. Hence, this recognized capacity of ruderal species to accumulate heavy metal can be used in contaminated sites phytoremediation which is one of the biological soil remediation technologies (Anoliefo *et al*., 2008).

In the city of Maradi in Niger, sanitation is one of the environmental priorities. Indeed, solid waste management, particularly that of household waste, is considered by the city officials as the main sanitation challenge in this city. Solid waste generation sources are mainly households, shops (markets) and industries. Waste disposal techniques and dumpsite management is insufficient in order to avoid contamination of soil, water, biodiversity and humans in this city. Abdourahamane *et al* (2015) even found that in the city of Maradi, the storage of solid waste on the different dumpsites and landfills types is not efficient against the heavy metal contamination such as zinc, cadmium and lead. The limited financial and technical resources also contribute to the mismanagement of the sites. It is in this context that the present study was carried out to investigate the role of ruderal flora on the municipal solid wastes dumpsites and landfill sites in Maradi city. The specific objectives of this work are to determine the floristic diversity and distribution of ruderal flora on the municipal solid waste disposal sites, and to identify potential species that can play an important role in the phytoremediation of these sites.

II. MATERIALS AND METHODS

2.1. Study area

The study was carried out in Maradi city which is the main economic centre of Niger. This city is located between latitudes 13° 32' N to 13° 26' N and longitudes 7° 40' E to 7° 13' E, and covers 8269 hectares (Fig. 1). The climate is of sahelo-sudanian type with an average temperature varying from 23.21°C in cold period to 40°C in hot period, an average relative humidity of 40.10% (Direction Nationale de la Météorologie, 2013). The mean annual rainfall, calculated over the past thirty years is around 476.89 mm. The hydrographical network is dominated the valley of Goulbi Maradi and some semi-permanent water points. This city is located on a plateau with an average altitude of around 400 m. The soils of Maradi region are tropical ferruginous, rich in silt in the Goulbi zone.

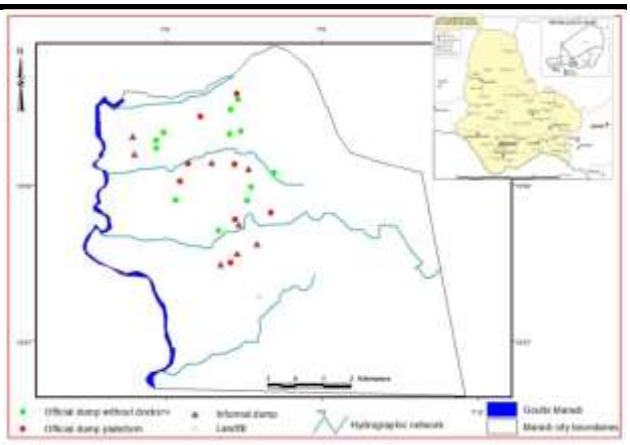

Fig.1: Study area and the sampling sites location

Maradi City has a population of 264,897 inhabitants with an annual growth rate of 4.3% in 2012 (INS, 2013). The availability of water, during a large part of the year allow the development of rainy and irrigated agriculture which is practiced by about 40% of the population of this city.

Waste management in Maradi city is provided by municipal services. The waste disposal is done in three steps (Abdourahamane *et al*., 2015). Firstly, the waste is temporarily stored in containers on the production sites which include homes, markets, main streets, hospitals, etc. Secondly, they are transferred by local people to the dumpsites where they are finally collected by municipal services to be stored in the landfill sites and very rarely buried. Recycling is hardly practiced. However, there are a few collectors of objects from landfills and dumpsites in order to transform into useful domestic tools or sell them to other interested users

2.2. Sites description

The sampling sites are dumps and landfills (Fig. 2).

- Dumpsites: They are of two types: official and informal dumpsite or uncontrolled.

Official dumps are places equipped with dumpsters or garbage containers for waste collection (Concept, 2007). These dumpsters have a capacity of 5.5 m^3 and more or less regularly removed and disposed of in landfills or other sites. There are two categories of official dumpsites in Maradi city: (i) the "dumps platforms" that are equipped with a three-compartment system with a dock for easy access to the dumpster, a terrace of about 5 m^3 in the downstream portion in contact with two or three containers with a small hut for on-site caretaker, and (ii) the "official dumps without docks", the most abundant in the city, which are simply characterized by the presence of a dumpster or other container. They are officially recognized by the municipality. Informal or uncontrolled dumps, unlike officials, are waste consolidation sites without official municipality permission, created by neighbouring inhabitants because of the lack of formal

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dumpster or the relatively long distance to an official dumpsite, making it inaccessible (Concept, 2007). They are usually located in inappropriate places, in the streets, often alongside health or education infrastructure, etc.

- Landfills: Last link in the waste disposal chain, these are quarries or areas adjacent to the city where the collected waste from dumpsites are eliminated. They are often very large uncontrolled dumpsites.

Fig.2: Different waste disposal sites
(adopted from Abdourahamane *et al*., 2015)

2.3. Sampling and Data collection on the herbaceous flora

Herbaceous plants sampling was carried out on six official dumpsites, six informal dumpsites and four landfill sites. Data collection was done in a 100 m2 sampling plots (10m x10m) on every site. Surveys were conducted along transects considering the main roads that run alongside these sites as main line. Transects were established perpendicular to this line towards landfills. The equidistance between adjacent transects is 15 m and 10 m between neighbouring plots on the same transect. Given the area covered by vegetation on dumps and landfills sites, plots were established to cover areas with vegetation as representative of the study site, as well as possible. Thus, on each plot, 2 transect lines were used for data collection. A total of 30 records were made on dumpsites and landfill sites.

Phytosociological records were made using the Braun-Blanquet sigmatiste method (1932). This method has the advantage to draw up an exhaustive list of all the plant species present in the plot. These records were completed by the Daget and Poissonet linear method (1971). Two lines of quadrats points were performed in each plot. Each line has 20 m of length and includes 100 points of contact.

2.4. Data analysis

Phytosociological records of the herbaceous layer collected were formed into a matrix of plant species abundance-dominance. This expresses the number of individuals of the same species and their degree of recovery. This matrix has been subjected to an Ascending Hierarchical Classification (AHC) using PC ORD software version 5. The resulted information table is summarized in a dendrogram. Analysis allows discriminating plant communities on the basis of similarity at the Sorensen index level (Legendre and Legendre 1998). Shannon and Weaver diversity (1949) and Pielou equitability (1966) indexes were calculated in order to analyze vegetation diversity of dumpsites and landfill sites.

III. RESULTS AND DISCUSSION

In this environmental context, floristic richness analysis of dumpsites and landfill sites of Maradi city revealed a relatively high floristic diversity despite the hash living conditions for many species. On the study area, 65 species belonging to 52 genera and 24 families were recorded (Table 1). Fabaceae and convolvulaceae were the most represented families with 6 species each, followed by Cucurbitaceae and Poaceae with 5 species each, the Malvaceae with 4 species. The families of Amaranthaceae, Acanthaceae, Cyperaceae, Solanaceae, Pedaliaceae and Rubiaceae are represented by 3 species each. Caesalpiniaceae, Aizoaceae, Commelinaceae, Euphorbiaceae and Tiliaceae families have 2 species each. Other families are Asclepiadaceae, Asteraceae, Capparidaceae, Lamiaceae, Molluginaceae, Nyctaginaceae and Zygophyllacea represented by one single species.

Table.1: Floristic list of dumpsites and landfill sites of Maradi city

Species	Families
Acanthospermum hispidum DC.	Asteraceae
Alysicarpus ovalifolius (Schum. Et Thonn.) J. Léonard.	Fabaceae
Amaranthus spinosus L.	Amaranthaceae
Amaranthus viridis L.	Amaranthaceae
Boerhavia erecta L.	Nyctaginaceae
Borreria scabra (schum.EtThonn.) K.Schum	Rubiaceae
Borreria stachydea (DC) hutch. Et Dalz	Rubiaceae
Cassia mimosoides L.	Caesalpiniaceae
Cassia occidentalis L.	Caesalpiniaceae
Celosia trygina L.	Amaranthaceae
Cenchrus biflorus Roxb.	Poaceae
Ceratotheca sesamoides Endl.	Pedaliaceae
Citrullus colocynthis (L.) Schard.	Cucurbitaceae
Citrullus lanatus (thunb.)Matsumara et makai	Cucurbitaceae
Cleome viscosa L.	Capparidaceae
Commelina benghalensis L.	Commelinaceae

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Commelina forskalaei Vahl.	Commelinaceae
Corchorus tridens L.	Tiliaceae
Cucurbita pepo L.	Cucurbitaceae
Cyperus esculentus L.	Cyperaceae
Cyperus rotundus L.	Cyperaceae
Dactyloctenium aegyptium (L.) Willd.	Poaceae
Datura innoxia Mill.	Solanaceae
Digitaria horizontalis Wild.	Poaceae
Echinochloa colona (L.) Link	Poaceae
Eleusine indica (L.) Gaertn	Gramineae
Eragrostis tremula Steud.	Poaceae
Euphorbia hirta L.	Euphorbiaceae
Evolvulus alsinoides (L.) L.	Convovulaceae
Gisekia pharnacioides L.	Aizoaceae
Hibiscus asper Hook. f.	Malvaceae
Hibiscus sabdariffa L.	Malvaceae
Indigofera astragalina DC.	Fabaceae
Indigofera pulchera Willd.	Papilonaceae
Ipomoea coptica (L.) Roth. ex. Roem. et Schult.	Convolvulaceae
Ipomoea dichroa Hachst.Ex Choisy	Convolvulaceae
Ipomoea vagans Bak.	Convolvulaceae
Jacquemontia tamnifolia (L.) Griseb.	Convolvulaceae
Kyllinga squamulata Thonn.Exvahl.	Cyperaceae
Leucas martinicensis (Jacq.) R. Br.	Lamiaceae
Merremia tridentata (L.) Hallier. f.	Convolvulaceae
Mitracarpus scaber (Sw.) DC.	Rubiaceae
Mollugo nudicaulis Lam.	Molluginaceae
Momordica balsamina L.	Cucurbitaceae
Monechma ciliatum (Jacq.) Milne. Red.	Acanthaceae
Mukia maderaspatana (L.) Roem.	Cucurbitaceae
Pennisetum pedicellatum Trin.	Graminea
Pergularia tomentosa L.	Asclepiadaceae
Peristrophe bicalyculata (Retz)Nees .	Acanthaceae
Peristrophe paniculata (Forssk.) Brum mitt	Acanthaceae
Phyllanthus pentandrus Schum. et Thonn.	Euphorbiaceae
Physalis angulata L.	Solanaceae
Physalis lagascae Roem. Et Schult.	Solanaceae
Ricinus communis L.	Euporbiacea
Rogeria adenophylla J. Gay.	Pedaliaceae
Sesamum alatum Thon.	Pedaliaceae
Sesbania pachycarpa DC.	Fabaceae
Sida alba L.	Malvaceae
Sida cordifolia L.	Malvaceae
Solanum lycopersicum L.	solanaceae
Tribulus terrestris Viv.	Zygophyllaceae
Trienthema portulacastrum (L.) L.	Aizoaceae
Triumfetta pentandra A. Rich.	Tiliaceae
Vigna unguiculata (L.) Walp. Subsp. Unguiculata	Fabaceae
Zornia glochidiata Reichb. Ex DC.	Fabaceae

The dendrogram derived from Ascending Hierarchical Classification (AHC) has allowed individualizing the different plant groups. Analysis divided the records into two groups (Fig. 3) at the level of 25%: *Amaranthus viridis* and *Cucurbita pepo* group (G1) and *Datura innoxia* and *Cucumis melo* group (G2).

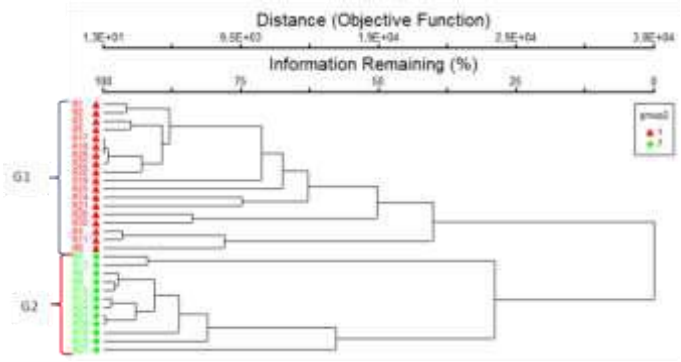

Fig.3: Dendrogram of plant group of species found on the different dumpsites and landfills in Maradi city

The group 1 of *Amaranthus viridis* and *Cucurbita pepo* is composed of 44 species (18 records) (Table 2). *Amaranthus viridis*, *Cucurbita pepo*, *Amaranthus spinosus*, *Corchorus tridens* are the characteristic species of this group. The companion species are *Sida cordifolia*, *Trianthema portulacastrum* and *Cleome gynandra*. This group belongs to the class of *Ruderali mahihotetea* Taton 1949, class containing anthropic and nitrophilic groups, trampled, from rubble and roadsides. The Shannon-Weaver index, of 4.46 bits, reveals a high diversity within this group. The equitability index, of 0.94 bits, indicates that several species participate in the recovery.

Table 2: Floristic list of the Group 1

Species	**Families**
Acanthospermum hispidum DC.	Asteraceae
Alysicarpus ovalifolius (Schum. Et Thonn.) J. Léonard.	Fabaceae
Amaranthus spinosus L.	Amaranthaceae
Amaranthus viridis L.	Amaranthaceae
Boerhavia erecta L.	Nyctaginaceae
Borreria scabra(schum.EtThonn.)K.Schum	Rubiaceae
Borreria stachydea (DC) hutch. Et Dalz	Rubiaceae

Cassia mimosoides L.	Caesalpiniaceae
Cassia occidentalis L.	Caesalpiniaceae
Celosia trygina L.	Amaranthaceae
Cenchrus biflorus Roxb.	Poaceae
Citrullus lanatus (thunb.)Matsumara et makai	Cucurbitaceae
Cleome viscosa L.	Capparaceae
Commelina benghalensis L.	Commelinaceae
Commelina forskalaei Vahl.	Commelinaceae
Corchorus tridens L.	Tiliaceae
Cucurbita pepo L.	Cucurbitaceae
Cyperus esculentus L.	Cyperaceae
Dactyloctenium aegyptium (L.) Willd.	Poaceae
Datura innoxia Mill.	Solanaceae
Digitaria horizontalis Wild.	Poaceae
Eragrostis tremula Steud.	Poaceae
Euphorbia hirta L.	Euphorbiaceae
Hibiscus sabdariffa L.	Malvaceae
Indigofera pulchra Willd.	Fabaceae
Ipomoea coptica (L.) Roth. ex. Roem. et Schult.	Convolvulaceae
Ipomoea dichroa Hachst.Ex Choisy	Convolvulaceae
Ipomoea vagans Bak.	Convolvulaceae
Kyllinga squamulata Thonn.Exvahl.	Cyperaceae
Leucas martinicensis (Jacq.) R. Br.	Lamiaceae
Mitracarpus scaber (Sw.) DC.	Rubiaceae
Monechma ciliatum (Jacq.) Milne. Red.	Acanthaceae
Pennisetum pedicellatum Trin.	Poaceae
Pergularia tomentosa L.	Asclepiadaceae
Peristrophe bicalyculata (Retz)Nees .	Acanthaceae
Ricinus communis L.	Euphorbiaceae
Rogeria adenophylla J. Gay.	Asclepiadaceae
Sesamum alatum Thon.	Pedaliaceae
Sesbania pachycarpa DC.	Fabaceae
Sida cordifolia L.	Malvaceae
Solanum lycopersicum L.	Solanaceae
Tribulus terrestris Viv.	Zygophyllaceae
Trienthema portulacastrum (L.) L.	Molluginaceae
Zornia glochidiata Reichb. Ex DC.	Fabaceae

The group 2 of *Datura innoxia* and *Cucumis melo* is composed of 40 species (12 records) (Table 3). The characteristic species of this group are *Datura innoxia*, *Cucumis melo*, *Physalis lagascae*, *Sida cordifolia*, *Ricinus communis*. The companion species are *Amaranthus viridis*, *Corchorus tridens*, *Cyperus rotondus*, *Cleome gynandra*. Like the previous one, this group belongs to the syntaxon of the *Ruderali manihotetea* Taton 1949. The value of the Shannon-Weaver index, of 3.39 bits, shows that the diversity within this group is moderate. The equitability index, of 0.96 bits, shows that a several species participate in the recovery.

Table 3: Floristic list of the Group 2

Species	**Families**
Acanthospermum hispidum DC.	Asteraceae
Alysicarpus ovalifolius (Schum. Et Thonn.) J. Léonard.	Fabaceae
Amaranthus spinosus L.	Amaranthaceae
Amaranthus viridis L.	Amaranthaceae
Boerhavia erecta L.	Nyctaginaceae
Borreria stachydea (DC) hutch. Et Dalz	Rubiaceae
Cassia occidentalis L.	Caesalpiniaceae
Celosia trygina L.	Amaranthaceae
Cenchrus biflorus Roxb.	Poaceae
Citrullus lanatus (thunb.)Matsumara et makai	Cucurbitaceae
Cleome viscosa L.	Cappparaceae
Cleome gynandra L.	Capparaceae
Commelina benghalensis L.	Commelinaceae
Commelina forskalaei Vahl.	Commelinaceae
Corchorus tridens L.	Tiliaceae
Cucumis melo L.	Cucurbitaceae
Cucurbita pepo L.	Cucurbitaceae
Cyperus esculentus L.	Cyperaceae
Dactyloctenium aegyptium (L.) Willd.	Poaceae
Datura innoxia Mill.	Solanaceae
Digitaria horizontalis Wild.	Poaceae
Eragrostis tremula Steud.	Euphorbiaceae
Euphorbia hirta L.	Euphorbiaceae
Indigofera pulchra Willd.	Fabaceae
Ipomoea vagans Bak.	Convolvulaceae
Ocimum gratissimum L.	Lamiaceae
Mariscus squarrosus (L.) C.B. Cl.	Cyperaceae
Pennisetum typhoides Stapf.	Poaceae
Pennisetum pedicellatum Trin.	Poaceae
Peristrophe bicalyculata (Retz)Nees .	Acanthaceae
Physalis lagascae Roem. Et Schult.	Solanaceae
Ricinus communis L.	Euphorbiaceae
Rogeria adenophylla J. Gay.	Pedaliaceae
Sesamum alatum Thon.	Pedaliaceae
Sesbania pachycarpa DC.	Fabaceae
Sida cordifolia L.	Malvaceae
Solanum lycopersicum L.	Solanaceae
Tribulus terrestris Viv.	Zygophyllaceae

Trienthema portulacastrum (L.) L.	Aizoaceae
Triumfetta pentandra A. Rich.	Tiliaceae

The Sorensen index of similarity between the two groups is of 42.98%, thus showing that they are each other independents. The analysis shows a relatively high floristic richness depending on the considered group. Thus, comparison between groups showed a high diversity within G1 than in G2. Comparison between the floristic lists of dumps and landfills sites with those of Maradi Tannery (Mahamane, 2012) and Maradi (Saadou, 1990) was done using the similarity Sorensen index (Table 4). Analysis of the results shows very low similarities between the three floristic lists. The highest rate is obtained between the floristic lists of dumps and landfills sites and Maradi Tannery.

Table 4: Similarity matrix between the floristic lists(in %)

Floristic lists	Dumps and landfills sites	Maradi Tannery	Maradi list
Dumps and landfills sites	-		
Maradi Tannery	31,25	-	
Maradi list	20,74	17	-

Some of herbaceous species found on the dumps and landfills sites are not included in the Maradi floristic list. These are ruderal species, namely *Amaranthus spinosus* L., *Amaranthus viridis* L., *Celosia trygina* L. and *Datura innoxia* Mill., and an introduced woody species, *Cuphea hyssopifolia* Kunth..

The very low similarities found between the floristic list of dumps and landfills sites with those of Maradi Tannery (from 2012) and Maradi (from 1990) is due to the urbanization effect. Therefore to ecological conditions change that occurs in the city over the years and the existence of particular conditions at dumps and landfills sites. The specific ecological conditions of these sites are characterized, firstly, by an overabundance of organic matter and a sun exposure throughout the year and, secondly, by the existence of some heavy metals contamination (Abdourahamane *et al.*, 2015). In fact, ruderal species are the best represented in the two groups.

Also, the toxicity of heavy metals operates a very thorough selection, eliminating many species that are found in these places. Indeed, the very severe selective screening imposed by metal toxicity can cause rapid evolution towards high tolerance levels. Although the flora of dumps and landfills habitats comes from ordinary habitats, species that compose it gather in particular vegetation compared to local ordinary habitats (Ernst, 1974 in Falcon (2012)). Plant colonization of these habitats is partly driven by the populations of existing non-tolerant plants nearby. The pH of these sites plays an important role in this selection. It act, on one hand, directly on the level of nutrients availability in the substrate, but also, one the other hand, on soil microbial activity causing organic matter decomposition and its mineralization. A pH between 6.5 and 8 is favorable for the installation of ground vegetation cover of herbaceous types (Henry *et al*., 2011). Characteristically, the growth of woody species (trees and shrubs) is inhibited, leading to the development of plant groups purely herbaceous or slightly shrub (Falcon, 2012). This would explain the predominance of ruderal species in the floristic composition of the dumps and landfills sites vegetation. In addition, some species, like *Datura innoxia*, *Amaranthus viridis* and *Amaranthus spinosus* of the ruderal flora, found exclusively in these sites, have the ability to tolerate heavy metals presence and accumulation at variable rates including those found by Abdourahamane *et al.* (2015) in the study area (Kouamé *et al*., 2006; Jean, 2007; Dazy, 2008; Atayese, 2009; Messou *et al*., 2013; Messou *et al*., 2015). In fact, several studies have showed that *D. innoxia* tolerates many heavy metals accumulation: Jean (2007) for zinc, nickel and chrome; Salt *et al.* (1995) for cadmium and Vaillant *et al.* (2005) for zinc. While many others have shown that the heavy metals tolerance and phytoaccumulation capacity of *A. viridis* and *A. spinosus*: Messou *et al.* (2013) for cadmium, lead, zinc, iron etc.; Prassad (2001) for cadmium, zinc, lead, cooper and iron; Abe *et al.* (2001) for cadmium, etc. These are metallicolous or metallophytes species. They develop special strategies to survive and colonize these environments with contrasting ecological conditions. Indeed, metallicolous species, tolerant to metals, have the ability to survive and reproduce on toxic or adverse soil to most of others organisms because of metal contamination, and metallophytes would be endermic, species associated with soil contrasting chemical conditions.

Finally, the study of the ecology of species counted on landfills and dumps sites in Maradi city reveals the preponderance of ruderal species. That illustrates the impact of landfills and dumpsites on the herbaceous vegetation which tends towards a homogenization in ruderal flora.

IV. CONCLUSION

The preponderance of those exclusive ruderal species in the floristic composition of the majority of sites illustrates the natural capacity of pollutant removal. Waste management through phyto-remedation is an alternative

that should be further investigated by the municipal sanitation services. *Datura innoxia*, *Amaranthus viridis* and *Amaranthus spinosus*. species are suitable candidates in phytoremediation project and an ecological waste management system in this city of Maradi.

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Biological Removal of Malachite Green and Congo red by Some Filamentous Fungi

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***Abstract*—** *Four strains of filamentous fungi were studied to a removal of Malachite green (MG) and Congo red (CR). These fungi were Aspergillus niger ,Aspergillus flavus , Aspergillus versicolor and P. funigulosum. P. funigulosum showed that decolorization activity was higher than other fungi on solid medium containing MG and CR . The stastical method obtained that there was no significance between fungi . All these fungi were able to degradation dyes to other metabolites . The dry weight (Biomass) of P. funigulosum reached to 1.10 ,1.02 in mineral salts medium (MSM) with MG and CR respectively , and the stastical methods obtained that there was no significance in dry weights between fungi .*

Keywords*— *aquatic life , degradation , dyes ,marsh , efficiency , filamentous fungi , metabolites.

I. INTRODUCTION

Various industries discharge effluents containing unused dyes directly into the water bodies causing serios threat to the environment (Zollinger ,1987) . These dyes effect the life of living organisms in the ecosystem by damaging the health of humans , plants , animals and microorganisms . They also added to the continuously increasing load of environmental water and soil pollutants (Dong et al.,2011) . Treatment of dyeing wastewater was very important before its safe discharging into environment (Hazrat ,2010). A large number of physiochemical methods are available for treatment of dyes wastewater but these methods posses a constraint due to their limited versatility , high cost , low efficiency and interference by other wastewater constituent (Banat et al .,1996). These physicochemical method also produce a lot of sludge posing a threat as secondary pollutant (Du et al ., 2011). However, biological methods are available which are eco-friendly and completely mineralize organic pollutants (Pandey et al., 2007). These methods are inexpensive have wide range applicability , low running cost , complete mineralization of dye to anontoxic compound and ecofriendly (Forgacs et al., 2004) . Malachite green (MG) is a water- soluble triphenylmethane cationic dye which is used to color fabrics (Zhou et al ., 2015) . It is also utilized in food and medical industry (Chowdhury et al ., 2011) . Also this dye was toxic on aquatic and terrestrial animals and elicits cytotoxicity on mammalian cell and causes formation of liver tumors (Srivastava et al ., 2004). Congo red dye (sodium salt of benzidinediazo-bis-1 naphtylamine-4-sulfonic acid, ($C_{32}H_{22}N_6Na_2O_6S_2$) is atypical diazo dye with two chromophoric groups (azo group) in its structure . It is highly soluble in water and persistent when once discharged into anatural environment (Tapalad et al ., 2008 ; Jalandoni-Buan et al ., 2009 ; Tang et al ., 2011) . The use of fungi is apromising alternative to replace or supplement current treatments (Fu and Viraraghavan ,2001 ; Dos santos et al ., 2004) . Several fungi are able of mineralizing pollutant compounds through their highly oxidative and non- specific ligninolytic enzymes , which are also responsible for the decolorizaion and degradation of many different dyes (Dos santos et al , 2004) . Studies on non-basidiomycetes fungi that degrade dyes are reduced ,neverthe less these fungi are also very efficient for metabolizing awide range of compounds , particularly by demethylation and oxidation (Cha et al ., 2001). *Aspergillus* species (EI-Rahim and Moawad ,2003 ; Jin et al ., 2007) *Cunninghamella elegans* (Ambrosio and Campos-Takaki , 2004) , *penicillium geastrivorus*(Yang et al ., 2003) ; *P. ochrochloron* (Shedbalkar et al., 2008) ; *Fusarium solani* and *Penicillium funigulosum* (Al-Jawhari , 2015) . Thus , the aims of the present study were to investigate the ability of *A. niger , A. flavus , A. versicolor and P. funigulosum* to removal malachite green and congo red

II. MATERIAL AND METHODS

Organisms and culture conditions

A.niger ,A.flavus ,A. versicolor and P. funigulosum were obtained from Marshes Research center , Environment laboratory , Thi-qar University ,Iraq . These fungi isolated by Dr. AI-Jawhari from the upper surface of a sediments in Abo-subat marsh in AL-Nasiriya governerate (south of Iraq) . stock cultures were maintained on the potato Dextrose Agar (PDA) slant sub cultured periodically and stored at 4 c^o .

Chemicals :

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The common names of the two dyes have been used for convenience . Malachite green (MG) , Congo red (CR) were from Merch (Germany) . All other chemical used in the present study produced by Himedia (India) .

Decolorization of MG and CR Dyes in solid medium

A disc (5mm) of fungal mycelium was inoculated into the center of petri dishes (85 mm) with the previously mentioned culture medium with agar. The medium is containing (2.5 mg/l) of each dye separately in triplicate. The plates were incubated at 25 c^o for 14 days , after which the mycelium diameter (MD) and decolorization diameter (DD) were determined .The ability of the fungi to decolorize the dye was then expressed as the docolorization index (DI), which was calculated using the following formula :

DI= DD/MD

Each test was replicated 3 times .

Biomass production

One disc (5mm) of *A.niger ,A.flavus ,A. versicolor and P. funigulosum* were transferred to 250 ml Erlenmeyer flasks containing 100 ml of autoclaved culture medium (MSM) contained in g/l : yeast extract 0.3 , k_2HPO_4 0.75 , $KH_2PO_4$0.75 , $MgSo_4.7H_2O$ 0.05 , $CaCl_2.2H_2O$ 0.05 and $FeSo_4.7H_2O$ 0.02 at PH 7.0 supplemented with 0.5 mg/l of each dye separately ,in triplicate . The flask were incubated at 25 c^o for 7 days and shaking manually every day . The biomass was determined by calculated the dry mass of mycelia . Mycelia were harvested from the cultivation liquid medium by filtration using whattman No.1 filter paper and dried of 65c^o at 30 min and weighted (mg/10ml) .

Biodegradation of dyes in liquid medium

After incubation (14 days) of one disc (5mm) from *A.niger ,A.flavus , A. versicolor and P. funigulosum* in mineral salts medium (MSM) . Mycelia were harvested from the cultivation liquid medium by filtration using whattman No.1 filter paper and the filter was used to determined the biodegradation (MG) and (CR) by using FourierTransform Infrared (FTIR) spectroscopy .

Stastical analysis :

The present study conducted an Anova (analysis of variance) which was performed on all the treatments and done using the spss (version 10.0) package to determin whether or not significance difference .

III. RESULT AND DISCUSSION

Decolorization of MG and CR dyes in solid medium

The dyes evaluated in this study contain aromatic compounds that are degraded by filamentous fungi during secondary metabolism . The growth and degradation efficiency of the test fungi as determined based on the their decolorization ability in solid medium are shown in Table 1 , Figure 1 ,of the 4 fungi cultured on solid medium with MG and CR.

Table.1:Decolorization of aromatic dyes on solid medium by filamentous fungi

Name of fungi	Malachite green			Congo red		
	MD*	DD	DI	MD	DD	DI
A.niger	28	50	1.8	65	9.0	0.14
A.flavus	26	44	1.7	74	4.0	0.10
A.versicolor	28	51	1.8	54	17.0	0.31
P.funigulosum	29	56	1.9	80	3.5	0.40

*: (mm) , MD : Mycelial diameter , DD: Decolorization diameter , DI : Decolorization index = DD/MD . The mycelia diameter and decolorization diameter were measured (mm , n=3) after 14 days of incubation .

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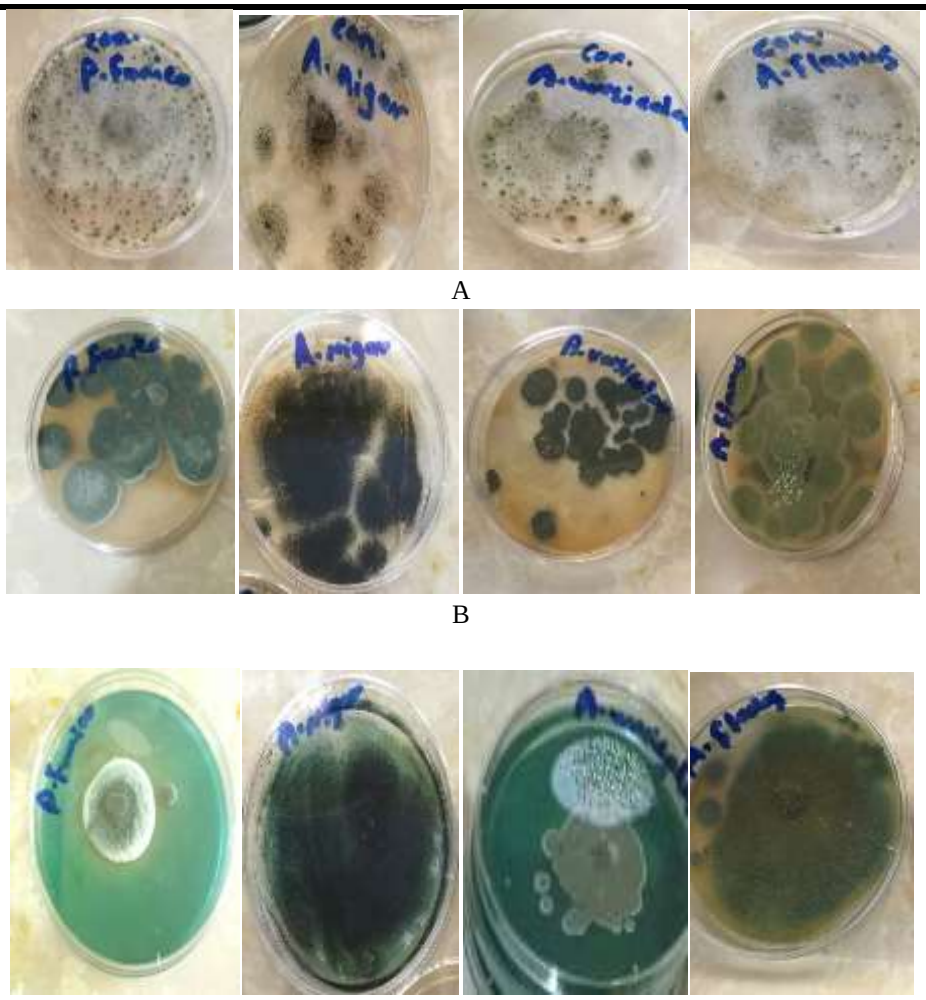

Fig.1:Decolorization of Malachite green and Congo red by filamentousfungi on solid medium .
A:Control , B: Congo red , C: Malachite green

P. funigulosum showed that decolorization activity was higher than that of remaining 3 fungi , these ability due to that this fungus have unique systems enzymes for breaking complex organic structures into simple fragments , however the mycelia of the *P. funigulesum* was higher than other fungi on solid medium contain MG .In the same time all the remaining fungi can able to decolorization this dye .

Table1 show also that *P. funigulosum* was higher decolorization activity than other fungi on solid medium contain CR and the mycelia of the *P. funigulosum* was higher than other fungi . The value of decolorization of MG and CR on solid medium by the selected fungi was not considerably higher . The stastical methods obtained that there was no significance between fungi . The same results were obtained by (Chandana et al ., 2008) , in this study show that when the white rot fungi *Cariolus versicolor*was good mycelia growth on solid media contained MB , but the efficiency of decolorization was very low , and in the same time the decolorization index with this fungus reached to 0.11 , but the results obtained by (Rania ,2008) were differ with results in present study , when studied the decolorization of crystal violet and malachite green by using *Fusarium solani* ,this fungus decolorized dyes quickly with the radial growth and the decolorizatin halo of CV and that of MG occupied nearly the entire diameter of the plate after 3 days of incubation at 30 c^o . The results in present study was similar with the results obtained by (Al-Jawhari , 2015) when show that *F.solani and P. funigulosum* appear higher ability to decolorization of MB and CV in solid media . In addition the results of study conducted by (Abo-state et al ., 2011) also showed that the ability of *pleurotus ostratus* to decolorize MB also increased , so the removal % increased for awide range of concentrations (25-700 mg/l) MB ,and in the same time (Abo-state et al ., 2011) refer that this result due to may attributed to the increasing in production of lignolytic enzymes as the concentration of MB increased due to their stress on the mycelia cells of *P. ostratus* . The results in present study were similar with results obtained by (Chandana et al ., 2008) , in this study showen that all the 10 fungi evaluated were grow

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slowly on solid media that contain Malachite green and poor ability to decolorize these fungi , but in the present study , the results were not agreed with the results obtained by (Hazrat et al ., 2013) , in this study showen that *Alternaria solani* is quite tolerant to crystal violet and decolorize and degrade relatively higher concentrations of the dye.

Biomass production

Growth study revealed that biomass and dye removal are directly proportional ,which may be attributed to the fact , the increase of biomass gave more surface area for sorption of the dye molecules available , and may be due to the shaken of flask , this result was agreement with the results of (Mohorcic et al ., 2004) , In this study showen that the most effective fungus in shaken flask experiment was *Bjerkandera adusta* , which was able to decolorize the dye from black- blue to yellow color in less than 10 days .

Table 2 explain that the dry weight of *P. funigulosum* was higher than other fungi with MG and CR , the dry weight of this fungus reached to 1.10 gm with MG dye , this extraordinary absorption value may have been due to areaction of MG with enzymes secreted by the fungal mycelia (Chandana et al ., 2008) . And in the same time the dry weight of *P. funigolosum* reached to 1.02 gm with CR . The stastical methods obtained that there was no significance in dry weights between fungi.

Table.2:Mycelial dry weight of fungal strains in liquid medium containing Malachite green and Congo red .

Fungi	MG	CR
A.niger	0.78*	0.91
A.flavus	0.70	0.85
A.versicolor	0.69	1.01
P.funigulosum	1.10	1.02

*Mean of triplicate , Dry weight calculated with (gm)

The same results were obtaind by (Muthezilan et al ., 2008) , in this study showen that the dry weight reached to 0.49 gm with *Penicillium citrinum* in liquid media containing CV and the low dry weight reached to 0.22 gm with *Mucer racemosus* and *Trichoderma viride* . (Haglund , 1999) refer that in liquid culture , rapid dye decolorization by the fungal strain was observed within 24 h . It was mainly due to the high adsorption of the dye in the mycelium . In subsequent dyes , dye decolorization may be due to production of extrucellular enzymes.

Bioderadation of dyes in liquid medium

Degradation of MG and CR was confirmed by FTIR analysis of MG and CR and its degraded metabolites . FTIR spectrum of MG showed distinct peaks in the finger print region (1500-500 cm $^{-1}$) , which corresponds to mone and para substituted benzene ring and were distinct to MG . The peaks were observed at 700 , 800 cm $^{-1}$ corresponding to aromatic ring structure . Also peak at 1100 cm $^{-1}$ corresponds aromatic C-N stretching vibration (Fig. 2). Fig. 3 showed degraded metabolites by *A. niger* , new peak were appear at 1400, 1450 cm^{-1} for aromatic group , also new peak appear at 3000 cm $^{-1}$ for CH_2 bond at 1600 cm $^{-1}$ for aromatic ketones . Reduction of peaks at 700, 800 and 1500 cm $^{-1}$ indicated loss of aromaticity of metabolites.

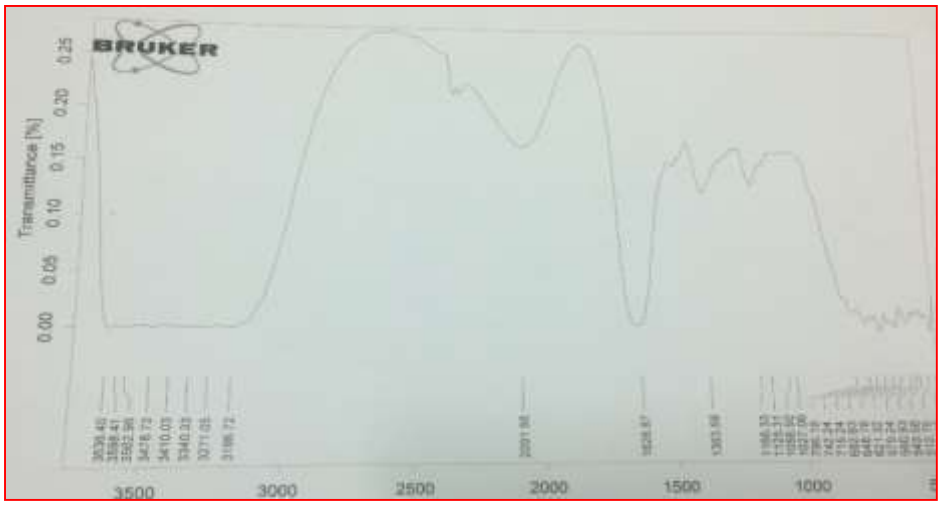

Fig.2: Malachite green (Standard) – uninculated .

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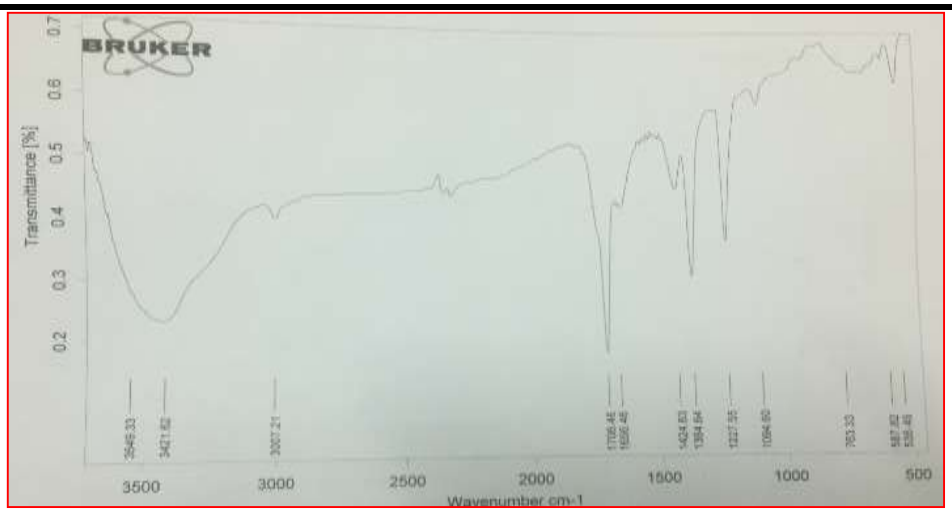

Fig.3: Biodegradation of malachitegreenby A.niger after 14 day incubation .

Fig. 4 showed degraded MG by *A. flavus* , new peaks also appear at 1200, 1300 cm $^{-1}$ for CH_2 stretching band and new peak appear at 1650 cm $^{-1}$ for aromatic ketones , also Fig. 4 showed reduction of peaks at 700 , 800 and 1500 cm $^{-1}$.

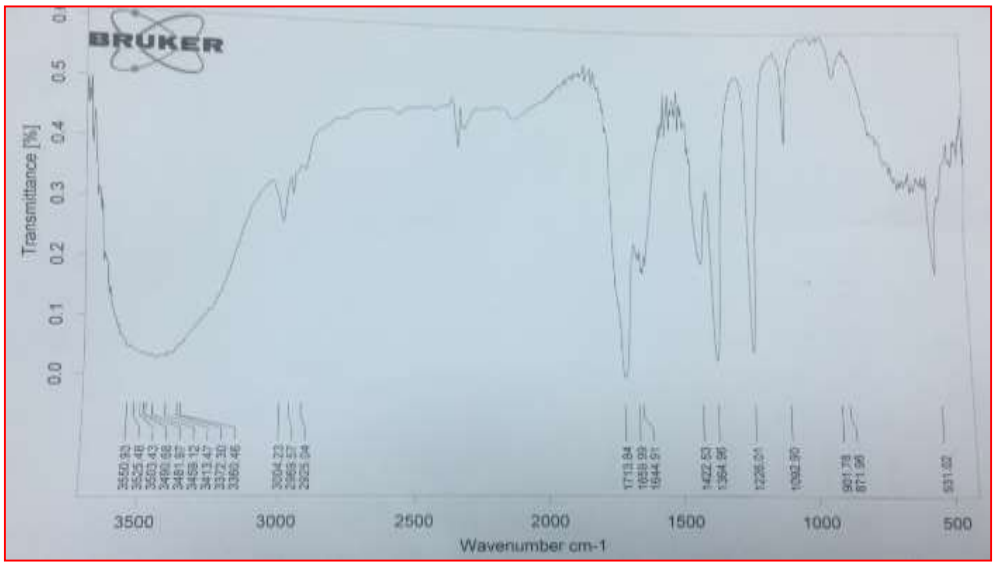

Fig.4: Biodegradation of malachite green by A.flavus after 14 day incubation .

Fig. 5 showed degraded metabolites by *A. versicolor* , new peaks were appear at 2400 and 3000 cm $^{-1}$, also many peak were reduction at 700 ,800 ,1500 cm $^{-1}$ and in the same time Fig.5 showed the ability of *A. versicolor* to degraded MG . Fig. 6 showed reduction of peaks at 700 ,800 , 1500 cm $^{-1}$, this result refer the loss of aromaticity of metabolites and this fungus was able to degraded MG .

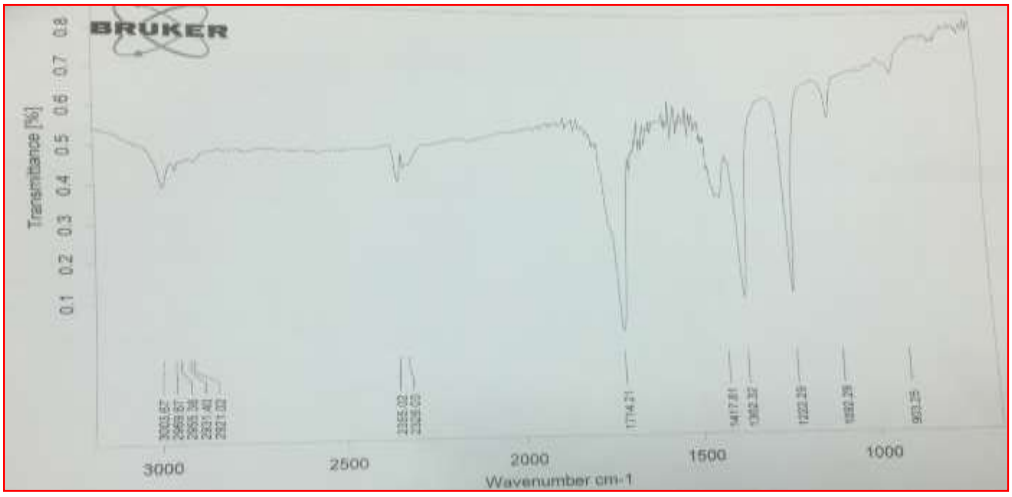

Fig.5: Biodegradation of malachite green by A.versicolor after 14 day incubation .

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Fig. 6 showed degraded metabolites by *P. funigulosum* , new peaks were appear at 1200 , 1300 cm $^{-1}$ and new peak appear at 1650 cm $^{-1}$, also many peaks were reduction at 700 , 800 ,1500 cm $^{-1}$. Fig. 6 showed high ability of this fungus to degraded MG .

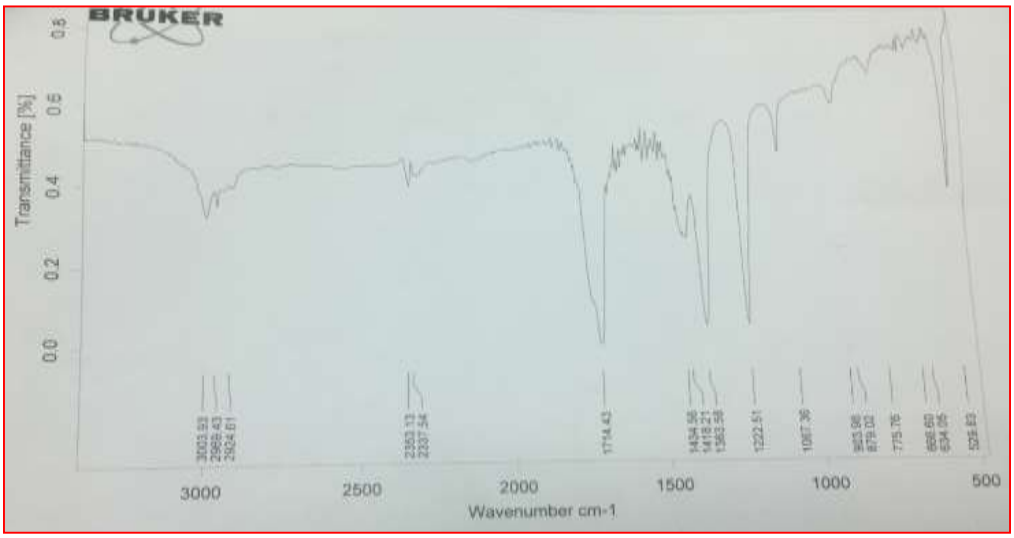

Fig.6: Biodegradation of malachite green by P. funigulosum after 14 day incubation .

Fig. 8 showed the ability of *A. niger* to degraded CR , new peak were appear at 1100 , 1200 ,1300 cm $^{-1}$ and also reduction of peaks at 1500 cm $^{-1}$ when compared control (Fig. 7) . Fig.9 showed the ability of *A.flavus* to degraded CR , new peak were observed at 500 , 1650 cm $^{-1}$.

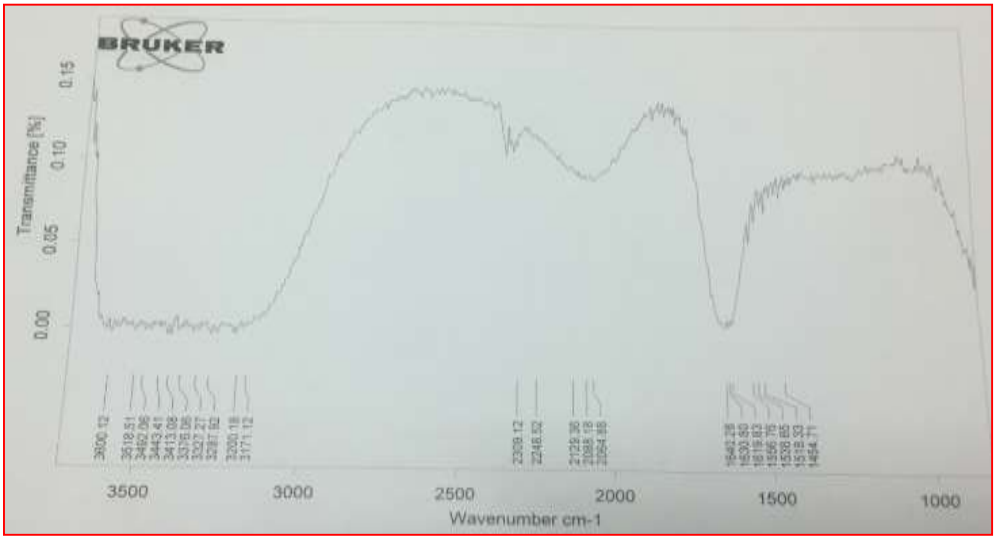

Fig.7: Congo red (Standard)-uninculated .

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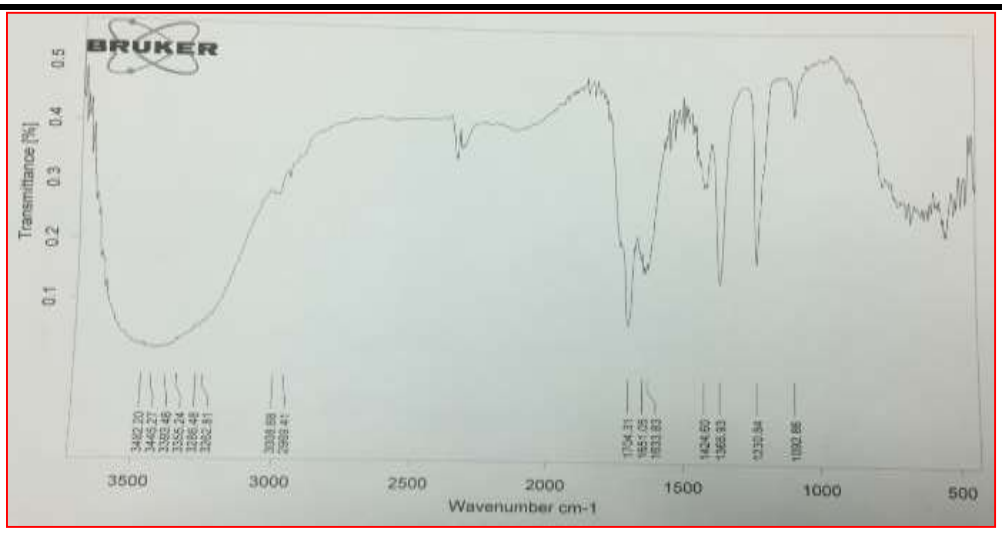

Fig.8: Biodegradation of congo red by A.niger after 14 day incubation.

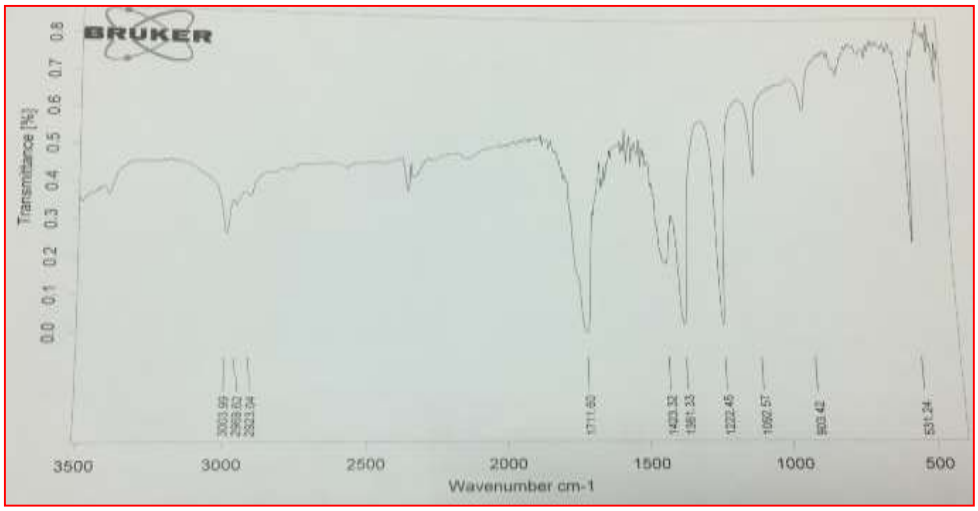

Fig.9: Biodegradation of congo red by A.flavus after 14 day incubation.

Fig. 10 showed the ability of *A.versicolor* to degraded CR , new peak were observed at 500 , 1650 cm $^{-1}$. Fig. 11 showed the ability of *P. funigulosum* to degraded CR , new peak were observed at 500 , 1650 cm $^{-1}$. These results are in accordance with previos reports of (Ayed et al ., 2009; Kalyani et al ., 2009 ; Chaturvedi et al ., 2013) (Du et al ., 2011) reported that degraded product formed by bio degradation of MG by *Pseudomonas aeruginosa* NCIM 2074 are non toxic . similarty (arshetti et al ., 2006) reported that biodegradation product of MG formed by action of *kocuria rosea* MTCC 1532 was non toxic .

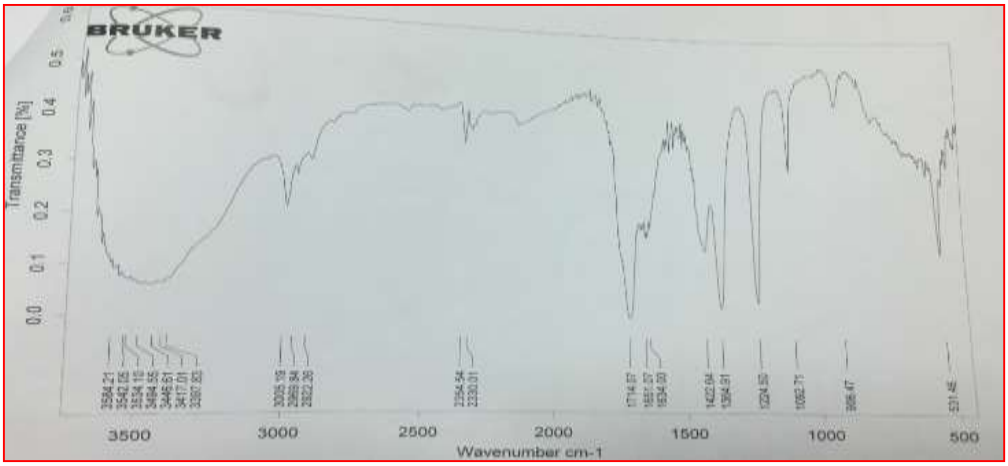

Fig.10:Biodegradation of congo red by A.versicolor after 14 day incubation .

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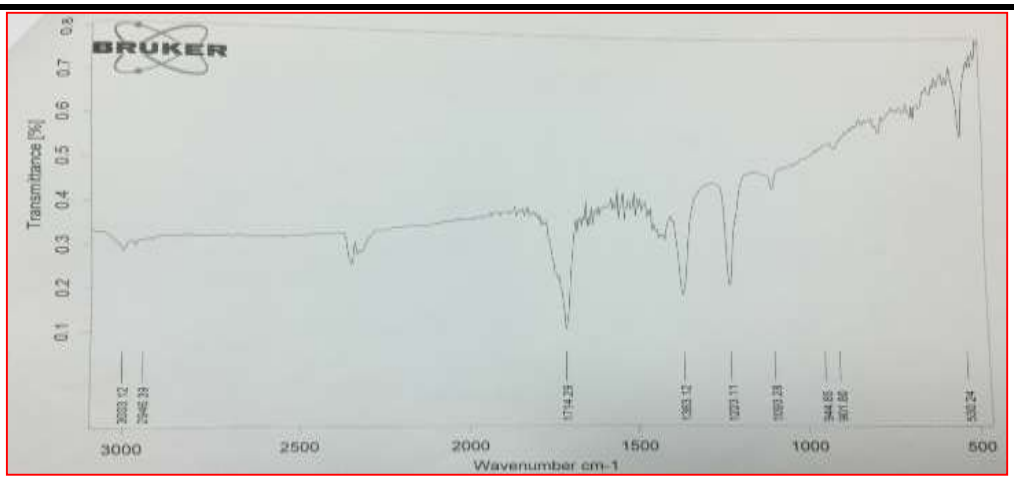

Fig.11: Biodegradation of congo red by P.funigulosum after 14 day incubation .

IV. CONCLUSIONS

The study concluded that, these fungal strains on their own can offer a costeffective, easily applicable and an environmently sound solution to dye effluents. Rehabilitation of MG and CR dyes contaminated rivers, Marshes water by the culture of these fungi were promising as it can reduce and removal the dyes pollution.

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Physiological Role of Humic Acid, Amino Acids and Nitrogen Fertilizer on Growth of Wheat under Reclaimed Sandy Soil

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Abstract**—** *In order to evaluate productivity of some wheat cultivars grown in sandy, saline soil under foliar spraying with humic acid, amino acids and nitrogen fertilizer levels. Highest chlorophyll b and carotenoid content, percentage of sodium and calcium, tallest plants and higher number of tillers/plant were achieved from Gemiza 9 cultivar. The highest relative growth rate (RGR) and net assimilation rate (NAR), chlorophyll and total chlorophyll values, higher flag leaf area and stem diameter were resulted from Giza 168 cultivar. While, higher percentages of proline, total phenols and potassium percentages were found from Shaka 93 cultivar. The earlier for a number of days to heading and flowering were resulted from Shaka 93 cultivar. Application of humic and amino acid mixture significantly enhanced total leaf area/plant, plant dry weight after 75 and 95 days from sowing (DFS), RGR, NAR, photosynthetic pigments, i.e. chlorophyll a, chlorophyll b and carotenoids and proline contents. In addition, highest total phenols, potassium and calcium percentages, height flag leaf area, tallest plants, highest stem diameter and number of tillers/plant compared with other foliar spraying treatments. Accordingly, it could be recommended that foliar spraying wheat plants Giza 168 cultivar with the mixture of humic acid and Amino acids with addition, mineral fertilizing with 262 kg N/ha to obtain the best growth characters of wheat under newly reclaimed sandy saline soil conditions.*

Keywords**—** ***Wheat Cultivars, Humic Acid, Amino Acids, Nitrogen Fertilizer levels.***

I. INTRODUCTION

There is a huge shortage in production of wheat in Egypt, it imported more than 50% of our consumption [1]. The extreme increase in population in Egypt needs to increase wheat production in order to overcome this lack in production through its cultivation in the new reclaimed soils especially under saline conditions of such soil. To increase the cultivated area of wheat plant it is necessary to go to newly reclaim soils. However, most of the newly reclaimed soil suffers from salinity problem. Salinity is a major abiotic stresses in arid and semi-arid regions that sustained decreases the yield of major crops by more than 50%. Considerate the influences between a plant's initial response and the downstream events that establish a successful adjustment to its altered environment is one of the next grand challenges of plant biology [2]. Salinity restrictions, soil fertility in irrigated regions of the world, this effect due to low rainfall in these areas besides soil leaching does not occur [3]. Soils contain soluble salts of multifarious nature, when soil and environmental conditions allow the concentrations in soil profiles to a high level, soil salinity becomes a severe threat to land degradation and crop productivity [4]. According to FAO about 20 to 30 million hectares of irrigated land are currently seriously damaged by salinity, and 0.25 to 0.50 million hectares are lost from production every year as a result of salt accumulation. So, it could be achieved through using suitable agronomic practices. Chosen the high yielding ability cultivars undoubtedly is very important to raise wheat productivity per unit area. For this reason, this study is aimed to evaluate the new promising cultivars for scooping light on the best cultivar that can be used under the environmental conditions of newly reclaimed sandy saline soils. Gemmiza 7 cultivar at the three stages (75, 96 and 117 days after sowing) of growth produced tallest plants, number of tillers/plant, dry weight/plant, leaf area/plant and flag leaf area and also crop growth rate (CGR) and relative growth rate (RGR) at growth intervals of 96-117 days after sowing than Sakha 93 cultivar [5]. The largest flag leaf area was obtained from Sakha 93 and Gemmeiza 9 cultivars. Though, Sakha 94 cultivar significantly exceeded all studied cultivars in plant height [6]. Sohag-3 cultivar produced tallest plants. While, BaniSweef-3 recorded the highest number of tillers/plant. Whereas, Sohag-2 recorded highest leaves area/plant [7]. Total phenols increased in Sakha 93 cultivar as compared with those of Gemiza 9 cultivar

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grown under salinity stress [8]. Gemmiza 10 cultivar exceeded (Gemmiza 9 and Sakha 93) cultivars in number of days to heading, flag leaf area and plant height [9].

Foliar fertilization is a widely used practice to correct nutritional deficiencies in plants caused by improper supply of nutrients to the roots. Foliar application stimulates the plants to create exudates in the roots which excite microbes to work harder and thus increases nutrient uptake from the soil. Sprayings are a great supplement to boost flavors, sweetness, mineral density and yield of crops [10]. Humic acid is a principal component of humic substances, which are the major organic constituents of soil (humus). Humic substances have many beneficial effects on soil physical structure and soil microbial populations as well as increase, modify mechanisms involved in plant growth stimulation, cell permeability and nutrient uptake and increasing yield [11,12]. Humic acid foliar spraying significantly increased photosynthesis process and antioxidant metabolism under water stress conditions [13]. Humic acid foliar spraying significantly affected dry weight and the uptake of mineral elements. Dry weight was higher in humic acid spraying when compared with the control treatment. The highest dry weight was obtained from 0.1% dose of humic acid [14]. Foliar spraying with humic acid significantly increased plant height [2,15].

Amino acids enhanced chlorophyll concentration leading to higher degrees of photosynthesis, which makes crops lush. Amino Acids act as a cytoplasm osmotic agent on stomata cell, which help plants improve absorption of macro and trace nutrients as well as gasses through favoring the opening of stomata. It helps the absorption and transportation of micronutrients inside the plant getting easier. Also, it actions as equilibrium of soil microbial flora to improve mineralization of the organic matter and formation of a good soil structure and fertility around the roots [16]. Applied of nitrogen to the plant will affect the amount of protein, protoplasm and chlorophyll formed. In turn, this influences cell size, leaf area and photosynthetic activity. Availability of nitrogen increased tiller number, the number and weight of the grains, consequently yields of wheat [17]. Nitrogen fertilizer has a good effect on plant productivity, nevertheless it's also having a polluting effect on the environment. Increasing nitrogen fertilizer up to 90 kg N/fed significantly exceeded other levels in photosynthetic pigments, growth characters, yield components, yield and quality characters [18]. Increasing nitrogen fertilizer level up to 100 kg N/fed significantly increased flag leaf area, plant height, spike length, grain and straw yields/fed and protein content of grains [19]. Plants grown-up under the control treatment produced significantly lesser of total dry matter than those treated with 90, 120 and 150 kg N /ha [20,21]. Increasing nitrogen fertilization rates up to 80 or 120 kg N/ha significantly increased number tillers/plant and spike length. High and economical increases of study parameters was 80 kg N/ha, which gave the highest number of tillers/plant and spike length. Maximum grain yield resulted from the application of 100 kg N/ha [22]. Therefore, this study was aimed to decide the effect of foliar application with humic acid, amino acid under nitrogen fertilizer levels on the growth, yield and its attributes and chemical constituents of some cultivars of bread wheat grown in newly reclaimed sandy saline soil conditions in conditions.

II. MATERIALS AND METHODS

2.1. Research time and location:

Two field experiments were conducted at Station Farm of Kalabsho and Zayan district, Faculty of Agriculture, Mansoura University, Egypt. The experiments were set to find response of wheat cultivars (Shaka 93, Gemiza 9 and Giza 168) to foliar spraying (Spraying with water, spraying with Actosol source of humic acid at the rate of 5 ml /liter water, spraying with Amino-Cat source of amino acids at the rate of 5 ml/liter water and spraying with the mixture of Actosol and Amino-Cat at the rates of 5 + 5/liter water, respectively) and nitrogen fertilizer levels (166, 214 and 262 kg N/ha as ammonium nitrate 33.5 % N).

The experimental soil was sandy in texture, pH was 8.5, E.C. was 9.11 dSm^{-1}, available nitrogen was 4.1 ppm, available phosphorus was 5.53 ppm and organic matter was 0.65%. Each experimental unit area was 3 × 3.5 m occupying an area of 10.5 m^2. A split-split plot design with three replicates was followed. Wheat cultivars were assigned to the main plots, whereas foliar spraying and nitrogen fertilizer were allocated in the 1st and 2nd order sub plots, respectively. Each experiment included thirty-six treatments comprising, three wheat cultivars, four foliar spraying and three nitrogen fertilizer levels. The foliar solution volume was 475 liter/ha and spraying was conducted by hand sprayer (for experimental plots) until saturation point three times after 30, 45 and 60 days from sowing. Tween-20 was used as a wetting agent at 0.02% concentration. While, Nitrogen fertilizer was applied at the aforementioned levels as a side-dressing in four equal doses prior every irrigation and finished before heading. All plants received full irrigation and maintained weed free by hand weeding after sowing whenever necessary. Phosphorus fertilizer was applied during soil preparation as calcium super phosphate (15.5% P_2O_5) at the rate of 476 kg/ha. Potassium fertilizer was broadcasted in one dose before the second irrigation as potassium sulphate (48 % K_2O) at the rate of 178 kg/ha. Grains of wheat

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cultivars were sown at the rate of 190 kg/ha, during the last week of November by using hand drilling in both seasons. Plants were harvested on 5th and 9th of May for growing seasons.

2.2. Studies characters:

At the emergence of approximately 50% spikes/plot and 50% flowering approximately of the spikes/plot, it was calculated the number of days to 50% heading and number of days to 50% flowering, respectively. At 75 and 95 days after sowing samples were taken for determination total leaf area/plant (cm^2): Determined according to [23]. The dry weight of plant (g): All plant fractions were air-dried, then oven dried at 70°C till constant weight obtained. 3-Crop growth rate (CGR): Determined according to [24]. 4-Relative growth rate (RGR): Determined according to [24]. 5-Net assimilation rate (NAR): Determined according to [25]. Samples of flag leaf were taken after 90 days from sowing for the following chemical analysis. 6-Photosynthetic pigments: Both chlorophyll and carotenoid contents in fresh leaves were estimated using the method of [26]. 7-Proline content: Proline content was determined in flag leaf by the modification of ninhydrine method of [27]. 8-Total Phenols: Total Phenols was assayed according to the method described by [28]. 9-Sodium and potassium percentages: Sodium and potassium percentages were estimated by using flame photometer according to [29]. 10-Calcium percentage: Calcium percentage was determined by using the atomic absorption spectrophotometer as a method of [30] After 125 days from sowing, where five guarded plants were chosen from each sub-plot to determine 6-Flag leaf area (cm^2): Calculated according to [23]. 7-Plant height (cm). 8-Stem diameter (mm). 9-Number of tillers/plant.

2.3. Experimental analysis:

All obtained data were statistically analyzed according to the technique of analysis of variance (ANOVA) for the split – plot design to each experiment (row spacing), then combined analysis was done between row spacing trails as published by [31] by using "MSTAT-C" computer software package. A Least significant of the difference (LSD) method was used to test the differences between treatment means at the 5 % level of probability as described by [32].

III. RESULTS AND DISCUSSION

3.1. Cultivars Performance:

Results presented in Tables 1, 2 and 3, the results clearly showed that there were significant differences in most the growth, physiological and chemical characters among wheat cultivars in the two growing seasons. Under conditions of this study, Gemiza 9 cultivar caused a significant increase in characters, *i.e.* chlorophyll b, carotenoids, sodium, calcium percentages, plant height (cm) and number of tillers/plant in both seasons. The highest values of relative growth rate (g/g/week), net assimilation rate (g/m^2/week), chlorophyll a (mg/g fresh weight), total chlorophylls (mg/g fresh weight), flag leaf area (cm^2) and stem diameter (cm) were resulted from Giza 168 cultivar. Whereas, the highest values of proline (mg/g fresh weight), total phenols (mg/100 g fresh weight) and potassium percentages were obtained from Shaka 93 cultivar. From obtained results, earliness characters (numbers of days to 50% heading and numbers of days to 50% flowering) significantly affected by different wheat cultivars. The earlier in heading and flowering were resulted from Shaka 93 cultivar in the first and the second seasons, respectively. The variation among wheat cultivars may be due to the genetically variation among them as a result of the differences of wheat cultivars pedigree (Table 1). Similar results were obtained by [5,6,7,8,9].

3.2. Effect of foliar spraying treatments:

Foliar spraying treatments using from humic acid, amino acids and the mixture of humic and amino acids were associated significant effect on most growth, physiological and chemical characters in both seasons (Tables 1, 2 and 3). Foliar spraying with a mixture of humic and amino acids significantly improved most characters and induced the highest values of total leaf area/plant (cm^2), plant dry weight (g) after 75 and 95 DFS, relative growth rate (g/g/week), net assimilation rate (g/m^2/week), flag leaf area (cm^2), plant height (cm), stem diameter (cm) and number of tillers/plant. These findings associated with increasing of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids mg/g fresh weight), Proline content (mg/g fresh weight), total Phenols (mg/100 g fresh weight), potassium percentage, calcium percentage and decrease of sodium percentage of wheat plants compared with other foliar spraying treatments (humic acid or amino acids) and spraying with water (control treatment) in both seasons. From obtained results, earliness characters (numbers of days to 50% heading and 50% flowering) significantly affected by foliar spraying treatments in both seasons. Foliar spraying wheat plants with the mixture of humic acid and amino acids significantly surpassed other foliar spraying treatments (humic acid or amino acids) and spraying with water (control treatment) and produced the highest values in the first and second seasons. This increase in attributes under study by foliar spraying treatments with nutrient compounds that contains macro and micronutrients may be due to the role of macro and micronutrients in increasing meristematic activity and production of some growth regulators such as Indole Acetic Acid (IAA), which is essential for the elongation of the internodes

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reflecting increases in these traits. These findings were proportionately with those reported by [12,14,15].

3.3 Effect of nitrogen fertilizer levels:

With respect to the effect of nitrogen fertilizer levels on most growth, physiological and chemical characters in both seasons (Tables 1, 2 and 3). The results clearly indicated that a significant in the two growing seasons of these characters as shown in Tables 1, 2 and 3. All studied characters of wheat plants gradually increased as a result of increasing nitrogen fertilizer levels from 166 to 214 and 262 kg N/ha in both seasons, it was evident that, under the environmental conditions of newly reclaimed sandy, saline soil, wheat plants still responded to more levels of nitrogen fertilizer up to 262 kg N/ha. Generally, maximum means of all studied characters were produced from fertilizing wheat plants with 262 kg N/ha in the first and second seasons. On the contrary, the lowest values of these characters were obtained from plots that received lowest nitrogen fertilizer levels (166 kg N/ha). This increase in wheat growth characters due to increasing nitrogen fertilizer levels might have been due to nitrogen, which considers as one of the major elements which is essential for plant growth, and plays an important role as division and elongation of cells are concerned, thus increasing cell number and size and also via activation metabolic and photosynthesis processes. Similar results were obtained by [18,18,20,21,22].

3.4- Effect of interactions:

Regarding the effect of interactions, there are many significant effects of the interactions on the studied characters. We present only the effect of significant interactions on the studied characters in both seasons (Tables 1, 2 and 3). Means of relative growth rate (RGR) were insignificantly affected by various interactions among studies factors, *i.e.* cultivars, foliar spraying and nitrogen fertilizer levels in both seasons, except the interaction between cultivars × nitrogen fertilizer levels in the first season and foliar spraying × nitrogen fertilizer levels in both seasons (Table 2). The results graphically illustrated in Fig.1 showed that foliar spraying with humic and amino acids in mixture and increasing nitrogen fertilizer levels up to 262 kg N/ha significantly recorded highest relative growth rate (RGR). The interaction among wheat cultivars, foliar spraying treatments and nitrogen fertilizer levels had a significant effect on the total chlorophylls percentage in flag leaf in both seasons, except the interaction between cultivars × foliar spraying treatments, cultivars × nitrogen fertilizer levels (in the second season) and foliar spraying treatments × nitrogen fertilizer levels (in the first season). The results graphically illustrated in Figs. 2 showed that foliar spraying Giza 168 cultivar with humic and amino acids in mixtures and increasing nitrogen fertilizer levels up to 262 kg N/ha significantly recorded highest total chlorophylls percentage in flag leaf in both seasons. However, the lowest total chlorophylls percentage in flag leaf was produced fertilizing Sakha 93 cultivar with 166 kg N/ha without foliar spraying (control treatment).

IV. CONCLUSION

Accordingly, it can be recommended that foliar spraying wheat plants Giza 168 cultivar with the mixture of humic acid and Amino acids with addition, mineral fertilizing with 262 kg N/ha to obtain the best growth characters of wheat under newly reclaimed sandy saline soil conditions.

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Table.1: Total leaf area/plant, plant dry weight, crop growth rate, relative growth rate and net assimilation rate as affecte d by foliar spraying and nitrogen fertilizer levels of some wheat cultivars as well as their interactions during 2013/2014 and 2014/2015 seasons.

Characters / *Treatments*	*Total leaf area/plant (cm^2)*				*Plant dry weight (g)*				*CGR (g/week)*		*RGR (g/g/week)*		*NAR (g/m^2/week)*	
	75 DFS	95 DFS	75 DFS	95 DFS	75 DFS	95 DFS	75 DFS	95 DFS						
	2013/2014	2014/2015	2013/2014	2014/2015	2013/2014	2014/2015	2013/2014	2014/2015	2013/2014	2014/2015	2013/2014	2014/2015	2013/2014	**2014/2015**
A- Cultivars:														
Shaka 93	212.4	216.0	238.7	243.2	6.05	6.28	11.36	11.82	1.86	1.93	0.18	0.18	10.73	10.82
Gemiza 9	238.8	248.6	270.1	281.3	7.85	8.67	13.21	14.29	1.87	1.96	0.20	0.19	12.21	12.22
Giza 168	213.3	216.1	246.0	251.8	7.43	7.80	12.88	13.36	1.90	1.94	0.23	0.22	12.22	12.74
F. test	*	*	*	*	*	*	*	*	NS	NS	*	*	NS	*
LSD at 5 %	9.5	9.6	10.6	10.1	0.47	0.30	0.80	0.78	-	-	0.02	0.02	-	0.68
B-Foliar spraying:														
Control treatment	183.5	189.3	209.3	215.6	6.28	6.82	11.70	12.50	1.89	1.98	0.19	0.18	9.65	9.43
Humic acid (HA)	207.3	212.2	237.5	246.0	6.72	7.36	12.16	13.05	1.90	1.99	0.21	0.20	12.37	12.56
Amino acids (AA)	231.0	233.0	262.0	265.2	7.49	7.70	12.77	13.15	1.84	1.90	0.19	0.19	10.90	11.47
Mixture of HA + AA	264.4	273.2	297.7	308.3	7.94	8.45	13.31	13.90	1.87	1.90	0.22	0.21	13.96	14.24
F. test	*	*	*	*	*	*	*	*	NS	NS	NS	*	*	*

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LSD at 5 %	15.6	15.6	15.7	16.2	1.13	1.01	1.00	1.08	-	-	-	0.02	1.23	1.51
C- Nitrogen fertilizer levels:														
166 kg N/ha	216.3	219.2	243.2	248.1	5.36	5.98	10.99	11.87	1.80	1.86	0.16	0.16	10.77	10.80
214 kg N/ha	218.2	224.9	248.0	256.5	6.82	7.41	12.16	12.89	1.86	1.92	0.20	0.19	11.73	11.73
262 kg N/ha	230.2	236.6	263.7	271.7	9.15	9.37	14.30	14.70	1.97	2.06	0.25	0.24	12.67	13.23
F. test	*	*	*	*	*	*	*	*	*	*	*	*	*	*
LSD at 5 %	8.9	9.7	8.0	9.8	0.34	0.27	0.35	0.32	0.06	0.08	0.01	0.01	0.58	0.37

Table.2: Chlorophyll a, chlorophyll b, total chlorophylls, carotenoids, proline percentage, total phenols, sodium percentage, potassium percentage and calcium percentage as affected by foliar spraying and nitrogen fertilizer levels of some wheat cultivars as well as their interactions during 2013/2014 and 2014/2015 seasons.

Characters Treatments	*Chlorophyll a (mg/g fresh weight)*		*Chlorophyll b (mg/g fresh weight)*		*Total chlorophylls (mg/g fresh weight):*		*Carotenoids (mg/g fresh weight)*		*Proline percentage (mg/g fresh weight)*		*Total phl. (mg/100 g fresh weight)*		*Na (%)*		*K (%)*		*Ca (%)*	
	1st	*2nd*	*1st*	*2nd*	*1st*	*2nd*	*1st*	*2nd*	*1st*	*2nd*	*1st*	*2nd*	*1st*	*2nd*	*1st*	*2nd*	*1st*	*2nd*
A- Cultivars :																		
Shaka 93	*0.780*	*1.144*	*0.367*	*0.439*	*1.146*	*1.583*	*0.439*	*0.494*	*14.22*	*15.21*	*95.68*	*96.44*	*0.092*	*0.101*	*2.192*	*2.392*	*0.313*	*0.323*
Gemiza 9	*0.778*	*1.056*	*0.472*	*0.520*	*1.251*	*1.576*	*0.611*	*0.684*	*12.41*	*13.61*	*93.49*	*95.46*	*0.107*	*0.116*	*1.870*	*2.070*	*0.248*	*0.298*
Giza 168	*1.026*	*1.151*	*0.422*	*0.488*	*1.448*	*1.639*	*0.430*	*0.485*	*13.07*	*14.47*	*93.92*	*95.95*	*0.099*	*0.109*	*2.025*	*2.225*	*0.254*	*0.304*
F. test	*	*	*	*NS*	*	*NS*	*	*	*	*	*	*NS*	*	*	*	*NS*	*	*
LSD at 5 %	*0.073*	*0.074*	*0.067*	-	*0.127*	-	*0.060*	*0.050*	*0.40*	*0.58*	*1.40*	-	*0.011*	*0.006*	*0.054*	-	*0.004*	*0.007*
B-Foliar spraying:																		
Control treatment	*0.806*	*0.939*	*0.387*	*0.453*	*1.205*	*1.393*	*0.411*	*0.455*	*11.15*	*12.67*	*91.45*	*93.84*	*0.133*	*0.143*	*1.310*	*1.510*	*0.335*	*0.372*
Humic acid (HA)	*0.853*	*1.075*	*0.399*	*0.455*	*1.240*	*1.529*	*0.505*	*0.568*	*14.11*	*15.04*	*95.15*	*96.64*	*0.090*	*0.100*	*2.274*	*2.474*	*0.250*	*0.357*
Amino acids (AA)	*0.864*	*1.164*	*0.407*	*0.469*	*1.271*	*1.633*	*0.469*	*0.543*	*12.12*	*14.04*	*94.22*	*95.26*	*0.113*	*0.123*	*1.802*	*2.002*	*0.320*	*0.286*
Mixture of HA + AA	*0.922*	*1.289*	*0.488*	*0.551*	*1.411*	*1.841*	*0.588*	*0.651*	*15.54*	*15.98*	*96.64*	*98.07*	*0.061*	*0.069*	*2.729*	*2.929*	*0.181*	*0.218*
F. test	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
LSD at 5 %	*0.011*	*0.010*	*0.011*	*0.009*	*0.011*	*0.009*	*0.002*	*0.001*	*0.93*	*0.79*	*0.98*	*0.86*	*0.020*	*0.015*	*0.189*	*0.152*	*0.023*	*0.030*
C- Nitrogen fertilizer levels:																		
166 kg N/ha	*0.830*	*1.080*	*0.387*	*0.449*	*1.217*	*1.529*	*0.462*	*0.523*	*13.11*	*14.45*	*94.25*	*95.75*	*0.090*	*0.099*	*1.931*	*2.131*	*0.275*	*0.312*
214 kg N/ha	*0.864*	*1.120*	*0.421*	*0.483*	*1.285*	*1.603*	*0.496*	*0.557*	*14.02*	*14.88*	*94.25*	*96.14*	*0.108*	*0.118*	*2.123*	*2.323*	*0.276*	*0.313*
262 kg N/ha	*0.890*	*1.151*	*0.453*	*0.515*	*1.343*	*1.666*	*0.521*	*0.583*	*12.57*	*13.96*	*94.59*	*95.96*	*0.101*	*0.109*	*2.033*	*2.232*	*0.263*	*0.300*
F. test	*	*	*	*	*	*	*	*	*	*	*NS*	*NS*	*	*	*	*	*	*
LSD at 5 %	*0.007*	*0.005*	*0.005*	*0.005*	*0.006*	*0.005*	*0.001*	*0.001*	*0.36*	*0.43*	-	-	*0.005*	*0.004*	*0.065*	*0.077*	*0.009*	*0.010*

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D- Interactions:																		
A ×B	*NS*	*	*NS*	*	*	*NS*	*NS*	*	*	*NS*	*	*NS*	*NS*	*NS*	*NS*	*	*	***NS***
A ×C	*NS*	*	*NS*	*	*	*NS*	*NS*	*	*	*NS*	*	*NS*	*	*NS*	*NS*	*NS*	*NS*	***NS***
B ×C	*NS*	*	*	*NS*	*NS*	*	*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*	*	*NS*	***NS***
A ×B × C	*	***NS***	*	***NS***	*	*	*	*	***NS***	***NS***	***NS***	***NS***	***NS***	*	***NS***	***NS***	*	***NS***

Table.3: Flag leaf area, plant height, stem diameter, number of tillers/plant, number of days to 50% heading and number of days to 50% flowering as affected by foliar spraying and nitrogen fertilizer levels of some wheat cultivars as well as their interactions during 2013/2014 and 2014/2015 seasons.

Characters Treatments	***Flag leaf area (cm^2)***		***Plant height (cm)***		***Stem diameter (cm)***		***Number of tillers/plant***		***Number of days to 50% heading***		***Number of days to 50% flowering***	
	1st	***2nd***	***1st***	***2nd***	***1st***	***2nd***	***1st***	***2nd***	***1st***	***2nd***	***1st***	***2nd***
A- Cultivars:												
Shaka 93	*25.79*	*26.71*	*80.84*	*84.18*	*3.20*	*3.60*	*3.69*	*3.88*	*77.8*	*78.1*	*84.1*	*84.1*
Gemiza 9	*30.74*	*32.22*	*93.30*	*96.30*	*3.77*	*4.03*	*4.76*	*4.93*	*87.9*	*88.9*	*92.9*	*95.9*
Giza 168	*32.20*	*35.18*	*90.25*	*93.25*	*3.66*	*3.98*	*4.38*	*4.38*	*82.1*	*82.6*	*89.5*	*90.6*
F. test	*	*	*	*	*	*	*	*	*	*	*	*
LSD at 5 %	*1.68*	*1.24*	*1.77*	*1.67*	*0.09*	*0.13*	*0.45*	*0.44*	*1.1*	*1.0*	*1.7*	*1.6*
B-Foliar spraying:												
Control treatment	*25.24*	*25.84*	*85.02*	*85.02*	*3.10*	*3.28*	*3.31*	*3.27*	*82.0*	*82.4*	*88.0*	*89.4*
Humic acid (HA)	*29.71*	*33.30*	*88.64*	*92.32*	*3.66*	*4.02*	*4.70*	*5.00*	*82.7*	*83.0*	*88.7*	*90.0*
Amino acids (AA)	*30.55*	*31.73*	*87.45*	*91.97*	*3.51*	*3.84*	*4.33*	*4.25*	*82.8*	*83.2*	*88.8*	*90.2*
Mixture of HA + AA	*32.82*	*34.59*	*91.42*	*95.66*	*3.90*	*4.33*	*4.77*	*5.07*	*83.0*	*84.3*	*89.9*	*91.3*
F. test	*	*	*	*	*	*	*	*	*	*	*	*
LSD at 5 %	*1.77*	*1.26*	*0.80*	*0.97*	*0.12*	*0.19*	*0.53*	*0.79*	*0.6*	*0.5*	*0.8*	*0.6*
C- Nitrogen fertilizer levels:												
166 kg N/ha	*26.40*	*28.43*	*83.39*	*88.64*	*3.32*	*3.59*	*3.93*	*3.76*	*81.9*	*81.7*	*87.8*	*88.7*
214 kg N/ha	*29.36*	*31.08*	*88.82*	*91.09*	*3.51*	*3.87*	*4.36*	*4.47*	*82.5*	*83.4*	*89.0*	*90.4*
262 kg N/ha	*32.98*	*34.59*	*92.18*	*94.00*	*3.79*	*4.14*	*4.55*	*4.97*	*83.4*	*84.6*	*89.8*	*91.6*
F. test	*	*	*	*	*	*	*	*	*	*	*	*
LSD at 5 %	*0.72*	*0.80*	*0.63*	*0.56*	*0.09*	*0.09*	*0.35*	*0.24*	*1.0*	*0.7*	*0.6*	***0.5***
D- Interactions:												
A ×B	*NS*	*NS*	*	*	*NS*	*	*NS*	*NS*	*NS*	*NS*	*NS*	***NS***
A ×C	*	*NS*	*	*NS*	*NS*	*NS*	*NS*	*	*NS*	*	*NS*	*
B ×C	*NS*	*	*	*	*	*NS*	*	*NS*	*NS*	*NS*	*	***NS***
A ×B ×C	*	***NS***	*	***NS***	*	***NS***	*	***NS***	***NS***	***NS***	*	***NS***

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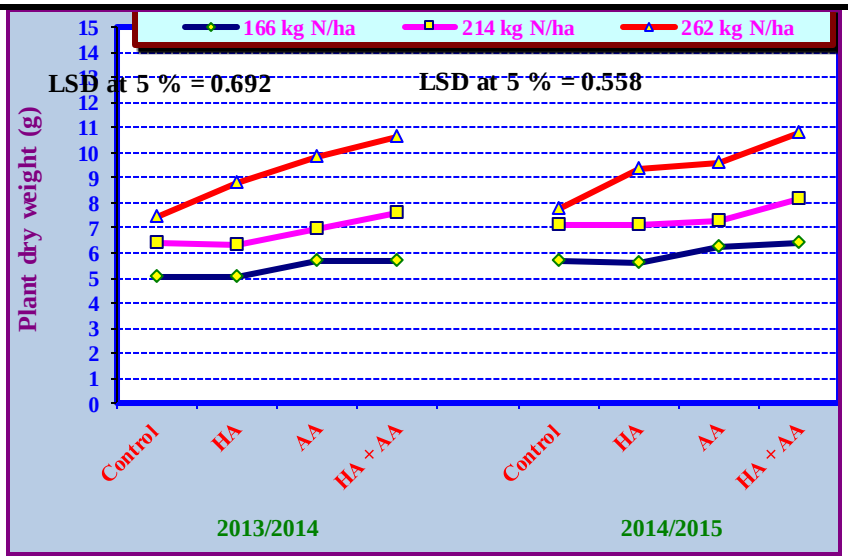

Fig. 1: Plant dry weight (g) after 75 DFS as affected by the interaction between foliar spraying and nitrogen fertilizer leve ls during 2013/2014 and 2014/2015 seasons.

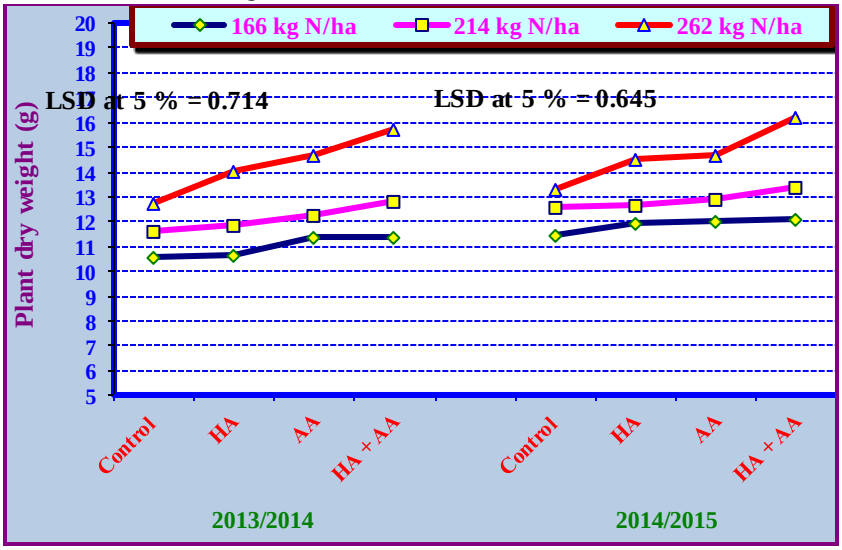

Fig. 2: Plant dry weight (g) after 95 DFS as affected by the interaction between foliar spraying and nitrogen fertilizer levels during 2013/2014 and 2014/2015 seasons.

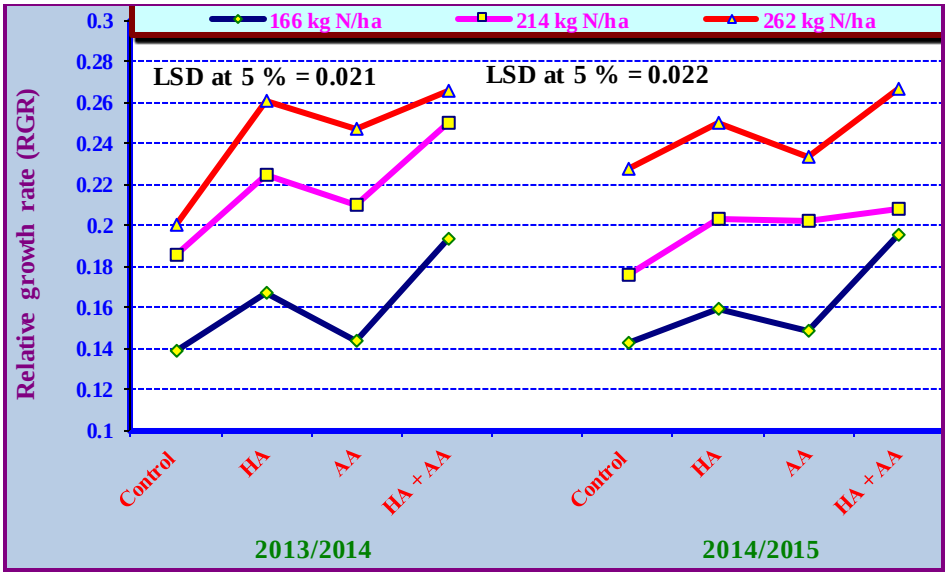

Fig. 3: Relative growth rate (RGR) as affected by the interaction between foliar spraying and nitrogen fertilizer levels during 2013/2014 and 2014/2015 seasons.

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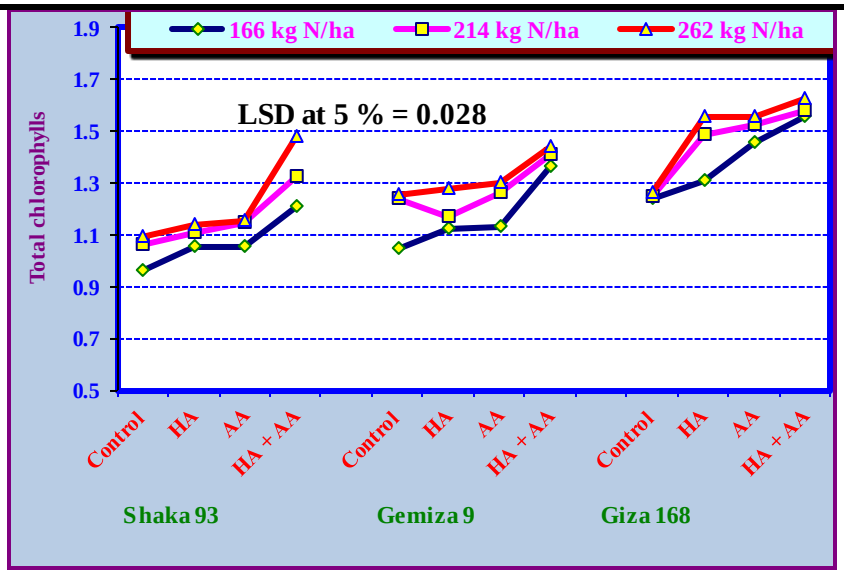

Fig. 4: Total chlorophylls content as affected by the interaction among wheat cultivars, foliar spraying and nitrogen fertilizer levels during 2013/2014 season.

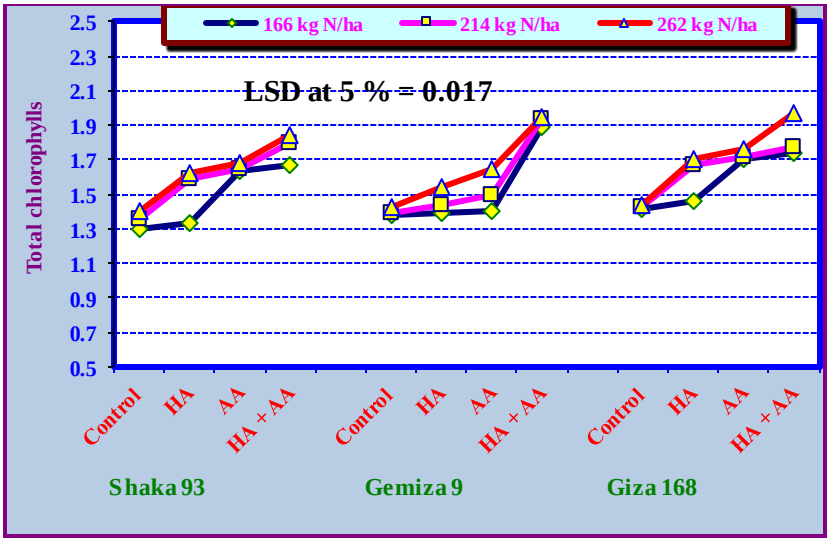

Fig. 5: Total chlorophylls content as affected by the interaction among wheat cultivars, foliar spraying and nitrogen fertilizer levels during 2014/2015 season.

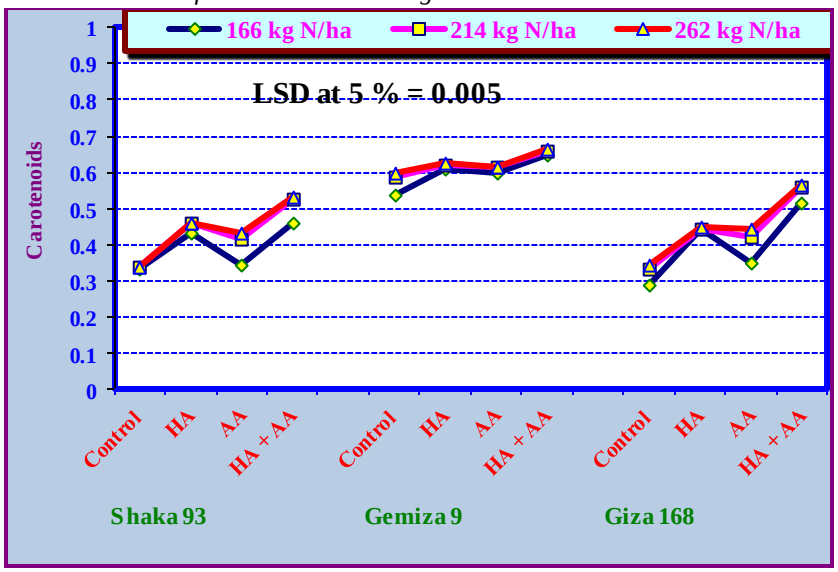

Fig. 6: Carotenoids content as affected by the interaction among wheat cultivars, foliar spraying and nitrogen fertilizer levels during 2013/2014 season.

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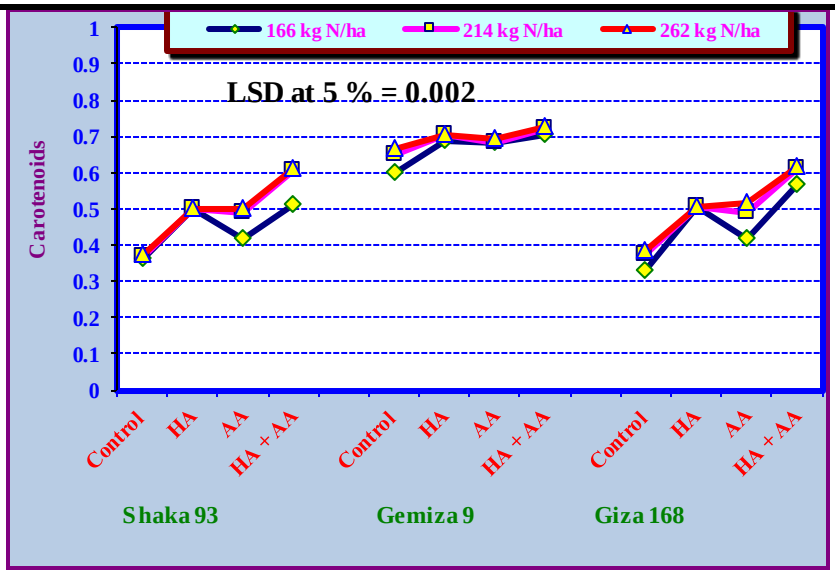

Fig. 7: Carotenoids content as affected by the interaction among wheat cultivars, foliar spraying and nitrogen fertilizer levels during 2014/2015 season.

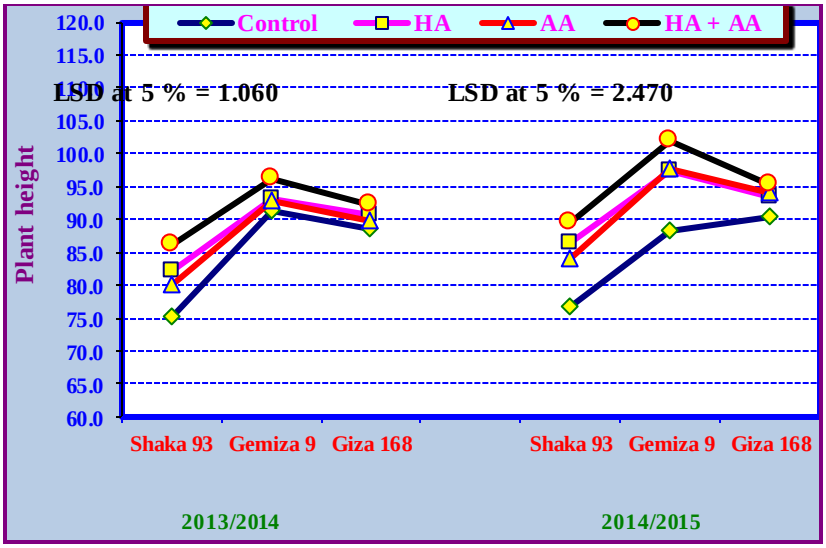

Fig. 8: Plant height as affected by the interaction between wheat cultivars and foliar spraying during 2013/2014 and 2014/2015 seasons.

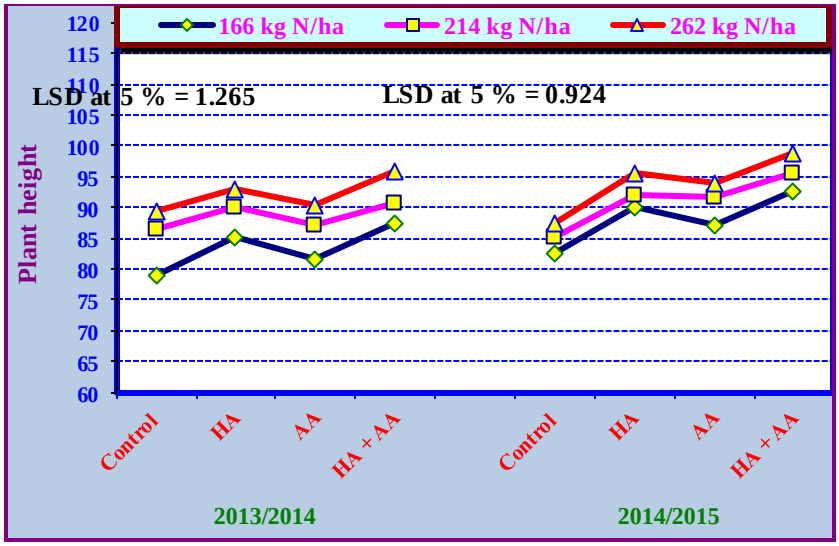

Fig. 9: Plant height area as affected by the interaction between foliar spraying and nitrogen fertilizer levels during 2013/2014 and 2014/2015 seasons.

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Validation of reference genes in leaf-cutting ant *Atta sexdens rubropilosa* in different developmental stages and tissues

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***Abstract*—** *Atta sexdens rubropilosa is an important leaf-cutting ant species considered as a pest in agricultural crop or reforestation areas. Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-qPCR) is a technique that can help us to understand the regulation and the function of a gene. However, its reliability depend on the data normalization. Different normalization strategies can be adopted for qPCR, reference genes has been cited as one of the most effective methods. It has not been identified a universal reference for all organism and experiment. In this way, the validation of reference gene is crucial step. This is the first study to evaluate reference genes for leaf-cutting ants. To this, we analyzed the expression levels of candidate reference genes (act, ef1-alpha, ef1-beta, GAPDH and rpl18) in different developmental stages (larva, pupa and worker) and tissues (head, mesosoma and worker without gaster) of A. sexdens rubropilosa. Four different algorithms (BestKeeper, geNorm, NormFinder and comparative ΔCt method) were used in statistical analysis of the stability of the genes and RefFinder was used to propose a consensus list for ranking the reference genes. Our results showed that the most suitable combinations of reference gene candidates were rpl18 and ef1-alpha for the different developmental stages and rpl18 and ef1-beta for the different tissues. In this work, we also report the obtaining from a putative acetylcholinesterase from A.sexdens rubropilosa (GenBank KY464935), which was used as a target gene to confirm the reliability of reference genes suggested.*

Keywords*— *Acetylcholinesterase, Atta sexdens rubropilosa, Developmental stages, Reference gene, RT-qPCR, Tissues.

I. INTRODUCTION

Insects are the dominant animals in most terrestrial ecosystems, both in number of species and biomass. Among them, social insects present colonies with large numbers of individuals and, consequently, greater biomass [1]. The diversity of ant species indicates that they rank among the most successful insects. It is estimated that 40.000 ant species exist in the world, of which about 16.000 species and subspecies have already been formally described [2]. All ants are considered eusocial. The ecological significance of ants is indisputable; however, as mankind changes the environment for agricultural or forestry development or for the construction of cities, the environment becomes less complex and there is a decrease in biodiversity; although, on the other hand, opportunistic animals (generalists) are favored [3]. Among them are some species of ants, which increase in population density and can adversely affect human activities [4, 5].

Although only few ant species are considered pests (less than 1% of the known species), the economic losses caused by them can be large, especially considering those that occur in silviculture and agriculture, both in the production and storage of food [6]. Among the economically important ant species in Brazil, leaf-cutting ants stand out. They are distributed throughout the Americas and cause major damage, particularly in South America [4, 5]. Leaf-cutting ants are the main herbivores present in the Neotropics and are also considered as pests in agricultural crop or reforestation areas [7].

In addition to the losses that leaf-cutting ants cause to agriculture, silviculture, and pastures, there are the environmental problems and poisoning of other animals, including humans, caused by excessive use of pesticides in the attempt to control these ants. One approach for the development of new ways to control this problem, while minimizing the damage to the environment, is causing the silencing of a specific gene.

Reverse transcription - quantitative real-time polymerase chain reaction (RT-qPCR) is a technique that can help us to understand the regulation and the function of a gene. Different normalization strategies can be adopted for

qPCR; however, the use of a reference gene has been cited as one the most effective methods, since the reference gene undergoes the same steps as the target gene, correcting errors and differences in the sample [8, 9]. Several works have demonstrated the importance of choosing a proper reference gene and impact of using such not appropriated gene. Incorrect results might be obtained due to misinterpretation of RNA transcription levels especially for low abundance gene transcripts [10, 11]. To date, several works were developed to determine the reference gene in Insecta, which demonstrate that is impossible to find a universal reference gene able to covering all organism and conditions [11, 12, 13, 14, 15, 16, 17].

The choice of a gene as reference gene is not trivial and starts with the selection of candidate reference genes to be analyzed [18]. Housekeeping genes (HKG) are usually first selected to be investigated as reference genes due to the assumption that they are involved in essential processes for the survival of cells and are expected to be expressed in a stable and nonregulated level [19]. A reliable reference gene should exhibit an expression level not affected by experimental factors, with minimal variability between tissues and physiological states and a Ct (Cycle Threshold) similar to the target gene [9]. The most studied reference genes, GAPDH and 18s rRNA, are not always expressed in a constant manner. In addition, their expression can be altered depending on the organisms and their life stages [9, 12].

A good strategy for selecting potential candidate reference genes is based on previous data from species relative to the studied specie due to the high degree of similarity between genomes and the expectation of a similar expression level [9]. However, differences in stability have been verified in the analysis of a reference gene in Insecta for organisms from the same order [11, 12, 14, 15], family [20, 21] and even for those of the same genus [14, 16, 21]. This justifies studies to validate reference genes for an organism and experimental conditions before the analysis for precise mRNA quantification [22].

We believe that this is the first study to evaluate reference genes for leaf-cutting ants. Genome from *Acromyrmex echinatior* [23] and *Atta cephalotes* [24], both leaf-cutting ants, are available in the database but there is no validated gene(s) for leaf-cutting ants. In the present study, seven candidate reference genes from *A. sexdens rubropilosa* were selected: actin (*act*), elongation factor 1-alpha (*ef1-alpha*), elongation factor 1-beta (*ef1-beta*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L18 (*rpl18*), TATA box binding protein (*tbp*), and 18S ribosomal RNA (*18S rRNA*). The expression level and stability of them, except *tbp* and *18S rRNA* were examined. The stability of these candidates was investigated in three *A. sexdens rubropilosa* developmental stages (larva, pupa and worker) and in the following parts of the insect: head, mesosoma, gaster, and worker without gaster.

To validate the results the expression profile of a putative target gene, acetylcholinesterase from *A. sexdens rubropilosa*, was investigated. Acetylcholinesterase (AChE, EC 3.1.1.7) is a serino hydrolase that hydrolyzes and inactivates the neurotransmitter acetylcholine controlling the cholinergic signal transmission in the synapse [25]. The evaluation with the target gene emphasize the importance to validate the reference gene as internal control in genomic research and the results presented will be useful for further works in this field for leaf-cutting ants.

II. MATERIAL AND METHODS

2.1 Biological samples

The *A. sexdens rubropilosa* Forel (Hymenoptera: Formicidae) was collected from laboratory nest localized in the Center of Studies on Social Insects (UNESP, Rio Claro, Brazil). The nest was supplied daily with leaves of *Eucalyptus alba***,** oat seeds and occasionally with the leaves of other plants such as *Hibiscus* sp., *Ligustrum* sp. or rosebush petals**.**

Developmental stages samples were picked from the nest: 10 larvae, 10 pupae and 10 workers were collected for each replicate, washed with RNase-free phosphate-buffered saline (PBS) and stored at -80 ^{0}C until used. Tissue samples were dissected from workers: 10 heads, 10 mesosomata, 10 gasters and 10 workers without gaster for each replicate, followed by wash with PBS and stored at -80 ^{0}C until RNA extraction. All samples were collected in triplicate (biological triplicate).

2.2 RNA extraction and cDNA synthesis

Total RNA from larvae, pupae and workers without gaster was extracted using a combined method with TRIzol® (Thermo Fisher Scientific) and PureLink® RNA mini Kit (Thermo Fisher Scientific). For tissue samples from workers, head, mesosoma and gaster, only the PureLink® kit was used. The manufacturer's protocol was followed for both applications. Total RNA from each sample was diluted in 10 mM Tris-HCl pH 7.5 and the quantity and quality of the samples were determined by the 260/280nm and 260/230nm ratio using a BioSpec-nano (Shimadzu-biotech). The RNA integrity was analyzed by agarose denaturing gel 1.2 % (w/v) and confirmed by the intense ribosomal RNA bands and the absence of smears. The total RNA was treated with DNase (DNaseI, RNase-free -Thermo Fisher Scientific) to eliminate potential genomic DNA contamination.

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First-strand complementary DNA (cDNA) was synthetized using 1.35 µg of total RNA with SuperScript® VILO Master Mix (Thermo Fisher Scientific) with 20 µl final reaction volume, following instructions from the manufacturer. The synthesis of cDNA was performed in triplicate for each sample (replicate) and the product was stored at -20°C for later use.

2.3 Selection and procedure for obtaining the sequence of candidate reference genes and the putative acetylcholinesterase gene

Seven genes were selected as candidate to reference genes: actin (*act*), elongation factor 1-beta (*ef1-beta*), elongation factor 1-alpha (*ef1-alpha*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), ribosomal protein L18 (*rpl18*), TATA box binding protein (*tbp*), and 18S ribosomal RNA (*18S rRNA*). Acetylcholinesterase (AChE) was used as a target gene.

A. sexdens rubropilosa genome is not yet known, for this reason, the sequences of most of these genes are not yet deposited. Sequence alignments for every gene from several species of ants were performed and conserved sequence regions were used to design specific and/or degenerated forward and reverse primers (Table 1).

The DNAs were amplified by PCR performed with 1 µL cDNA; 1 µM for the specific primer and 2 µM for the degenerated one; 0.2 mM of dNTPs and 1.25 U of Pfu DNA Polymerase (Thermo Fisher Scientific) in 25 µL final volume. PCR amplification was performed using the following program: 3 min at 95 °C followed by 40 cycles of 1 min at 95 °C, 90 s at 52 or 62 °C, 6 min at 72 °C and a final extension step of 10 min at 72 °C. The amplification products were evaluated on 1% agarose gel and the bands were extracted and purified. Samples were quantified by absorbance in 260 nm and then submitted for sequencing analysis (ABI 3730 DNA Analyser - Thermo Fisher Scientific) with the same primers used for amplification. The sequences were analyzed with *BioEdit* (v7.2.5-http://www.mbio.ncsu.edu/bioedit/page2.html) and the search for similarity was carried out using the BLAST *tool.* The amplicons were compared with ants' sequences and led to an identity of over 90 %.

Acetylcholinesterase sequence from *Acromyrmex echinatior* (GenBank GL888116.1) was used to design primers with the inclusion of site for restriction enzymes and exclusion of signal peptide (Table 1). The PCR reaction was performed similar as described above with 0.2 µM of each primer, an annealing temperature of 63°C in 30 cycles with an extension time of 3 min. The reaction product was analyzed on 1% agarose gel, purified and sequenced.

2.4 Primer design for quantitative real-time RT-PCR (RT-qPCR)

Using the sequence from the amplicons, new primer pairs were designed, for each gene, using Primer Express® Software Version 3.0 and selecting the amplicon length between 50 and 150 pb. Among the various possibilities of primers provided by the software output, the selection of the primer pair was based on the low score penalty and smaller size of the amplicon. Primer sequences and amplicon characteristics are summarized in Table 2 for each candidate gene and for the AChE gene.

2.5 Quantitative Real-time PCR (qPCR)

The minimal primer concentration was determined using two-by-two combinations of forward and reverse primers in 100, 150 and 300 nM, in duplicate, and a non-template control for each combination. RT-qPCR was performed in an Applied Biosystem StepOnePlus™ system (Thermo Fisher Scientific) with a total reaction volume of 12 µL, containing 6 µL *Power* SYBR® Green PCR Master Mix (Thermo Fisher Scientific), 3 µL of forward and reverse primers in the appropriate concentration to give the relation described above and 3 µL of cDNA previously 30-fold diluted. Cycling conditions were: 10 min at 95 °C (polymerase activation) followed by 40 cycles at 95 °C during 10 s (denaturation) and 60 °C during 1 min (annealing/extension). For each reaction, the dissociation of the PCR products (melting curve) was analyzed from 60 to 95 °C to ensure the specificity of the amplified product.

The appropriate primer concentration was used to determine the RT-qPCR efficiency by a relative standard curve for each candidate reference genes. For this purpose, a 5-fold serial dilution of the cDNA was used as a template molecule for candidate reference genes and a 2-fold serial dilution for acetylcholinesterase. Samples were analyzed in triplicate plus a negative control. RT-qPCR efficiency was calculated according to the equation 1, in which the slope comes from the plot of Ct values against the logarithm of cDNA concentration [10]. Efficiencies between 90% and 110% were used for further statistical analysis (Table 2).

$$E = (10^{-1/\text{slope}} - 1) \times 100 \qquad (1)$$

Once the optimum primers and cDNA concentrations were determined for each gene, the gene expression analysis was performed by RT-qPCR in the conditions already described above using 3 µL of cDNA diluted 60 times. The reaction was performed in triplicate and with a non-template control for each conversion reaction of cDNA.

2.6 Data Analysis and Statistics

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2.6.1 Expression level analysis

The expression level of candidates for reference gene was analyzed by standard deviation, coefficient variation and Student's *t*-test. The t-test was used to verify if the mean value of expression levels between two different stages of development are statistically different or not. The same procedure was adopted to analyze if there is significant difference among the expression levels between different parts of the ant body. For this comparison, the first procedure is to calculate the pooled estimate of standard deviation (2), followed by the calculation of the experimental t-value (3), where *S*, *n* and $\bar{x}$ are the standard deviation, degrees of freedom and means, respectively, for the two analyzed genes. If the experimental t-value is lower than the critical t-value then there is no significant difference between the mean values of the gene expression at a 95% of confidence level [26].

$$S_p = \sqrt{\frac{S_1^2(n_1+1)+S_2^2(n_2+1)}{n_1+n_2-2}} \quad S_p = \sqrt{\frac{S_1^2(n_1+1)+S_2^2(n_2+1)}{n_1+n_2-2}} \qquad (2)$$

$$t_{exp} = \frac{|\bar{x}_1-\bar{x}_2|}{S_p}\sqrt{\frac{n_1 n_2}{n_1+n_2}} \quad t_{exp} = \frac{|\bar{x}_1-\bar{x}_2|}{S_p}\sqrt{\frac{n_1 n_2}{n_1+n_2}} \qquad (3)$$

In addition, the source of the standard deviation was compared in the analyses by comparing the variability of data from replicates with data related to the different body parts or different life cycle stages.

The autoscaling preprocess also was used in order to obtain a better visualization of the most similar variables. This strategy is well known in chemometrics to normalize all the variables (expression levels, in this case) in order to minimize the differences in the intensity among them [27]. This preprocess is performed by subtracting the mean value of the genes expression in each developmental stage from the total mean of the same gene for all developmental stages, followed by division by the standard deviation of the gene for all developmental stages.

All of the tests were performed at 95% confidence level using the Microsoft Excel® software.

The relative expression level of the target gene was obtained according to the relative quantification by $2^{-\Delta\Delta Ct}$ method [28]. For developmental stage larva was used as calibrator and mesosoma for tissue. The data was plot as mean ± SEM and the analysis with 95% confidence using GraphPad Prism 5.

2.6.2 Selection of reference genes

The selection of the reference gene was performed using four algorithms, BestKeeper© version 1 [29], geNorm version 3 [30], NormFinder version v0.953 [18] and the comparative ΔCt method [31]. RefFinder was used to compare and rank the reference gene candidates [32]. In addition, the results from these software were compared with the statistical analysis performed.

2.6.3 BestKeeper

BestKeeper is an Excel based spreadsheet software that uses raw data (Ct values) and reaction efficiency (E) to identify the best-suited standards and combines them into an index [29]. The output Table shows descriptive statistics for each reference gene candidate: the geometric mean (geo Mean), arithmetic mean (ar Mean), minimal (min) and maximal (max) value, standard deviation (SD), and coefficient of variation (CV). The x-fold over- or under-expression of individual samples are calculated based on the geometric mean. These results are corrected via RT-qPCR efficiency to exhibit minimal and maximal values considering the x-fold ratio and their SD (SD [± x-fold]). The stability of the reference gene candidate can be evaluated by the user considering the calculated variation, such as SD and CV. Reference genes can be ordered from the most stable (lowest variation) to the least stable (highest variation). Candidate genes with SD [± Ct] higher than 1 (= starting template variation by a factor of 2) can be considered inconsistent and it is recommended to exclude them from the calculation index [29].

BestKeeper also tests individual samples for their integrity. To do this, x-fold values are used through an intrinsic variation (InVar) for a single sample. It has been suggested that samples with 3-fold over- or under-expression should be removed from the analysis due to high deviation that can be attributed to inefficient sample preparation, incomplete reverse transcription or sample degradation [29].

2.6.4 geNorm

geNorm uses relative quantification data from raw Ct values by $2^{-\Delta Ct}$. This software determines the expression stability of candidate reference genes based on the gene-stability measure [30]. The internal control gene stability measure M is defined as the average pairwise variation for that gene with all other tested reference genes, where the lowest value for M corresponds to the most stable candidate, and the highest corresponds to the least stable. Values that surpass the cutoff value of 1.5 are not considered stable. The program enables stepwise exclusion of the gene with the highest value of M and recalculation of M for the remaining genes ranking them according their expression stability.

The second important parameter calculated by geNorm is the pairwise variation (V_n/V_{n+1}) between two sequential normalization factors (NF_n and NF_{n+1}) to obtain the minimal number of reference genes [30]. The cutoff value of 0.15 indicates that no additional gene, beyond the *n*

most stable genes, needs to be included for a reliable analysis.

2.6.5 NormFinder

NormFinder, a model-based approach for the estimation of expression variation. It is able to identify stably expressed genes in a set of reference gene candidates. The Ct values were transformed to a linear scale by the same method used for geNorm in order to prepare input data. The mathematical model of gene expression presented on a Visual Basic application for Microsoft Excel estimates the intragroup variation as well as the intergroup variation in all groups [18]. These variations are combined into a stability value, representing a practical measure of the systematic error that will be introduced when using the investigated gene. The software requirements are 8 samples/groups and at least 3 candidate reference genes; 5-10 candidates are recommended in order to obtain reliable results. The best reference gene candidates are ranked in an index that is based on stability values; a low stability value indicates the most stably expressed gene.

2.6.6 Comparative ΔCt method

This method compares the relative expression of "pairs of genes" within each sample to identify a useful reference gene [31]. The variation ΔCt for each two genes is obtained by the difference of Ct values. The mean, standard deviation and mean of the standard deviation related to the ΔCt are obtained and used to rank genes. Two genes are stably expressed or co-regulated if a constant ΔCt value is observed between two genes. A low deviation value shows a more stable expression due to a short variability.

2.6.7 RefFinder

RefFinder is a web-based tool (http://fulxie.0fees.us/?type=reference) that considers the four algorithms described before to rank the candidate reference genes. It uses raw Ct as input data to obtain the rank provided by each program. Then, based on the ranks, a weight for each individual gene is calculated to obtain the final overall rank [32].

Table.1: Primer pairs sequence to identify the sequence of candidate reference genes.

Genes	Function	Primer sequence[a] (5'-3')	Amplicon size sequenced (bp)
act	Cytoskeletal structural protein involved in cell motility, structure and integrity	F: GYGACGACGAMGTAGC R: TGCCAGATCTTCTCC	259
ef1-alpha	Elongation during polypeptide synthesis in the ribosome	F: GACATTGCCTTGTGGAAG R: CAGTTGGCCTGGTAGGTGGC	498
ef1-beta	Elongation during polypeptide synthesis in the ribosome	F: GTGGCAACCAACTCAGG R: GTGGACGAAGCTGGG	177
GAPDH	Carbohydrate metabolism	F: CAACTTYGARRTYSTCGAGG R: CCRWAYTCGTTGTCATACC	436
rpl18	Encode a ribosomal protein that is a component of the 60S subunit	F: CGATATTAATCATAAGCATGATCGGA R: CTTATAACCGCAGCTGCGTC	481
tbp	Coordinate the initiation of transcription by RNA polymerase II promoter	F: ATGGATCAGATGCTTCCG R: AGACCTGGAAATAGCTCTGG	677
18S rRNA	Structural RNA constituent of subunit 40S of the ribosome	F: AGCCATGCATGTCTCAGTGC R: CGCGACGGGATATTAGTTGG	648

[a] F and R indicate forward and reverse primers, respectively.

Table 2: Primer sequences and amplicon characteristics for reference gene candidates used in RT-qPCR analysis.

Gene	Sequence (5'-3')	Product Lenght (bp)	Efficiency (%)[b]	R^{2} [c]
act	F: TCCTCGCGCCGTCTTTC R: TTGACCCATACCGACCATCA	69	98.2	0.990

ef1-alpha	F: AGCCGCTGTTGCATTCGT R: TGACGGATACTTCCAACATATTGTC	64	95.1	0.993
ef1-beta	F: GGCAACCAACTCAGGCTGAT R: CAACGGAGTACATGAGGATTCG	82	99.8	0.900
GAPDH	F: ATGACGACTGTACATGCGATTACA R: TCACGCCATAGCTTGCTTGA	70	97.4	0.990
rpl18	F: CGAGATCATCACGTTCGATC R: CTGCATCAAGACTGTACGTTTTCC	66	97.9	0.988
tbp	F: CAGCAGTCACAACAATTTCAACAA R: TCATTAGCATGCCACTCTGCAT	75	*	
18S rRNA	F: CTGATCGCACGGTCTTAGCA R: CAGAACCTACCATCGACAGTTGAT	73	*	

[a] F and R indicate forward and reverse primers, respectively
[b] RT-qPCR efficiency, calculated by the standard curve method
[c] Determination coefficient
[*]Results will be discussed in item 3.3

III. RESULTS

3.1 Sample quality

Despite the accurate validation of reference genes, several problems can directly influence the results during the sample processing and preparation. In general, these problems can be associated to factors such as sample storage, RNA extraction and quality, synthesis of cDNA with transcriptase reverse, primer design and normalization [33]. Agarose gel electrophoresis was used to confirm the integrity of the RNA extracted from *A. sexdens rubropilosa* (Fig. 1). As previously described for insects [34], only one intense RNA band can be seen in the denaturing gel, which corresponds to the two fragments of the 28S rRNA that co-migrate with 18S rRNA.

The RNA extracted from worker and from gaster using the Trizol method was degraded. Valles and collaborators detected the presence of an endogenous component located in the abdomen of adult ants (terminal abdominal segments) from *Nylanderia pubens* Forel (Hymenoptera: Formicidae), and also in queens and alate ants, capable of degrading RNA [35]. This report has also showed that the addition of at least 50 mM EDTA leads to intact RNA. However, EDTA can inhibit subsequent transcription and the PCR reaction, which could include one more variable in RT-qPCR experiments [36]. Therefore, new RNA extraction was carried out with the PureLink® RNA mini Kit producing intact RNA from the worker (Fig. 1, lane 3) and partially intact from the worker's gaster (Fig. 1, lane 6). RNA extracted by combining Trizol with the kit (from lavae, pupae and workers without gaster) (Fig. 1, lanes 1, 2, and 7, respectively) and only with the kit (head and mesosoma) (Fig. 1, lanes 4 and 5) showed characteristic bands of intact RNA.

There are divergent discussions about the influence of RNA integrity on RT-qPCR experiments. Some authors [36] suggest that RNA degradation can be tolerated since an amplicon with 70-250 bp is obtained, while other authors indicate that partially degraded RNA can give an imprecise result of genic expression [37]. Because of this, the RNA from the ant's gaster was excluded from the analysis with exception of the BestKeeper algorithm that also analyze the sample integrity.

3.2 Selection and procedure for obtaining the sequence of reference gene candidates and AChE gene

The lack of genome information for *A. sexdens rubropilosa* was not an obstacle for gene validation: the sequences alignment of other ant nucleotides and the analysis of the conserved regions enabled the design of primers, which were used for obtaining amplicons from *A. sexdens rubropilosa* cDNA (Table 1). The choice of a reference candidate for analysis was made based on the reference gene for *Solenopsis invicta*, the closest insect (Formicidae) with described reference genes [12] and other insects [9, 13, 15, 21, 38, 39, 40].

All seven candidate reference genes (*act*, *ef1-beta*, *ef1-alpha*, *GAPDH*, *rpl18*, *tbp* and *18S rRNA*) were amplified by PCR using these primer pairs. The amplicons were sequenced and these sequences were used in a sequence similarity search, confirming the identity of the genes. The AChE sequence from *A. sexdens rubropilosa* without signal peptide can be accessed in GenBank KY464935. The DNA sequence amplified from the candidate reference genes and AChE was used to design specific primers for the RT-qPCR (Table 2).

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3.3 Standardization of the conditions for Quantitative Real-time PCR (qPCR)

The minimum primer concentration for each target gene was determined to minimize non-specific amplifications and to reach the maximum amplification efficiency [41]. The proper combinations of primers were considered those that introduced the melting curve with a single peak, resulting in amplification reactions with lower Ct values and greater ΔRn. All samples showed a single peak in the melting curve. However, it was also observed one peak in the melting curve of the non-template control for the *tbp* gene suggesting the formation of a primer dimer. The primers pairs designed for the *tbp* gene could provide unreliable results and, therefore, this gene was excluded from this study. Nevertheless, analysis would be possible through the design of new primers for this gene.

The best primer concentrations determined for the remaining six genes were used in the determination of the RT-qPCR efficiency for each gene. The *18S rRNA* showed a high abundance of transcripts due to the low value of Ct (data not shown). The sample (cDNA) was diluted by a factor of 60 to verify the reaction efficiency, but the results were not satisfactory. The discrepancy between rRNA and mRNA has been discussed as a negative point in the use of rRNA in reference genes studies [30]; in addition, the necessity of high sample dilution prior to qPCR can lead on dilution errors [42]. For these reasons, studies that analyzed this gene as a reference gene in insects also suggested the elimination of *18S rRNA* from the list of consensus genes [17, 21]. Therefore, *18S rRNA* gene was excluded from this study.

RT-qPCR efficiency for a*ct, ef1-alpha, ef1-beta, GAPDH, rpl18 and AChE* was between 95.1 – 103.6 %, showing that they can be used for RT-qPCR analysis (Table 2). The relative expression level of the target was obtained by $2^{-\Delta\Delta Ct}$ method, to this the target and reference should have amplification efficiencies approximated equal, the observation of how ΔCt varies with template dilution showed that the method can be used for analysis (data not shown) [28].

3.4 Statistical analysis

3.4.1 Transcription profile of candidate reference genes

Fig. 2a shows the genes plotted as function of their gene expression average at different developmental stages. The autoscaling preprocess was used in order to obtain a better view of the correlation among the variables. Fig. 2a shows that the most correlated genes are *ef1-alpha* and *ef1-beta*, followed by *rpl18*, being the genes *act* and *GAPDH* more intercorrelated. Therefore, the variables *ef1-alpha*, *ef1-beta*, and *rpl18*, are the most correlated variables and they present the low variability with the development stage, making these variables good candidates for reference genes.

Fig. 2b shows the genes plotted as function of their gene expression average in different parts of the body using autoscaling, as explained before. As showed in this Fig., the most correlated genes with respect to different body parts are *rpl18* and *ef1-beta*, followed by *GAPDH* and *ef1-alpha*. In this case, although the genes *GAPDH* and *act* present the best SD and CV values (data not shown), they do not present good correlation compared to the other variables. Then, the best choice for a reference gene will depend if the algorithm used seeks lower SD and CV values or the two most correlated variables.

The Student's t-test showed no significant difference for all genes, with a confidence level of 95%, when larvae and pupae were compared. However, there were significant differences in the expression of the genes when larvae and pupae were compared to workers. *ef1-alpha*, *ef1-beta* and *rpl18* presented the most constant expression with the development stage. Similar results were obtained for the different tissues, where there were not significant differences for all genes expression levels when head and torax were compared. In addition, act was the only gene that didn't present significant differences comparing any tissue by t-test at 95% of confidence. Again, the best selected gene will depend of the algorithm used for the genes evaluation.

The comparison between the standard deviation for replicates from RT-qPCR experiment and replicates of converting RNA into cDNA showed that the deviation of the last one is 1.6 and 3.6 times higher than the first. This was expected since it is well known that the conversion of RNA into cDNA is the main source of data variability.

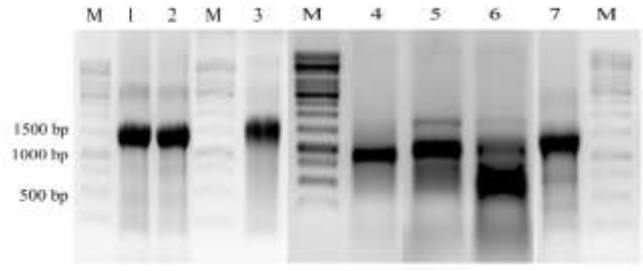

Fig. 1: Analyzes of RNA integrity by denaturing agarose gel electrophoresis 1.2% (w/v). (M) molecular marker; RNA extracted from: 1) larvae; 2) pupae, 3) workers; 4) head; 5) mesosoma; 6) gaster; 7) worker without gaster.

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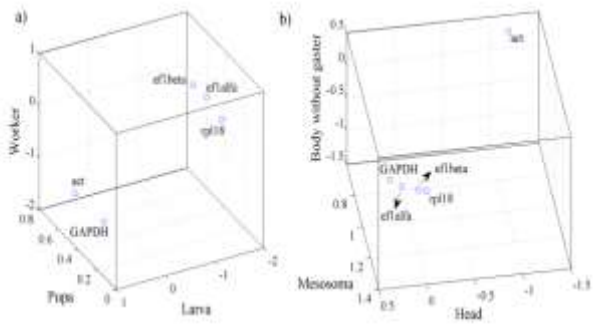

Fig. 2: Averages of gene expression as function of the developmental stage (a) and tissue (b) for each candidate. The data was auto-scaled before the plot without gaster.

3.4.2 BestKeeper

Using the output values for Ct variation (SD [± Ct]), the expression level of the candidates reference genes was analyzed and the ranking was constructed. As the SD [± Ct] are below 1 for all of the five candidates from the developmental stages (larva, pupa and worker), it means that they can be considered stably expressed (Table 3). The gene stability in decreasing order for developmental stages is: *GAPDH*, *rpl18*, *ef1-alpha*, *ef1-beta*, and *act*. The intrinsic variance (InVar) of expression for a single sample is below 3 and the highest value obtained was 1.03. This result confirms the integrity of total RNA extracted from specimen of every developmental stage. In addition, the results agree with the statistical analysis where was verified that GAPDH present the lowest standard deviation.

The tissue samples (head, mesosoma, and worker without gaster) including gaster the Ct variation (SD [± Ct]) and up/downregulation (SD [± x-fold]) showed higher values than 1 and 2, respectively. In this way, none of the candidate reference genes from the tissues could be used. The InVar [± x-fold] values for the samples were higher than 3 for the gaster confirming RNA degradation as observed on the denaturing agarose gel (Fig. 1) and justifying the exclusion of this tissue from analysis. The gaster samples were eliminated and the data were analyzed again; this has led to acceptable values of SD [± Ct], SD [± x-fold] and InVar [± x-fold] for all reference gene candidates. This proceeding also was adopted by Ponton and collaborators that also identified InVar [± x-fold] > 3 when analyzing different treatments together from *Drosophila melanogaster* [17]. To overcome this, the authors suggested a separate analysis of the samples with different treatments.

The stability of the genes obtained by BestKeeper, in decreasing order for tissues (head, mesosoma, and worker without gaster), was *GAPDH*, *act*, *rpl18*, *ef1-1beta* and *ef1-alpha* (Table 3).

3.4.3 geNorm

Vandesompele and collaborators described this robust and innovative strategy to identify the most stably expressed control genes in a given set of tissues, and to determine the minimum number of genes required to calculate a reliable normalization factor [30]. These authors also suggested the use of at least three reference genes to increase the confidence of the analysis when the suggested number of genes is too high or the sample limited.

First geNorm calculates the gene stability measure (M); the genes presenting M < 1.5 are considered stable. Here, the five candidate reference genes, considering both the development stages and different tissues, could be considered for use as reference genes.

The candidate reference genes were ranked after stepwise exclusion of the highest M value (Table 3, Fig. 3), which results in a combination of two constitutively expressed genes that exhibit the most stable expression in the tested samples. The decrease of the M value during this analysis reflects the differences in the stability of reference gene candidates associated with the highest stability of the remaining genes. In this way, it is clear that *act* and *GAPDH* present an unstable expression, represented by the decrease of the M value after removal of these genes (Fig. 3) in both groups of analyzed samples (development stages and tissues).

Therefore, the most stable genes are *ef1-alpha* and *ef1-beta* for the different developmental stages and *ef1-beta* and *rpl18* for the different tissues. These results perfectly agree with the spatial representation of the gene expression levels presents in Fig. 2.

Vandesompele and collaborators also demonstrated the large errors associated with the use of a single gene as reference gene [30]. To obtain reliable results for gene expression analysis, geNorm determines the minimum number of genes to be used as reference genes in a particular experiment. To do that, the pairwise variation was individually determined for each gene starting with the two most stable genes (n=2) with the sequential addition of the other least stable genes ($V_{n/n+1}$). The optimum number of reference genes was determined by the levels of variation in the average reference gene stability. $V_{2/3}$ values are below the threshold value of 0.15 (Fig. 4). Then, geNorm tool indicates that the use of only two genes, the most stable ones, is sufficient to obtain accurate results for normalization experiments in RT-qPCR analysis from the different developmental stages and tissues for *A. sexdens rubropilosa*.

3.4.4 NormFinder

The mathematical model of NormFinder considers the inter- and intra-group variation to estimate the gene stability and rank genes with minimal variation,

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eliminating problems associated with the selection of co-regulated genes [18]. For the different developmental stages, the most stably expressed candidate gene was *rpl18* with the lowest variability value. The decreasing order of gene stability is *rpl18*, *ef1-alpha*, *ef1-beta*, *GAPDH* and *act* (Table 3). For different tissues, the decreasing order was *rpl18*, *ef1-beta*, *GAPDH*, *ef1-alpha* and *act.*

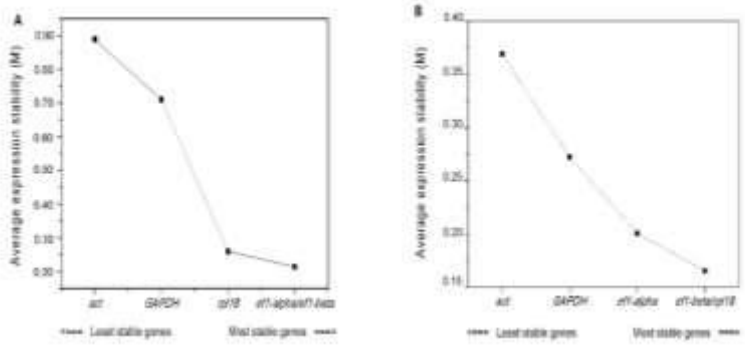

Fig.3: Gene expression analysis by geNorm. Expression stability and ranking of 5 candidate reference genes. The M value (indicates the average expression stability) is lower for the most stable expression. A) Developmental stages; B) Tissues

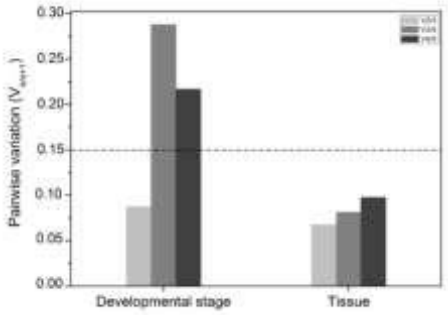

Fig. 4: Pairwise variation of candidate reference genes for determination of the optimal number of control genes for accurate normalization. Pairwise variation ($V_n/_{n+1}$) analysis between the normalization factors NF_n and NF_{n+1}

Andersen and collaborators that elucidated the discrepancies caused by the differences between the approaches, due to the tendency of pairwise comparison to select genes with highest degree of similarity in their expression profile [18], foresaw the difference in rank obtained by NormFinder and geNorm for developmental stages. This is a problem when there are co-regulated genes between the candidates, they usually have a tendency to show very similar expression profiles and be top ranked, independently of their expression stability [18]. This can be the reason for which geNorm ranked *ef1-alpha* and *ef1-beta* genes as the best genes to be used as reference for different developmental stages.

3.4.5 The comparative ΔCt method

The ΔCt method compares pairs of genes, similarly to geNorm, and uses ΔCt to estimate the gene variability [31]. Changes in gene variability were observed by the increase or decrease on the deviation of ΔCt among all possible combinations between candidate reference genes. The analysis of the most stable gene was done comparing the mean of the standard deviation of ΔCt. The lowest values correspond to lower variability for this gene, which establishes it as the most stable gene. For the developmental stages, *rpl18*, *ef1-alfa*, and *ef1-beta* genes showed the lowest and similar deviation (Table 3). The rank in decreasing order of stability for the developmental stages was *rpl18*, *ef1-alpha*, *ef1-beta*, *GAPDH* and *act*. For the tissue samples the decreasing order of stability was *rpl18*, *ef1-beta*, *ef1-alpha GAPDH* and *act*.

3.4.6 RefFinder

The stability of the *candidate reference genes* was evaluated with four different algorithms (BestKeeper, geNorm, NormFinder and comparative ΔCt method). Differences in the mathematical model for each one result in different ranks for gene stability, but the methods are equally important [9, 17]. RefFinder was used to propose a consensus list for ranking the reference gene for *A. sexdens rubropilosa* (Table 3), showing, for the developmental stages, in a stability decreasing order: *rpl18, ef1-alpha, ef1-beta, GAPDH and act.* For the tissues, the decreasing order of stability was *rpl18, ef1-beta, GAPDH, ef1-alpha and act.*

3.4.7 Expression of the AChE from *A. sexdens rubropilosa*

The RT-qPCR data for AChE normalized by each of the candidate reference genes are presented in Fig. 5a and Fig. 5b, for developmental stages and for tissues, respectively. As predicted, the results show a difference in quantification depending on the gene used to normalize. In worker the AChE expression level normalized with *act* and *GAPDH* was about 7.3-fold and about 4.2-fold lower than those normalized with *rpl18* and *ef1-alpha* (P <0,0001). The great difference in tissues was in worker without gaster when the data were normalized with *act*, exhibiting an expression level 2-fold higher when compared with *rpl18* (P=0,0012). However, the statistical analysis for the two top ranked genes by RefFinder for developmental stages (*rpl18* and *ef1-alpha*)

and for tissues (*rpl18* and *ef1-beta*) showed no significant difference (P>0,05).

Table.3: Rank of reference genes in decrease order based on their expression stability according to BestKeeper, geNorm, NormFinder, comparative ΔCt method and RefFinder. The values were obtained after individual analysis of each software.

Developmental stage					Tissues				
BestKeeper	geNorm	NormFinder	ΔCt method	RefFinder	BestKeeper	geNorm	NormFinder	ΔCt method	RefFinder
GAPDH (0.50)	ef1-alpha/ ef1-beta (0.21)	rpl18 (0.135)	rpl18 (0.73)	rpl18 (1.57)	GAPDH (0.33)	ef1-beta/ rpl18 (0.16)	rpl18 (0.028)	rpl18 (0.30)	rpl18 (1.32)
rpl18 (0.58)	rpl18 (0.26)	ef1-alpha (0.143)	ef1-alpha (0.74)	ef1-alpha (1.86)	act (0.38)	ef1-alpha (0.20)	ef1-beta/ GAPDH (0.050)	ef1-beta (0.31)	ef1-beta (2.00)
ef1-alpha (0.59)	GAPDH (0.71)	ef1-beta (0.176)	ef1-beta (0.79)	ef1-beta (2.45)	rpl18 (0.41)	GAPDH (0.27)	ef1-alpha (0.060)	ef1-alpha (0.34	GAPDH (2.83)
ef1-beta (0.71)	act (0.89)	GAPDH (0.218)	GAPDH (1.02)	GAPDH (2.83)	ef1-beta (0.44)	act (0.37)	act (0.140)	GAPDH (0.39)	ef1-alpha (3.41)
act (0.74)		act (0.254)	act (1.16)	act (5.00)	ef1-alpha (0.49)			act (0.51)	act (3.98)

The parameter for each software was standard deviation of the Ct (SD [±Ct]) for BestKeeper, expression stability value for NormFinder, M value after stepwise exclusion of the highest M value for geNorm, mean of standard deviation of ΔCt for ΔCt method and geomean of ranking values for RefFinder.

The expression level for AChE in developmental stages, when the AChE data were normalized considering the two top ranked gene, enhance from larva to worker (Fig. 5a). AChE from *Anopheles gambiae* also showed a similar expression pattern and some works have shown that this enzyme also exhibit a noncholinergic functions associated with insect development [43, 44, 45]. In tissues, there is a small variation in the expression of AChE in the head compared to the mesosoma, while in worker without gaster, that is the junction of the other two, the expression is almost 2-fold higher than in mesosoma (Fig. 5b). In *A. gambiae* a higher AChE2 expression was observed in abdomen than in head [45]. Until now, none classification has been done for AChE from *A. sexdens rubropilosa* and we are working in another analysis for an accurate classification.

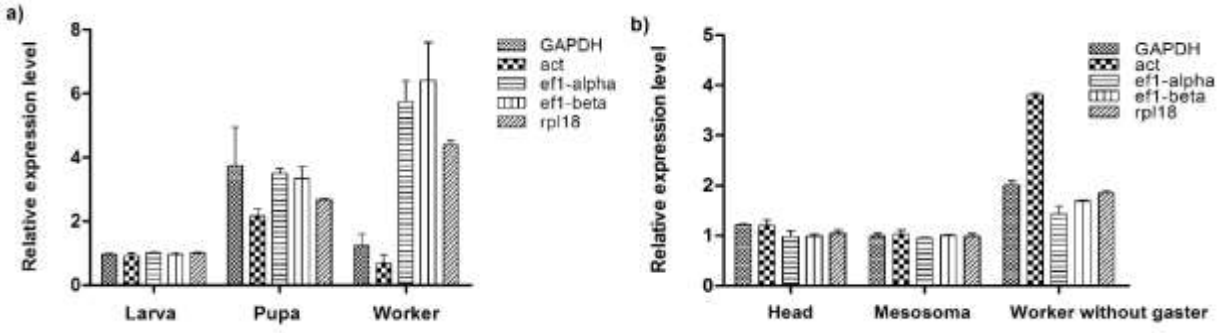

Fig. 5:Relative quantification of AChE in A. sexdens rubropilosa. Expression profile of target gene normalized with different candidate reference genes in three A) developmental stages and B) tissues. Data are presented as mean±SEM of biological triplicate.

IV. DISCUSSION

RT-qPCR has been widely used for gene expression analysis due to the high accuracy, however, the reliability of the results are strictly correlated with the genes used as reference genes. Due to this, the validation of reference gene is necessary and there are several works suggesting the importance of the validation for each organism and experimental condition. This is the first work to validate reference gene for *A. sexdens rubropilosa* under biotic condition for different developmental stages and tissues. The results obtained here can support research in this field once leaf-cutting ants is considered as pests in

agricultural crop or reforestation areas mainly in South America.

For the validation for reference gene was done statistical analysis of the data, beside this, different statistical algorithms such as BestKeeper, geNorm, NormFinder, the comparative ΔCt method and RefFinder were used to verify the stability of the genes selected. Once the reference gene can be regulated to some extent, a combination of reference genes should be used, and as indicated by geNorm two reference genes are enough to obtain accurate results. So, we suggest the use of *rpl18* and *ef1-alpha* for developmental stages and *rpl18* and *ef1-beta* for tissues for genomic analysis in *A. sexdens rubropilosa*, based on consensus list provided by RefFinder. For *S. invicta rpl18* and elongation factor (*beta*) were the most stable genes for expression in different developmental stages, castes and tissues [12]. The similar results from these two studies was not obvious and experimental results were necessary, once the expression stabilities of HKGs were not conserved among evolutionarily close species [11, 14].

The expression stability values for candidate reference genes are higher for samples from developmental stage than tissue for all algorithm analyzed (Table 3) and this result can be associated with higher complexity of the sample [21]. The transcript profiles from adult stage can change during eclosion process from pupa to adult, as predicted for *S. invicta* resulting in an increase of sample complexity [46]. Moreover, the fact that mature leaf-cutter ant colonies have one of the most complex polymorphic worker caste within ants can contribute to this pattern [24, 47].

Analysis using standard deviation (statistical analysis and BestKeeper algorithm) ranked GAPDH as one of the most stable genes for both development stages and different tissues. All other algorithms listed *rpl18/ef1-alpha* (developmental stages) and *rpl18/ef1-beta* (tissues) as the most stable ones. On the other hand, *act* was classified as the worst for almost all approach. *act* has not been ranked for other Hymenoptera [12, 14], but showed a controversy results for insects from Lepdoptera [11]. This result can be validate by the large number of genes involved in actin cytoskeleton organization identified in *A. cephalotes* compared to other hymenopteran genes that are associated to the extensive cytoskeletal changes that occur during caste differentiation in *Atta* adults [24].

In conclusion, we analyzed five candidate reference genes in two different samples from *A. sexdens rubropilosa* with different statistical approaches, a consensus list from stability of genes was obtained and the two top ranked gene were suggested as reference genes for this insect. The AChE expression pattern normalized with different candidate reference genes emphasize the importance of validation to obtain reliable and accurate results from gene expression analysis. Beside this, the expression analysis from AChE suggest that this enzyme is important in developmental stage growing from larva to worker and is spread on insect body. The results presented are essential to gene expression analysis in this leaf cutting ant associated with low genome information and the growing interest in pest management control.

AUTHORS' CONTRIBUTIONS

DHFS and OCB designed the research and provided guidance; RLC and ACM performed the statistical analysis; ACM and AMS performed the RT-qPCR experiments. DHFS wrote the manuscript. All authors read and approved the final manuscript.

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Impact on Income Farmer Debt Bondage System Cengkeh (Case Study in Liwutung Village Pasan District Southeast Minahasa Regency)

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Abstract— *Until recently known as the clove plantation crop that can provide the greatest revenue among plants cultivated population in Southeast Minahasa Regency. Although the level of selling price per kilo of dry high compared to most other plantation crops among the results, but the reality in practice is still a lot of farmers who still fall into the practice of debt bondage system that is very detrimental to farmers. The purpose of this research is: (a). Knowing the dynamics of debt bondage system applicable at the farm level. (2). Assessing the value of the losses suffered by farmers with their debt bondage system. Research was conducted during 3 months from July to September 2015. The location is determined purposive sampling with consideration as production centers. Respondents were selected by 20 farmers. Data collected included primary and secondary data. The data type for the primary data are: recording made by every owner of the garden at the time of measurement of the crop in the form of a notebook crops and types of other expenses such as shopping lists staple for the harvest progresses, wages harvest, pre-harvest such as ladders, rope as media binder stairs and media container yields while secondary data obtained from the department or the relevant authorities are technically fully understand the business development of the crop. The data were analyzed descriptively. The results showed that the source of bonded labor system is productive cycle, farmers' lack of funds to finance farming, relatively high maintenance costs as well as costs of urgency needs of school children especially in the beginning of the new school / college or religious holidays. The amount of bonded labor is determined by the length of time the next harvest or the condition indicator ovary. Great value losses experienced by farmers is Rp. 8,122,056.25 per harvest period.*

Keywords— Boundage, Losses, Clove.

I. INTRODUCTION

Clove is one of the plantations of highly dependable government in increasing the national income is mainly derived from the cigarette tax. The revenue contribution can be seen from the large cigarette excise tax that is produced can reach Rp.23.2 trillion (Anonymous, 2017).

Currently, the needs of cloves in the country reached 110 thousand tons per year. Of these 93 percent of the crop was purchased by the industry as a mixture of clove cigarettes (perkebunannews.com, 2016). Therefore it is not surprising that the clove cigarette factory becomes the single largest consumer of cloves users.

In line with the still fairly high demand for fruit clove affect the development of farming which is characterized by the growing folk back clove plantations. Still quite dominant clove plantation developments such as the impact of higher selling prices.

Based on the monitoring of price developments in some areas the production of cloves in Indonesia generally have levels of selling prices were quite high but varied among others: Aceh between Rp.105,000 - Rp.130,000 per kg, Bali Rp.100,000 per kg (perkebunannews.com, 2017) and East Java Rp. 110,000-130,000 per kg (kominfo.jatimprov.go.id, 2017).

Panca, (2016) says that in line with increased selling prices significantly expand the area eventually sparked many clove plantations whether conducted through its own efforts and the help of outsiders. One program outside help farmers is: Hub project and Clove Intensification System (CIS) launched since 2013 with the aim of supporting increased productivity cloves in Indonesia by providing full support in terms of input supply, agricultural training and infrastructure development. Until now there are 21 locations in clove production centers in Indonesia. (sampoerna.com, 2016).

Puslitbun (2007) says that there are four varieties of cloves namely: siputih, zangsibar, ambon and zanbon (clove composite). Nationally, the area of commodities clove until now has reached about 400 thousand hectares of productive land area of 300 thousand hectares, (Panca. 2016).

Relation to the development of existing farming Mamonto (2012) reported this farm should be developed because they have a value of R/C ratio ranged from 4.90 to 6.94 with the ROI is 390 - 598. Therefore, the clove farming is

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feasible to be developed as it has the value of R/C ratio> 1 and the efficiency of capital usage.

Based on the user benefits from the fruits of cloves, among others, besides a main source to serve as the manufacture of cigarettes is also capable of ridding the body of toxins and harmful microbes, protecting the damage caused by cells in the body, helping to stimulate the production of energy in all parts of the body, heal the sick gear, mosquito repellent, anti-inflammatory, digestive problems, a healthy digestive system, helps men in terms of sexual and prevent colds or influenza can also be used as an anthelmintic and anesthesia (merdeka.com, 2016; de Lin, et. al, 2016), while by-products such as dried clove oil can be used as raw materials of pharmaceutical industry, food flavorings and fragrances (Danarty, 1993).

Table.1: Development of production and the selling price of cloves in the level of farmers in North Sulawesi (2008-2013).

Year	Production (Ton)	Price (Rp)
2008	461.00	55,000
2009	1,663.00	34,000
2010	20,166.00	40,000
2011	338.93	170,000
2012	327.00	120,000
2013		145,000

Source: North Sulawesi Plantation Office (2014)

North Sulawesi (Sulawesi) has been very well known as one of the potential for being a production center because it has been able to provide a substantial contribution to the income of farmers. This is most apparent in Table 1.

From Table 1 shows that production is generated during the time span from 2008 to 2013 is quite varied with the highest production in 2010 reached 20,166 ton in 2009 followed by 1,663 tons while others < 1,000 ton. Similarly to the sales price achieved is the highest reached Rp.170,000 /kg that occur after 2011.

Some of the main challenges is the development of farming sustainability of natural resources, climate change and the decline in factor productivity. Besides the downward trend in the size of land holding is a serious challenge to the profitability of agriculture. (Behera and France, 2016)

Southeast Minahasa (Mitra) is known as one of the districts of considerable potential in North Sulawesi in contributing to the production of next Minahasa and Bolaang Mongondow. This is apparent from the development of farming are still very dominant cloves. BPS, (2015) reported that the total area of the development of cloves in the District Partners covering 5,375.85 ha distributed over 1,240.29 ; 3,892.96 ha immature produce and 242.6 do not produce (damaged).

In line with the development of farming, the bonded labor system is one of the dynamics which influenced the income of farmers clove especially at harvest time. This condition is noteworthy because it is very detrimental phenomenon due to the farmers only receive income in very small amounts as a result suffered a loss that ultimately affect the financing, the development of farming and the cost of daily living. Therefore, it has carried out research aimed at : (1). Knowing the dynamics of debt bondage system applicable at the farm level. (2). Assessing the value of the losses suffered by farmers with their debt bondage system.

II. RESEARCH METHODOLOGY

Research carried out for 3 months ie from July to September 2015. The research location is determined intentionally (purposive sampling), the Liwutung One village Pasan districts which is a regional production center. The number of respondents who selected farmers by 20 farmers who harvest cloves. Data collected included primary and secondary data. The data type for the primary data are: recording made by each owner of the garden at the time of measurement of the crop in the form of a notebook crops and types of other expenses such as shopping lists staple for the harvest progresses, wages harvest, pre-harvest such as ladders, rope as media binder stairs and media container yields while secondary data obtained from the department or the relevant authorities are technically fully understand the business development of the crop. The data were analyzed descriptively.

III. RESULTS AND DISCUSSION

Bonded system applicable to operations at Minahasa clove harvest.

Understanding Bonded by Indonesian dictionary are loans granted to farmers, fishermen or small businesses which payment is made to the yields or production by poor prices.

Bonded usually occurs due to urgency of farmers to meet the needs of everyday life. The types of needs associated with debt bondage system is usually the primary or the absolute, the numbers are quite large and are in cash.

Until now bonded labor is one form of lending and borrowing system that applies in almost all lines of business agriculture. A form of borrowing that do usually in the form of cash that will be returned in the form of goods in this case the yield of agricultural commodities.

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This type of business is long-standing in the midst of everyday life peasants even more detrimental to their own but these losses tend to be regarded as normal due to the urgency of subsistence.

According Sondakh (2014) have bonded labor system in the farming clove is considered normal for many indigenous communities who practiced in many plant crops such as cloves in North Sulawesi in Minahasa, Sangihe Talaud, Ambon (Maluku) and Central Sulawesi. Area production center of this system is a tradition that has been done in the form of agreements that are normally associated with buying and selling crops or selling fruit with cloves. Therefore, the agreement in the bonded labor system is already included in the Minahasa culture oldest such Mapalus (Taulu,1997).

Agreements usually done between farmers and traders when see the plant has shown any indication of vegetative and generative growth that will bear fruit within the next 6 months. Farmers as owners usually look for or offer to ordinary traders who buy and sell the capital. The meeting between traders and farmers is very synonymous with the supply and demand which resulted in an agreement of sale and purchase transaction but still within the bounds of reasonableness, or in other words mutually beneficial. It is important to remember the form of the transaction model is not regulated in the Act.

Characteristic of respondents

Characteristic respondent is required to determine the extent of the resource potential of internal support that farmers had helpful in supporting the implementation and development of the business.

Table 2 shows that the age of the respondent farmers still on productive criteria, despite being on the verge of unproductive (55 years old). Therefore, the picture indicates the farm is plagued with labor because of the potential labor force in the family is constrained age, the job is interrupted or need help labor sourced from outside. Therefore, for the development of farming is to the better, advanced and productive, the farmer is an absolute need support greater costs, especially the money that is used in cash for finance workers coming from outside the family considering the type of farming is classified as the type of farming solid capital.

Formal education achieved average farmer just graduated from high school (9.85%). Educational attainment on clove farmers can be said to be better because generally rural farmers in the average primary school do not graduate school not even at all cause high levels of illiteracy at the level of village farmers. This aspect must be considered absolute, especially in supporting the successful implementation of the farm is mainly related to the speed of innovation adoption because until now there's been a lot of advanced innovations being introduced to farmers through both formal and non-formal media but less adopted or develop properly. One reason is the low level of education of farmers.

To experience known to be one of the critical success factors of farming as it relates to the quality of use of production facilities and measures anticipatory especially in anticipation of the obstacles encountered. From Table 2 it appears that the length of experience in the development of farming clove farmers can be said is long enough. Therefore, the cloves have a quality farm farming better than similar types of farm commodities as farmers in the region have had a long experience in dealing with the constraints of farming cloves. One solutions that do include exchanging experiences overcome pests/diseases like caterpillars that attack the stem cloves, and others.

The land area is medium size farms to be done. The importance of this aspect because it is correlated with the amount of financing means of production such as labor, fertilizer, seeds and pesticides and others. The more land that farmers have greater opportunities to raise revenue rather than just have a small land area, but if one manages this land there will be inefficient utilization of the budget due to incorrect prediction time allocation outpouring of financing.

Table.2: Characteristic farmer respondents

No	Component	Unit	Amount	The Range
1.	Age	Year	53.05	38 – 68
2.	Education	Year	9.85	6 - 18
3.	The Number of dependents	Soul	1.6	0 - 4
4.	Experience	Year	36.35	15 - 51
5.	Large	Ha	1.29	0,8 - 225
6.	The Number of Persil	Place	1.65	1 - 3
7.	The Number of productive plants	Tree	158	85 - 240
8.	Proportion of plants : - Local Variety - Zangsibar	 Percent Percent	 24.36 75.64	

Source: Results Analysis.

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The number of productive clove plant is one indicator of success because it includes an annual plant that empirically dire need of attention, especially treatment that can produce well. The plants will produce well they are maintained too well through optimalization resource use of existing support and vice versa, the plant will not produce well if not adequately supported by the level of maintenance of the plants a good result is not optimal maintenance affecting crops such as damage or death.

From Table 2 appears that the number of plants that are able to produce is an average of 158 trees. Seeing this number, it can be said that farmers have been able to develop this clove farming is well characterized by the high number of plants which production has reached 75.64 percent, while the proportion of plants that did not produce only the remaining 24.36 percent.

System implementation Bonded.

In this clove farming system mechanisms longstanding debt bondage once, especially when started development of business, especially as we enter the productive phase. Opportunities bonded labor system is very open at the farm level clove caused by several factors, among others: (a). Relatively long productive cycle reaches 4-5 years other hand there are periods of low production and farmers need to budget for living expenses, (b). Maintenance costs are relatively high and (c). Absolute urgency financing needs such as school children, the sick, a family event, the cost of farming labor, etc.

This debt bondage occurs when farmers in dire need of funds that are cash. Usually the moneylenders are traders, employees or fellow farmers who have substantial funds. The timing of the transaction is relatively dependent bondage time when the funds it needed people who do bonded. The time usually has the highest proportion of demand when entering the new school year in children enter school or college or face religious holidays such as Christmas eve.

The value of the transaction depends on the length of time of harvest. The longer the time of harvest, the value transactions getting smaller, and vice versa if it is getting close to harvest the bonded transaction value is likely to be even greater. Usually used as indicators of the transaction is raw fruit crops or dried. Returns in dry form intended for the moneylenders are not bothered anymore with additional work or fear of loss due to the harvest season sometimes happens to coincide with the rainy season so that when agreements are transactions conducted in the form of raw fruit (raw material) it is necessary to additional costs resulting in traders' profits become smaller. If the period of harvest time is still quite long (8-10 months), the deal value of transactions typically between 10 percent to a maximum of 20 percent, and vice versa when the harvest season the rest of the 1-2 months, the amount of the transaction value of bonded labor will be greater and its value reaches a maximum of 80 percent of the real selling prices in the market.

Returns bounded usually held to coincide with the completion of the harvest season. This activity is usually done by the people who do bonded (farmers) while the moneylenders usually tends to be a wait. If within a period of 1-2 months after completion of harvest has been no indication of the recipient bondage want to bring back some fruit clove agreed upon commitments, then the giver will charge directly bonded. What is interesting in this return is when the people who do bonded returns on time and in numbers that commitment then the people who do bonded will be awarded a gift/rewards as motivators to further strengthen the relationship like money or goods. Returns this model usually occurs bonded labor system that has little value (between 10 to 50 kilograms of dried cloves) whereas> 50 kg is usually done in stages.

Farmer benefits to people who do bonded

The benefits of this system of bonded labor as contained in Table 3 below are quite varied between 5-30 percent with a high of 30 percent, which is used to finance school need existing family members, while the other needs such as finance day-farming and living costs today only 15 percent. From this utilization shows that the needs related to non technical farming such as the financing of school children do not correlate directly with farming can become a major factor causing the system process doing bonded in large scale not only in the region Partners but also a long-standing, especially in the areas of production centers clove like districts Minahasa, South Minahasa as the center and the largest producer in North Sulawesi. The overall picture shows that the fulfillment of the daily needs of the family members and cash is a major factor in the creation of a system of bonded labor is not related to the success of farming and management itself.

Some off-farm factors that helped influence the occurrence of bonded labor system are: finance the needs of everyday life, ceremonial occasions family members/the marriage and to fund business interests together three each by 10 percent.

Table.3: Benefits of bonded labor system on farmers

No	Perception	Amount (%)
1.	Farmyng cost	15
2.	Cost of daily living	15
3.	Buy cattle	5
4.	Bussines	10
5.	School children	30
6.	Events marriage family	10
7.	Buy farm equipment	5

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8.	Transportation/mean of transport	10

Source: Results Analysis.

Returns bonded.

Bonded known as a business that is very detrimental to farmers. Said to be very harmful because usually giver bonded only appreciate the value of production is very low. As was the case in other commodities, in determining the amount of the transaction value of debt bondage is usually correlated with the length of fruit trees where the longer the plants bear fruit, the value of debt bondage will be smaller, whereas the closer the season reaping the value of the transaction will be of great value as appreciated bigger but remain the same as the market price of cloves fruit when the transaction took place.

Some things that are usually used as indicators of assessing the feasibility of giving bonded to the receiver bounded clove fruit are:

- Planting conditions.

Bonded labor will be provided by the moneylenders when planting conditions have been shown to have been producing well. It is seen in addition apparent from household conditions routinely harvesting every year, as well as from information sourced from fellow farmers. Volum corelated with the amount of debt bondage with planting conditions. The better planting the cloves then bonded amount to be awarded will be greater but the conversion value per kg, the same as other farmers.

- Level of confidence

- Although farmers have a pretty good crop, but his good name has been tarnished as a result often do not pay off for cheating/outsmart merchants then bonded labor will not be given, but on the contrary if they always keep its promise on time then bonded labor will always be given.

Furthermore, for those who do not return the fully bonded several conditions commonly used as reason for the delay in paying off bondage as shown in Table 4. The Major reason major non-fulfillment of repayment obligations of bonded labor is dominated by reason of installments of 30%, followed by the reason plants are not producing in line with expectations (less) and will pay off when the production lot with each of 25%. While others tend to be no more than 10%.

Table.4: The reason farmers do not return the appropriate commitment bonded

No	Perception	Amount (%)
1.	The production not suitable/less	25
2.	Burnt/damaged	5
3.	Traders profit	10
4.	Payed in installments	30
5.	Production of new lots of pay	25
6.	off	5
	There is a family connection	

Source: Results Analysis.

The impact of debt bondage system on farmers' income

Indicators of the impact of this system is a strategic information for each farmer as it relates to the value of the loss caused by the small value of return.

As seen in Table 5 shows that the volume is quite large because of generally amounted to 134.75 kg or greater than 100 kg were taken between 2012 to 2013 ago. Newly restored volume reached 73.75 kg or with new proportions reached 54.99% with a grace period of a maximum of 5 years has fully repaid the predictions and expectations of farmers. The magnitude of the price of what happens when a return of Rp.96,025/kg dry. This figure is relatively far in value compared to the value of the deal happens when bonded only priced at Rp.35.750/kg dry. Overall it can be said that the value of the transaction when bonded done only Rp.4,817,312.5 whereas an obligation that must be returned to Rp.12,939,368 or in other words, farmers suffered losses reached Rp.8,122,056.25 (as well as traders' profits) are distributed each are: already paid Rp. 4,466,318.73 while unpaid (up to 5 years ahead) of Rp.3,655,737.52.

Table.5: Benefits of bonded labor system on farmers

No	Component	Unit	Amount (%)
1.	Amont bondage	Kg	134.75
2.	Year bondage	year	2,010.95
3.	Long-duration debt bondage	year	5.05
4.	Year refund		
	- Amount	Kg	73.75
	- Percentage	percent	54.99
5.	Download current prices ijon	Rp	35,750
6.	Submit current prices Boundage result		
	Which must returned	Rp	96,025
7.	Total debt bondagewere taken farmers	Rp	12,939.368

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8	Farmers losses people who do bonded		
	- Which has been repid	Rp	4,817,312.5
9	- Unpaid		
		Rp	8,122,056.25
		Rp	4,466,318.73
		Rp	3,655,737.52

Source: Results Analysis

Note: * = Losses farmers = trader profits

IV. CONCLUSION

1. The cause of bonded labor system at the farm level is the productive cycle, farmers' lack of funds to finance farming, relatively high maintenance costs as well as costs of urgency needs of school children especially in the beginning of the new school/college or religious holidays. The amount of bonded labor is determined by the length of time the next harvest or with indicators of the condition of the ovaries.
2. Great value losses experienced by farmers is Rp.8,122,056.25 per harvest period.

V. SUGGESTION

Attention is needed in the form of the policy of the local government in helping to providing capital for farmers who really needed to finance the operational cost of harvesting is best done alone by the Government through the relevant agencies and institutions/offices/entities competent as cooperatives and farmer groups that return is performed after the harvest takes place with no interest or interest, but has a repayment scheme with the financing are clear, but not burden the clove farmers.

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An Experimental Investigation on Treatment of Tannery Effluent Using Azadirachta Indica

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Abstract**—** *A preliminary investigation was carried out for the feasibile use of Azadirachta indica leafs powder as a natural coagulant to the treatment of tannery effluent. In this paper, Azadirachta indica leafs powder of 1, 2, 3 and 4mg/L dosages were used. Floc formation in coagulation process had been studied in the laboratory scale to determine the optimum dosage of natural coagulant. The above dosages were used in pre-treated tannery effluent with coagulants were considered to evaluate the percentage removal efficiency on the major pollutants of concern in in tannery effluent such as turbidity, TSS, TDS, COD and BOD. From the observed results, dosage of 3 mg/L gives better removal efficiencies with respect to turbidity, TSS, TDS, COD and BOD and appears to be suitable for tannery effluent treatment, when compared with other dosages.*

Keywords**— *Azadirachta indica(Neem leaf), Coagulation, Chemical oxygen demand (COD), Tannery, Turbidity.***

I. INTRODUCTION

Indian leather industry has established to a large range and is the second larger producer following to China. The industry is equipped mostly with a potential for employment generation, growth and exports, with the annual export touching 2 billion USD. Presently it is on an ever increasing phase with optimum utilization of available raw materials and returns from exports.

Ever increasing industrialization and rapid urbanization have considerable increased the rate of water pollution. The dwindling supplies of natural resources of water have made thus a serious constraint for industrial growth and for reasonable standard urban living. Tanning industry is one of industries, which considered as highly polluting industry [1]. Tannery effluents contain lot of hazardous elements which can affect human immunity when it is directly discharged in water bodies [2]. Tannery generate effluent in the range of 30 – 35 L/kg skin or hide processed with variable pH and high concentration of suspended solids, BOD, COD [1].

In effluent treatment, coagulation has been practiced since earliest time and the main objective is to remove colloidal impurities hence also removing turbidity from water. Coagulant is a chemical used that is added to the water to withdraw the forces that stabilizes the colloidal particles and causing the particles to suspend in water. Once the coagulant is introduced into the water, the individual colloids must aggregate and grow bigger so that the impurities can be settled down at the bottom of the beaker and separate from the water suspension. Aluminium and iron coagulants are commonly used in most industries. However, when aluminium is used as a coagulant in waste water treatment, it can caused several bad effect on human health such as intestinal constipation, loss of memory, convulsions, abdominal colic's, loss of energy and learning difficulties [3].

In recent years there has been considerable interest in the development and usage of natural coagulants which can be produced or extracted from microorganisms, animal or plant tissues. The coagulants should be biodegradable and less voluminous sludge that amounts only 20 – 30% that of alum treated counterpart [4]. Therefore this study is carried out to analyze the effect of Azadirachta indica leafs powder as a primary coagulant in clarifying tannery effluent in coagulation process at its optimum dosage. The optimum dosage and its removal efficiencies of Azadirachta indica leafs powder on pH, turbidity, TSS, TDS, COD and BOD were determined.

II. MATERIAL AND METHODOLOGY

2.1 Collection of Azadirachta indica leafs

In this study Azadirachta indica leafs(Neem leaf) was used as natural coagulant. It was collected from road side of Tiruchengode city. Figure 1 shows the pictorial view of Azadirachta indica.

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Fig.1: Azadirachta indica

2.2 Preparation of Natural Coagulant

Azadirachta indica leafs were picked from the branches of the tree and dried in oven at 60º for 24 hours. Then dried leafs were ground to fine powder and sieved to get particles of size 600 µm. Figure 2 shows the pictorial view of Azadirachta indica leafs powder.

Fig.2: Azadirachta indica leafs powder

2.3 Collection of raw water

The raw effluent was collected from the tannery industry at Brahmana Periya Agraharam in Erode District. Sample was taken from the equalization tank and their initial parameter shown in table 1. Figure 3 shows the pictorial view of equalization tank.

Fig.3: Equalization Tank

Table.1: Initial tannery effluent characteristics

Sl. NO	PARAMETERS	RAW EFFLUENT	BIS LIMITS IS 2490-2009
1	pH	10.92	5.5 – 9.0
2	Turbidity(NTU)	1283	-
3	TDS(mg/L)	13300	2100
4	TSS(mg/L)	520	100
5	COD(mg/L)	960	250
6	BOD(mg/L)	768	30

2.4 Coagulation Studies

Jar test is the most wildly used experimental methods for coagulation-flocculation. A conventional jar test apparatus was used in the experiment to coagulate sample of tannery effluent using natural coagulant.

Fig.4: Jar test apparatus

This apparatus consists of four beakers to be agitated simultaneously. 500ml of tannery effluent sample is taken into one-liter beakers and placed under the jar test apparatus. Previously prepared powder of Azadirachta indica leafs powder is taken into various dosages i.e., 1, 2, 3 and 4 mg/L was added simultaneously and stirred for 10min at 180 rpm, followed by 10 min slow stirring for flocculation [5]. Then the solution is allowed to settle for twenty four hours and measure the turbidity, pH, COD, BOD, TSS and TDS.

2.5 Optimization of Coagulant Dosage by turbidity and pH

This is found by using jar test apparatus. The turbidity and pH was measured after 24 hours, the turbidity and the pH for dosage of 1, 2, 3, 4 mg/L was found to be 510, 348, 184, 440 NTU and 7.84, 7.98, 7.79, 8.07. Optimum dosage from above is found as 3mg/L.

III. RESULTS AND DISCUSSIONS

3.1 Effect of Azadirachta indica leafs powder on the removal of turbidity

It was found by using CL 52D Nephelometer. For the various dosage of natural coagulant with sample and their

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percentage of removal is shown in table 2 and chart 1. Initial turbidity is noted as 1283NTU before coagulation. At various dosages like 1, 2, 3 and 4mg/L the percentage of removal of turbidity was 60.26, 72.88, 85.66 and 65.71 % respectively. The turbidity removal percentage was higher in dosage of 3mg/L.

Table.2: Turbidity of sample

Sl. No	Volume of sample (ml)	Dosage (mg/L)	Turbidity reading (NTU)		Turbidity removal %
			Initial	Final	
1	1000	1	1283	510	60.25
2	1000	2	1283	348	72.88
3	1000	3	1283	184	85.66
4	1000	4	1283	440	65.71

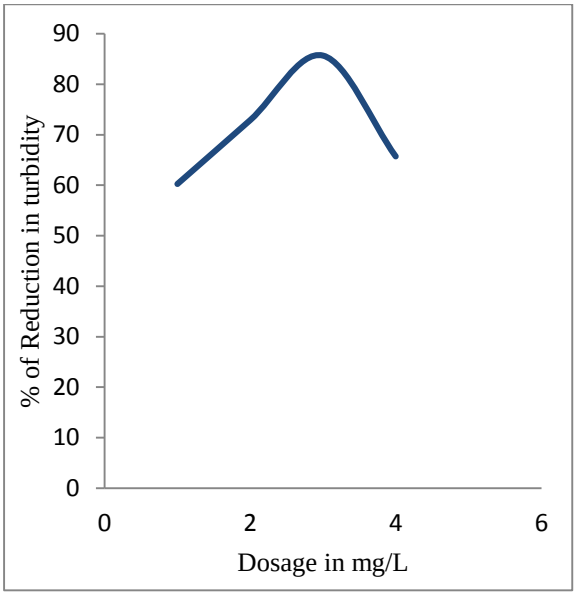

Chart.1: Azadirachta indica dosage vs. Removal of turbidity

3.2 Effect of Azadirachta indica in pH of sample

pH of sample was found by using LI120 pH meter, for various dosage of Azadirachta indica with sample is shown in table 3 and chart 2. At various dosages like 1, 2, 3 and 4mg/L the pH of the sample was 7.84, 7.98, 7.79 and 8.07 respectively. The maximum reduction in pH was found at dosage of 3mg/L.

Table.3: pH of sample

Sl. No	Volume of sample(ml)	Dosage (mg/L)	pH	
			Initial	Final
1	1000	1	10.92	7.84
2	1000	2	10.92	7.98
3	1000	3	10.92	7.79
4	1000	4	10.92	8.07

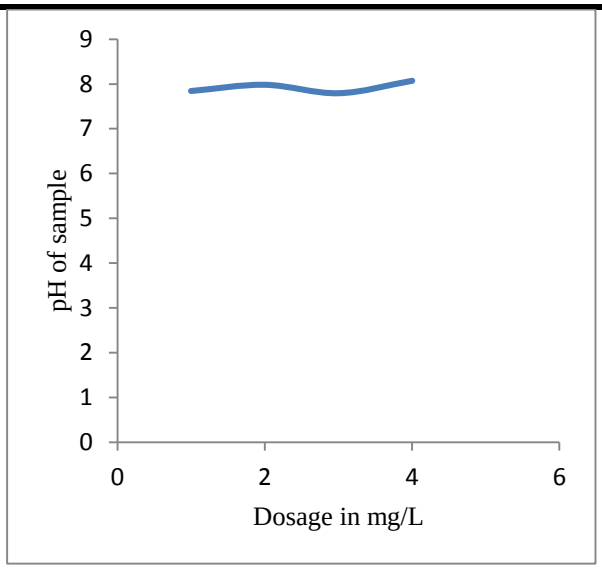

Chart.2: Azadirachta indica dosage vs. pH

3.3 Effect of Azadirachta indica leafs powder on removal of COD of sample

For the various dosages of Azadirachta indica leafs powder with sample and their effect on removal of COD is shown in table 4 and chart 3. At dosages like 1, 2, 3 and 4mg/ L the percentage of removal of COD of the sample is 72.81, 74.79, 80.42 and 75.83%. The maximum removal percentage of COD was found in 3mg/L.

Table.4: COD of sample

Sl. No	Volume of sample (ml)	Dosage (mg/L)	COD (mg/L)		COD removal %
			Initial	Final	
1	1000	1	960	260	72.81
2	1000	2	960	242	74.79
3	1000	3	960	188	80.42
4	1000	4	960	232	75.83

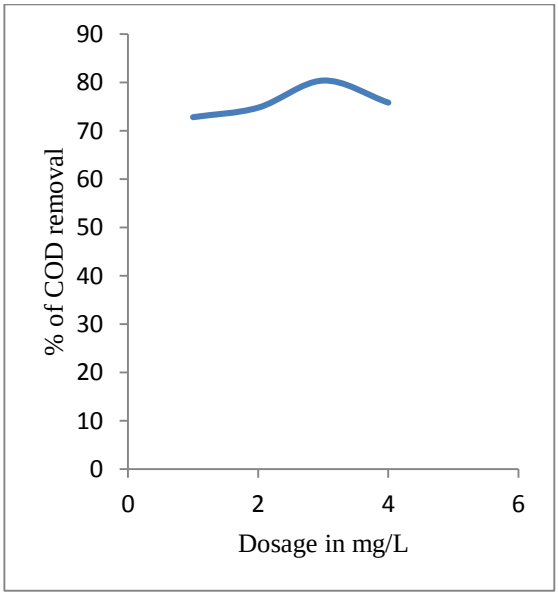

Chart.3: Azadirachta indica dosage vs. % of COD removal

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3.4 Effect of Azadirachta indica leafs powder on removal of BOD of sample

For the various dosages of Azadirachta indica leafs powder with sample and their effect on removal of BOD is shown in table 5 and chart 4. At various dosages like 1, 2, 3 and 4mg/ L the percentage of removal of BOD of the sample is 79.42, 87.23, 96.74 and 89.71%. The maximum removal percentage of BOD was found in 3mg/L.

Table.5: BOD of sample

Sl. No	Volume of sample (ml)	Dosage (mg/L)	BOD (mg/L)		BOD removal %
			Initial	Final	
1	1000	1	768	158	79.42
2	1000	2	768	98	87.23
3	1000	3	768	25	96.74
4	1000	4	768	79	89.71

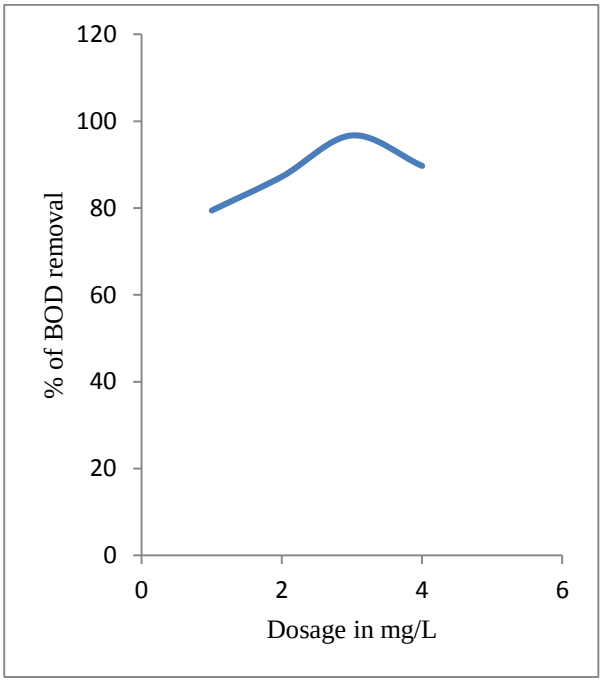

Chart.4: Azadirachta indica dosage vs. % of removal of BOD

3.5 Effect of Azadirachta indica leafs powder on removal of TSS of sample

For the various dosages of Azadirachta indica with sample and their effect on removal of TSS is shown in table 6 and chart 5. At dosages like 1, 2, 3 and 4mg/ L the percentage of removal of TSS of the sample is 76.35, 81.15, 84.81 and 71.73%. The maximum removal percentage of TSS was found in 3mg/L.

Table.6: TSS of sample

Sl. No	Volume of sample (ml)	Dosage (mg/L)	TSS (mg/L)		TSS removal %
			Initial	Final	
1	1000	1	520	123	76.35
2	1000	2	520	98	81.15
3	1000	3	520	79	84.81
4	1000	4	520	147	71.73

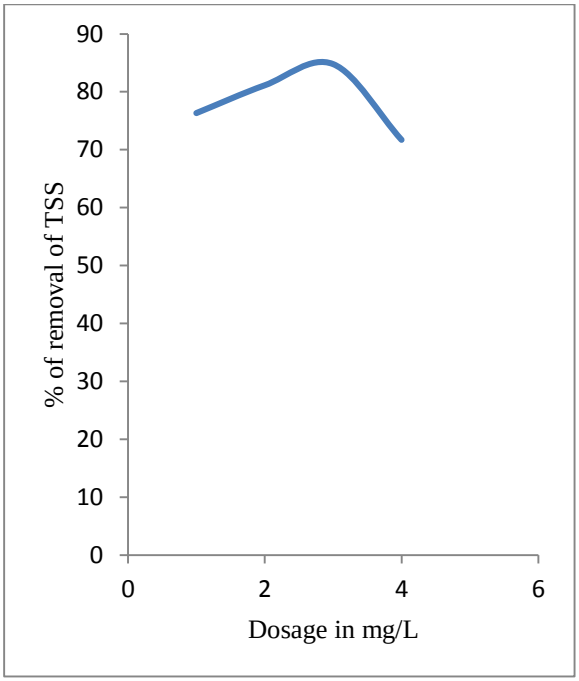

Chart.5: Azadirachta indica dosage vs. % of removal of TSS

3.6 Effect of Azadirachta indica leafs powder on removal of TDS of sample

For various dosage of Azadirachta indica with sample and their effect on removal of TDS is shown in table 7 and chart 6. At dosages like 1, 2, 3 and 4mg/ L the percentage of removal of TDS of the sample is 75.64, 77.52, 87.06 and 82.11%. The maximum removal percentage of TDS was found in 3mg/L.

Table.7: TDS of sample

Sl. No	Volume of sample (ml)	Dosage (mg/L)	TDS (mg/L)		TDS removal %
			Initial	Final	
1	1000	1	13300	3240	75.64
2	1000	2	13300	2990	77.52
3	1000	3	13300	1720	87.06
4	1000	4	13300	2380	82.11

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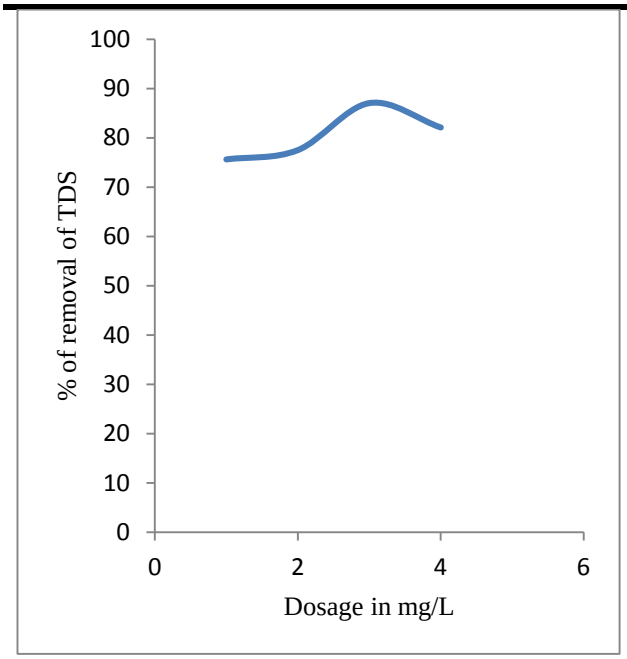

Chart.6: Azadirachta indica dosage vs. % of removal of TDS

IV. CONCLUSION

The tannery effluent collected from Erode district was examined for various parameter like Turbidity, pH, TDS, TSS, COD and BOD were not in permissible limit and in need of elimination. The feasibility in the treatment of tannery effluent using natural coagulant Azadirachta indica leafs powder had been taken for investigation. Optimum dosage for maximum removal (%) in turbidity, COD and BOD using the dosage of Azadirachta indica leafs powder was 3mg/L. When Azadirachta indica leafs powder was used as coagulant and added the dosage of 3mg/L found that percentage of removal of turbidity, COD, BOD, TSS and TDS were 85.66%, 80.42%, 96.74%, 84.81% and 87.06%. As compared to the other dosages it has more potential for the removal of tannery effluent.

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Bacteriological Assessment of Meat Pie Sold at Ochanja Market Onitsha, Anambra State

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Abstract— *Ten meat pie samples were purchased from different eatery points in Ochanja Main Market, Onitsha and analyzed for the presence of pathogenic bacteria using standard microbiology and biochemical techniques. The following bacteria genera were isolated and identified from the meat pie; Escherichia coli (39%), Staphylococcus aureus (35%), and Bacillus cereus (26%). The percentage distribution showed that Escherichia coli were the most prevalent in the meat pie samples while Bacillus cereus was the least. The meat pie samples sold within Ochanja Main Market were considered fit for human consumption since the distributions of the bacteria isolates were below standard threshold limit.*

Keywords— Meat Pie, Bacteria and Food borne diseases.

I. INTRODUCTION

There has been a notable and remarkable increase in the consumption of convenience and ready to eat foods by the people in recent times. Ready to eat foods can be described as foods that were meant for immediate consumption at the point of sale. It could be raw or cooked, hot or chilled and can be consumed without further heat treatment [Tsang, 2002]. Different terms have been used to describe such ready to eat foods include, convenient, ready, instant or fast foods. Examples of such ready to eat foods include pastries, meat pie, sausage rolls, burger, moi-moi, salad, fried meat, fried chicken, milk and milk products [Alexander and Tittiger, 1971]. Meat and meat products have been a constant food for man as far back as there has been any evidence of civilization on the face of earth.

Meat pie is a food product that comprises of savory pie with a filling of meat and other savory ingredients [Clarkson, 2009]. It is made up of meat enclosed in a biscuit dough or pastry. Meat pie contains at least 25% meat, protein boosters such as soya protein thus giving it a high protein content. A locally made meat pie usually comprises of meat, salt, water, nutmeg, egg thyme, olive oil, onions, garlic and some other savory ingredients [Adesiyun, 1995; Oluwafemi and Simifaye, 2005]. Meat pie contributes about 20-40% of daily recommended intakes of sodium for adults. It is a good source of carbohydrates. The flour content of meat pie provides high percentage of carbohydrates to the consumers [Bickert, 2010].

The susceptibility of meat pie to spoilage by micro-organisms gives it a shelf-life of 72 – 96 hours (3-4days) [Adesiyun, 1995].

Micro-organisms play an important role in the quality of meat products before, during and after processing by limiting many undesirable biological changes in it [Ukut *et al.*, 2010]. Meats and meat products undergo spoilage as a result of microbial action on the fats and proteins [Adesiyun, 1995]. Food contamination is the introduction or occurrence of a contaminant (any biological or chemical agent, foreign matter or other substance not internationally added to food which may compromise food safety or suitability) in food or food environment [Omoloya and Adeleke, 2013]. Food is prone to contamination at every stage in the food chain. The consumption of food contaminated by micro-organism will result in food borne illnesses. These are usually either infectious or toxic in nature, caused by agents entry into the body through ingestion of food [WHO, 2002]. A food borne infection involves the ingestion of pathogen, followed by growth in the host, including invasion and/or release of toxin [Brener, 2005]. Food supply issues of processed meat are usually as a result of contamination (bacterial) introduced exogenously during activities such as harvesting, processing and preparation [WHO, 2002]. Food borne illnesses have continued to form a significant part of morbidity and mortality of Nigerians and have been in the increase in recent times. The international impact of food borne illnesses is difficult to estimate. Bacteria are the causative agents of food borne illnesses in 60% of cases requiring hospitalization [Mead *et al.*, 1999]. Enterotoxigenic

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Staphylococcus, *Escherichia coli*, *Clostridium perfrigens*, *toxoplasma gontu* and *salmonella pp* have been isolated from foods implicated in illnesses [Bello *et al*., 2013; Cencil *et al*., 2003]. Research has shown that food and water is the vehicle for many illnesses [WHO, 2002].

Street foods are frequently associated with diarrhea diseases, which occur due to improper use of additives, the presence of pathogenic bacteria, environmental contaminants, disregard of good manufacturing practices and food hygiene. [WHO, 2002] reported that vendors are often poorly educated, unlicensed, untrained in food hygiene and they work under crude unsanitary conditions with little or no knowledge about the causes of food borne diseases .Data on issues of food borne are well documented worldwide[Thomas *et al*.,2006].Food borne illnesses is a major international health problem with consequent economic reduction. In Nigeria, a number of foods have been reported to have high incidence of bacteria [Adestan, *et al*., 2013]. Constant bacteriological surveillance is required to ensure wholeness and quality of ready to eat foods consumed by the people.

The need by the vendors to focus more on food hygiene as well as regulatory agencies to ensure compliance with approved standards underscored the reason behind this experiment.

II. MATERIALS AND METHODS

Sample Collection

Ten meat pie samples were obtained from different fast food retail and vending centers in Ochanja market, Onitsha. The samples were immediately wrapped in sterile aluminium foil to prevent contamination and then transported to microbiology section in spring board laboratory, Awka for microbial analysis.

Preparation and Inoculation of Samples

10g of each food sample was weighed out and homogenized into 10ml of sterile distilled deionized water using a sterile warming blender. Tenfold dilutions of the homogenates were prepared and up to 10^{-7} dilution factors of the homogenate were plated in triplicates on the Mueller Hinton agar, Mac-Conkey agar and Manniton salt agar using the spread plate techniques. The plates were then incubated at 37^{o}C for 24 – 48hrs. Mac-Conkey agar was used for coliform enumeration while mannitol salt agar was used for isolation of *S. areus*. Total viable bacteria count was performed in Mueller Hinton agar. At the end of the incubation periods, colonies were counted using illuminated colony counter. The count for each plate were expressed as colony forming unit per gram of the sample (cfu/g).

Identification of Isolates

Colonies identifiable on the Hinton agar were carefully examined macroscopically for control characteristics such as color, size e.t.c. Gram staining as well as appropriate biochemical tests according to (Mead *et al*., 1999) were carried out.

III. RESULTS AND DISCUSSION

Table.1: Plate count of viable bacterial isolates from the meat pie samples sold at Ochanja market, Onitsha.

Sample	Bacteria counter per gram
1	86
2	65
3	81
4	67
5	80
6	57
7	76
8	58
9	65
10	68
Mean	**69.5**

The mean bacteria isolates from the samples was 69.5cfu/g. The mean viable bacteria isolates was found to be within 10^{2}cfu/g threshold limits of foods fit for human consumption.

Table.2: Plate count for different colonial forms from the meat pie samples

Sample	Designated of colonies (cfu/g)		
+	A	B	C
1	34	35	17
2	21	23	21
3	26	32	23
4	25	26	16
5	30	30	21

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6	13	19	19
7	27	29	20
8	17	23	16
9	24	26	16
10	27	25	16
Mean	**24.4**	**26.8**	**18.4**

Where A = White coloured colonies
B = Red coloured colonies
C = Pink coloured colonies

The mean bacteria isolates were macroscopically counted for cultural characteristics in colour. The colour count of bacteria isolates decreased as follows:
B > A > C.

Table.3: Cultural characteristics and gram reaction for bacterial identification

Sample	Colonial characteristics	Gram reaction	Probable identification
A	Opaque, smooth, white coloured, colonies measuring 0.1 – 0.3mm on Mac Conkey Agar.	Gram positive cocci, occurring in sample.	*Staphylococcus aureus*
B	Smooth red coloured colonies on Mac Conkey Agar measuring 0.2, 0.5mm.	Gram negative straight rods occur in sample.	*Escherichia Spp.*
C	Pink coloured colonies, irregular and flat, measuring 1 – 2mm on the Mac Conkey Agar.	In chains	*Bacillus cereus*

Table.4: Biochemical tests for identification of bacterial isolates

Identified bacteria	Biochemical Tests									
	Catalase	Coagulase	Citrate	Oxidase	Urease	Indole	Glucose	Lactose	Sucrose	Maltose
Staphylococcus aureus	+	+	-	-	+	-	+	+	+	+
Escherichia Spp.	+	-	+	-	-	+	+	+	+	+
Bacillus cereus	+	N/A	+	N/A	N/A	-	N/A	-	N/A	N/A

Table.5: Percentage distribution of the bacteria isolate

Identified bacteria	Mean conc. per gram	Percentage distribution
Staphylococcus aureus	9	35
Escherichia Spp.	9.1	39
Bacillus cereus	6	26
TOTAL	**23**	**100**

Table 5 showed the percentage distribution of the identified bacterial isolates in the studied sample. *Escherichia coli* was the most prevalent bacteria isolated from the meat pie samples with highest percentage of 39% while the least isolated bacteria was *Bacillus cereus* with percentage distribution of 26%. The result of this finding is in accordance with the reports of [Adesiyun, 1995; Bickert 2010] were they isolated similar organisms from sausages and sea food processors respectively. The presence of these organisms in meat pies depicts a deplorable state of poor hygienic and sanitary practices employed in the processing and packaging of these food products. From the results obtained, the meat pie samples were contaminated with *Escherichia coli, Staphylococcus aureus*and *Bacilus cereus* however the bacterial distribution were within the permissible threshold limits of foods fit for human

consumption. *Escherichia coli* and *Staphylococcus aureus* are named flora in human and animals. Their presence in food products is an indication of excessive human handling [WHO, 2002]. The result obtained in this research agrees with [Adesiyun, 1995; Okonko *et al*., 2009] that foods of animal origin either cooked or uncooked were predominantly contaminated with *Escherichia coli* and *Staphylococcus aureus*. They further stated that the presence of *Escherichia coli* in food products is an indication of fecal contamination of the water sources that were utilized during the processing of the food products. The presence of *Bacillus cereus* in the meat pie samples could be due to improper handling of raw materials from harvesting to processing points [WHO, 2002]. All the three isolated bacteria in the meat pie samples have been incriminated to contribute to life threatening food borne illnesses.

IV. CONCLUSION

The study shows that the meat pie samples sold within Ochanja market,Onitsha,were fit for human consumption since the bacterial load of the three isolates were below the permissible level in sausage foods (10^2cfu/g). This could be attributed to adherence to proper personal and environmental hygiene, use of portable water, proper cooking of the products and use of efficient storage techniques. Since the three bacteria isolates have seen implicated in many life threatening food borne illness, it is good news that their distribution in the meat pie consumed by the people were below recommended threshold limits.

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Hydrological Risk Assessment at Praia, Cape Verde

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Abstract*— *Hydrology modeling became a relevant topic for the Cidade da Praia, Cabo Verde, Africa, due to negative impact risk to local population and its assets. The modeling via Geographical Information Systems (GIS) can help the decision-making process of space occupation and characterization for this type of risk. Under the municipalities of Praia, the phenomenon of flash flood is common, causing soil erosion and landslide. This constitutes a risk for the local habitat, particularly in districts with a lack of strong human infrastructures. To simulate, analyze and generate risk maps using GIS to help this county governance authorities for decision-making, thus, becomes the main aim of this article.

Keywords*— *Hydrology, population, Cape Verde.

I. INTRODUCTION

The county of Praia in Cape Verde features a large morphological diversity and some adverse weather conditions [1]. Environmental vulnerability and hydrological risk have always existed in this county due to its own territorial morphology derived from the local climate and aggravated by human intervention upon the physical environment. On the other hand, the economic and social changes on the Praia county over several decades and the accelerated pace of the economic growth in recent years have led to strong pressures on the environment, leaving behind a negative imprint on the territories, which sometimes requires huge financial costs to reverse the situation [2].

GIS have the advantage of allowing simulations and develop spatial support systems in the sense of finding a balance in the development of human activities. For example, rainfall takes place with increased frequency in Cape Verde in an intensive and irregular manner. Between the months of September and October, there are records of, at least, one high intensity rain event, causing extensive damage and endangering the lives and safety of the population.

This writing goals the characterization of hydrological risk (flash flood) that the municipality of Praia (133,000 inhabitants) is subject to, especially in urban areas, a phenomenon that frequently occurs in regions featuring this dry tropical climate [3]. With this purpose in mind, we intend to produce a set of information that allows to (A) Model the physical environment of Praia county and determine the associated hydrological risks; (B) Provide the mapping of hydrological risk areas based on a surface runoff model; (C) Analyze and quantify the various factors of soil occupation risks associated with climatic, environmental and social aspects; (D) Highlight the importance of GIS in the planning urban areas at Praia.

This research paper is divided into eightmain sections. Starting with this introduction and followed by an overview of hydrological risk, the third section recalls the fundamental concepts of digital terrain models (DTM) and the theoretical references to the current drainage models (D8, NGFLOW and SCS). Section four addresses a review of the literature towards the importance of remoting sensing in risk assessment, particularly on precipitation topics. The following two sections include an overview of the county of Praia.Section 7 reveals the hydrological methodology used for the realization of rainfall simulation, analysis and generation of risk maps. The last chapter expresses some recommendations to the local government of Cape Verde.

II. HYDROLOGIC RISK

Territory disordering reflects on the potential danger of flooding, caused by the surface runoff increased and on the dry cargo effect (destruction of vegetation, increased erosion of slopes, soil sealing). However, for a correct assessment of the hydrological risk, it is essential to know the existing rainfall regime in the area. It is known that precipitation is a natural event, which has great variability in terms of its distribution in the planet's surface. This is a phenomenon that feeds the hydrological cycle and is the major factor for surface runoff, infiltration, evaporation, aquifer recharge and alike. Thus, precipitation is an essential element in studies of infrastructures planning and projection. Certainly, measuring rainfall data is crucial for identifying fields with conditions for agriculture, design of water resources, environmental assessment and quantification of soil erosion [4].

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Within arid regions, the phenomenon of flash flooding can be particularly dangerous for several reasons. Although they are rare, these storms can discharge a large amount of water in a short time. Secondly, these rains usually fall in low permeability soils such as clay by greatly increasing the amount of runoff, overloading the rivers and drainage channels. Sometimes these regions do not have adequate infrastructural conditions to divert water such as manholes, underground holding tanks and retention basins, either due to lack of population, poverty or because the residents still ignore the real risk of flooding. Moreover, the lack of regular rain water to clean water channels can cause flooding because of a large amount of accumulated debris throughout the dry period which are then carried by floods.

Generally, the hydrological risk approach can be broken down into four stages, according to [5]: (A) Analysis of the region morphology by considering their historical values; (B) Survey on the changes and introduced by man (it can serve to counteract the effects or minimize the likely impacts); (C) Survey of existing vulnerabilities due to the presence of man (urban areas) or due to the economic lacking infrastructures; (D) Presentation of the risks/dangers chart and their respective spatial weighting. Note that this last step requires a thorough analysis, taking into account the evaluation of the intensity of risks, vulnerabilities and environmental conditions.

III. DIGITAL MODEL OF LIFTING (DML) AND DRAINAGE NETWORK (D8, NGFLOW AND SCS)

Digital Elevation Model (DEM) is a major key element in any hydrological phenomena study. Undoubtedly, the topography of the terrain influences the hydrological flow. However, the advent of GIS based on DEM has facilitated the hydrological modeling at different scales of watershed areas [6]. Figure 1 depicts the DEM in the southern region of the island of Santiago (municipality of Praia), where water spatial distribution in the basin requires the use of spatial data, particular at the borders of the river basins and sub-basins, slopes and drainage channels. Certainly, all these topographic attributes are determined by the DEM.

With regard to the dynamics of hydrological processes and movement of soil, slope is another significant factor. The definition of a slope, [4] considers the formula $\varphi = \Delta Z \frac{1}{100x}$*, where φ stands for slope, ΔZ for the altitude variation and x equals the distance between the center of any GIS cells. Thus, it is possible to determine, at the pixel level, the current direction of the water that will take, allowing the generation of an image with flow directions. These channels are identified as cell lines, whose flow accumulation exceeds a specific number of cells. By computing the number of cells above a particular threshold setup for the drainage network, one can determine the accumulated flow in that cell [7]. The algorithm that describes the flow direction became known as the Deterministic Algorithm 8 or D8 [8]. This methodology is based on the fact that water is able to move in eight possible directions, as shownin Figure 2.*

Fig.1: DEM of Praia County.

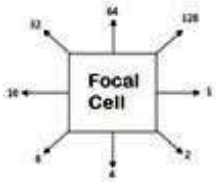

Fig.2: Directions codification of the flow at the cell level.

During the creation of the drainage system with GIS, the first step consists in filling small depressions existence on the model (fill sinks) that arise for geographical data entry errors. The correction of these depressions is an important step for the generation of the river system within the DEM. Thereafter, one delimits the contribution basins that are identified as a set of all cells that flow to a certain target cell from which drain lines are defined (Figure 3).

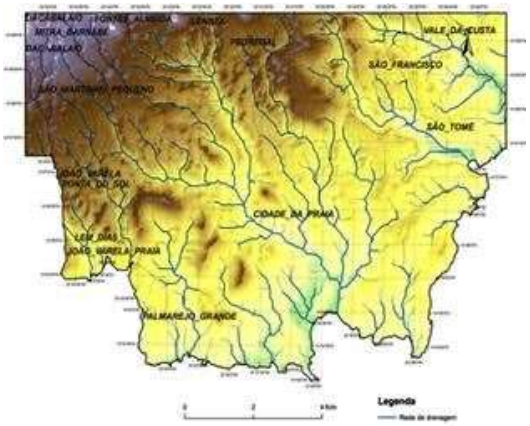

Fig.3: Draining network generated from a DTM.

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In turn, the NGFlow model is based on the DEM as a spatial representation of weather stations, providing the precipitation data records and rainfall time series [9]. In situations where dams can be found, an increase of timely information is needed about the volume, length and release of water. To the input data, one adds the estimated water balance based on the precipitation data, soil storage capacity and potential evaporation. Therefore, the excess flow is formed by water that does not evaporate or infiltrates the soil. In order to calculate the excess runoff flow, we took into consideration equation 1. The left side of the formula describes the water absorption by the soil in a given time interval. Note that the absorption at t time corresponds to the absorption occurred in the previous time, plus the amount of precipitation minus evaporation. The right side of the equation describes the flow. While the soil capacity to absorb water is not met (w(t)≤w'), the flow rate is zero. At the moment that the soil infiltration capacity is reached (w(t)>w'), it is then possible to determine the flow rate that corresponds to the water surplus (not absorbed by the soil in a given time interval).

$$\frac{W(T)}{Ä} = \frac{W(t-1)}{Ä} + P(t) - E(t)\ S(t) = \frac{(W(t) - W')}{Ä} (1)$$

Under this equation, S(t) stands for the flow rate at a given t time of the simulation, P(t) indicates the precipitation, E(t) represents the evaporation; W(t) means the soil capacity to absorb water while W specifies the water holding capacity of the soil where Ä denotes the considered time interval.

The SCS-CN (Soil Conservation Service - Curve Number) method is widely used in hydrology for its simplicity and quality of results in estimating the direct surface runoff from a precipitation event [10]. In this model, the main elements that determine the surface runoff volume (effective precipitation) are the retention of rain on the ground depressions and infiltration. This effective precipitation estimate considers three variables: (A) precipitation in the time interval; (B) soil characteristics and moisture which defines the retention potential; (C) loss on early rainfall. As expected, the flow rate has an influence on the hydrological dynamics of soil due to its action on the surface, on the mass movement and on the flow time or concentration. The evaluation of this speed can be carried out by the Soil Conservation Service (Table 1).

Table.1: Speed in m/s based on the slope and soil occupation (Source: Soil Conservation Service, 1972).

Slope in %	Forests	Natural grasslands	Slope in %	Almost bare grounds
0-4	0.3048	0.4572	0-2	0.6096
4-8	0.6096	0.9144	2-4	0.6096
8-12	0.9144	1.2192	4-6	0.6096
12-15	1.0668	1.3716	6-10	0.9144
			10-12	1.2192
			12-15	1.52

The estimated speed computation shown in the previous table can, hence, be performed by equation 2 (S represents the slope or gradient). In turn, the coefficient K is estimated taking into account Table 2.

$$V = K \times S^{0.5} (2)$$

Table.2: K coefficient value of the flow speed assessment (Source: Soil Conservation Service, 1972).

Types of areas	Speed
Forest with a lot of foliage on the soil	0.076
Area with little cultivation	0.152
Meadow-grasses	0.213
Cultivated land	0.274
Bare ground	0.305
Flowlines	0.457
Paved surface	0.61

The time of flow path (the water flow distance from one location to another) is also an important variable in hydrological modeling. According to NRC [11], the flow time between two points in a water basin is determined on the basis of the principle of equation 3(Tp: flow path time; n: roughness coefficient; l: stream length;$P_{24}^{0.5}$: 24 hours average of rainfall over the last two years; S: slope or gradient).

$$Tp = \frac{0{,}007x(nl)^{0.8}}{P_{24}^{0.5} \times S^{0.4}} (3)$$

The determination of the roughness coefficient presumes the assessment of soil conditions and occupation. To this end, the NRSC has set a coefficient, complying with these factors as it is described in Table 3.

Table.3: Index of roughness, according to NRSC[8].

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Hard surfaces (concrete, asphalt...)	0.011
Fallow soil	0.05
Cultivated land	
With coverage < a 20%	0.06
With coverage > a 20%	0.17
Formation of meadow-grassland	
Grassland	0.15
Dense grassland	0.24
Bermudagrass	0.41
Forest	
Relatively high density	0.8
Low density	0.4

The calculation of the discharge assumes this rational method for determining the specific discharge of each pixel in the runoff basin. It can be computed with the SCS estimate as described in equation 4 (Tp: time; Q: discharge peak for each cell (m^3/s); S: pixel area (ha); I: precipitation average intensity (mm/min); α: flow coefficient).

$$Q = 0.167 \times S \times I \times \alpha (4)$$

By definition, flow rate equals the volume of a fluid flowing through a given section of a free channel per time unit (a free channel can be a river or a pipe, for instance). Thus, one can determine the flow rate as $F = A \times V$, in which A stands for the area in question and V for the speed of flow expressed in m^3/sec. In order to measure this speed rate of a water course, one typically uses the windlass (an apparatus provided with a propeller and a rotation meter). This measurement is universally used to determine the flow of a natural watercourse and consists in determining the cross-sectional area and the average speed in any section.

Finally, the surface runoff in rivers and channels is represented mathematically by two differential equations that describe mass or volume preservation and the quantity of flow movement, named the equations of Saint Venant [12] where Q equals volumetric flow rate, A is the area of the wet section, X stands for the distance in the longitudinal direction, t denotes time and qL represents the input or output flow per unit of width.

$$\frac{\partial Q}{\partial X} + \frac{\partial A}{\partial T} = q_L (5)$$

IV. IMPORTANCE OF REMOTE DETECTION IN RISK ASSESSMENT

Remote sensing refers to the obtaining process of information about objects or areas via electromagnetic radiation without being in direct contact with them [13]. This practiceis based on the principle that all electromagnetic radiation presents fundamental properties, behaving in a predictable way, according to the wave theory. It is achieved through a series of steps that go through the detection and recording of energy reflected or emitted by the objects and subsequent processing, analysis and application of the recorded information. Sensors capture the fractions of the emitted spectrum of electromagnetic energy from objects of the earth's surface, converting them into a paper image or a digital numerical signal.

In order to be able to extract information from the remote sensing data, it is vital to know the behavior of the spectral reflectance of the different bodies as well as the factors that interfere in its behavior.It is called the electromagnetic spectrum.Technically, this spectrum is the complete range of all types of electromagnetic radiations, ranging from radio waves to gamma radiations. The figure below shows the different bands of the electromagnetic spectrum.

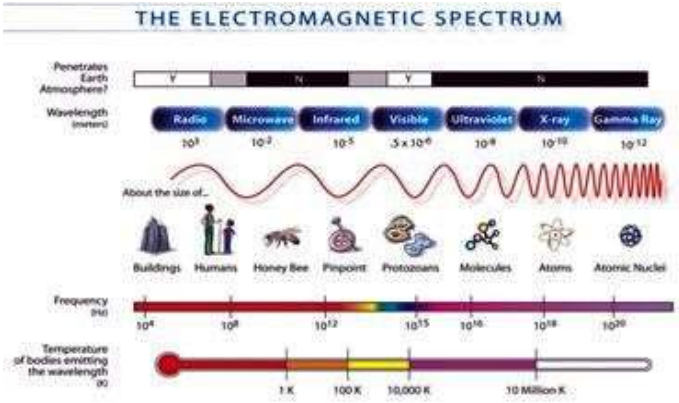

Fig.4:The range of visible light to the human eye is also a component of this electromagnetic radiation picture. However, its range is very small compared to that of other wavelengths.

Remote sensing has become an important alternative for research and practical applications in various domains including the environmental sector such as geography, geology, water resources, agriculture, forestry, meteorology, oceanography and civil engineering. This is particularlynotablefor collecting data on unreachable regions such as high altitude mountains and cold regions where continuous monitoring by humans is usually quite impossible.

Information by remote sensing can be acquired through satellites or airplanes. Again, remote sensors use electromagnetic spectra for global observations of objects on the Earth surface or even on the limit of the Earth's atmosphere (in the case of suspended aerosol particles, for instance). Each object has specific properties of absorption and reflection of the energy received by the sun. Technically, remote sensors pick up that electromagnetic energy reflected by the surface of objectsto determine the type of cover and its properties.

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4.1. Remote detection and precipitation

Precipitation is the phase of the hydrological cycle that exhibits the greatest spatial variability in this study. Its measurement is conventionally measured by pluviometry stations which provide data for a limited region. The World Meteorological Organization (WMO) recommends the ideal number of rainfall data logs per square Km. For small islands, the recommendation is for a registration station for 250 square Km. For urban areas, a check-in station is recommended for each 10 to 20 square Km.

The precipitation measurement at the record stations is made from the height of the water slide which would be accumulated on a flat surface if no loss occurred. Typically, intense precipitation is considered to be one originating from the same meteorological disturbance, whose intensity exceeds a certain value in millimetres (mm). Generally the variation of this disturbance goes from a few minutes to a dozen hours and the area affected by it can range from a few square Km to thousands of ones. In extensive continental regions, the collection of precipitation data by land stations is hampered by the low density of collection points, making spatial estimates of precipitation as a useful and low-cost resource.

Estimatesgive a good impression of the overall distribution of cloud cover and precipitation. Satellite images have been an important means of obtaining precipitation data. Its importance is even greater in places where there is a low density of ground stations. The continuous data for a wide area is obtained only by means of geostationary satellites of high temporal and spatial resolution. In these regions, according to [14], meteorological satellites are the only realistic means of monitoring spatial and temporal distribution of precipitation with a high level of accuracy.

The estimation of precipitation by satellites has been done using images of several satellites and in several bands of the electromagnetic spectrum. The use of these spatial images for the meteorological forecast was the first civil application of satellite remote sensing [15]. The physical and optical properties such as reflectivity and emissivity depend on both the characteristics of the clouds and the wavelength of the radiation observed by the satellite. However, there is no direct relationship between the electromagnetic spectrum data measured on the clouds surface and the amount of precipitation observed [16]. As an example, techniques developed for tropical regions may not be applicable to extratropical regions.

A paradigmatic example is the principle of the precipitation estimation by satellite images in the visible spectrum of electromagnetic radiation. This method is based on the fact that the brightness of the sun reflected by clouds can be a reasonable indication of its thickness and, consequently, of the volume of water in its interior. Similarly, temperatures at the top of the clouds by sensors in the infrared range are indicative of their thickness or vertical development where the greater the thickness,the higher precipitation rate [17].

The top cloud information reveals the temperature and reflectance that are treated by statistical methods to estimate cloud precipitation over its entire length. This estimate is two-dimensional (VIRS sensors). Under the microwave spectrum (frequency lower than 50 GHZ), a great emission sensitivity of water vapor, clouds, rain and properties of the Earth surface can be found. These frequencies are very useful for the discrimination of surface type, cloud content (hydrometeors) and precipitation intensity [18].

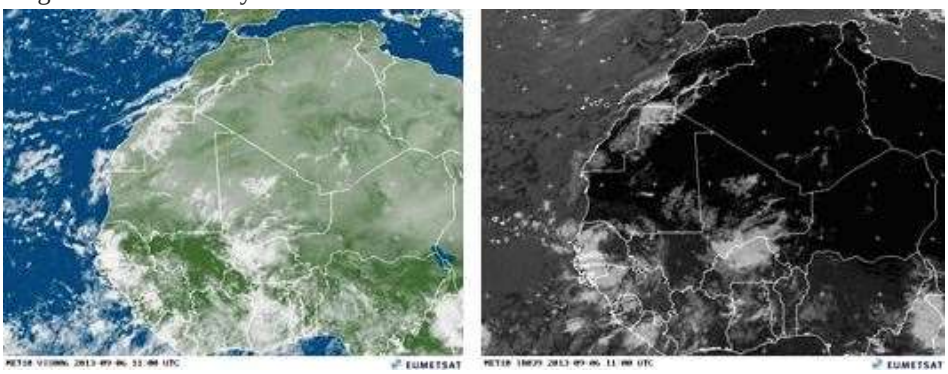

Fig.5: Image of the Meteosat satellite of the visible and infrared spectrum.

Precipitation indices are usually derived by reading the temperature through infrared radiation. Yet, there is some uncertainty about the data presented. The hypothesis that cloud height is tied to its thickness works well for convective ones but is problematic for nimbostratus ones whose rainfall estimates are often underestimated because of the relatively high temperature at the top [18]. However, it has become evident that not all the clouds with a cold top layer effectively produce precipitation. Therefore, the infrared estimates can be made locally only, providing poor results when transposed to other regions [14].

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4.2. Meteorological satellites

Meteorological satellites are used to monitor Earth's weather and climate but can also serve to monitor human activities such as city lighting, burning, pollution levels and deforestation, for instance.The Geostationary Operational Environmental Satellite (GOES) satellite series is the leading meteorological satellite family managed by the United States National Oceanic and Atmospheric Administration (NOAA). GOES images and data seek to provide a steady stream of reliable information used for weather forecasting and surveys. These satellites are geostationary, that is, they have the same Earth speed of rotation and are stationed on a fixed point of the equator, which allows them to observe continuously the same area.To date, NASA has already launched 15 GOES and is in development to release GOES Q in 2015.

The GOES are located in a geostationary orbit about 35,800 km above the Earth's surface. Under these conditions,the GOES family satellites provide important information on Atlantic and Pacific weather conditions, especially on precipitation estimates, tropical storm movements and snow cover in extra-tropical regions. These satellites carry two types of components, namely a sensor that captures multiple wavelengths of the electromagnetic spectrum (visible and infrared) and an aerosol sensor of the Earth's atmosphere to obtain temperature and humidity data.

In addition to the GOES series, there is the Tropical Rainfall Measuring Mission (TRMM), considered the first global precipitation measurement equipment (GPM). Launched in November 1997, it is a joint project between NASA (American Space Agency) and (JAXA), Japanese Agency for Aerospace Exploration, with the specific objective of monitoring rainfall in the tropics and verifying its influence on the global climate.

The TRMM satellite holds a 405 km high, with a very steep orbit between 35ºN and 35ºS latitude and holds a translation period of 91 minutes, allowing a relatively high spatial and temporal resolution. The TRMM satellite sensors sweep a path with several hundred Kmwidth, monitoring most of the tropics daily. This orbit allows the satellite to remain over the tropics most of the time [19].There are four main sensors on board that use different spectra and bands to collect information [20]:

- Visible Infrared Radiometer (VIRS) is a passive transverse range scanning radiometer with five channels centred at 0.63, 1.6, 3.75, 10.8 and 12 μm wavelengths, providing high resolution at cloud cover, cloud type and cloud top temperature. Under this band, the radiation does not penetrate the clouds so the temperature values collected refer to the top of the clouds.
- Microwave Imager (MI) is a passive multi-channel (10.65, 19.35, 37.0 and 85.5 GHz) microwaves radiometer with vertical and horizontal polarization. This sensor provides information on the integrated content of precipitation column, liquid water and cloud ice, rainfall intensity and types of rain.
- Precipitation Radar (PR) is a scanning radar (active) operating at 13.8 GHz and measures the distribution of precipitation in three dimensions. In parallel, it defines the depth of the precipitation layer.
- Lightning Imaging Sensor (LIS) is an optical (passive) sensor that detects and locates lightning events under the neutral oxygen range (0.777 μm).

Despite the importance of the satellites in the provision of precipitation data and the great advances achieved since the launch of the first meteorological satellite, there is still some imprecision in the estimation of precipitation values.Unsurprisingly, the accuracy of the spatial and temporal distribution of precipitation is fundamental for a wide range of applications of climate modelling and generalization, from global to local [21].

One approach of measuring the accuracy of satellite precipitation data is to collect and compare data series in situ. It is assumed that the cold top clouds obtained in the infrared (11μm) range of the GEOS satellite is correlated with the high precipitation rates. In general, this indirect approach provides good results when precipitation estimates are made for a high spatial and temporal scale and the tendency for increased uncertainties for instantaneous estimates of precipitation at smaller scales [22]. However, the hydrometeor information provided by the satellite in the microwave range on oceans allows accurate information on precipitation estimates.

As well, the extrapolation of data associated with the continuous feature in space allows the estimation and spatial distribution of the phenomenon to areas far from the point where historical series are recorded. For example, rainfall is characteristically a continuous phenomenon in a limited space but, however, rainfall measurements are made only at some points on the terrain. Of course, the extrapolation of the data observed for the whole area must be done in a very careful way.

V. THE CITY OF PRAIA

The archipelago of Cape Verde is located in the Atlantic Ocean, approximately 450 km from the West Coast of Africa, between the latitude of 14º48'00 and 17º12'13 "

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North and the meridians 22º23'59 " and 25º20'40 " West . This archipelago consists of 10 islands and 8 islets, organized into two groups, according to each geographic location: Windward and Leeward. The archipelago has a total land area of 4033 km^2 whose corresponding coast line extent is 1,020 km.

Cape Verde's climate is part of an extensive range of arid and semi-arid climates in the heart of the Sahelian region (situated between the subtropical high pressures of the Atlantic and the equatorial low pressures). This track of African territory (Senegal, Mauritania, Mali, Burkina Faso, Chad, Sudan, Ethiopia, Erithrea, Djibouti and Somalia) has a precipitation regime, ranging from 150-300 mm per year.

The county of Praia is located in the south of the Santiago islandand covers an area of 102 km^2 (about 10% of the surface of the island of Santiago). It is bordered to the North by the mountain of Antonia Peak and to the South by the limits of the maritime coastline. Administratively, it has borders with the municipalities of São Domingos and Ribeira Grande de Santiago.

Towards morphology, the elevations of the islands of Santo Antão, St. Nicholas, Santiago and Fogo reach an altitude above 1,300m. Yet, the highest altitude corresponds to Pico on Fogo Island, with 2,829m, followed by the Crown Top on the island of Santo Antão with 1,985m. The Antonia peak in Santiago reaches 1,395m and Monte Gordo, in St. Nicholas, presents 1,304m. By considering the geomorphological characteristics of Praia, there were sharp erosions lately and, along with the poor vegetation cover, it creates a positive situation for dynamic erosion (Figure 6). The existing valleys in the area are relatively well embedded between the highlands (achadas), among which some relevant elevations stand out, such as the Hill of Vacas with 392m, the Ilhéu or Hill of São Filipe with 274m, Red Hill with 195m, Hill of Gonçalo Afonso with 235 m and the Hill of Ilhéu with 259 m.

The drainage networks extend from the highlands of the North (Hill of Rui Vaz) to the South of the county, flowing into the Atlantic. As it can be observed from Figure 6 and 7, there is a main basin where there is a set of flow networks that converge downstream. It turns out that the urban area of the municipality is located at the crossroads of these drainage networks. However, the existing security infrastructure cannot always withstand the water discharges coming from upstream areas.

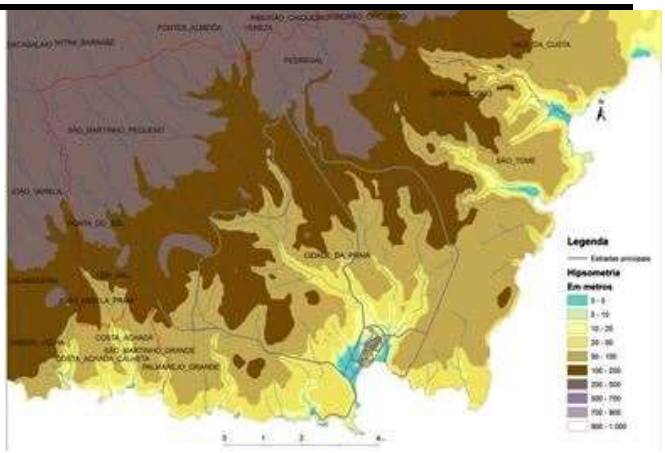

Fig.6: Hypsometric southern map of Santiago Island.

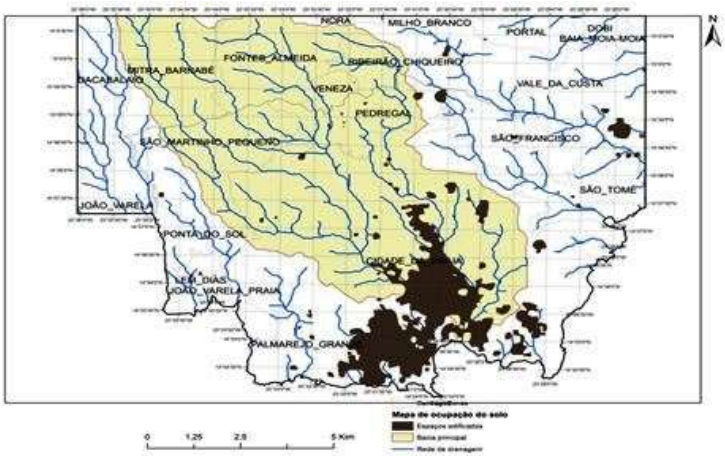

Fig.7: Drain main basin, edification areas and network drain.

The average annual rainfall in the Cape Verde archipelago is no more than 300mm in the lowlands (less than 400m high). According to [23], the precipitation of downward trend has been recorded since the 60s, reflected negatively on the conditions of farming and water supply. The areas under the influence of hot and dry air masses that blow from the eastern quadrant during the Harmattan (December/January) are the driest, corresponding to an average rainfall of only 150 mm. In spots located more than 500m above sea level and exposed to the trade winds, rainfall can exceed 700 mm. In relation to water balance, it is considered that about 20% of the precipitation water is lost by surface runoff, 13% goes towards aquifers recharging and 67% will disappear by evaporation [23].

However, extreme precipitation in the form of flash floods is a phenomenon that occurs in all latitudes, including arid regions and desert climates. This phenomenon has been a reason for concern to the scientific community due to its increasing frequency as a result of recent climate changes. These effects of extreme precipitation are sometimes more serious due to the existing lack of

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vegetation cover that could reduce the impact on the ground. Moreover, notwithstanding the existence of side protection dikes, certain cross-sectional passages of circulation in the city streams do not hold the sufficient height to allow the flow of water when intensive precipitation occurs (Figure 8).

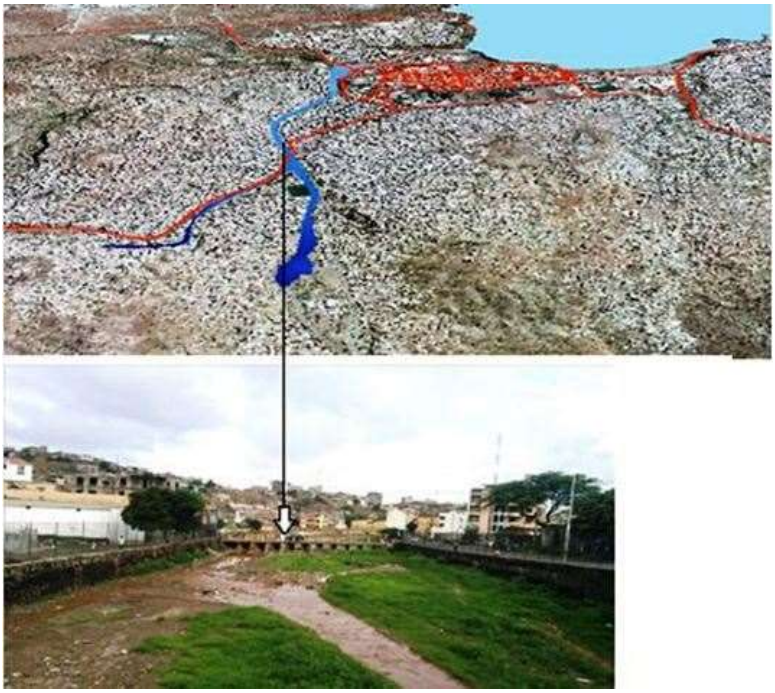

Fig.8: Superficial draining at Safende riverside.

VI. THE HYDROLOGICAL METHODOLOGY

The present hydrological risk simulation is based on a model with three main components: (A) Flow module, in which considers the various layers of soil types/occupation and of precipitation; (B) Flow path time module, in which the DEM and flow rate are considered; (C) Discharge module, which results from the interaction of the fallen precipitation and the pedagogical and geomorphological processes characteristics. As expected, the different layers interact together, having an influence on the runoff dynamics. For this research, the water discharge at the end of the drainage basin was simulated. The graphical user interface provided by the Model Builder is significant, as most of the functions are already integrated within ArcGIS (Figure 9).The first component is the flow. Based on the SCS procedure, several parameters are taken in consideration such as the Curve Member, the initial infiltration and the potential one. These elements play a key role in the final activity when determining the flow coefficient that is obtained by the ratio between the total precipitation and the flow amount. This calculation presupposes the raster format associated to all layers: soil type, foregoing moisture of soil (amount of moisture before precipitation), land use, precipitation and DEM. The intermediate results obtained are the curve number, initial infiltration and maximum potential of moisture retention.

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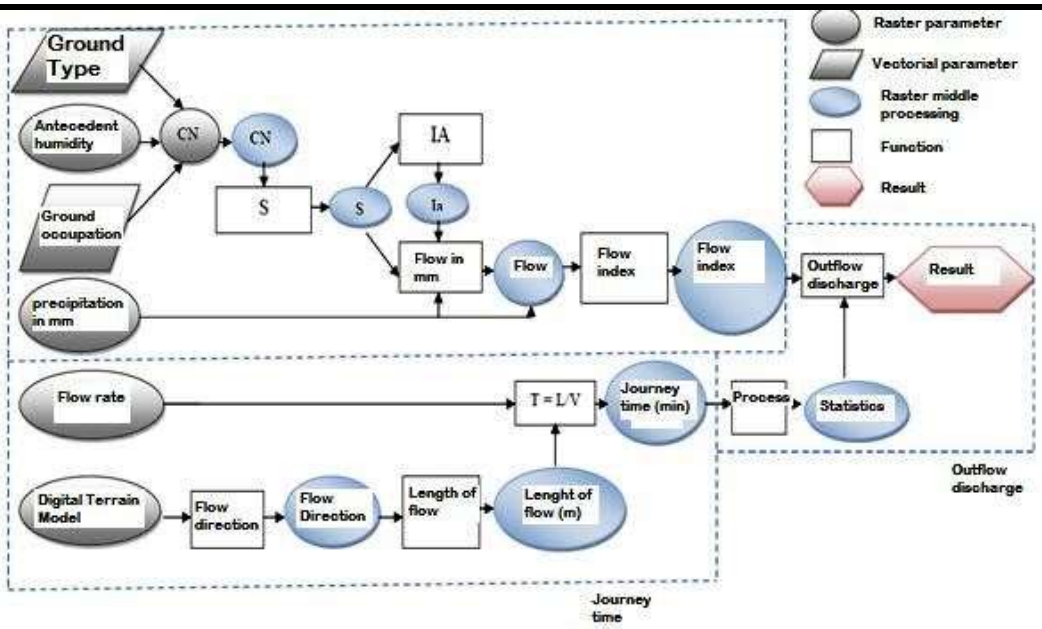

Fig.9: Simulation model for the hydrologic flow.

For framing soil types, it was considered the different soil groups according to the FAO classification [24]. The map of Figure 10 shows the predominant soil types of the county of Praia. As demonstrated, there is a predominance of the Xerossol class that is distributed throughout the northern and northwestern regions of the county of Praia. These soils display a reddish coloring of great thickness. Next, in terms of expressiveness, there are the Cambisol soils, which are little developed soils from non-limestones formations. These soils are usually associated with rocky outcrops, often being characterized by a high proportion of stony elements. One can also identify lithosols belonging to the group of non-climatic erosion mineral soils, which are young, little evolved and associated with outcrops of hard consolidated rocks and basalts. There is still a small portion of undefined soils, which occupies the Plateau neighborhood of Praia.

The foregoing humidity is a parameter that can be modified, depending on the time acclimatization: condition I for dry soils, condition II for moderate soils and condition III for wet soils. Under the land layer drawn from Landsat TM5 images (Figure 11), one can see a predominance of bare ground and rocks in over 80% of the county of Praia's area. However, there is some scattered green area, consisting of bushes and acacia trees, a species that was introduced in the last 40 years, framed in the national afforestation program.

The discharge makes up the final phase of the flow process. This index takes place at the pixel level (see equation 4 and 5) and, subsequently, it is followed by the accumulation of the discharges, resulting on the flow output. As expected, the total flow in the drainage basin is obtained by the sum of discharges. Finally, we proceed to the discharge values reclassification by the equidistance of the flow path time. In this case, we selected a temporal equidistance of every 30 minutes.

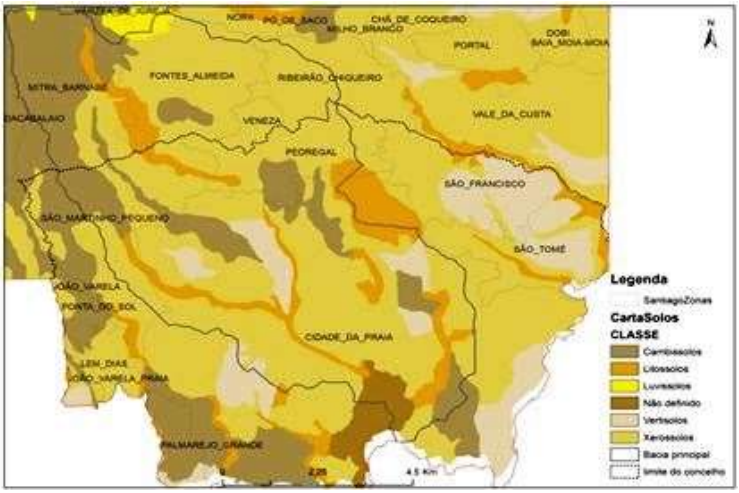

Fig.10: Soil southern map of Santiago Island.

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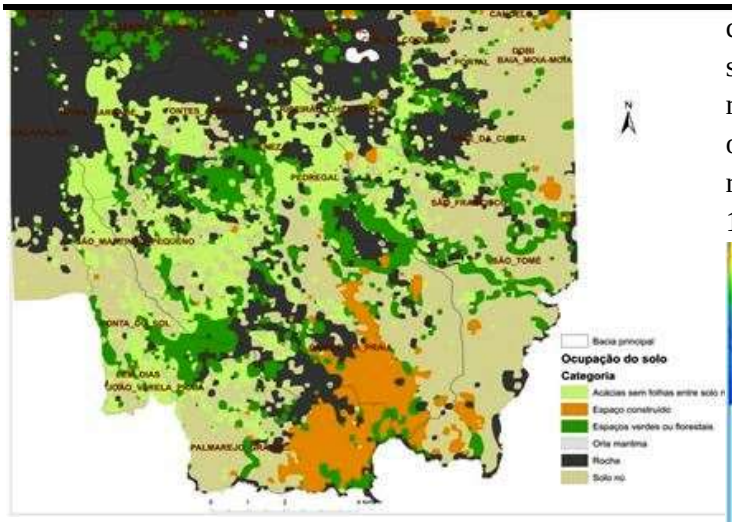

Fig.11: Soil map occupation in the southern island of Santiago.

VII. The county of Praia: simulation findings

The precipitation data was obtained from the sensor 3B42 of the TRMM satellite. The historical rainfall records indicate some variation of rainfall occurring in the county. As a rule, higher areas receive a greater amount of rainfall as shown in Figure 12[4]. The central region of the island matches with the areas of higher altitudes whilst lower precipitation rates are situated along the coast. As a reference, the county of Praia presents an annual variation between 73-163 mm of rain.

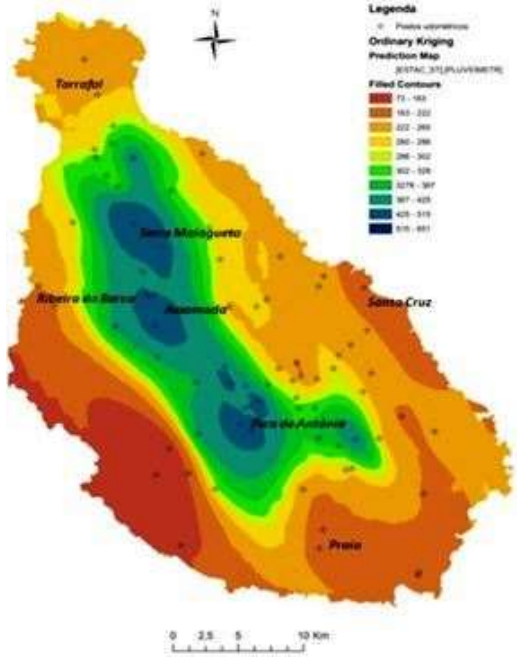

Fig.12: Average precipitation map of Santiago Island [4].

It is particularly notable that the TRMM satellite estimates precipitation in a resolution of 0.25 x 0.25, corresponding to an area of 2,162 km^2. Given the size of Santiago, approximately 900Km^2, this resolution covers 2.4 times the island of Santiago. In order to carry out this study, it was considered the rainfall data occurred on October 22, 2010 (Figures 13 and 14).

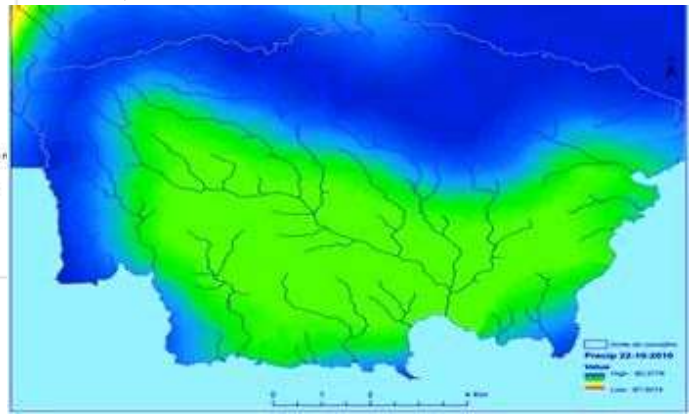

Fig.13: Precipitation map on October 22, 2010.

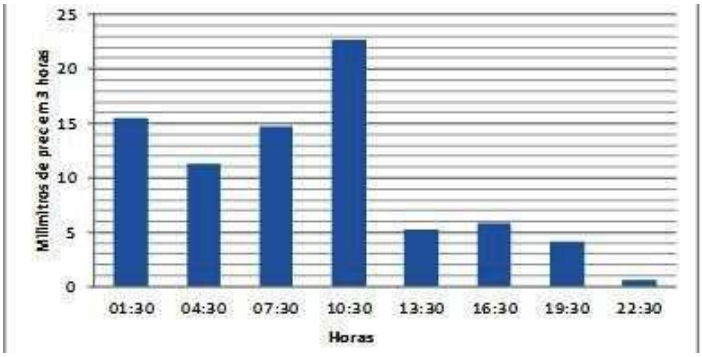

Fig.14: Time precipitation distribution on October 22, 2010.

By definition, the potential infiltration presents the soil's capacity to retain water. Figure 15 illustrates the sectors with greater retention capacity, according to the existence of some dispersion towards the infiltration potential level. These differences are linked to the pedagogical features, degree of vegetation cover, soil occupation and land use. As one might expect, the built-up areas, rocky zones and bare ground spaces are generally of lower potential retention, implying a higher degree of surface runoff.

The flow path time is another significant variable as far as it influences the flow volume in a specified section of the basin, either in volume or in terms of duration. The approach used for determining the flow time formula of section 3. In a simplified manner, it defines the flow time as the ratio between the flow length and the average speed over the entire

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area of the basin. Subsequently, the stream length is used to estimate the flow time for its relationship with the average velocity of the basin flow. Afterwards, this flow time is reclassified every 30 minutes and applied to the basin for the delimitation purposes of the different patterns. Thus, spaces are shown after having been reclassified according to the flow path time. Figure 16 characterizes the flow time of surface runoff generated from the ratio between the watercourses length in different parts of the basin and the average speed recorded across the basin. Certainly, the flow time is shorter as it moves towards downstream.

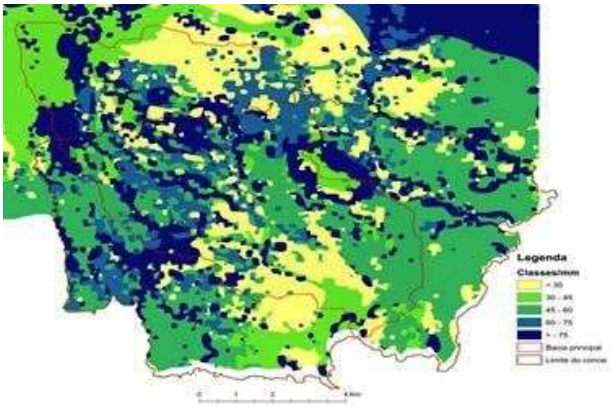

Fig.15: Potential infiltration map in mm at Praia County.

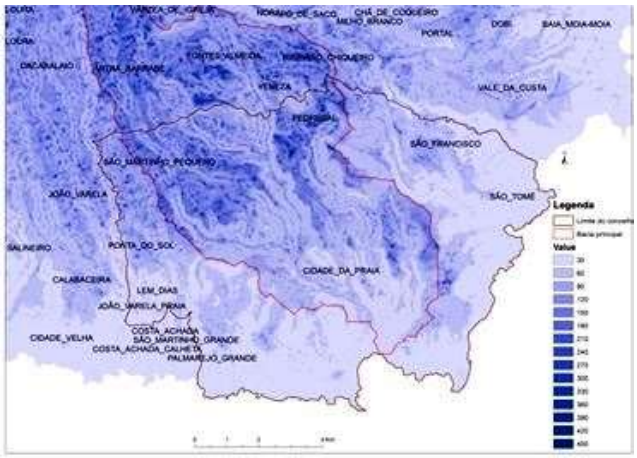

Fig.16: Time map of flow route.

The flow speed involves the*K* coefficient and the relief slope of the basin. After an analysis of the basin conditions for determining this constant, we chose the value 0.305, resulting from the fact that most of the space within the drain basin is almost a bare soil. It is noted that the average flow velocity is 1.9 m^{-1}, with the minimum speed of 0.053 m^{-1} and a maximum of 6.49 m^{-1}. As expected, higher speeds occur in the areas of greater slope, which are well represented in regions further north of the county, matching with the most mountainous areas.

The discharge is the last element obtained from the overall model. This is obtained by the interaction of rainfall parameters in mm/min, flow time and infiltration potential (soil capacity to retain water). From this latter factor, we may obtain the flow coefficient (effective precipitation). Subsequently, the precipitation that was initially in mm per hour is now converted to mm per minute in order to make the discharge reclassification in every 30 minutes easier. It should also be noted that the discharge is initially obtained at the level of the pixel and reclassified in areas depending on the flow path time by ArcGIS 10 Spatial Analyst (Zonal Statistics function).

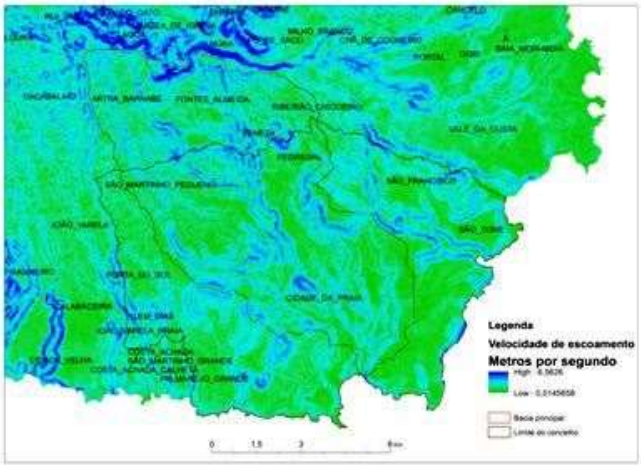

Fig.17: Flow speed.

Fig.18: Basin flow at Praia county and the water volume drained in m^3 during 450 minutes.

Figure 18 shows the discharge result from the precipitation data. This allows the water volume

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evaluation in cubic meters that crosses throughout the final section at intervals of 30 minutes for 8 hours. It is demonstrated that 76% of the precipitation volume stems from the flow coefficient that passed through the final part of the basin in 120 minutes and 99% crossed in 300 minutes.

Finally, Figure 19 shows the flow to the main basin, already reclassified at an equidistance of 30 minutes in terms of flow time. There is, however, some inconsistence of the results for the sectors with lower discharge that occurs further within the basin region, supposedly associated with areas of low permeability and rapid surface runoff.

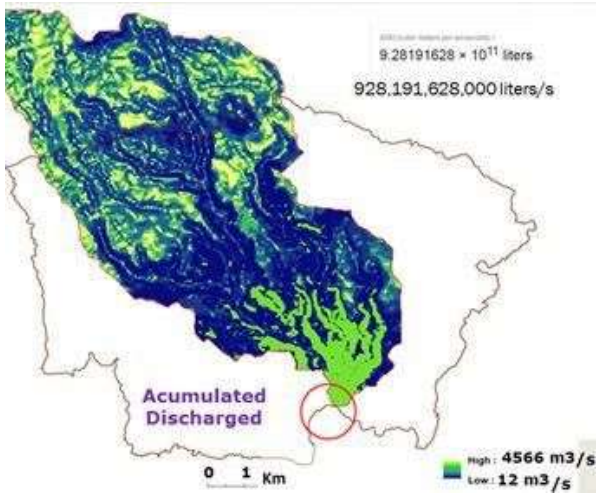

Fig.19: Accumulated drain map where almost one trillion litters of water per second was the top accumulated discharged assessed by this study on the mouth of the Safende river.

VIII. CONCLUSION

In the county of Praia, there is a large population growth living under a hydrological risk area, especially along the water streams. The morphology and configuration of the surface runoff network in the surrounding areas of this county increase that risk mainly due to the confluence of several basins that flow into the urban area. Indeed, the areas of greatest hydrological risks in the county of Praia are in the lowland areas (along the flow streams). Despite the existence of lateral protection dikes in these streams, there is water overflow in some zones (particularly in Vila Nova creek) because of the large amount of material carried from the slopes and the highland areas of Fontes Almeida, St. Martinho and Mitra Barnabé. Moreover, there is a certain shortage of infrastructures for torrential correction in several ramps of the city and along the side protection dikes delimiting the rivers, where one can find a high density of soil occupation, especially in the settlements of San Pedro, Safende, Calabaceira, Vila Nova and Fazenda.

In this sense, it is recommended interventions in these risk areas in order to protect households that are at the edge of these flood lines, despite the financial costs. The intervention on these slopes becomes a priority in order to change the arrangement of the surface runoff lines. This involvementmay consist of afforestation and construction of grooves to prevent excessive transport of soil debris toward the low lying areas of the city during precipitation. *Certainly, GIS solutions can play an essential tool for the surveillance and prevention of hydrological phenomena in this municipality. GIS adds a set of spatial information, facilitating the identification of existing vulnerabilities. For example, in this project we tried to define a model to* characterize the hydrological risk flexible enough to operate at different scales or to be applied in other geographical areas with the necessary adjustments. Municipal authorities are, therefore, in a position to setup a warning and risk prevention system, as well.

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Biosynthesis and Degradation of Carotenoids in Ornamental Crops with specific reference to Chrysanthemum

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Abstract— *Carotenoids are lipophilic secondary metabolites derived from the isoprenoid pathway, accumulated in most plant organs and widely used as an antioxidant. Carotenoids synthesized in chloroplasts are essential for protecting tissues against photo-oxidative damage in the green tissues of higher plants. The importance of carotenoids for plant growth and development is evident since at least two major phytohormones, strigolactones and abscisic acid, are derived from carotenoid precursors. In flowers, carotenoids synthesized in the chromoplasts provide colour to the petals, ranging from yellow to red, in order to attract pollinators and determines the commercial value of ornamental plants. On analysis in chrysanthemum, β, ε-carotenoids, lutein and its derivatives, reflecting the high expression levels of lycopene ε-cyclase (LCYE) were found in yellow petals compared to the ratio of β, β-carotenoids to total carotenoids found in leaves reflecting the high expression levels of lycopene β-cyclase (LCYB). Petals of the yellow-flowered cultivar Yellow Paragon showed increased accumulation and drastic componential changes of carotenoids as they mature, compared to petals of the white-flowered cultivar Paragon that showed drastically decreased carotenoid content during petal development.The white petals of chrysanthemum (Chrysanthemum morifolium Ramat.) contain a factor that inhibits the accumulation of carotenoids. All the white-flowered chrysanthemum cultivars tested showed high levels of CmCCD4a transcript in their petals, whereas most of the yellow flowered cultivars showed extremely low levels indicating that in white petals of chrysanthemums, carotenoids are synthesized but subsequently degraded into colourless compounds, which results in the white colour. Studying the regulatory mechanisms underlying carotenoid accumulation in ornamental plants at the molecular level will help in producing novel coloured cultivars by plant transformation.*

Keywords— Ornamental crops, Chrysanthemum, Carotenoids, Biosynthesis, Degradation.

I. INTRODUCTION

1.1 Pigments:

Plant compounds that are perceived by humans to have colour are generally referred to as 'pigments'. Their varied structures and colours have long fascinated chemists and biologists, who have examined their chemical and physical properties, their mode of synthesis, and their physiological and ecological roles. Anthocyanins, a class of flavonoids derived ultimately from phenylalanine, are water-soluble, synthesized in the cytosol, and localized in vacuoles. They provide a wide range of colours ranging from orange/red to violet/blue. In addition to various modifications to their structures, their specific colour also depends on co-pigments, metal ions and pH. They are widely distributed in the plant kingdom. The lipid-soluble, yellow-to-red carotenoids, a subclass of terpenoids, are also distributed ubiquitously in plants. They are synthesized in chloroplasts and are essential to the integrity of the photosynthetic apparatus. Betalains, also conferring yellow-to-red colours, are nitrogen-containing water-soluble compounds derived from tyrosine that are found only in a limited number of plant lineages. In contrast to anthocyanins and carotenoids, the biosynthetic pathway of betalains is only partially understood. All three classes of pigments act as visible signals to attract insects, birds and animals for pollination and seed dispersal. They also protect plants from damage caused by UV and visible light (Fig.1).

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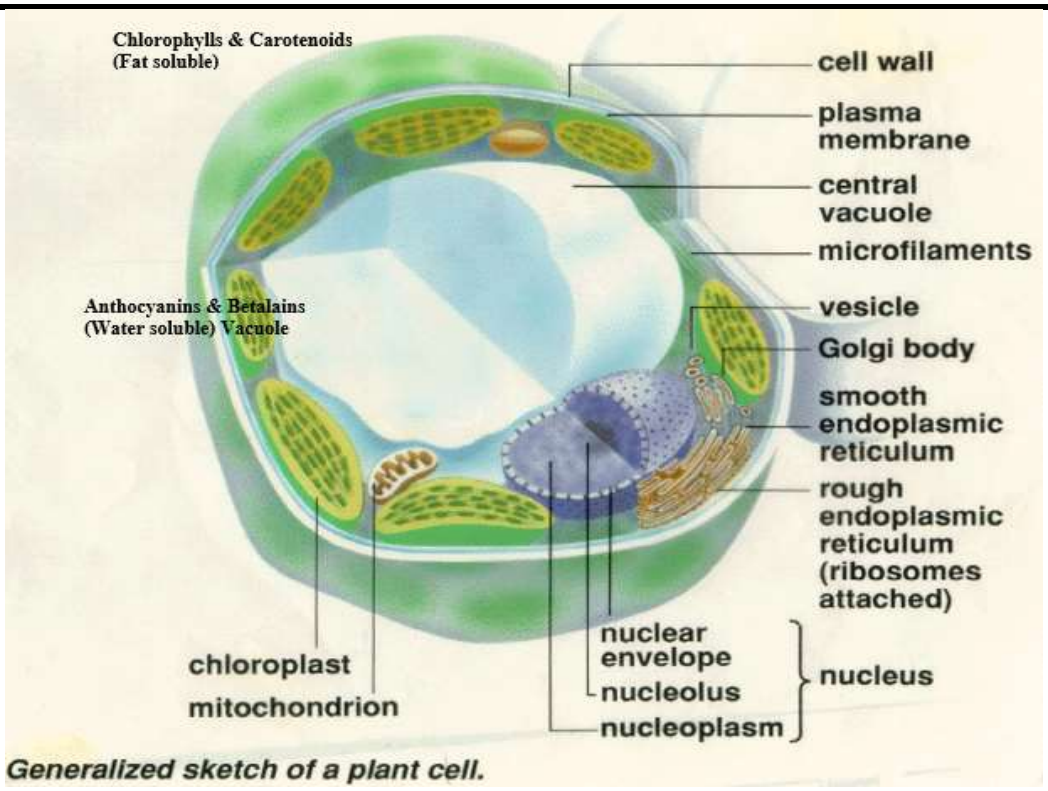

Fig.1: Site of colour accumulation

II. CAROTENOID BIOSYNTHESIS AND REGULATION IN PLANTS

Carotenoids are lipophilic secondary metabolites derived from the isoprenoid pathway and are accumulated in most plant organs (Howitt & Pogson, 2006). They contribute to the red, yellow and orange colours of many fruits and flowers, and are a factor in attracting pollinators to flowers. Carotenoids can exert important physiological functions in a wide range of organisms, including plants and humans. They are essential components of the photosynthetic machinery, and play a critical role in preventing photo oxidative damage (Howitt & Pogson,2006). Carotenoid catabolism products, such as β-ionones, are involved in plant-insect interactions. The importance of carotenoids for plant growth and development is evident since at least two major phytohormones, strigolactones and abscisic acid, are derived from carotenoid precursors (Cazzonelli & Pogson, 2010). Some carotenoids are precursors of **vitamin-A**, and prevent human age-related macular degeneration. Others, like lycopene, a red carotenoid pigment contained in tomato and watermelon, is a potent antioxidant and is considered to prevent prostate cancer. Astaxanthin, another red carotenoid, is found mainly in red sea animals and some algae, is likely to prevent cardiovascular disease and UV-light aging in the human body. Carotenoids are also widely used as colourants in the food and cosmetic industries, and some are important supplements in livestock and fish feed formulations.

Although carotenoid biosynthesis in plants has been well investigated, extensive studies on its regulation are relatively limited. Knowledge of carotenoid biosynthesis and regulation has led to a plethora of successful attempts at metabolic engineering of carotenoids in economically important crops (Giuliano*et al*. 2008). Here, we briefly summarize carotenoid biosynthesis, and identify steps that control the flux through the pathway. In addition to the rate of biosynthesis, sequestration and the storage capacity of the cell, along with the rate of carotenoid catabolism, all play a significant role in determining the levels of carotenoid accumulation in plant tissues and organs.

2.1. Carotenoid biosynthesis

Since the landmark review paper by Cunningham and Gantt (Cunningham &Gantt, 1998), major discoveries have been made in the characterization of carotenoid biosynthesis and its regulation in plants. The core carotenoid pathway is conserved in most plant species although some plants accumulate special and rare carotenoids via unique biosynthetic routes (Fig.2). As isoprenoids, carotenoid compounds originate in the plastid-localized 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway that starts with the reaction between pyruvate and glyceraldehyde-3-phosphate. The first steps in the MEP pathway are regulated by 1-deoxy-D-xylulose-5-phostaphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) (Fig.2). The second key regulatory step is catalysed by 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR)

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eventually leading to the production of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Fig.2). Geranyl-geranyl diphosphate (GGPP) synthase catalyses the condensation of three molecules of IPP and one molecule of DMAPP to produce GGPP – a 20-carbon molecule. The first committed step in carotenoid biosynthesis is the condensation of two molecules of GGPP by phytoene synthase (PSY) to form phytoene (Fig.2). GGPP is also the precursor for several other groups of metabolites, including chlorophylls, ubiquinones and tocopherols. Phytoene then undergoes four sequential reactions to form lycopene.

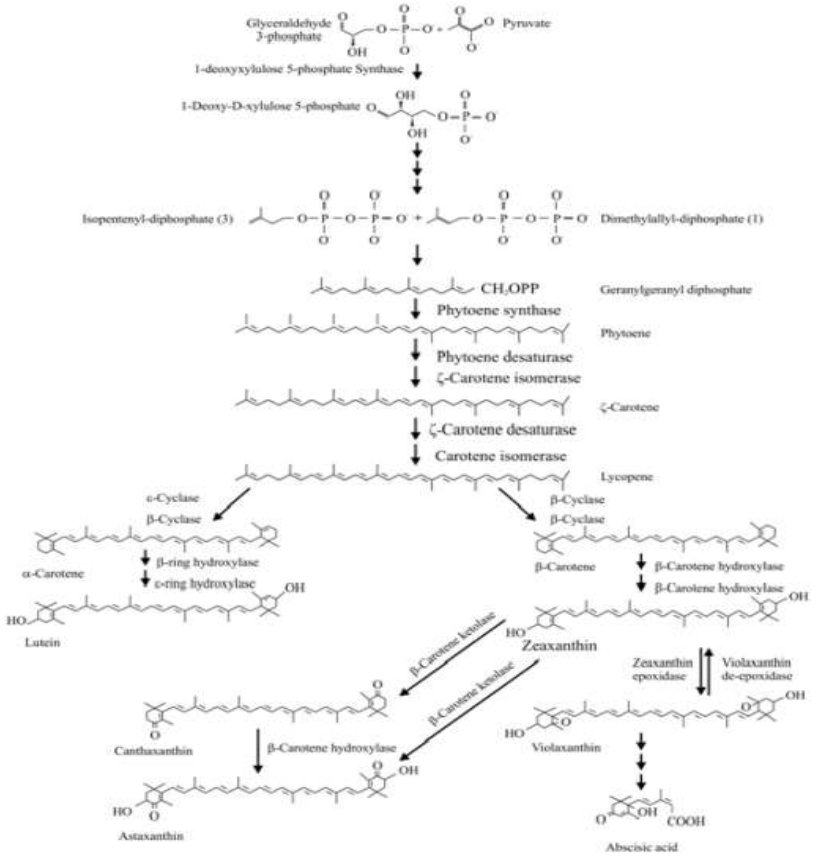

Fig.2: Carotenoid biosynthesis pathways

In bacteria, only one phytoene desaturase, crtI, catalyzes the conversion of phytoene to lycopene; however in plants, at least four enzymes are required for this step. These enzymes are phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS) which produce respective poly-*cis*-compounds, which are then isomerized to transforms by zeta carotene isomerase (ZISO) and carotenoid isomerase (CRTISO) to produce lycopene.In higher plants, the cyclization of lycopene with lycopene ε- and β-cyclases is a critical branch-point in carotenoid biosynthesis (Cazzonelli & Pogson,2010,Fig.2). In one branch, a single enzyme, lycopene β-cyclase (β-CYC), introduces a β-ring at both ends of lycopene to form β-carotene in a sequential two-step reaction. The first dedicated reaction in the other branch, leading to lutein, requires both ε-CYC and lycopene β-cyclase (β-CYC) to introduce one β- and one ε-ring into lycopene to form α-carotene. α-Carotene is acted upon by a β-ring hydroxylase to form zeinoxanthin, which is then hydroxylated by an ε-ring hydroxylase to produce lutein. Unlike plants, the cyanobacterium *Prochlorococcus-marinus* MED4 encodes a ε-CYC that can simultaneously catalyze the formation of α-, β- and ε-carotenes. Carotenoids with two ε-rings are rare in plants and algae, with an exception in lettuce, wherein a single ε-CYC adds two ε-rings to lycopene to form lactucaxanthin (Cunningham & Gantt, 2001). β-Carotene can be hydroxylated in a two-step reaction to zeaxanthin, with β-cryptoxanthin as an intermediate product. In green tissues, zeaxanthin can be epoxidized to violaxanthiun, and a set of light- and dark-controlled reactions known as the xanthophyll cycle rapidly optimize the concentration of violaxanthin and zeaxanthin in the cell through the action of zeaxanthin epoxidase and violaxanthin de-epoxidase, respectively, via antheraxanthin (Demmig & Adams,2002).

2.1.1. Regulation of carotenoid biosynthesis

Carotenoid accumulation occurs in most plant tissues, including green shoots, flowers, fruits, seeds and roots. Although the contents and types of carotenoids of green tissues are relatively conserved across most plant species,

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the levels of carotenoids and their profiles in non-green tissues, such as flowers, fruits and seeds, vary considerably, and are influenced by many factors, including the developmental stage, environment, stress or a combination of these (Howitt & Pogson ,2006). In general, the steady-state levels of carotenoids are determined by the rate of biosynthesis, storage capacity of the cell and the rate of catabolism and degradation. Combined, these factors have made the study of carotenoid regulation challenging. Here, we discuss the important steps that has been used in the metabolic engineering of carotenoid biosynthesis in plants.

2.1.2. Isopentenyl pyrophosphate (IPP) biosynthesis

The fact that the first reaction in the MEP pathway is catalyzed by the DXS enzyme makes it a presumptive regulatory step in carotenoid biosynthesis. The initial evidence in support of this came from results of overexpressing and silencing of the *DXS* gene in Arabidopsis seedlings. Overexpression of *DXS* was shown to result in up to 112–131% increase in the total carotenoid content, whereas silencing of this gene reduced the carotenoid content by 75–87% relative to the wild type control. *DXS* has been exploited in biotechnological applications to improve the carotenoid content of crops.

2.1.3. Phytoene biosynthesis

Phytoene biosynthesis is the first committed step in carotenoid biosynthesis, and has long been considered a 'bottleneck' in the pathway. Except for Arabidopsis, most other plant species express multiple functionally redundant copies of phytoene synthase (*PSY*), although different *PSY* genes appear to be differentially expressed and regulated. In Arabidopsis, its single copy *PSY* gene is tightly regulated by light. A phytochrome-interacting transcription factor, RIF, binds to the *PSY*promoter and maintains it in a repressed state under dark conditions. Under light conditions, RIF degrades and dissociates from the *PSY* promoter, thus allowing for its active expression (Toledo & Rodríguez, 2010). Another transcription factor, RAP2.2, was also shown to bind to the *PSY* promoter and regulate *PSY* expression and carotenoid levels.

2.1.4. Lycopene biosynthesis

The first step in lycopene biosynthesis is catalyzed by phytoene desaturase (PDS). The promoter of the Arabidopsis *PDS* gene was found to have a regulatory region similar to that of the binding site of the RAP2.2 transcription factor to the *PSY* promoter, and analysis showed that RAP2.2 does indeed bind to the *PDS* promoter and affect carotenoid accumulation. Carotenoid isomerization catalyzed by CRTISO, which isomerizes *cis* bonds to all-*trans* lycopene, is another step with regulatory impact on carotenoid biosynthesis. Characterization of the *ccr1* locus of Arabidopsis revealed that it encodes a histone methyl-transferase (SET DOMAIN GROUP, SDG8). This enzyme catalyzes the methylation of chromatin histones, and when the expression of *SDG8* gene was disrupted, it caused a reduction in the levels of *CRTISO* transcript and carotenoid content (Cazzonelli *et al*. 2010). In addition, Arabidopsis plants with reduced *SDG8*expression showed altered shoot and root branching, which were possibly caused by changes in levels of branch-inhibiting strigolocatone hormones that are derived from carotenoid catabolism products (Cazzonelli & Pogson, 2010). This was the first report on epigenetic regulation of carotenoid biosynthesis; this breakthrough has the potential to open new avenues for engineering of carotenoids in plants.

2.1.5. Lycopene cyclization

The carotenoid biosynthesis pathway branches after the formation of lycopene. One branch forms carotenoids with two β-rings, while the other introduces both β- and ε-rings to lycopene to form α-carotene, which is then converted to lutein. Thus, the relative activities of β-CYC and ε-CYC would be expected to determine the proportion of lycopene channeled to the two branches of the carotenoid pathway; i.e. β, β- and ε,β-carotenoids . Flux through the carotenoid pathway may play a role in controlling ε-CYC.

2.1.6. Sequestration and storage

In addition to the rate of carotenoid biosynthesis, other factors, such as sequestration and availability of storage compartments, play a significant role in determining levels of carotenoid accumulation. Even though carotenoid biosynthetic enzymes are nuclear-encoded, they are all located in the plastids, where carotenoids are synthesized and accumulated. In chloroplasts, most carotenoids accumulate in the form of chlorophyll–carotenoid–protein complexes in the thylakoid membranes associated with light-harvesting antenna. These complexes play an important role in stabilizing plant light–harvesting complexes (LHCs) and in assembling a functional photosystem II (PSII). Seed carotenoids are compartmentalized to elaioplasts (lipid–storing plastids), which use specialized lipoprotein-sequestering structures to store large quantities of carotenoids (Howitt & Pogson 2006). In chromoplasts, significant amounts of carotenoids may be stored in membranes, oil bodies or other crystalline structures within the stroma (Howitt & Pogson 2006, Cunningham & Gantt 1998).Reports in the literature have shown cases where enhanced carotenoid accumulation was accompanied by changes in the anatomical structure of plastids, resulting in enhanced ability to store carotenoids (Paolillo *et al*. 2004). Further analysis revealed changes in

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the thylakoid membranes of the plastids, thus allowing them to store large quantities of carotenoids (Paolillo*et al*. 2004). The authors therefore suggested that enhanced carotenoid accumulation could be due to changes in the cell structure, thus allowing for storage of more carotenoids.

2.1.7. Catabolism and degradation

A study revealed that carotenoids are constantly synthesized and degraded (Beisel, K.G.*et al*. 2010).This presumably maintains carotenoids at physiological levels, especially in green tissues, where a constant ratio of chlorophylls to carotenoids has to be maintained to ensure the integrity of the photosynthesis system. Carotenoid degradation by enzymatic oxidative cleavage produces an array of terpenoid products collectively known as apocarotenoids. These include abscisic acid and strigolactones, and other volatile and non-volatile compounds, which are well known in respective industries for their use as aromas, flavours and fragrances. Some apocarotenoids, e.g. β-ionone, are also known to play a role in plant-insect interactions.In Arabidopsis, the gene family that encodes carotenoid cleavage enzymes is comprised of at least nine members. Four of these encode carotenoid cleavage dioxygenases (CCD), and the remaining five encode 9-*cis*-expoxycarotenoid dioxygenases (NCED), with many of these enzymes exhibiting substrate promiscuity *in vitro* (Vogel, J.T. *et al*. 2010). The functions of some of these enzymes have been reported to be associated with certain apocarotenoids in several plant species. For example, CCD7 and CCD8 act in a coordinated manner in strigolactone biosynthesis, CCD1 is involved in β-ionone biosynthesis, and NCED2, NCED3, NCED5, NCED6 and NCED9 are associated with abscisic acid production (Walter & Strack, 2011). In chrysanthemums, CCD activity is important for petal colour, and elevated transcript levels of CmCCD4a is reported to play a role in the degradation of yellow carotenoid pigments, which resulted in flowers with white petals.

III. CAROTENOID BIOSYNTHESIS IN ORNAMENTAL CROPS

Carotenoids are synthesized in chloroplasts and are essential for protecting tissues against photo-oxidative damage in the green tissues of higher plants (Britton, 1998). In flowers, carotenoids synthesized in the chromoplasts provide colour to the petals, ranging from yellow to red, in order to attract pollinators (Grotewold, 2006 and Tanaka *et al*. 2008). The colour of a flower is an important character that determines the commercial value of ornamental plants.

3.1. Carotenoid composition and carotenogenic gene expression during *Ipomoea* petal development.

Japanese morning glory (Ipomoea nil) is a representative plant lacking a yellow-flowered cultivar, although a few wild Ipomoea species contain carotenoids in their petals such as Ipomoea sp. (yellow petals) and I. obscura (pale-yellow petals). In the present study, carotenoid composition and the expression patterns of carotenogenic genes during petal development were compared among I. nil, I. obscura, and Ipomoea sp. to identify the factors regulating carotenoid accumulation in Ipomoea plant petals. In the early stage, the carotenoid composition in petals of all the Ipomoea plants tested was the same as in the leaves mainly showing lutein, violaxanthin, and β-carotene (chloroplast-type carotenoids). However, in fully opened flowers, chloroplast-type carotenoids were entirely absent in I. nil, whereas they were present in trace amounts in the free form in I. obscura. At the late stage of petal development in Ipomoea sp., the majority of carotenoids were β-cryptoxanthin, zeaxanthin, and β-carotene (chromoplast-type carotenoids). In addition, most of them were present in the esterified form. Carotenogenic gene expression was notably lower in I. nil than in Ipomoea sp. In particular, β-ring hydroxylase (CHYB) was considerably suppressed in petals of both I. nil and I. obscura. The CHYB expression was found to be significantly high in the petals of Ipomoea sp. during the synthesis of chromoplast-type carotenoids. The expression levels of carotenoid cleavage genes (CCD1 and CCD4) were not correlated with the amount of carotenoids in petals. These results suggest that both I. obscura and I. nil lack the ability to synthesize chromoplast-type carotenoids because of the transcriptional down-regulation of carotenogenic genes. CHY B, an enzyme that catalyses the addition of a hydroxyl residue required for esterification, was found to be a key enzyme for the accumulation of chromoplast-type carotenoids in petals were analysed by HPLC. V, violaxanthin; N, neoxanthin; L, lutein; Z, zeaxanthin; A, antheraxanthin.

3.1.1. Changes in carotenoid composition during petal development in *Ipomoea.*

HPLC chromatograms of the carotenoid extracts obtained from the leaves of *Ipomoea* sp., *I. obscura*, and *I. nil* were similar. A representative chromatogram of *Ipomoea* sp. is shown in (Fig. 3A). The majority of the carotenoids in leaves were lutein, violaxanthin, and β-carotene, which are essential for photosynthesis. Carotenoids in the non-saponified extract from leaves exhibited an HPLC chromatogram similar to those in the saponified leaf extract, except that chlorophyll *a* and chlorophyll *b* were detected in the non-saponified extract (Fig. 3B). The carotenoid composition in leaves was designated

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'chloroplast-type carotenoid'. The total carotenoid content in the leaves of all tested cultivars was around 300 µg g FW. Changes in the HPLC chromatograms of carotenoid extracts during petal development in *Ipomoea* plants are shown in (Fig. 4B and C). At stage 1, all petals tested were pale green and showed the same chromatograms as chloroplast-type carotenoids, mainly showing lutein, violaxanthin, and β-carotene, albeit at lower levels than in leaves (<10 µg g FW). In petals of *I. nil* at stage 4, the carotenoid content decreased below the detection limit, whereas small amounts of chloroplast-type carotenoids remained in *I. obscura*, and the carotenoids existed in the free form just as in leaves. In petals of *Ipomoea* sp. at stage 4, the carotenoid composition (designated 'chromoplast-type carotenoid') was completely different from the chloroplast-type carotenoids: lutein and violaxanthin levels were drastically reduced, and approximately 85% of the total carotenoids were made up of β-cryptoxanthin, zeaxanthin, and β-carotene. In addition, xanthophylls such as β-cryptoxanthin and zeaxanthin existed in the esterified form (Fig. 4C).

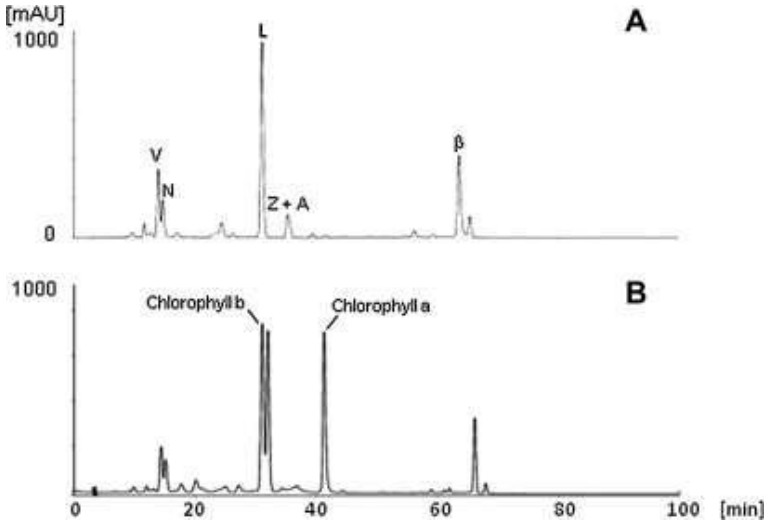

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Fig.3: Carotenoid analysis in leaves of Ipomoea plants. Saponified (A) and nonsaponified (B) carotenoids extracted from 0.1 g fresh weight (FW) of leaves of Ipomoea sp.

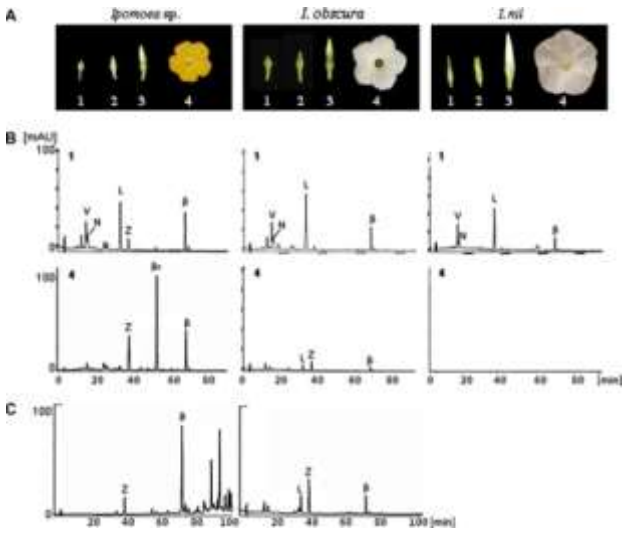

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Fig. 4:Changes in carotenoid composition during petal development in Ipomoea plants. (A) Photographs of flowers at stages 1 and 4. HPLC elution profiles of saponified (B) and non-saponified (C) carotenoids extracted from petals of each species at various stages.

3.2.Carotenoid components in petals of yellow flower camellia species

Carotenoid components of eight yellow flower Camellia species were analyzed by HPLC (Fig.5). Carotenoid contents of these flower petals ranged from 0.8 to 11.3 µg

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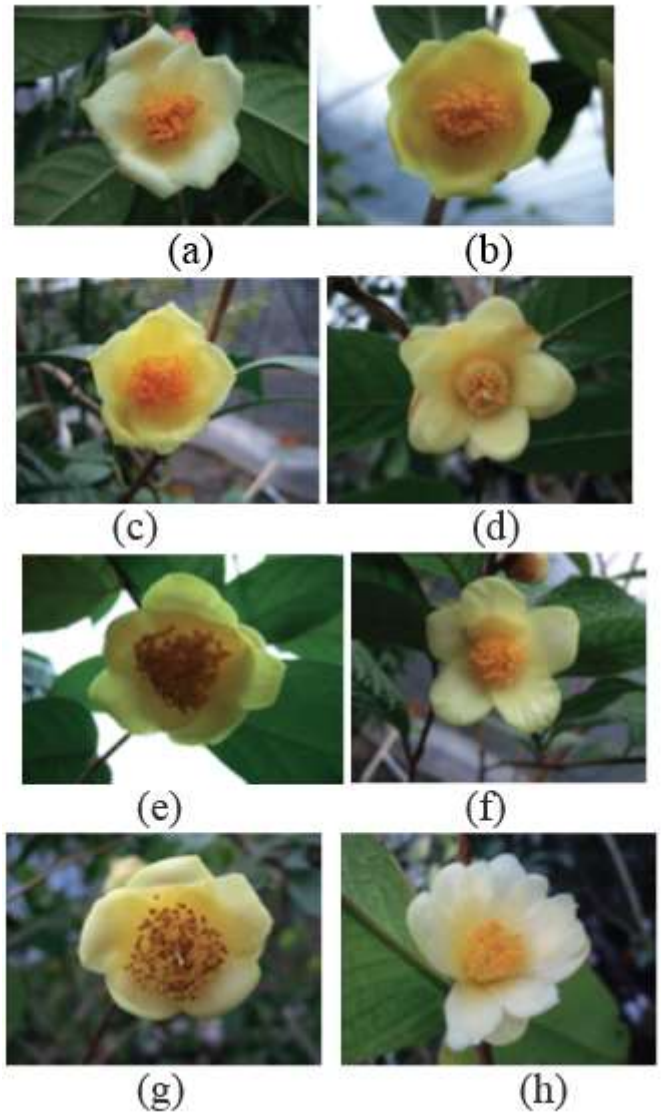
lutein equivalent • g^{-1}Fresh Weight (FW).Violaxanthin, (9Z)-violaxanthin, luteoxanthin, antheraxanthin, β-cryptoxanthin, and β-carotene were identified as carotenoid components and these compositions were similar among all of the examined yellow flower Camellia species. These carotenoids were accumulated as esterified forms in the petals (Natsu Tanikawa *et al*. 2010).



Natsu Tanikawa et al.2010

Fig. 5: Yellow flower Camellia species (a) C. chrysantha (b) C. chrysantha (c) C. chrysantha var. phaeopubisperma, (d) C. cucphuongensis, (e) C. fusuiensis, (f) C. impressinervis, (g) C. ptilosperma, (h) C. quephongensis.

3.3. Isolation, Stabilization and Characterization of Xanthophyll from Marigold Flower- Tagetes Erecta-L.

Marigold (*Tagetes Erecta L*), an ornamental plant belonging to the composite family, has a rich source of natural antioxidant-Lutein. A natural pigment, xanthophylls offer an alternative to synthetic dyes as a food colourant, due to its non-toxicity. Chromatographic separations of saponified and unsaponified oleoresin were performed and Trans-Lutein identified as the major constituent. Well-preserved flowers exhibit a high yield of Xanthophyll content (105.19 g/Kg) in contrast to the unpreserved flower sample (54.87 g/Kg), emphasizing the significance of flower preservation in the extraction of xanthophyll. The stability and amount of xanthophyll also increased from 105.19 g/Kg to 226.88 g/Kg on saponification and subsequent purification with Ethylene Dichloride (Pratheesh*et al.*2009).

Carotenoids are abundant in fruits and plants and are widely used an antioxidant and may be useful in the prevention of diseased including cancer. The consumption of lutein and zeaxanthin reduces 40 % of the age related macular degeneration .The xanthophylls because of their yellow to orange-red colouration and natural occurrence in human foods, also finds its use as a food colourant. Therefore there exist a high demand for the significantly pure Xanthophyll that can be used as a food colourant and a nutrient supplement. Flowers such as *Tagetes* comprise different species about 33 in number, helenium, helianthus, sunflower, dandelion and many others. Of these, most concentrated source of xanthophylls is of the order 4500mg/lb (Verghese, 1998b) in the petals of *Tagetes Erecta* L (African marigold, Aztec marigold, Zempasuchil). Marigold flower petals are a significant source of the Xanthophyll and have a much higher concentration of this pigment compared to other plant materials (Verghese, 1998a). Marigold flower (*Tagetes* Depending on the varieties, cultivar and horticulture practices, the yield of flower showed remarkable variations in number and in flower weight (from 11 to 30 ton/hectare). Although flower is made up of petals, calyx pedicle, seeds etc., approximately 40% to 50% of the flower consist of petals. Extraction studies of petals and total flower with hexane showed that only flower petals contain xanthophylls. Calyx contains chlorophyll which in-turn affects absorption of xanthophylls by broilers and layers (Verghese. J , 1998b). Hence, only the petals are used for the isolation of oleoresin. The main colouring component of Marigold flower is lutein ($C_{40}H_{56}O_2$). Free Lutein hardly exists in the flower and it naturally occurs in the acylated form. The Lutein ester concentration in fresh Marigold flowers varies from 4 mg/Kg in greenish yellow flowers to 800 mg/Kg in orange brown flowers (Sowbhagya *et al.* 2004). Dark-coloured flowers contain about 200 times more Lutein esters than the lightcoloured flowers. Xanthophyll content varies in the range of 9 to 11 g/Kg. The concentration of Lutein varies in different shades of marigold flowers, viz.; greenish yellow to bright yellow and orange brown (Gregory*et al.*1986). Total Lutein esters have been reported to be in the range of 3.8 to 791 mg/Kg of flower (Sowbhagya *et al.* 2004). Lutein palmitate is the major ester in the flower. The other esters of lutein identified in

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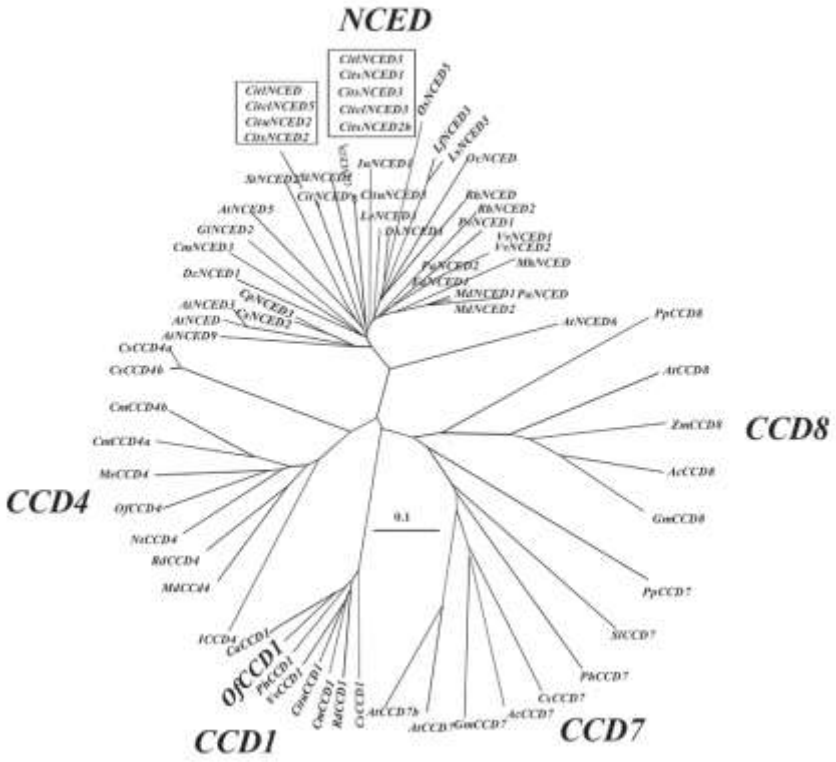
the flower are dimyristate, myristate palmitate, palmitate sterate, and distearate. A purified extract of marigold petals mainly containing Xanthophylls dipalmitate is marketed as an ophthalmologic agent (Sowbhagya et al. 2004, Gau *et al*. 1983). Lutein is stable in pH range 3 to 9. At extreme pH and in the presence of light, lutein undergoes isomerization resulting in colour loss. Lutein structure consists of conjugated bonds, which when react with the oxygen present in air, cause oxidation to take place and lead to colour loss. Oxidation products of xanthophylls are mono and di-epoxides, carbonyls, alcohols etc. and extensive oxidation results in bleaching of carotenoid pigments. To minimize colour loss, it is safe to pack lutein-containing products in tin or opaque containers.

3.4. Biosynthesis of α- and β-ionone, prominent scent compounds, in flowers of *Osmanthus fragrans.*

Carotenoid derived volatiles are important fragrance compounds, which contribute to the scents of flowers from diverse taxa. A famous example is represented by the flowers of Osmanthus fragrans where apocarotenoids account for more than 20% of all volatiles (Fig.6). Bio-degradation of carotenoids has been shown to be an important route for apocarotenoids formation. Here it has been reported on the contribution the O. fragrans carotenoid cleavage dioxygenase 1 to the synthesis of the two predominant C13-apocarotenoids, α- and β-ionone, derived from α-and β-carotene, respectively ,Susanne *et al.*, 2012.

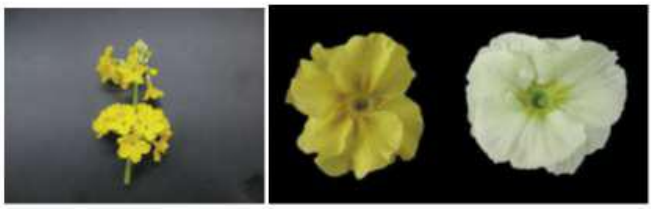


Susanne baldermann et al.2012

Fig.6: Unrooted phylogenetic tree of cDNA sequences of CCDs involved in the cleavage of carotenoids and apocarotenoids in Osmanthus fragrans

3.5. Carotenoid Composition in the Yellow and Pale Green Petals of *Primula* Species.

The carotenoid composition in the yellow petals of *Primulaxpolyantha* and *P. helodoxa*, and in the pale green petals of *P.* ×*polyantha* was analyzed by high-performance liquid chromatography(Fig. 7 a,b). The major carotenoids detected in the yellow petals were (9*Z*)-violaxanthin, (all-*E*)-violaxanthin, lutein, and antheraxanthin. The carotenoid composition in the pale green petals was completely different from that in the yellow petals; the former accumulated predominantly lutein and β-carotene. Carotenoids in the yellow petals were present in the esterified form, while those in the pale green petals were in the free form (Chihiro Yamamizo *et al.*2011).



7a.*P.* ×*polyantha* (Yellow)

7b. *P.*× *polyantha* (Pale green)

Chihiro yamamizo et al.2011

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IV. REGULATION OF CAROTENOID BIOSYNTHESIS IN PETALS AND LEAVES OF CHRYSANTHEMUM (CHRYSANTHEMUM MORIFOLIUM)

Carotenoid composition and the content and expression of genes encoding isoprenoid and carotenoid biosynthetic enzymes in petals and leaves of chrysanthemums were analyzed. Most of the carotenoids in yellow petals were β, ε-carotenoids, lutein and its derivatives, reflecting the high expression levels of lycopene ε-cyclase (LCYE). In contrast, the ratio of β, β-carotenoids to total carotenoids in leaves were higher than that of β, εcarotenoids, reflecting the high expression levels of lycopen β-cyclase (LCYB). Petals of the yellow-flowered cultivar Yellow Paragon showed increased accumulation and drastic componential changes of carotenoids as they mature. In petals of the white-flowered cultivar Paragon, carotenoid content was drastically decreased during petal development and became less than the detection limit late in development. Transcript levels of most genes tested increased during petal development in Yellow Paragon. All genes except that for 1-deoxyxylulose 5-phosphate synthase (DXS) showed similar expression patterns in Paragon. Between-cultivar comparison of the expression of these genes in the petals at mid-development showed no distinct differences between petal colour. It is possible that the formation of white petal colour is due to neither down-regulation nor destruction of the carotenoid biosynthetic pathway. We presume that another factor inhibits carotenoid accumulation in chrysanthemum petals.

The green tissues of most plants show similar carotenoid profiles, containing both β,ε-carotenoids (α-carotene derivatives) and β,β-carotenoids (β-carotene derivatives) (Goodwin and Britton 1988). The essential carotenoids for plant photosynthesis, such as zeaxanthin, violaxanthin, and antheraxanthin, are invariably found in the green tissues. In contrast, carotenoids in flowers show distinctive compositions that depend on the plant species (Deli *et al.* 1988, Eugster and Märki-Fischer 1991, Kull and Pfander 1997, Maoka *et al.* 2000, Tai and Chen 2000, Kishimoto *et al.* 2005). For example, petals of tiger lily (Lilium lancifolium) contain only β-carotene derivatives (Deli *et al.* 2000). Compositae plants tend to accumulate mainly lutein, an α-carotene derivative, and lutein derivatives in their petals; for example, marigold (Tagetes erecta) accumulate a large amount of lutein (Khachik *et al.* 1999).

In general, transcriptional activation of carotenoid biosynthetic enzymes is thought to be the major factor in the upregulation of carotenoid accumulation in many fruits and flowers (Hirschberg, 2001; Fraser and Bramley 2004; Taylor and Ramsay 2005). However, various other post-transcriptional factors coming into play during the regulatory process were reported recently (Al-Babili *et al.* 1996, Liu *et al.* 2004). A variety of factors might be involved in the regulation of carotenoid biosynthesis, therefore, further work in this area is required. It is well known that some plant species, especially horticultural crops, have a wide variety of carotenoid contents in petals even within the same species. Moehs *et al.* (2001) reported that DXS and PSY might be responsible for the colour development from pale yellow to deep yellow in marigold petals. In Sandersonia, petals of pale yellow-flowered cultivars showed lower expression of PDS than those of yellow-flowered cultivars, and levels tended to be proportionate to carotenoid accumulation level (Nielsen *et al.* 2003).

Chrysanthemum (C. morifolium Ramat.) is one of the most important ornamental plants in the world, and the petal colour of yellow-flowered cultivars originates mainly from carotenoid pigments. Kishimoto *etal.* 2004 showed the uniqueness of the carotenoid composition in petals of chrysanthemum: approximately 92% of the total carotenoids isolated from petals of yellow-flowered chrysanthemum were lutein and its derivatives. This yellow colouration due to carotenoids in chrysanthemum petals is a recessive trait against a white one (Langton, 1980). Hattori (1991) suggested that a single dominant gene inhibiting carotenoid biosynthesis exists. The gene has not yet been identified, and the factor regulating carotenoid accumulation in chrysanthemum petals is still unknown.On analysis the carotenoid composition and content in petals and leaves of yellow- and white flowered chrysanthemum cultivars during their development showed the expression of genes encoding carotenoid and isoprenoid biosynthetic enzymes in those cultivars. By comparing the behaviour of carotenoid pigments and biosynthetic genes, clarified the factor that determines the characteristic accumulation of carotenoids in chrysanthemum petals.

4.1. Materials and methods

4.1.1. Plant materials

Three white-flowered cultivars (Paragon, White Marble, and Fiducia) and three yellow-flowered cultivars (Yellow Paragon, Florida Marble, and Sunny Orange) of chrysanthemum (Chrysanthemum morifolium Ramat.) were grown in greenhouses at the National Institute of Floricultural Science (Tsukuba, Ibaraki, Japan) (Fig.8 B). Yellow Paragon and Florida Marble are bud variants of Paragon and White Marble, respectively. Petal development was divided into four stages from which RNAs and carotenoids were extracted: very early (VE), early (E), middle (M), and late (L) (Fig.8 A). The lengths of petals were ca. 2–3 mm at stage VE, ca. 8–10 mm at stage E, ca. 15–18 mm at stage M, and ca. 30–35 mm at stage L. Flowers

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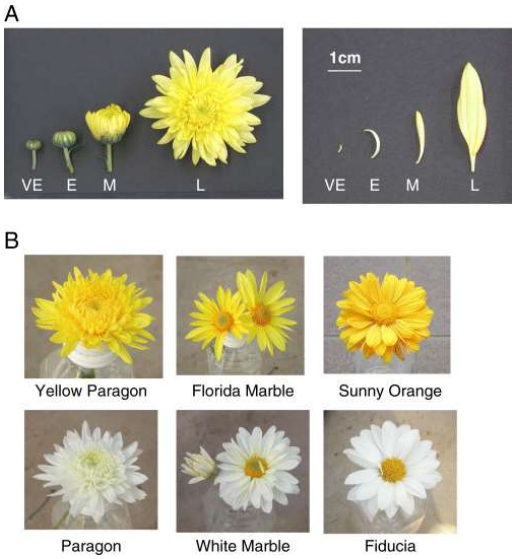
were fully opened at stage L. Leaf development was divided into three stages from which carotenoids were extracted: early (E), middle (M), and late (L). RNAs were extracted from stage L. The lengths of leaves were ca. 15–25 mm at stage E, ca. 35–45 mm at stage M, and ca. 60–70 mm at stage L. Leaves were fully developed at stage L.



Kishimoto and Ohmiya et al.2006

Fig.8. (A) Sampling stages of chrysanthemum petal development and (B) fully expanded flowers of chrysanthemum cultivars used for the experiment. VE = very early, E = early, M = medium, and L = late.

4.1.2. Carotenoid extraction and HPLC analysis.

Each extract was analysed by HPLC with a Jasco MD-915 photodiode array detector (Jasco, Tokyo, Japan). HPLC analysis was performed under the following conditions: column, YMC Carotenoid (250 × 4.6 mm i.d., 5 μm; YMC, Kyoto, Japan); solvent A, methanol (MeOH) / methyl tert-butyl ether (MTBE) / H2O = 90:6:4 (v/v/v); solvent B, MeOH / MTBE / H2O = 25:71:4; gradient, 0/100, 12/100, 96/0 (min/% A); flow rate, 1.0 mL min−1; column temperature, 35 °C; UV/visible monitoring range, 200–600 nm. The amounts of total carotenoids were calculated according to the total peak area of HPLC chromatograms at a wavelength of 450 nm.

4.1.3. HPLC analysis of carotenoids:

The carotenoid components and concentrations at the four stages of petal development in Yellow Paragon and Paragon are shown in Figures 9 and 10, respectively. Petals of Yellow Paragon showed increased accumulation and drastic componential changes of carotenoids as they matured. Lutein, β-carotene, and violaxanthin were detected at stage VE, then violaxanthin and β-carotene disappeared and lutein was dominant at stage E. At stage M, (3*S*, 5S, 6R, 3′*R*, 6′*R*)-5, 6-dihydro-5, 6-dihydroxylutein, *cis*-forms of lutein ((9*Z*)-lutein and (9′*Z*)-lutein), and lutein epoxides ((9*Z*)-lutein-5, 6-epoxide and (9′*Z*)-lutein-5, 6-epoxide) were detected in addition to lutein. The chromatogram at stage L showed a large decrease in lutein and (9′*Z*)-lutein contents, and a drastic increase in (9*Z*) - lutein and (9′*Z*)-lutein-5, 6-epoxide contents.

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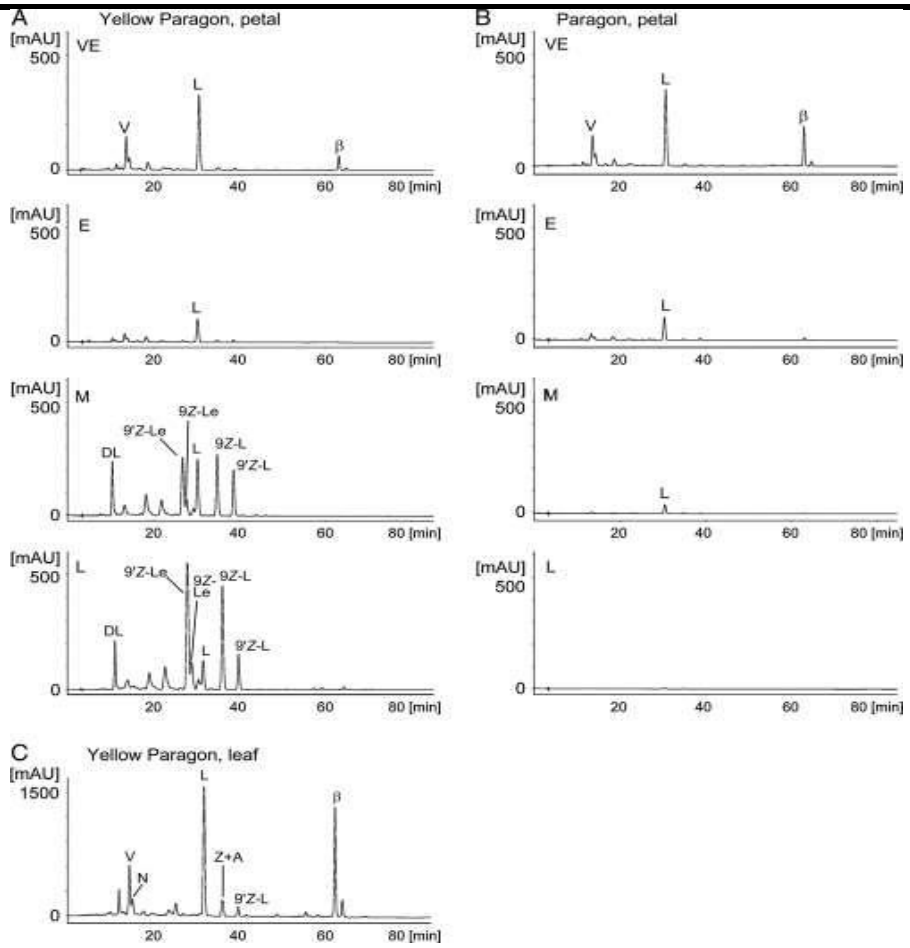
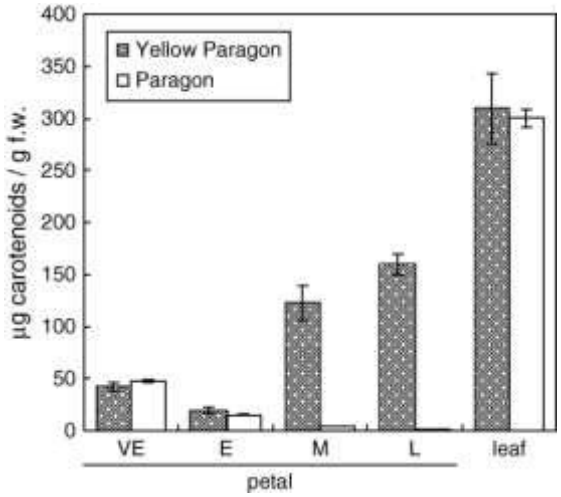


Kishimoto and Ohmiya et al.2006

Fig. 9: Carotenoid analysis in petals and leaves of Yellow Paragon and Paragon. Carotenoid extracts from 0.1 g f.w. of petals of (A) Yellow Paragon and (B) Paragon, and (C) leaves of Yellow Paragon were analysed by HPLC.



Kishimoto and Ohmiya et al.2006

Fig.10: Total carotenoid contents in petals and leaves of Yellow Paragon and Paragon.

4.1.4. Comparison of gene expression between petals and leaves.

In the comparison of petals and leaves by Northern analysis, the most striking feature was the difference in expression levels of LCYB and LCYE. Real-time PCR was performed to compare the expression levels of these genes. In petals of yellow-flowered cultivars, the levels of LCYE transcripts were remarkably higher than those of LCYB. In contrast, LCYE showed extremely lower levels in leaves, reflecting the lower ratio of β, ε-carotenoid content. Leaves showed higher transcription levels of ZEP and VDE, which encode enzymes that catalyse the synthesis of carotenoids that are essential for photosynthesis, than did petals . The expression level of

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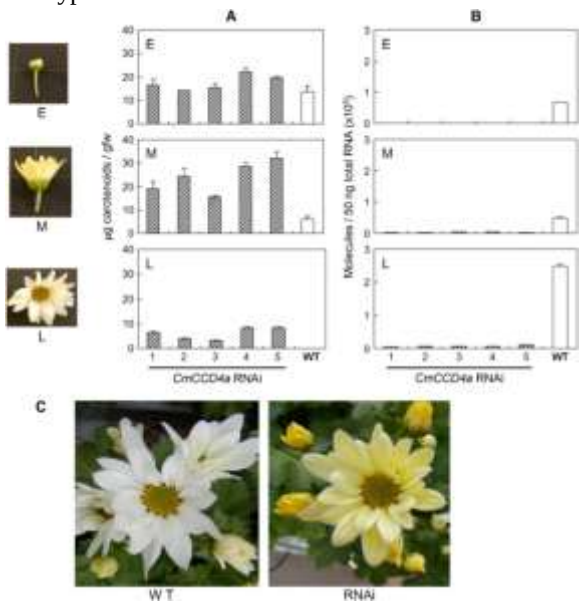
DXR was also remarkably higher in leaves. On the other hand, levels of DXS and CHYB were lower in leaves.

4.1.5. Carotenoid Cleavage Dioxygenase (CmCCD4a) Contributes to White Colour Formation in Chrysanthemum Petals.

The white petals of chrysanthemum (*Chrysanthemum morifolium* Ramat.) are believed to contain a factor that inhibits the accumulation of carotenoids. To find this factor, polymerase chain reaction- Select subtraction screening was performed and obtained a clone expressed differentially in white and yellow petals. The deduced amino acid sequence of the protein (designated CmCCD4a) encoded by the clone was highly homologous to the sequence of carotenoid cleavage dioxygenase. All the white-flowered chrysanthemum cultivars tested showed high levels of *CmCCD4a* transcript in their petals, whereas most of the yellow flowered cultivars showed extremely low levels. Expression of *CmCCD4a* was strictly limited to flower petals and was not detected in other organs, such as the root, stem, or leaf. White petals turned yellow after the RNAi construct of *CmCCD4a* was introduced. These results indicate that in white petals of chrysanthemums, carotenoids are synthesized but are subsequently degraded into colourless compounds, which results in the white colour. The chrysanthemum (*Chrysanthemum morifolium* Ramat.), which has been bred for more than 2,000 years, is one of the most important ornamental flowers in the world. The petal colour of yellow-flowered cultivars originates mainly from carotenoids. Understanding the mechanism that controls carotenoid accumulation in petals will not only contribute greatly to the breeding of chrysanthemums and other flowering plants but also provide important information about the molecular evolutionary mechanisms responsible for different petal colours. Cultivated chrysanthemums are thought to have originated from hybrids between white- and yellow-flowered wild species. On the basis of an experiment in which white- and yellow-flowered chrysanthemums were crossed, Hattori (1991) observed that the white petal colour is dominant over yellow and suggested that a single dominant gene that inhibits carotenoid accumulation may exist. The detailed function of such a gene, however, is still unknown. Kishimoto and Ohmiya (2006) demonstrated no significant difference between the expression levels of carotenoid biosynthetic genes in white and yellow petals during the course of development. In addition, the carotenoid content in immature white petals is almost equal to that in yellow petals, and the carotenoid content decreases to undetectable levels as the white petals mature. These results indicate that the formation of white colour is caused neither by down regulation nor by disruption of the carotenoid biosynthetic pathway.To find a factor that controls carotenoid content in chrysanthemum petals, PCR-Select subtraction screening was performed to search for cDNAs that were differentially expressed in white and yellow petals.

4.1.6. Suppression of *CmCCD4a* Expression by RNAi.

To determine the role of *CmCCD4a* gene products in the formation of petal colour, transgenic chrysanthemum plants were produced with reduced expression of *CmCCD4a*. The RNAi construct of *CmCCD4a was introduced under* the control of the tobacco (*Nicotiana tabacum*) elongation factor 1*α* promoter into the white-flowered chrysanthemum cultivar Sei-Marine. Five independently derived transgenic plants were analysed. Carotenoid content values in middle-stage petals of these transgenic lines were about 3 to 6 times the values observed in wild-type plants (Fig.11 A). During late-stage petal development, when petals of wild-type plants completely lost their carotenoids, the petals of transgenic lines contained 3 to 8 *μ*g/g fresh weight of carotenoids and looked yellow (Fig. 11 B). Quantitative real-time RT-PCR analysis showed that the petals of the transgenic lines expressed the *CmCCD4a* gene at only 2% to 4% of the wild-type level.



Akemi et al.2006

Fig.11: Suppression of CmCCD4a expression by RNAi. A, Changes in carotenoid concentrations during petal development of wild-type (WT) and five independent CmCCD4a RNAi plants (1–5).

4.1.7. *CmCCD4a* in Wild Chrysanthemum Species

Genomic PCR analysis was also performed in white- and yellow-flowered wild species of chrysanthemum (Fig.12). The bands that corresponded to *CmCCD4a* were observed in all the white-flowered species but not in the yellow-flowered species. In contrast, the bands that corresponded to *CmCCD4b* were observed in both white- and yellow-flowered species.

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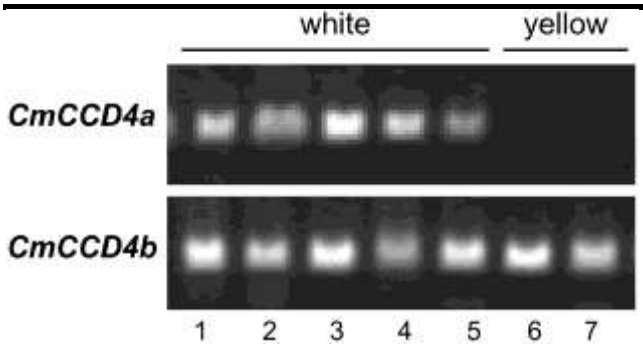

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Fig.12: Genomic PCR of white- and yellow-flowered wild species of chrysanthemum with CmCCD4a and CmCCD4b primers: 1, Chrysanthemum boreale; 2, Chrysanthemum indicum; 3, Chrysanthemum makinoi; 4, Chrysanthemum japonese; 5, Chrysanthemum yezoense; 6, Chrysanthemum sp.

4.1.8. Light Microscope Observation of Transverse Sections of Petals.

Among yellow-flowered cultivars, only Yellow Paragon expressed *CmCCD4a* in petals. It is possible that petals of Yellow Paragon are periclinal chimera, and either the L1 or the L2 layer may behave genetically in a manner identical to that of the white progenitor Paragon. Periclinal structures were determined by microscope examination of transverse sections of petals (Fig.13). In the sections of Yellow Paragon, yellow pigmentation was localized in the adaxial epidermis (L1 layer), and the underlying mesophyll (L2 layer) appeared to be white. In contrast, both the L1 and L2 layers were yellow in petals of Super Yellow.

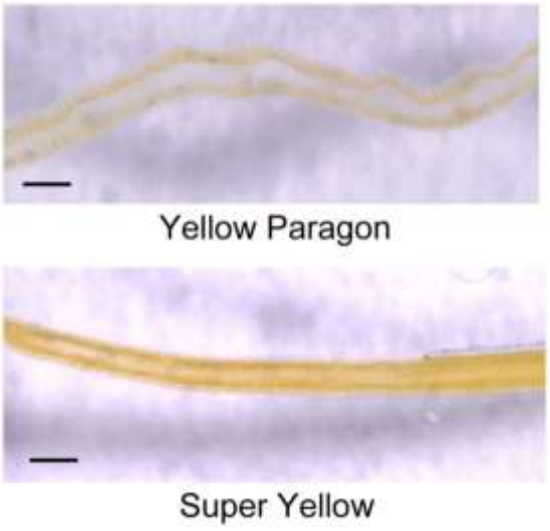

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Fig. 13: Light microscope observation of transverse section through petals of Yellow- flowered cultivars Yellow Paragon and Super Yellow.

V. CONCLUSION

- Knowledge at the biochemical and molecular level has made it possible to develop novel colour which are otherwise absent in nature.
- Metabolic cross-stock between different pathway makes the production of transgenic challenging.
- Petals of the yellow-flowered cultivar Yellow Paragon showed increased accumulation and drastic componential changes of carotenoids as they mature, compared to petals of the white-flowered cultivar Paragon that showed drastically decreased carotenoid content during petal development.
- All the white-flowered chrysanthemum cultivars tested showed high levels of CmCCD4a transcript in their petals, whereas most of the yellow flowered cultivars showed extremely low levels indicating that in white petals of chrysanthemums, carotenoids are synthesized but subsequently degraded into colourless compounds, which results in the white colour.
- Studying the regulatory mechanisms underlying carotenoid accumulation in Ipomoea plants at the molecular level will help in producing yellow-flowered cultivars by plant transformation.
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Nanocrystalline Nickel Zinc Ferrite as an efficient alcohol sensor at room temperature

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Abstract— *In the present communication, nanocrystalline nickel zinc ferrite (NZF) has been successfully synthesized by temperature and spin controlled coprecipitation techinique. The structural and surface morphological characterizations of the sample have been analyzed by means of Powder X-ray Diffraction (PXRD) and Field Emission Scanning Electron Microscopy (FESEM). The minimum crystalline size of prepared NZF sample calculated from Scherer's formula and is found to be 25 nm. FESEM images exhibit the porous nature of the sensing material with a number of active sites. In a comparative study on the sensing characteristics of nanostructured NZF pellet towards three primary alcohols viz. ethanol, propanol and butanol, the maximum sensitivity is found to be nearly 90% for 1000 ppm of the ethanol vapour at room temperature. The sensing response followed the order of ethanol > propanol > butanol with respect to time. The experimental results show that nanostructured NZF is a promising material for alcohol sensor. The sensor responses are quite stable and highly reproducible even at room temperature.*

Keywords—Coprecipitation synthesis, NiZn ferrites, nanostructural analysis, porosity, VOC sensor.

I. INTRODUCTION

The demand for portable vapour sensors are increasing now-a-days along with the progress in the electronics industry. There is also a need to enhance the quality of vapour sensors. Many of the studies reported that binary metal oxides exhibit a high sensitivity to alcohols, but are known to suffer from poor selectivity and high working temperature [1]. Recently, nanosized mixed metal ferrite materials have received considerable attention in vapour sensor application as they exhibit more selectivity and stability for a particular gas and organic vapour [2]. Spinel type oxide semiconductors with formula MFe_2O_4 have been reported to be sensitive materials to both oxidizing and reducing gases [3]. Liu et al [4] reported the high sensitivity of $CdFe_2O_4$ towards ethanol vapour, Reddy et al [5] investigated $NiFe_2O_4$ as sensor to detect Cl_2 in air. Chen et al [6] revealed that $MgFe_2O_4$ and $CdFe_2O_4$ are sensitive and selective to LPG and C_2H_2. Among the various ferrite materials, zinc ferrite is an important n-type semiconducting material widely applied for the detection of acetone, ethanol, hydrogen and H_2S because of its good chemical and thermal stability [7-10]. The review surveys revealed that the nanosized ferrite materials, which have high surface activity due to their small particle size and enormous surface area, have been widely studied in the field of vapour sensors in recent years. Mixed metal ferrites offer more sensitive, selective and long-term stable sensor materials [11].

The aim of the present work is to compare the prepared nanostructured NZF towards various primary alcohols like, ethanol, propanol and butanol at room temperature. Aliphatic primary alcohols like ethanol, propanol and butanol have been widely used in various industrial and scientific applications. Ethanol is a hypnotic solvent and it is widely applied in the manufacture of wine, medical processes and food industries. A continuous monitoring of ethanol is required in wine industry in order to determine the quality and flavour of wine. Ethanol can also be measured in breath analysis [12]. Propanol is used as a solvent for several organic compounds. It is widely used as a cleaning agent and especially in dissolving oils. Propanol is a skin irritant and its long term exposure can lead to a series of health complications [13]. Butanol is widely used as solvent mainly in textile and chemical industries. It has its application as paint thinner. Moreover, it is widely used in the manufacture of biofuels now-a-days. But its toxicity lies as a severe eye and skin irritant. Prolonged exposure to fumes can cause danger and affects the central nervous system. Hence there is a great demand for monitoring these primary alcohol vapours.

II. MATERIALS AND METHODS

2.1. Preparation

The sample with chemical composition $Ni_{0.5}Zn_{0.5}Fe_2O_4$ (NZF) has been prepared successfully by temperature and spin controlled coprecipitation technique [14-16]. A.R

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grade Zinc sulfate heptahydrate, Nickel chloride hexahydrate, and anhydrous Ferric chloride are dissolved in distilled water with appropriate molar ratio. Stirring is done on magnetic stirrer for one hour to obtain homogeneous solution. The precipitation of metal hydroxides has been occurred by adding 2M NaOH solution by maintaining pH at 12 throughout the reaction. The precipitation then washed with distilled water repeatedly with stirring till the pH attained a value of ~ 7. The resulting solution is then filtered and the precipitation is dried at 100ºC for 24 hours. By grinding the flakes in agate mortar, the powdered form of the material is then annealed at 1200ºC for 24 hours. Annealed sample is regrind and stored in a dry and cool place for further characterization and analysis.

2.2. Structural characterization

PXRD has been recorded using a Bruker´D8 Advance´ Diffractometer (funded by UGC-DRS (SAP-II) DST (FIST-II), at Jadavpur University), equipped with a Globel mirror using Cu Kα (λ = 1.54184A°) radiation. The generator setting was maintained at 40kV and 40mA.The diffraction patterns has been recorded at room temperature with a counting time of 2 s/step over a range of $2\theta=20^0$- 90^0.

The lattice constant a for the prepared NZF sample is calculated from diffraction planes by using formula:

$$a = d\sqrt{(h^2 + k^2 + l^2)} \quad (1)$$

where d is the interplane spacing, $h, k, and\ l$ are the Miller indices of the crystal planes [17].

The theoretical density of the sample is calculated from X-ray data according to the relation:

$$\rho_x = \frac{8M}{Na^3} \quad (2)$$

Where $\rho_x$the density is calculated from XRD data, M is the molecular weight, N is the Avogadro's number, and a is the lattice constant of the cubic unit cell [18]. The experimental density ρ_m of the sintered sample was calculated by considering the cylindrical shape of the pellet and using the relation:

$$\rho_m = \frac{m}{\pi r^2 h} \quad (3)$$

where m is the mass, r is the radius and h is the thickness of the pellet [19].

Porosity P of the ferrite pellet is determined using relation:

$$P = \frac{\rho_x - \rho_m}{\rho_x} \times 100 \quad (4)$$

Where ρ_x and ρ_m are the theoretical and experimental densities [20].

Surface morphology of the samples are investigated by Field Emission Scanning Electron Microscopy (FESEM) (FEI, INSPECT F 50) equipped with an energy dispersive x-ray spectrometer system, (configuration no. QUO-35357-0614 funded by FIST-2, DST Government of India), at the Physics Department, Jadavpur University.

2.3. Measurements

A measured quantity of annealed powder is mixed with 1-2 drops of freshly prepared saturated solution of polyvinyl alcohol (PVA) and pressed in the form of circular disc with a diameter of 10 mm and thickness of 2.5 mm. About 5 tonnes of pressure is applied on the die by means of a hand press machine. The prepared pellet is again heated at 800° C for 4 hours to remove the organic binder. The surface of the pellet is coated with two planner highly pure silver paste electrode. The VOC sensing characteristics of the prepared sample is measured using a static flow vapour sensing set up, developed in our laboratory [21]. Vapour sensing measurements are performed in a closed test chamber at a static atmosphere at room temperature. In order to improve vapour sensor stability the sensing element is kept in the sensing chamber for more than 12 hours before testing.

III. RESULTS AND DISCUSSION

3.1. Material characterizations

XRD pattern of annealed NZF sample is presented in Fig. 1 below. The sharp diffraction peaks indicate a high degree of crystallization for the obtained metal ferrite compound. The sensing response is calculated using the given formula.

$$S\% = \left(\frac{\Delta R}{R_{air}}\right) \times 100$$

$$\text{or } S\% = \left(\frac{|R_{air} - R_{gas}|}{R_{air}}\right) \times 100 \quad (5)$$

Where R_{air} and R_{gas} are resistance in air and in presence of test vapours respectively and ΔR is the resistance variation. The resistance variation of NZF sample is recorded at room temperature with different alcohol vapours. Heater with thermocouple is used for resetting of the sensor pellet to conduct the repetitive experiments. This is also aimed to study at optimized temperature for different VOCs in varied

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concentrations. Some crystalline properties of NZF are shown in Table 1. The low measured density and porosity >50%, satisfying the requirements for materials used as organic vapour sensors [22]. Fig.2 presents the FESEM micrographs for the ferrite sample which reveals that the sample has nanosized grains with open porosity. Presence of both nanostructured grain size and porosity, increases the specific surface area making it a suitable material for vapour sensing applications. It is known that samples with higher specific surface have higher response to the organic vapour [23].

Table.1: Properties of prepared NZF vapour sensor

Composition	a (Å)	ρ_x(g/cc)	ρ_m(g/cc)	P(%)
$Ni_{0.5}Zn_{0.5}Fe_2O_4$	8.386085	5.35393	1.99137	62.80534

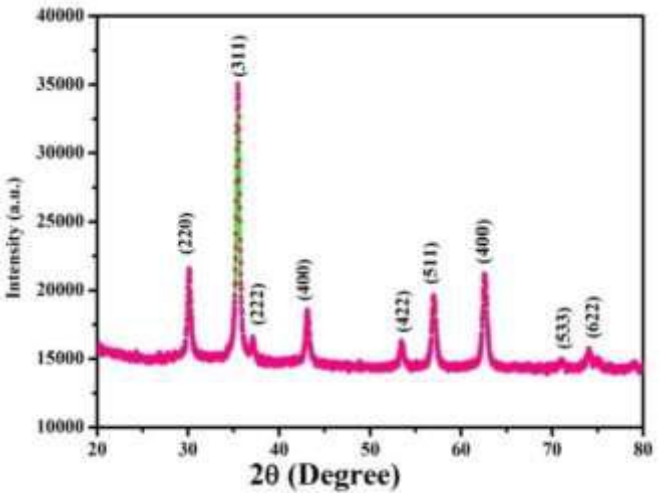

Fig.1: XRD pattern of nanocrystalline NZF

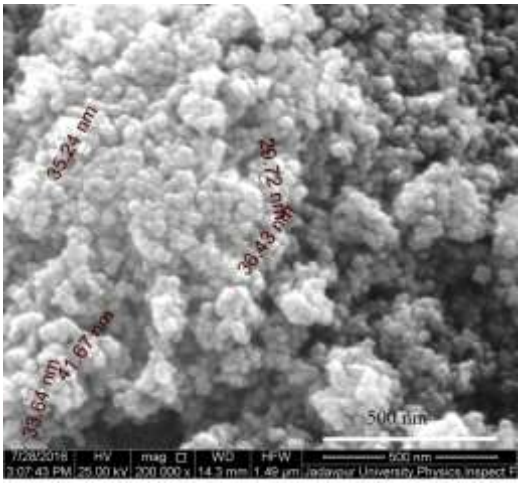

Fig. 2: SEM micrograph of nanocrystalline NZF

3.2. Alcohol sensing characteristics

3.2.1 Transient response study

The repetitive response of the NZF pellet towards 1000 ppm of ethanol, propanol and butanol vapours with time is shown in Fig. 3a, 3b and 3c. The resistance of the pellet sensor is measured, once in presence of air before the introduction of vapour and again after injection of a measured quantity of test vapours within the chamber every time. As soon as the alcohol vapours are inserted, the NZF pellet showed a decreasing trend in resistance response and thus an increase in the sensor response is observed. After reaching to the steady response, alcohol vapours are removed from the closed chamber by removing the lid and the resistance response is recorded again.

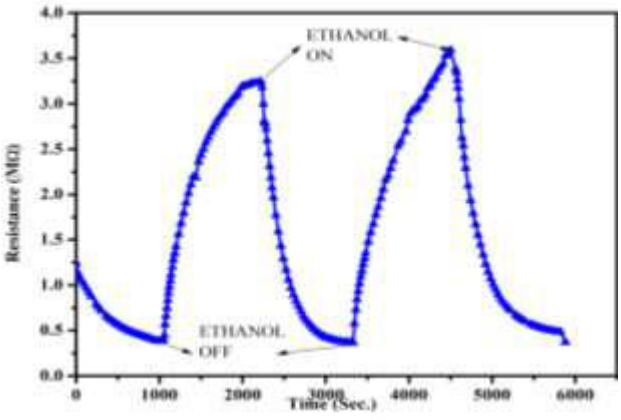

Fig.3a: Repetitive resistance response of ethanol

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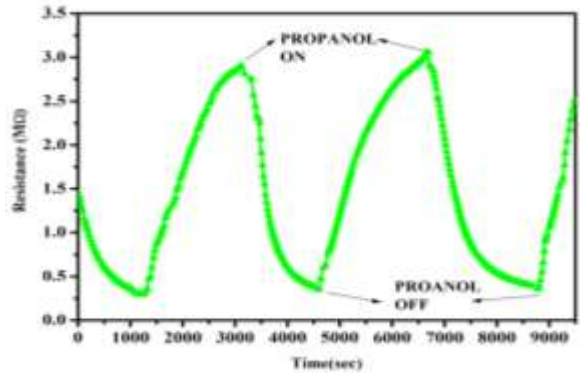

Fig.3b: Repetitive resistance response of propanol

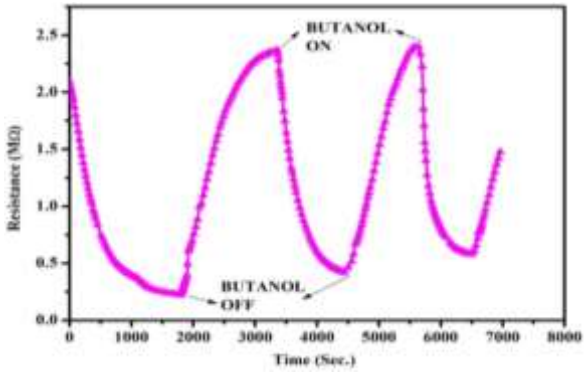

Fig.3c: Repetitive resistance response of butanol

3.2.2. Sensitivity study

The sensitivity response curves of the NZF pellet sensor obtained from 1000 ppm of ethanol, propanol and butanol is shown in Fig.4 whereas Fig.5 shows its response time towards different alcohols vapours at room temperature. The response time and recovery time of the sensor is shown in Table 2 where ethanol shows the lowest response and recovery time compared to other two test vapours. The stability data of the sensor is obtained under similar conditions at room temperature over a period of 30 days to confirm the reliability of the measurements. The response increases up to 80% within 8 mins and it reached to a steady value of 90% within 15 mins for 1000 ppm ethanol vapour. It took several minutes to recover the original resistance after removal of test vapours from the closed chamber. A long time recovery observed at room temperature is due to the agglomerated nature of the sensing element revealed by FESEM microstructure.

Table.2: Comparison for ethanol, propanol and butanol sensing characteristics of NZF at room temperature.

Type of Sensing Materials	Test vapours	Concentration (ppm)	Maximum response (%)	Response time (min)	Recovery time (min)
NZF	Ethanol	1000	≈ 90	≈ 15	≈ 20
	Propanol	1000	≈ 88	≈ 23	≈ 26
	Butanol	1000	≈ 81	≈ 25	≈ 28

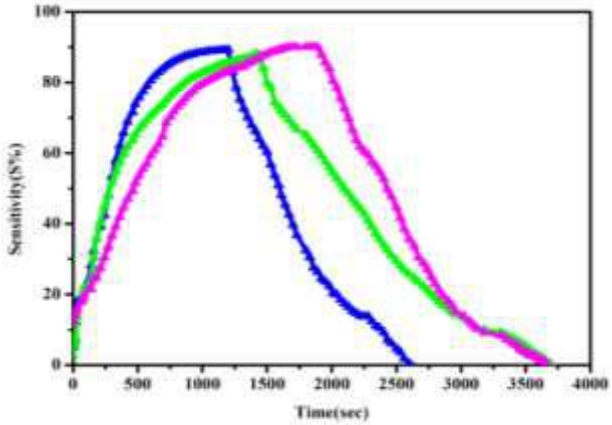

Fig.4: Sensitivity of NFZ towards ethanol (blue), propanol (green) and butanol (pink) at room temperature.

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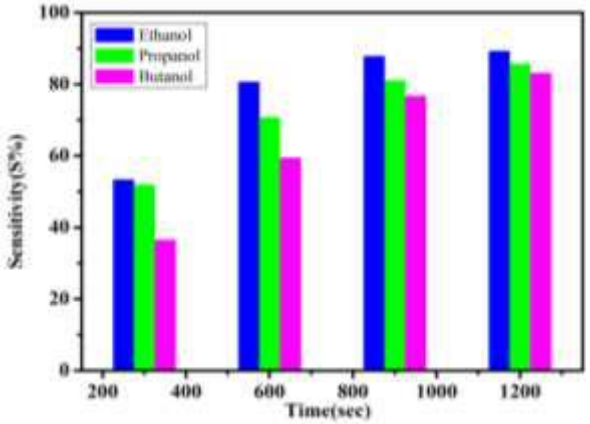

Fig.5: Response of NZF with time at room temperature.

1.2.2. Selectivity study

Selective detection of VOC is a big challenge for any commercial sensor. It is found that, the sample shows a very good and stable response towards aliphatic alcohol vapours at room temperature condition with a special mention on ethanol vapour sensing.

1.2.3. Sensing mechanism

It is observed that the resistance of the sensing element decreases when exposed to reducing vapours like ethanol, propanol and butanol which suggest that NZF behaves as an n-type semiconductor. The vapour sensing mechanism of the metal ferrite, described in the previous work, is a surface controlled phenomenon that is based on the surface area of the pellet sensor at which the vapour molecules adsorb and react with pre-adsorbed oxygen molecules [21, 23, and 24].

IV. CONCLUSION

When NZF is exposed to the primary aliphatic alcohol vapours, electrical resistance is shown to vary strongly with a wide range of response times and magnitudes at room temperature. Wide range of differential responses is observed across the various combinations of alcohol vapours, indicating the excellent potential for NZF to be used in the manufacture of primary aliphatic alcohol vapour detector, or electronic nose. The comparative study shows that the material is very much sensitive towards alcohol vapours especially in quick detection of ethanol vapours with high sensitivity and good stability at room temperature.

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Design and Fabrication of Densified Biomass Briquette Maker Machine

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Abstract— *The project we designing & fabricating is to reduce the problems of Cutting Trees for fire logs. "Leaf Log Maker Machine" is designed to make dry leaves get compressed and made to logs as a fuel. Typically, dead leaves are dumped a lot in landfills and one of the problems with leaving wet leaves to decompose like this is that they give off methane 20 times more poisonous gas than carbon dioxide. In contrast, when leaves are burnt, they only give off the carbon they absorb while on the tree they add nothing extra to the environment. This machine is compact, easily accessible & eco-friendly. It can also able to compress wood wastes, papers & tin cans. This machine is to make fuels from the natural resources like dry leaves, instead of cutting them. This machine is easy to understand the operation to user. It had come over many changes and modifications within it.*

Keywords— Briquetting, Biomass, Deforestation, Densification, Environmental friendly briquettes, Paper pulp.

I. INTRODUCTION

Leaf Log Maker Machine is a concept to make the dry leaves to be compressed for useful fire logs. Actually, the leaves which are left in landfills get decomposed and produce the methane gas (toxic level is 20 times more than carbon-di-oxide) harmful to human nature [1]. In contrast, when leaves are burnt, they only give off the carbon they absorb while on the tree they add nothing extra to the environment. So, we planned to make those leaves as fire logs instead of leaving in landfills, for the purpose of reducing the tree cutting for the fire logs which leads to one of the way of the Deforestation [1].

Biomass briquetting is the densification of loose biomass material to produce compact solid composites of different sizes with the application of pressure. Briquetting of residues takes place with the application of pressure, heat and binding agent on the loose materials to produce the briquettes. Two different types of densification technologies are currently in use. The first, called pyrolizing technology relies on partial pyrolysis of biomass, which is mixed with binder and then made into briquettes by casting and pressing. The second technology is direct extrusion type, where the biomass is dried and directly compacted with high heat and pressure [2].

Due to the present world's energy crisis and its related environmental issues as well as increasing trend of fossil fuel prices, renewable energy source is an essential matter. Biomass briquettes are a renewable source of energy and they avoid adding fossil carbon to the atmosphere. They are made from biomass and are a replacement for fossil fuels, and can be used to heat boilers in manufacturing plants, and also have applications in developing countries.

Biomass Briquetting Technology

Biomass densification represents a set of technology for the conversion of biomass residues into a convenient fuel. Depending on types of equipment used it could be categorised into five main types [3]:

1. Piston press densification
2. Screw press densification
3. Roller press densification
4. Pelletizing
5. Low pressure manual presses briquetting.

We use piston press densification technology to make log with the help of paper pulp as a binder.

II. MATERIAL AND METHODOLOGY

For the purpose of study, dry leaves was used for performance evaluation of machine. And also paper pulp was used as binding agent mainly to overcome the major problem of material compaction [4].

A. Design and construction

A biomass briquetting machine was designed and constructed (Fig.1). The briquetting machine was equipped with 20 tone hydraulic bottle jack with spring return mechanism. Hydraulic bottle jack applies pressure with the help of piston on biomass inside cylindrical

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mould measuring 105 mm internal diameter and 150 mm length.

The mould was splitted in two section for removing the briquette easily. The hydraulic bottle jack used applies pressure about 3000 psi on biomass. The machine was fabricated using EN8 carbon steel because of its properties like good tensile Strength and it often used for making shaft, gear, stressed, pins, studs etc.

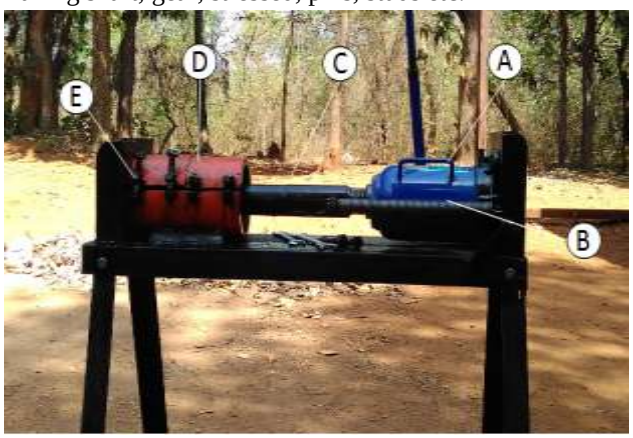

Fig.1 : The Leaf Log Maker Machine
A-Hydraulic Jack, B-Springs, C- Piston, D-Cylinder, E-Nut & Bolt

B. Preparation of briquette

The briquette was prepared at Rajendra Mane College of Engineering and Technology (Devrukh), University of Mumbai, India using manual hydraulic briquette machine with single cylindrical mould. The binders made with the help of waste paper which were manually torn to small pieces and sucked in water to form gelatinised paste. 3-5 days of socking in water was required to get a sticky solution.

Binder was added after each stroke of ram to get proper uniformity of log. The pressure was maintained at 3000psi [5] for 5 min for making each briquette . The choice of quantity of binder was used based on the optimum amount for production of briquettes best density high stability and calorific value.

C. Biomass binder mixture

Dry leaves was mixed with an already prepared paper pulp for carrying out various tests. First test were carried out without using binder. Second test were carried out with the help of addition of small amount of binder in between each stroke and in third test paper pulp is mixed with dry leaves in proportion of 100:30 and used for making briquette [4]. The biomass-binder mixture was hand fed into mould and compacted to form the briquettes.

III. PERFORMANCE EVALUATION

For the performance evaluation, three briquette samples were made from the dry leaves for evaluation. During the process of densification, the following statistic: time for loading biomass into moulds, t1, sec, time for compressing the biomass, t2, sec, and time for ejecting the biomass briquettes, t3, sec, were observed and recorded following after [5][6]. The production capacity of the machine in kg/hr was also recorded. On ejection of the briquettes from the moulds, the mass and the dimensions of the briquettes were taken to determine the density in g/cm3. The compressed density (density immediately after compression) of the briquette was determined immediately after ejection from the moulds as the ratio of measured weight to the calculated volume [5].

Fig. 2: Leaf Logs

IV. RESULTS

The mean biomass loading time, t1, mean biomass compaction time, t2, and the mean briquette ejection time, t3 as well as their percentages of the total production time were recorded as shown in Table 1.

Table.1: Production time

Mean production time		**Time (Sec)**	**production time in percentage**
Biomass loading time	t_1	60	11.5384
Biomass compaction time	t_2	270	51.9231
Briquette ejection time	t_3	190	36.5385
Total		520	100

The mean production time for making one biomass briquette is 520 seconds (8.6667 minutes). The production capacity of machine is about 2.5 kg/hr.

Density of biomass Briquette

The density of briquettes are shown in Table 2. The influence of binder level was significant on density of

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briquette. The compressed density ranged from 0.3926 to 0.7699 gm/cm^3 on different addition of paper pulp (binder). The maximum compressed density of 0.7699 gm/cm3 was reached by adding binder in between each stroke of piston for uniformity. Without addition of binder density of 0.3926 $gm/cm^3$was reached and mixing the binder 30% in dry leaves density found 0.5389 gm/cm^3.

Table.2: Physical properties of briquette

Biomass-binder mixture	**Compressed density(gm/cm^3)**
Without binder	0.3926
30% of binder	0.5389
Small amount binder mixed in between each stroke of piston	0.7699

Calorific values of briquettes formed having paper pulp binder was found as 18.14MJ/kg. The briquettes formed gave energy cost less than 0.16 Rs./MJ [4].

V. CONCLUSION

The study on development of leaf log making machine is of great importance to make briquette at lower cost. Agricultural residue and saw dust also provide an enormous untapped fuel resources.

The following conclusions were arrived at from study:

1. Leaf log making machine suitable for the production of dry leaves briquette on a small scale with production capacity 2.5 kg/hr.
2. The density of dry leaves briquette was found to be significantly affected by the binder level.
3. Briquette with satisfactory quality was produced using Leaf Log Making Machine. However briquette quality was decided with the help of compressed density.
4. Approximate value of energy cost of wood is 0.17 rupees per mega joule and approximate value of energy cost of briquette is less than 0.16 rupees per mega joule.

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Phyto-mediated Synthesis of Copper Nanoparticles by *Cassia auriculata* and its Characterization with reference to E-waste Management

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Abstract*—An eco-friendly loom has been taking up in the present study to synthesize copper nanoparticles using Cassia auriculata. The leaf extract of Cassia auriculata acts as reducing as well as capping agent. Synthesis of copper nanoparticles was initially confirmed by the visual observation i.e color change (dark green color). The synthesized copper nanoparticles were primarily characterized by UV-vis spectroscopy and Fourier Transform Infrared (FTIR) spectroscopy. Further, the formation of amorphous and crystalline phase was analyzed by X-Ray Diffraction pattern. The size and morphology of the synthesized Copper nanoparticles was characterized by Scanning Electron Microscopy (SEM) and the elemental composition was analyzed by EDAX. The present study is a preliminary investigation to know about the capability of Cassia auriculata to synthesize copper nanoparticles from its salts. The results of the present study confirmed that the leaf extract of Cassia auriculata be capable of recovering copper from printed circuit boards in the form of nanoparticles in near future.*

Keywords*— *Copper Nanoparticles; Cassia auriculata; Nanotechnology; Characterization and E-waste.

I. INTRODUCTION

Electronic waste is the fastest escalating wastes in all over the world. E-waste consists of heavy metals such as silicon, arsenic, iron, copper, aluminium, lead, zinc, chromium, cadmium, mercury and barium. In addition most of these components can be recycled and reused [1,2]. Several techniques especially landfill and incineration has been applied to an E-waste management and both are causing considerable contamination risks [3,4]. An alternate method needed for the better E-waste management. Nanotechnology plays a key role in the recycling of E-waste through environment friendly manner [5]. Biological approaches using plants extracts and microorganisms for the extracting of metals have been recommended as expensive alternatives to conventional methods [6,7,8].

Keeping the above all in view the present study is initially aimed to screen the ability of *Cassia auriculata* for the synthesis of copper nanoparticles from its salts. After confirming the capability of Cassia auriculata to synthesize copper nanoparticles, it would be used to recover the copper present in the printed circuit boards. Therefore, the present study gives a way out to bioremediation as well as the recovery of valuable metals from E-waste in nano-form.

II. EXPERIMENTAL METHODS

2.1 Preparation of plant extract

For the preparation of plant extract of *Cassia auriculata*, 25g of leaves have been taken and washed thoroughly in distilled water. The washed leaves were dried after that it was cut into fine pieces. The crushed leaves were added into 100ml sterile distilled water. Further it was heated at 60°C for 10 minutes and filtered through Whatman No.1 filter paper [9].

2.2 Synthesis of Copper nanoparticles

25ml of leaf extract was added into 100ml of 2.5mM copper sulphate solution. The culture flask was then incubated at room temperature for 24hrs. Reduction of cupric ions by leaf extract was observed by signatory color change (dark green color) which indicates the formation of copper nanoparticles. Further the reaction solution has been centrifuged for 15min at 10,000rpm and dispersed in double distilled water to remove any unwanted debris [9].

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2.3 Characterization of Copper Nanoparticles

The optical characteristics of the synthesized Copper Nanoparticles were recorded as well as confirmed by using Systronics 118 (UV-vis double beam spectrophotometer) by wavelength scan from 200- 800nm at a resolution of 2nm. To determine the functional groups present in the leaf extract, FTIR analysis was carried out which is responsible for the reduction of Copper ions with the spectral range of 400-4000 cm-1. FTIR spectrum was taken by NICOLET iS5 -Thermo Scientific, USA. The crystalline structure of the copper nanoparticles was determined by X-Ray diffraction spectroscopy (PW3040/60 X'pert PRO-PANalytical, Netherlands). The size and morphology of the synthesized SeNPs were analyzed using a Field Emission Scanning Electron Microscope (FESEM) (Quanta 250- FEG Co.Ltd) at an accelerating voltage of 10.0kv. The spectrum of the energy dispersive absorption X-ray spectroscopy (EDAX) of the sample was carried out using an Oxford IE150 instrument.

III. RESULTS AND DISCUSSION

Cassia auriculata is an evergreen shrub that grows in many parts of India and in other parts of Asia. The flower, leaves, stem, root, and unripe fruit are used for treatment, especially in Ayurvedic medicine. We can use Cassia auriculata for diabetes, eye infections (conjunctivitis), joint and muscle

pain (rheumatism), constipation, jaundice, liver disease and urinary tract disorders. As a preliminary attempt we have been selected *Cassia auriculata* for the synthesis of copper nanoparticles.

Fig. 1: Cassia auriculata

3.1 Synthesis of Copper Nanoparticles by *Cassia auriculata* and its Characterization

3.1.1 UV-Vis spectroscopy analysis

The optical characteristics of the synthesized nanoparticles obtained from UV-Visible spectroscopy analysis of the sample were presented in Fig 3. It is the most important method of analysis to detect the Surface Plasmon Resonance property of CuNPs [10]. The formation of CuNPs was confirmed from the peak at 300nm, this result of the present study similar with the results of Majumder, D.R (2012) [1] and the UV range was 300nm.

Fig. 2: Synthesis of CuNPs by Cassia auriculata

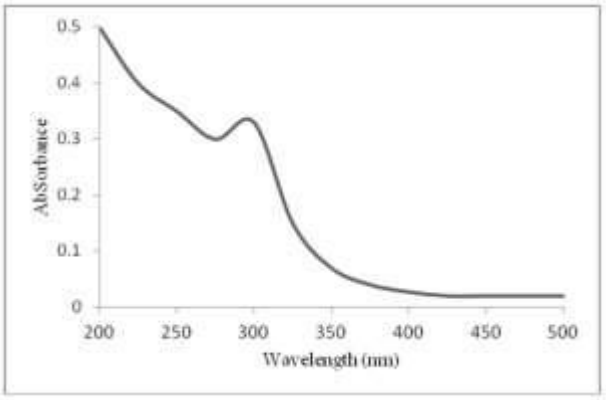

Fig.3: UV-visible spectrum of CuNPs

3.1.2 Fourier Transform Infra Red Analysis

The FT-IR measurements were carried out to identify the presence of biomolecules responsible for capping and efficient stabilization of copper nanoparticles synthesized by leaf extracts of *Cassia auriculata*. The infrared peaks were ranging from 1000- 4000cm^{-1} and thus confirmed the presence of polyphenols with aromatic ring [9].

3.1.3 Field Emission Scanning Electron Microscopy and Energy Dispersive Spectroscopy Analysis

The size and surface morphology of the copper nanoparticles were obtained by Scanning Electron Microscopy (SEM) analysis. The Figure 4 shows the CuNPs synthesized by the plant extract of *Cassia auriculata*.

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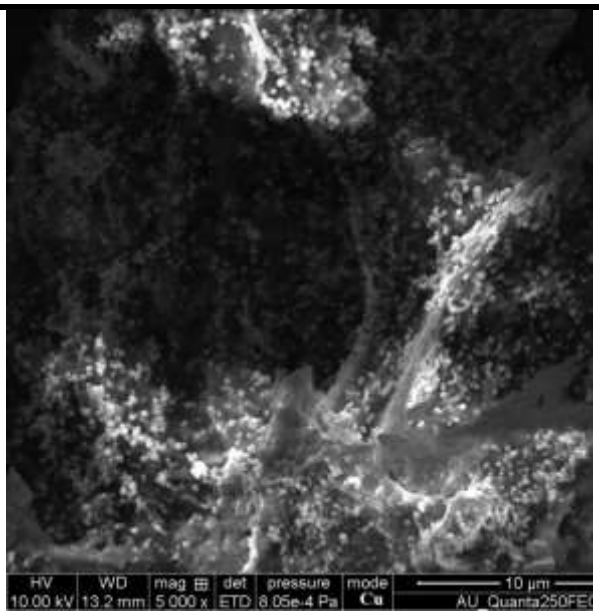

Fig.4: FESEM image of CuNPs

The electrostatic interactions and hydrogen bond between the bio-organic capping molecules bond were responsible for the synthesis of copper nanoparticles using plant extract [9]. It was shown that spherical and relatively uniform shape of the copper nanoparticles was confirmed in the range of 50-100nm. The results obtained from EDAX strongly confirmed that the synthesized nanoparticles were copper nanoparticles without any peaks of impurities [1].

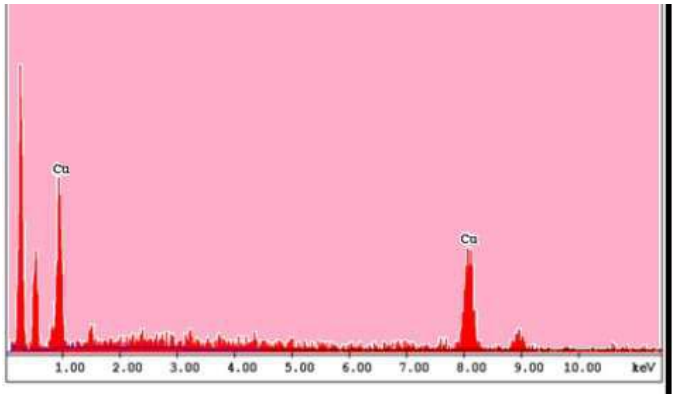

Fig.5: EDAX analysis of synthesized CuNPs

3.1.4 X-Ray Diffraction Analysis

The crystalline phases of the synthesized copper nanoparticles were confirmed by the characteristic peaks observed in the X-Ray Diffraction image (Figure 4). The diffraction peaks were observed at 2Θ angle and it was indexed as (111), (200), (220), (311) and (222). Similarly in the studies of Majumder D.R (2011)[1] and Abboud et al (2013)[12] the peaks of the copper nanoparticles were indexed as (111), (200), (220), (311) and (222). Hence the result of the present study strongly revealed that the synthesized nanoparticles were Copper Nanoparticles.

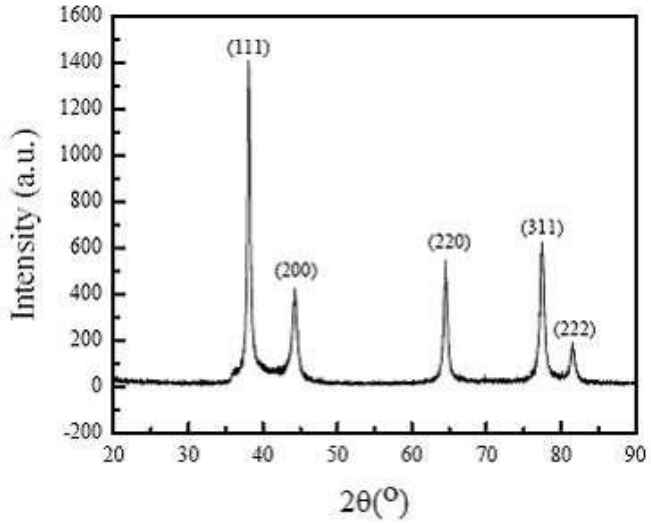

Fig.6: XRD pattern of synthesized Copper Nanoparticles

IV. CONCLUSION

The present study is a preliminary investigation to know about the capability of *Cassia auriculata* to synthesize copper nanoparticles from its salts. The results obtained from the characterization techniques confirmed that the leaf extract of *Cassia auriculata* be capable of recovering copper from printed circuit boards in the form of nanoparticles in near future. Hence the present study is going to be a solution to bioremediation as well as the recovery of Copper nanoparticles from Printed Circuit boards.

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Effects of used engine oil polluted-soil on seeds' germination and seedlings' growth characteristics of some tropical crops

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Abstract— *The ability of Phaselous vulgaris, Zea mays L., Solanum lycopersium and Sorghum saccharatum to germinate and grow in unpolluted soils, 1% w/w and 2% w/w used engine oil polluted soils were investigated. Twenty (20) seeds of each plant species were sown in the various polluted and unpolluted soils and germination were monitored for 7 days, and subsequent growth for 7 weeks. The numbers of germinated seeds were counted daily from the 2nd to the 7th day, and percentage germination recorded. Plants' growth parameters (shoot heights and leaf area) of the seedlings were assayed and recorded on the 3rd, 5th and 7th week. Percentage germination varied for the various plant seeds.* S. saccharatum *had the best germination in polluted and unpolluted soil with 100%, 95% and 90% germination as against the least germination 100%, 65% and 25% observed in S. lycopersium in unpolluted, 1% w/w polluted and 2% w/w polluted soils, respectively. In terms of growth, P.* vulgaris *had the best performance in unpolluted and polluted soils with mean shoot heights of 47.8 cm, 41.3 cm and 28.4 cm as against S.* lycopersium *with mean shoot heights of 10.8 cm, 5.8 cm and 3.6 cm in unpolluted, 1% w/w and 2% w/w polluted soils, respectively at the end of the study. The results of this study showed that used engine oil inhibited the germination of these seeds in a dose depended manner, and that inhibition of seeds' germination does not connote inhibition of subsequent growth. This highlights the need to prevent agricultural soil pollution with used engine oil.*

Keywords— Petroleum hydrocarbon pollution, plants' growth inhibition, seed germination, Used engine oil pollution.

I. INTRODUCTION

Used engine oil (UEO) is a brown/black liquid mixture of heavy metal contaminants (zinc, lead, and chromium that come from engine parts as they wear down), low to high molecular weight aliphatic and aromatic hydrocarbons, polychlorinated biphenyls, chlorodibenzofurans, lubricative additives and decomposition products [1]. UEO are usually improperly disposed after oil changing operations.

The oil which is not recycled in Nigeria is spilled by mechanics into runoff water drains and open farm lands [2], thereby polluting both soil and water bodies. Contamination of soil by UEO creates an unsatisfactory condition for life in the soil. This result from poor aeration it causes in the soil by displacement of air from the spaces between soil particles, immobilization of soil nutrients, loss of water-holding capacity, and lowering of soil pH. These leads to chlorosis of plant leaves, dehydration of plants and retardation of plant growth [3].

This study is aimed at ascertaining the effects of used engine oil polluted-soil on germination and early growth characteristics of some tropical crops commonly grown in Nigeria. These plants are beans *(Phaselous vulgaris)*, maize *(Zea mays L.)*, tomatoes *(Solanum lycopersium*) and guinea corn *(Sorghum saccharatum*).

II. MATERIALS AND METHODS

Sample collection

The soil sample used for this study was collected from a fallow farm land at the Federal University of Technology, Owerri. Surface soil (0-20 cm) was collected using a shovel, and was bulked to form composite sample. Used engine oil (UEO) was collected from the mechanic village Nekede in Owerri town, the oil was collected with a chemically clean aluminum pan and ran into a ten liter plastic container immediately after draining from vehicle engine. The seeds of the four different plant species were purchased from Ekeonunwa market in Owerri.

Soil pollution and experimental setup

The UEO was dissolved in acetone, and mixed with 10% of the total soil. The UEO laddered soil was then added to the bulk of the soil and mixed to obtain the final concentrations of 1% (10 g/kg) and 2% (20 g/kg) UEO in soil. The mixed UEO enriched soil was then stirred several times for two days to remove the acetone [4]. The soil samples were then moistened with tap water to bring the soil's moisture level to about 80% of its water holding

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capacity and allowed to undergo weathering for four weeks.

Weathering was by incubation in a green house with approximately 12 h daylight with intermittent moistening with tap water.

Twelve planting pots of approximately 8 cm diameter, each containing 1 kg of soil sample each were set up as follows:

i. Unpolluted soil samples - Four pots
ii. Polluted soil samples (1%) - Four pots
iii. Polluted soil samples (2%) - Four pots

Determination of % germination and plants' growth parameters

Twenty seeds each of the four different plant species (*Z. mays* L., *P. vulgaris*, *S. lycopersium* and *S. saccharatum)* were sown respectively in different unpolluted pots, 1% w/w polluted soil pots and 2% w/w polluted soil pots. The pots were kept in green house and watered three times a week using tap water. Germination in each pot was monitored and number of germinated seeds recorded daily for 7 days. On the 7th day, the germinated seedlings were thinned down to four, and subsequently, plants' growth parameters (shoot heights and leaf area) were determined at the 3rd, 5th, and 7th week of the study.

Germination Indices

The germination indices below were determined as follows:

Final germination percentage (FGP)

FGP = (No. of germinated seeds after 7days/No. of seeds tested) x 100

Germination index

Germination index = % Germination in treatment/ % Germination in control

Energy of germination (EG)

Energy of germination (EG) on Day 4 = % germination 4 days after planting (4DAP) relative to the number of seeds tested.

Measurement of plants' height

Plant's shoot height was determined by measuring from the shoot base to the apical tip using a meter rule. These were determined at the 3rd, 5th and 7th WAP.

Determination of plants' leaf area

The plants' leaf area was determined by calculating the product of its length and width taking at their broadest portions [5]. These were determined at the 3rd, 5th and 7th WAP.

III. RESULTS

Table.1: Z. mays L. seeds' germination in polluted and unpolluted soil (%).

% Oil in soil	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0%(control)	0	65	100	100	100	100
1%	0	0	0	70	75	75
2%	0	0	0	65	65	65

Table.2: P. vulgaris seeds' germination in polluted and unpolluted soils (%).

% Oil in soil	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0%(control)	0	15	100	100	100	100
1%	0	0	25	60	60	65
2%	0	0	0	15	35	40

Table.3: S. lycopersium seeds' germination in polluted and unpolluted soil (%).

% Oil in soil	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0%(control)	0	35	100	100	100	100
1%	0	0	0	40	45	65
2%	0	0	0	10	25	25

Table.4: S. saccharatum seeds' germination in polluted and unpolluted soil (%).

% Oil in soil	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0%(control)	25	85	100	100	100	100
1%	20	80	85	95	95	95
2%	0	10	35	60	85	90

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The percentage seed germination of the four different plant species namely *Z. mays L., P. vulgaris, S. lycopersium* and *S. saccharatum* were shown in Tables 1 to 4. The results revealed that percentage seed germination were higher in unpolluted soil samples (100% from day 4) than in polluted soil samples, and it decreased with the increase in the concentration of used engine oil in soil.

S. saccharatum had the best germination response in unpolluted and polluted soil samples with 100%, 95% and 90% germination respectively in 0%, 1% and 2% w/w oil in soil, followed by *Z. mays L*. with 100%, 75% and 65%, while the least percentage germination was observed in *S. lycopersium* with 100%, 65% and 25% germination respectively in 0%, 1% and 2% w/w oil in soil at the end of the 7 days incubation.

Table.5: Final germination percentages (FGP) of plants' seeds in unpolluted and polluted soil.

% Oil in soil	*Z. mays L.*	*P. vulgaris*	*S. lycopersium*	*S. saccharatum*
0%(control)	100	100	100	100
1%	75	65	65	95
2%	65	40	25	90

The results of the final germination percentages of the test crops (Table 5) show that all the crops had 100% final germination in unpolluted soil. The final germination percentages of the crops decreased as the concentration of oil in soil increased from 0% to 2%. *S. saccharatum* had best final germination percentages with 100%, 95% and 90% in unpolluted soil (0%), 1% w/w and 2% w/w oil in soil respectively. The least FGP was observed in *S. lycopersium* with 100%, 65% and 25% in unpolluted soil, 1% w/w and 2% w/w oil in soil respectively.

Table.6: Germination index of Zea mays L. within 7 days of seed sowing.

% Oil	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0%(control)	0	0	0	0	0	0
1%	0	0	0	0.70	0.75	0.75
2%	0	0	0	0.65	0.65	0.65

Table.7: Germination index of P. vulgaris within 7 days of seed sowing

% Oil	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0%(control)	0	0	0	0	0	0
1%	0	0	0.25	0.6	0.6	0.65
2%	0	0	0	0.15	0.35	0.4

Table.8: Germination index of S. lycopersium within 7 days of seed sowing.

% Oil	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0%(control)	0	0	0	0	0	0
1%	0	0	0	0.4	0.45	0.65
2%	0	0	0	0.1	0.25	

Table.9: Germination index of S. saccharatum within 7 days of seed sowing.

% Oil	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0%(control)	0	0	0	0	0	0
1%	0.8	0.94	0.85	0.95	0.95	0.95
2%	0	0.11	0.35	0.6	0.85	0.9

The results of the germination index of *Z. mays L., P. vulgaris, S. lycopersium* and *S. saccharatum* are shown in Tables 6 to 9. From the results, *S. saccharatum* had the highest germination index with 0.95 in 1% w/w and 0.9 in 2% w/w oil in soil at the end of the 7 days incubation, followed by *Z. mays* L. with 0.75 in 1% w/w and 0.65 in 2% w/w oil in soil. The least germination index was observed in *S. lycopersium* with 0.65 in 1% w/w and 0.25 in 2% w/w oil in soil at the end of 7 days incubation.

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Table.10: Energy of germination of Z. mays L., P. vulgaris, S. lycpersium and S. saccharatum seeds in unpolluted and polluted soil on day 4 of planting.

% Oil	*Z. mays L.*	*P. vulgaris*	*S. lycopersium*	*S. saccharatum*
0%(control)	5	5	5	5
1%	0	1.25	0	4.25
2%	0	0	0	1.4

The result of the energy of germination of these test crops (Table 10) revealed that unpolluted soil (0%) gave the highest energy of germination for all the crops tested. This decreased as the concentration of oil in soil increased from 1% to 2% in all the crops tested.

Table.11: Average shoot heights and percentage seedling height reduction of Z. mays L. seedling in unpolluted and polluted soil.

	3WAP		5WAP		7WAP	
% Oil	SH(cm)	% SHR	SH(cm)	% SHR	SH(cm)	%SHR
0%(control)	38.8	0%	43.1	0%	45.5	0%
1%	30.8	25.97	33.5	28.65	35.3	28.89
2%	28	38.57	26.4	63.25	30.4	49.67

Key: WAP-Week after Planting; SH- Shoot Height; SHR- Seedling Height Reduction

Table.12: Average leaf area and percentage leaf area reduction of Z. mays L. seedling in unpolluted and polluted soil.

	3WAP		5WAP		7WAP	
% Oil	LA (cm^2)	%LAR	LA (cm^2)	%LAR	LA (cm^2)	%LAR
0%(control)	22.8	0	32	0	37.8	0
1%	11.5	98.26	22.2	44.14	22.5	68
2%	6.2	267.74	15.1	111.92	15.2	148.68

Key: WAP-Week after Planting; LA- Leaf Area; LAR- Leaf Area Reduction

Table.13: Average shoot heights and percentage seedling height reduction of P. vulgaris seedling in unpolluted and polluted soil.

	3WAP		5WAP		7WAP	
% Oil	SH(cm)	%SHR	SH(cm)	%SHR	SH(cm)	%SHR
0%(control)	38.8	0%	47.3	0%	47.8	0%
1%	35.3	9.92	39.8	18.8	41.3	15.7
2%	25.1	54.6	28.1	68.3	28.4	68.3

Key: WAP-Week after Planting; SH- Shoot Height; SHR- Seedling Height Reduction

Table.14: Average leaf area and percentage leaf area reduction of P. vulgaris seedling in unpolluted and polluted soil.

	3WAP		5WAP		7WAP	
% Oil	LA(cm^2)	% LAR	LA(cm^2)	%LAR	LA(cm^2)	% LAR
0%(control)	12.4	0	35.4	0	40.1	0
1%	7.6	63.2	21.2	67.0	24.9	61.0
2%	3.2	287.5	13.9	154.7	16.1	149.1

Key: WAP-Week after Planting; LA- Leaf Area; LAR- Leaf Area Reduction

Table.15: Average shoot heights and percentage seedling height reduction of S. lycopersium seedling in unpolluted and polluted soil.

	3WAP		5WAP		7WAP	
% Oil	SH (cm)	%SHR	SH(cm)	%SHR	SH(cm)	%SHR
0%(control)	8.1	0	10.8	0	10.8	0

1%	3.1	161.29	5.8	86.20	5.8	86.20
2%	2.4	273.5	3.5	208.57	3.6	200

Key: WAP-Week after Planting; SH- Shoot Height; SHR- Seedling Height Reduction

Table.16: Average leaf area and percentage leaf area reduction of S. lycopersium seedling in unpolluted and polluted soil.

	3WAP		5WAP		7WAP	
% Oil	LA(cm^2)	% LAR	LA(cm^2)	% LAR	LA(cm^2)	% LAR
0%(control)	0.5	0	1.8	0	2	0
1%	0.1	400	0.7	157.14	0.7	185.71
2%	0.05	900	0.2	800	0.29	589.65

Key: WAP-Week after Planting; LA- Leaf Area; LAR- Leaf Area Reduction

Table.17: Average shoot heights and percentage height reduction of S. saccharatum seedling in unpolluted and polluted soil.

	3WAP		5WAP		7WAP	
Oil %	SH(cm)	%SHR	SH(cm)	%SHR	SH(cm)	%SHR
0%(control)	41.8	0	45.1	0	45.1	0
1%	21.3	96.24	27.1	66.42	27.9	61.64
2%	19	120	21.5	109.76	22	105

Key: WAP-Week after Planting; SH- Shoot Height; SHR- Seedling Height Reduction

Table.18: Average leaf area and percentage leaf area reduction of S. saccharatum seedling in unpolluted and polluted soil.

	3WAP		5WAP		7WAP	
% Oil	LA(cm^2)	%LAR	LA(cm^2)	%LAR	LA(cm^2)	%LAR
0%(control)	14	0	27.2	0	27.3	0
1%	2.2	536.36	8.8	209.09	10.6	157.54
2%	1.4	900	6	353.33	5.8	370.68

Key: WAP-Week after Planting; LA- Leaf Area; LAR- Leaf Area Reduction

The results of the shoot heights and percentage seedling height reduction, leaf area and percentage leaf area reduction of *Z. mays L.*, *P. vulgaris*, *S. lycopersium* and *S. saccharatum* are shown in (Table 11-18). The result show that shoot heights and leaf area of these crops were higher in unpolluted soil than in polluted soil, and they decreased with the increase in the concentration of used engine oil. *P. vulgaris* had the best shoot heights with 47.8 cm, 41.3 cm and 28.4 cm and leaf area with 40.1 cm^2, 24.9 cm^2 and 16.1 cm^2 in unpolluted soil (0% w/w), 1% w/w and 2% w/w used engine oil in soil respectively at end of the study, as against *S. lycopersium* with shoot heights of 10.8 cm, 5.8 cm and 3.6 cm and leaf area of 2 cm^2, 0.7 cm^2 and 0.29 cm^2 in unpolluted soil (0% w/w), 1% w/w and 2% w/w used engine oil in soil respectively at the end of the study. The percentage seedling reduction and percentage leaf area reduction tend to increase as the concentration of oil increased from 1% to 2%.

IV. DISCUSSION

Indiscriminate disposal of used engine oil has been found to be harmful for living organisms and vegetation. The adverse effects on plant growth may range from morphological aberrations, reduction in biomass to stomatal abnormalities [6].

In this study, the percentage seed germination results revealed that seeds' germination were higher in unpolluted soil samples than in polluted soil samples and it decreased with the increase in the concentration of used engine oil in soil. This could be as a result of the fact that the used engine oil components were toxic to the seeds, thereby inhibiting their complete germination in polluted soil samples. Wang *et al.* [1] and Adam and Duncan [7] suggested that the failure of seeds to germinate in the presence of hydrocarbon contaminants could be as a result of formation of polar compounds dissolved in water that could penetrate the seed coat, exerting polar narcosis. Used engine oil may also have prevented uptake of nutrient, water and oxygen required for seed germination. This finding is in accordance with the findings of Agbogidi and Ilondu [8] in which the inhibitory effect of spent engine oil on germination and seedling growth of *M. oleifera* was found to be dose dependent. Of the tested seeds, *S. saccharatum* had the best germination in used engine oil-polluted soil, followed by *Z. mays L*. The least percentage germination was observed in *S. lycopersium.*

This shows that the response of the seeds to the toxicity of the UEO differs.

The results of the final germination percentage, germination index and energy of germination of these test crops seeds shows that FGP also decreased in a dose dependent manner in all the seeds tested. *S. saccharatum* had the best FGP, followed by *Z. mays L*., while the least was observed in *S. lycopersium*. The germination index of these crops seeds also decreased as the oil concentration increased. *S. saccharatum* had the highest germination index, while the least was obtained in *S. lycopersium*. The energy of germination of these crops revealed that the highest energy of germination was obtained in unpolluted soil (0% w/w) and it also decreased in a dose dependent manner. These results show that used engine oil contaminant is inhibitory to seed germination. It also shows that different plant species reacted differently in the midst of this pollutant. This is in line with the work of Wiltse *et al*. [9] that reported that the potential to tolerate polluted soils differs among plants species and even among varieties.

The effect of the contaminant on the seedlings' growth parameters (shoot heights, percentage seedling height reduction, leaf area and percentage leaf area reduction) of the test crops revealed that the mean plant shoot height and mean leaf area of the crops decreased with the increase in the concentration of used engine oil . The mean heights of the unpolluted controls were greater than those of the plants grown in 1% w/w and 2% w/w used engine oil in soil in all the different plant species. This could be due to the absence of oil in the control which made the soil conducive for the growth of the plant crops. These findings agree with the work of Ekpo *et al*. [10] and Kayode *et al*. [11] on *Glycine max*, *Vigna uniguiculata* and *Z. mays L.*, and the findings of Adenipekun [12] that used engine oil affect plant height, stem girth, moisture content, leaf area and number of leaves in *Celosia argentea.* In this study the higher percentage seedling height reduction and percentage leaf area reduction observed as the concentration of oil increased in soil shows that the seedling heights and leaf area tend to reduce more as the concentration of used engine oil increased from 1% w/w to 2% w/w oil in soil. From the seedlings' growth indics *P. vulgaris* had the best growth response in the presence of the UEO pollutant. Nwoko *et al*. [13] also observed that *P. vulgaris* thrives well in the presence of this contaminant. Seedling growth was found to be adverse for *S. lycopersium*.

The results show that inhibition of germination does not necessarily lead to inhibition of growth. This was seen in *P. vulgaris* whose percentage germination on used engine oil polluted soil was reduced, but its seedlings had the best growth response in the presence of the contaminant.

V. CONCLUSION

Used engine oil polluted-soils are unsuitable for germination and growth of plants, hence there is need to enlighten the public on the hazards of indiscriminate disposal of this pollutant into environmental media. This will go a long way in ensuring human and environmental health, improved crop propagation and safety, and food security.

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Does fertilization practices increase residual nitrate nitrogen in soil irrigated with treated wastewater? An experimental trial on maize

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Abstract— *Treated wastewater has significantly improved DM yield compared to ground water. The form of nitrogen provided by the water was determinant in drawing yields. Irrigation with ground water (where nitrogen is as nitrate) induces a faster migration of nitrogen at depth. In contrast, using treated wastewater (where nitrogen is as ammonium), resulting in a relative distribution of the remaining nitric smaller in the lower profile and therefore higher in the surface, especially after the second year (2010). In addition, the relative distribution of nitrates in the soil surface is even more important in the presence of organic manure. All happens as if a certain amount of ammonium provided by treated wastewater is retained in the organic compounds of manure. Yields were significantly lower in irrigation with treated wastewater in the second year and especially when fertilization was given in additional. If the soil can be used for storage of the nitrogen supplied by the treated wastewater during the first year of irrigation (24 kg N-NO3/ha before irrigation to 115 kg N-NO3/ha after irrigation), to the second year the capacity drops (to 64 N-NO3/ha) and a significant increase in nitrate leaching occurs. Therefore, unlike the contribution of manure that seems enrich the topsoil nitrate nitrogen, at least during the first campaign, mineral fertilization unreasoning causes faster migration of nitrogen at depth.*

Keywords—Treated wastewater, Fertilizer, Nitrate leaching, Dry matter.

I. INTRODUCTION

In arid and semi-arid region, water has become increasingly rare source which by its lack alters the socio economic development. To preserve their fields and keep constant production of their crops to continue living, farmers are willing to use all types of water such as treated wastewater (TWW). In Tunisia, this method is an old (since 1960) and popular practice in agriculture. Despite of this long experience, a great effort remains to spend to convince farmers about fertilizers and economic potential of this water and to raise farmers' awareness of the drawbacks of poor use of these waters. Irrigation with TWW has been used for three purposes: (i) complementary treatment method for wastewater (Bouwer and Chaney, 1974); (ii) use of marginal water as an available water source for agriculture (Bouwer and Idelovitch, 1987; Al-Jaloud et al., 1995; Tanji, 1997) – a sector demanding ~ 83% of the consumptive water use in Tunisia (iii) use of TWW as nutrient source (Bouwer and Chaney, 1974**;** Vazquez-Montiel et al., 1996**;** Khelil et al., 2011) associated with mineral fertilizer savings and high crop yields (Smith and Peterson, 1982; Feigin et al., 1991; Khelil et al., 2005).

In many studies worldwide the use of TWW as water and nutrient sources in agricultural have been introduced as a viable alternative for TWW destination in the environment. However, various studies have revealed that the nutrient supply only by TWW irrigation was not sufficient to meet plant nutrient requirements resulting in yield decreases. The problem could be solved by an adapted effluent/fertilizer management (khelil et al., 2012; da Fonseca et al., 2007a). Due to the often observed accumulation of nitrogen losses (leaching, volatilization and denitrification) after TWW irrigation, the monitoring of these components is of crucial importance for a sustainable use. According to Rafael Marques Pereira Leal and al. (2009), throughout the irrigation period, high NO_3-N concentrations (up to 388 mg/ l with treatment receiving 200% of crop water demand) was measured in soil solution below the root zone, indicating the potential of groundwater contamination. Nitrogen (N) cycling in agro-systems can also be altered by TWW irrigation, mainly in the long-term (da Fonseca et al., 2007a). Several studies have shown increased total carbon (TC) and total nitrogen (TN) contents in the soil due to C and N input by TWW irrigation (Friedel et al., 2000; Ramirez-Fuentes et al., 2002). Other studies have found decreased contents of soil TC and TN (Speir et al., 1999; Snow et al., 1999), mainly attributed to enhanced mineralization

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and nitrification processes under effluent irrigation (da Fonseca et al., 2007b). Of greater concern, increasing concentrations of nitrate (NO_3-N) in soil solution due to TWW irrigation have often been reported (Polglase et al., 1995; Smith and Bond, 1999; Gwenzi and Munondo, 2008), representing one of the main challenges for the sustainable land application of effluents (Bond, 1998; da Fonseca et al., 2007a).

Otherwise, the soil-plant system, if adequately managed, encourages retention of TWW components mainly due to the incorporation of elements in the dry matter (DM) of plants (Bouwer and Chaney, 1974; Vaisman et al., 1981), leading to decreasing element concentrations in ground and surface waters (Feigin et al., 1978). Harvest and removal of plant material withdraw the accumulated elements, which further contribute to prevent leaching of elements, mainly nitrogen (N) and enrichments in the subsoil solution and the groundwater concentrations (Quin and Forsythe, 1978; Hook, 1981). Although irrigation with TWW may mitigate the damage and utilization of natural water resources and enables the diversion of nutrients from TWW and save the conventional inorganic and organic fertilizers including nitrogen fertilizers, it may result in risks that need to be considered in more detail, especially since farmers do not conceive reducing their fertilizer supply. With this in mind, a study of experimental was conducted at the Agricultural Experimentation Unit – Nabeul-Tunisia to study the impact of the different fertilizations practices adopted by farmers on maize yield and on nitrate status of the ground after harvest and to serve as farmer's awareness to convince them to reduce their contribution in terms of fertilizers, including irrigation with treated wastewater.

II. MATERIALS AND METHODS

This field study was conducted during the summer in 2009 and 2010, as part of a larger study in bilateral collaboration between the Agronomic research Center, (CRAg) Gembloux ABT(ULg) from Belgium and the Rural, Water and forest research Institute "INRGREF" of Tunisia. The field had been for maize in summer and vegetables in winter for three years prior 2009. Some physical and chemical properties of the experimental soil determined before sowing are presented in Table 1.

Table.1: Physicochemical and moisture characteristics of the soil

Paramètres			**Soil depth (Cm)**		
	0 /20	**20/40**	**40/60**	**60/80**	**80/ 100**
%					
Coarse silt	5	5	5	-	
Fine sand	29	24	30	-	
Coarse sand	64	68	64	-	-
Conductivity ds.m^{-1} 25° C	2.01	1.87	1.98	-	-
% Organic matter	0.4	0.3	0.2	-	-
% Total nitrogen	0.087	0.066	0.045	-	-
% Carbon	0.3	0.2	0.1	-	-
C/N	3.4	3.0	2.2	-	-
Humidity at pF 4.2	2.88	1.97	1.28	1.10	1.10
Humidity at pF 2.7	8.68	6.76	4.43	2.77	2.72
weight Density (da)	1.35	1.35	1.35	1.35	1.35
Ru (mm)	15.66	12.93	8.50	4.51	4.37

pF 4.2 corresponds to moisture at the point of wilting. 2.7 pF corresponds to moisture at field capacity. da, bulk density. Ru, reserves calculated in mm per layer (20 cm x 2 = da (Humidity in pF2.7 - humidity at pF 4.2)

These analyzes show that the soil is sandy type of low organic matter, with a C/N ratio, lower than 10. Moisture content expressed as% at pF 2.7 and pF 4.2 by 20 cm layer to a depth of 100 cm, were used to calculate the usable water reserves (Ru) for the soil (Table 1). From Table above, the Ru soil decreases with depth for both pF, indicating a low water-holding capacity of about 45mm on 1m soil depth. The use of a sheet of water over Ru, leads to a loss of water and solute by the system and automatically contributes to groundwater pollution.

The experimental protocol was designed to use fertilization practices used by farmers. The treatment comprised: (i) two irrigation water qualities, treated wastewater (TWW) and well water (WW), and (ii) four practices fertilization taken as treatments for each kind of water : (1) treatment without fertilizer (0N), (2) treatment with application of 120kgN/ha as ammonium nitrate, brought in two equivalent fractions, at raising and at elongation stage, (3) a treatment that corresponds to the application of 20t/ha of cow manure and (4) a treatment which represents the joint application of manure and

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mineral fertilizer (120kg N/ha + 20t/ha of manure). The experiment was organized in a randomized complete block design with four replications. Each treatment block was 2.25m by 4.2 m. TWW used in this study come from the wastewater treatment plant SE4 and WW used was a mixture of shallow wells on the experimental station. Water samples were collected ones a week in wells and outlet valves distribution of wastewater. The main characteristics of the two types of water are shown in the following table (Table 2).

Table.2: Characteristics of irrigation water

Parameters	Well water (WW)	Treated wastewater (TWW)
	mg/l	
NO3-	129 (±19.2)	< 5
N-NH4	2.36 (±0.26)	36 (±07.68)
HCO3-	219 (± 25.6)	344,94 (±37.24)
SO4--	487 (± 131.5)	426,51 (±148.6)
Cl-	729.2 (± 50)	548,57 (±55.14)
Ca++	238.8 (±13.6)	126,79 (±17.12)
Mg++	90 (± 6)	90,00 (±07.45)
P	-	5.37 (±01.99)
K+	60.45 (± 13.6)	31,64 (±03.40)
Na+	579.4 (± 40.7)	408,94 (±176.4)
pH	7.29 (± 0.14)	7,15 (±0.14)
Sels dissous (g/l)	2.86 (± 0.21)	2,17 (±0.26)
SAR	8.10 (± 0.66)	8,00 (±0.94)
Cd	-	0,009 (±0.01)
Co	-	-
Cr	-	-
Cu	-	-
Fe	-	0,005 (±0.02)
Mn	-	0,003 (±0.01)
Ni	-	0,006 (±0.005)
Pb	-	0,030 (±0.014)
Zn	-	0,009 (±0.005)

The TWW is rich in potassium and in nitrogen and poor in phosphor and nitrate and have salinity comparable to that of the WW. The concentration of heavy metals in TWW is below Tunisian standard (NT 106.03) on the use of TWW in agriculture. The N composition of TWW ranged from 28 to 51 mg $N\text{-}NH_4$/l, with an average of 36 mg $N\text{-}NH_4$/l and contained less than 2 mg/l of NO_3^-. However, WW were loaded with nitrate with an average of 25 mg $N\text{-}NO_3^-$ and accounted less than 2 mg/l of nitrogen as ammonium. Mineral composition of manure is comparable in 2009 and 2010 but with a lower phosphorus content in 2010 (Table 3).

Table.3: Chemical characteristics of manure

Parameters		**Manure 2009**		**Manure 2010**
			(%)	
Total nitrogen		0.707		0.779
Ammoniacal nitrogen		0.004		0.001
Organic nitrogen		0.703		0.778
Dry matter		53.25		32.18
Carbon		55.34		55.26
P		0.741		0.375
K		1.782		1.900
Ca		5.086		4.248
Mg		0.505		0.419
C/N		78.15		70.93

N composition was in the order of 0.7% which corresponds to a contribution of 140 kg N/ha. Manure is rich in calcium but low in magnesium with a C/N ratio of about 75.

Due to the sandy nature of the soil, a pre-irrigation was performed in order to fix the soil and to ensure optimum germination and emergence. Feed maize (zea mays) was planted on monthly statement on Mai in 0.65 m row spacing and with a spacing of 0.2 m within the row, with two seeds per hill. Plant was thinned before fertilization to keep one foot per hill shortly after emergence. Each repetition consisted of four lines representing 80 feet of Maize. Manure was spread and incorporated into the soil two weeks before planting, while nitrogen fertilizer was applied online at equal fraction at 3-leaf stage and at elongating stage.

Water irrigation levels were designed to approximate the seasonal evapotranspiration (ETP) minus precipitation deficit, according to the following formula "Water requirement = Kc x ETP – effective rainfall – R**",** where R is the stock of the soil moisture at planting time, assumed equal to zero. Water requirement was calculated on the basis of the average climatic parameters of the experimental station (ETP), calculated by the Penman-Monteith formula on 12-year period (1997/2008) and on the bases of crop coefficients (Kc) at various vegetative stages of maize, mentioned in Richard et al (1998)**.** The crop ETP requirement was 750mm from mid Mai to mid September under local conditions. According to Rebour and Deloye (1971), water use efficiency was estimated at 95% for drip irrigation, so that an additional of 5% (equal to 27 mm) excess water was applied to meet 100% water use efficiency. A total of 19 irrigations were made in

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2009 and 2010. Overall, the crop received a total amount of about 544 mm (5440 m^3/ha) for each year and for each kind of water (Table 4).

Table.4: Water requirement of maize and irrigation scheduling

Parameter		Months from May to September					Total (mm)
		M	J	J	A	S	
ETP (mm /Mois)		134	155	182	163	116	**750**
Kc		0.5	0.65	1.08	0.9	0.6	-
Besoin/Mois (mm)		67	100	197	147	70	**581**
number of irrigation		1	4	7	6	1	**19**
Rate/irrigation		24	27.4	31.4	27.4	25	-
Rate/month (mm)		**24**	**110**	**220**	**165**	**25**	**544**

Before sowing, a characterization of the content of nitric nitrogen in soil was performed. Then, after each campaign a soil sample is taken to determine the residual nitrate nitrogen. Only one plant sampling was carried out at pasty milky stage in 2009. While in 2010, to follow the dilution of nitrogen in the dry matter "DM" (N% DM), four sampling during the vegetative cycle were performed. The fourth sampling coincided with the pasty milky stage. All samples were made on the above ground part of ten corn feet taken on a surface of 1m². All plant portions were dried at 70°C, and weights were recorded. Soil sampling concerned a profile probed by 80 cm layer of 20 cm. Three carrots repetition were performed. For each depth, the three samples were mixed and an average sample depth was analyzed. Analyzes are concerned mainly nitrogen, either in the soil or in the plant. Mineral nitrogen in the soil was determined on the filtrate after extraction with KCl (0.5 M) with a dilution of 1/5, the nitrate analysis was performed at CRA-W by colorimetry using a continuous flow system. The principle is to reduce the nitrate by hydrogen sulphate then to cause the Griess reaction on the nitrite formed to give a purple compound. The plant tissue was ground and analyzed for total N using kjeldhal digestion method according to Bremner and Mulvaney (1982).

III. RESULTS AND DISCUSSION

3.1 Dry matter yield production

Irrigation with treated wastewater has greatly improved the production of DM especially in 2009 despite the significant amount of nitrate nitrogen provided by WW (Fig 1).

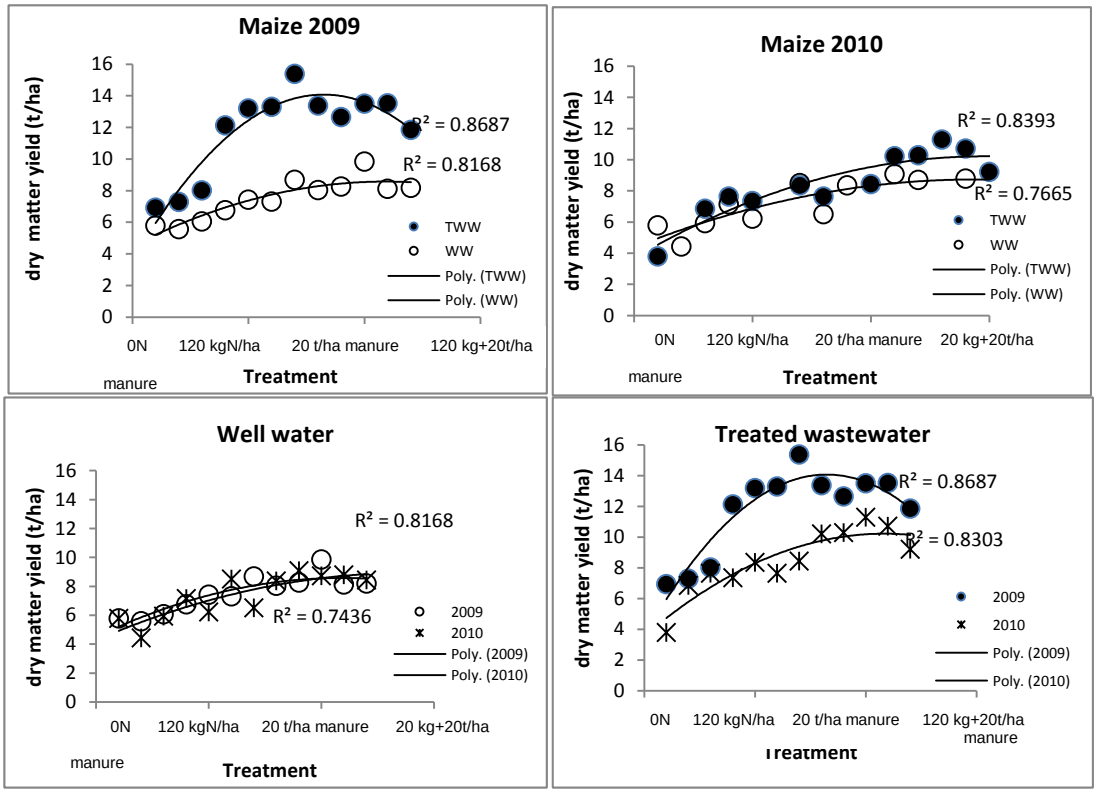

Fig.1: Effect of the kind of water and of fertilizer practices on the DM yield of feed maize.

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This improvement would explained either by the wealth of treated wastewater with other nutrients such as P, Ca, Mg, or also by the low efficiency of the nitrate nitrogen provided by TW throughout the vegetative cycle and even at times when the plant absorbs less. The nitrate nitrogen is also exposed to leaching, especially at the beginning and at the end the vegetative cycle. Vazquez-Montiel (1996) on maize, noted an increase in yield and N content (% N) and phosphorus (P%) in irrigation with TWW compared to WW. Moreover, the comparison between the two years shows that the yield response to different fertilization practices was more significant in 2009 than in 2010. It seems that the memory effect of previous contributions (in 2009 and even before planting) is produced on corn in 2010. In the WW treatment, the DM production was similar for both years. Whereas, we note a decrease in DM production in 2010 when TWW was used for irrigation, notably when a supplemental fertilization was added.

3.2 Effect of the treatments on the dilution of N in aboveground biomass during maize growth

The dilution curves of N in the aboveground biomass during maize growth in 2010 (Fig 2), were compared to the reference curve used for non-limiting nitrogen nutrition for grasses (Lemaire and Salette, 1984).

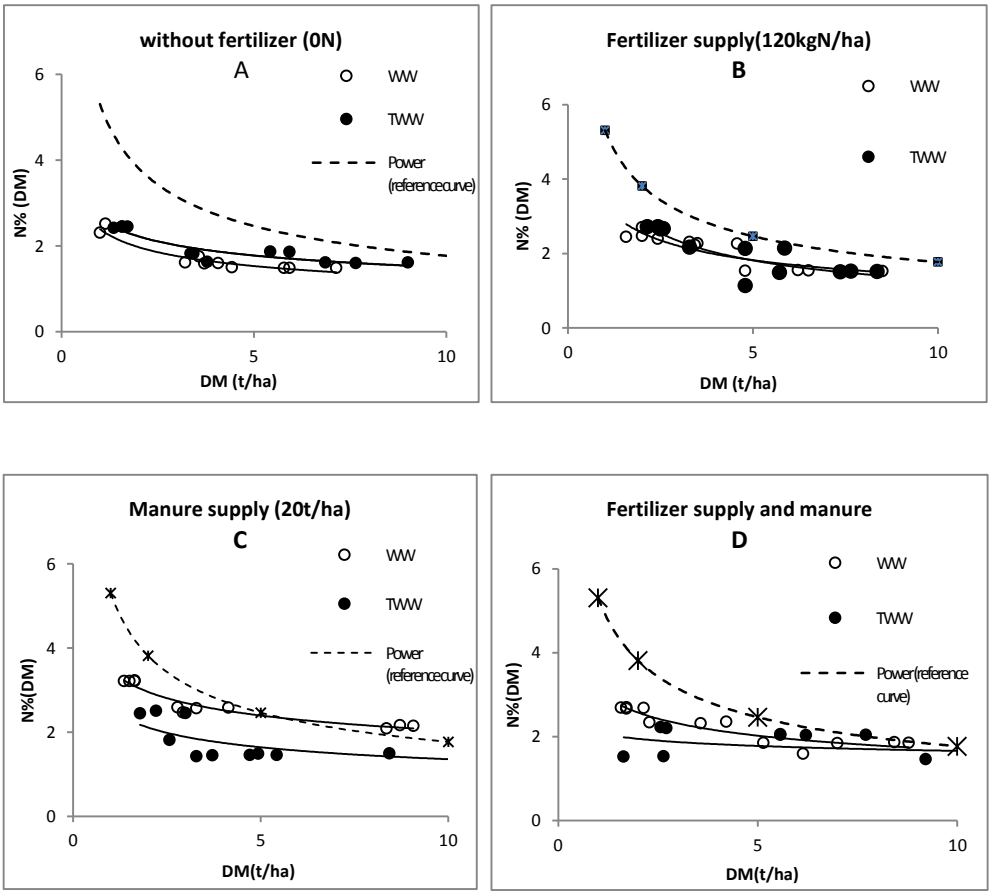

Fig.2: Dilution curve of nitrogen (N%) in the dry matter production (DM) depending on water qualities and fertilization practices

Regardless of treatment, nitrogen dilution in the DM during the growth cycle decrease with increasing dry matter production. The following mathematical relationship has been applied to our results:

$$\alpha = N\% \, (DM)^{-\beta} \qquad 1$$

In the absence of fertilizer, the dilution curve is below the reference curve and shows that nitrogen nutrition was significantly better with TWW compared to WW treatment (Fig 2A). By cons, when nitrogen fertilizer was added, N nutrition is rather better with WW irrigation. (Fig 2B), it even exceeds the reference curve in WW irrigation when manure (20 t/ha) was spread (Fig 2C), indicating a good nitrogen nutrition notably at final harvest. The difference between the two treatments WW and TWW is probably due to growth retardation observed on TWW plots. Independent on water irrigation quality, mineral fertilization improves nitrogen nutrition even in the presence of manure. However, improved nitrogen nutrition of maize was not followed by a significant increase in the DM production due to the luxury

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consumption of nitrogen especially on the plots irrigated with TWW. We assume therefore that irrigation with TWW with higher N content in addition to fertilizer input, leads to promote a situation of excess nitrogen resulting in a depressive effect on nitrogen nutrition and on DM production. All these observations support the thesis already advanced by Salette and Lemaire (1981) on the existence of a more or less closely relationship between the accumulation of nitrogen in shoots and their dry matter growth.

3.3 Effect of treatments on the relationship between dry matter produced and nitrogen uptake

The relationship between DM and N exported has been demonstrated for stands of grasses by Salette and Lemaire (1981). It can result in the following mathematical relationship:

$$N\ uptake = 10\ \alpha\ (DM)^{1-\beta} \qquad 2$$

This is in fact an allometric relationship between nitrogen uptake and DM production. The coefficient (1 - β) is the ratio of the relative rates of N uptake and relative growth rates, the coefficient 10α represents the amount of nitrogen absorbed for the production of the first tone of DM. However, although this relationship is much talking in terms of agronomic, since it describes the relationship between growth and nitrogen uptake. The comparison of the different relationships between them arises under Lemaire et al. (1985) a statistical problem not satisfactorily solved theoretically. In our work, we used the method Dagnelie (1969), cited by Lemaire et al. (1985), which is based on the determination of the confidence interval of the orthogonal regression coefficient. We have used this method to compare the values of the allometric coefficient (1 - β) and the coefficient values 10α. Though, the values used to calculate the regressions curves are the average of four samples which were used for determining the DM yield and N content.

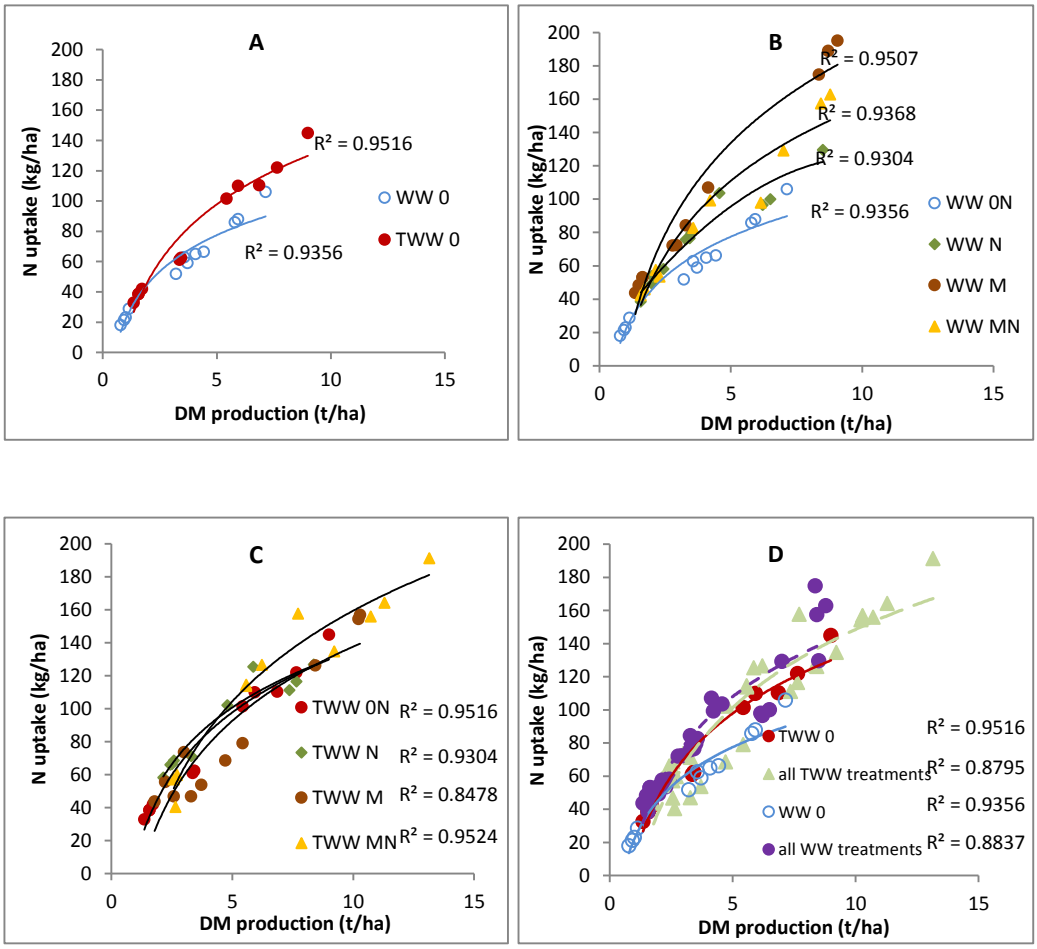

Fig.3: Relationship between allometric growth of DM and nitrogen uptake in maize (2010)

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For our work, the values were between 0.3 and 0.4% N for the WW and between 0.1 and 0.3% N for TWW. This precision corresponds to a variation of the coefficient 10α of about 3 to 4 kgN/ha and 1 to 3 kgN/ha for the well and the treated wastewater, respectively. In the absence of fertilization, irrigation with treated wastewater significantly increases the nitrogen and the DM production. However, when well water is used for irrigation we note a deflection of the allometric relationship reflecting a slowing of nitrogen uptake followed by DM production stagnation despite continuing input of N by these waters. This slowdown is probably linked to the nitric form of nitrogen supplied by well water that is relatively labile and more exposed to leaching than ammonium provided by TWW. Otherwise, in TWW irrigation, no effect of fertilizer used on the relationship between the dry matter produced / nitrogen input was observed (Fig 3). However, when manure and nitrogen fertilizer was applied together, we note a slight improvement in the absorption of N at the end of growth, represented by an accumulation of 15 kgN without being translated into DM. This excess nitrogen consumption at the end of culture could be the result of the mineralization of nitrogen from manure or also from soil under the action of microbial biomass. Unlike irrigation by TWW; a significant effect of fertilizing practices on nitrogen absorption and on the production of DM was recorded when WW was used for irrigation (Fig 3). This significant effect is all the more important as the total dose of N added is significant. Comparing the coefficients 10α and (1-β) at the same level of growth, shows that the lowest nitrogen uptake was recorded with WW0 treatment (Table 5) with the absorption of 23 kgN/ha for the production of the first ton of MS against 26-39 kg N/ha for the rest of the treatments. This difference is dependent on the treatment and is related to the amount of available nitrogen in the soil with treated wastewater irrigation.

Comparing the value of 10α we note that in irrigation with TWW, the value of 10α is the highest for the treatment with mineral fertilizer input split. Whereas, 10α value is significantly lower with manure. This could be explained by the organization of a certain amount of ammonia nitrogen supplied by TWW rendered inaccessible to the plant, especially since the value of the manure C/N ratio is well over 20. However, in irrigation with WW, the difference between the values of 10α for all treatments with fertilization does not exceed 4 kg/ha. It should also be noted that, at the end of growth, and particularly with treatments with an addition of 120 kg N/ha, a curve decline reflects a more marked slowdown in nitrogen withdrawals at the end of growth, which is explained by a more low coefficient value (1-β) (Table 5). This slowing of the absorption of N at the end of growth coincides with the depletion of the nitrogen reserves in the soil just after a phase of acceleration of N absorption following the applications of mineral fertilizer (ammonitrate). For the rest of the treatments, including control treatments, the value of (1-β) is similar, indicating a consistency in the nitrogen absorption during the vegetative cycle and also a constant supply of N by the environment.

Table.5: Comparison of the coefficients 10 α and 1-β of the relation $N_{exp} = 10\alpha\ (DM)^{1-\beta}$

Traitements	**10 α**	**1-β**	**R^2**	confidence interval of **(1-β) P = 5%**
TWW-0N	27	0.76	0.990	0.03
TWW-120N	39	0.53	0.689	0.02
TWW-20t Fumier/ha	26	0.73	0.870	0.12
TWW-120N+20t Fumier/ha	25	0.81	0.915	0.16
WW-0N	23	0.75	0.992	0.03
WW-120N	33	0.63	0.909	0.08
WW-20t Fumier/ha	35	0.77	0.976	0.01
WW-120N+20t Fumier/ha	31	0.74	0.994	0.02

The highest allometry coefficient (0.81) corresponds to the treatment receiving the highest total dose of N (450 kgN/ha), shows that the N content in the plant decreases little during growth, from 1.99 at the beginning growth at 1.46 at the end of growth. This coefficient would be a sign of luxury consumption of N and probably of growth retardation.

3.4 Effect of treatments on nitrate nitrogen in the soil

The profile before sowing was poor with nitrate nitrogen content of 15kgN-NO_3/ha at surface and practically nothing beyond 40cm depth (Fig 4). Only irrigation, without any addition of fertilizer, modifies and in the same way as well with the TWW and WW the nitric profile. About 40 kgN-NO_3/ha was found on the soil

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surface but also at depth after harvest (Fig 4), highlighting a deep migration of nitrate. Hence the lack of effect of the nitrogen form (NO^-_3 vs NH^+_4) provided by the two types of water on the nitric profile in the soil. It is often reported in the literature that ammonium ions contributed by the TWW is rapidly oxidized to nitrate after irrigation (Speir et al, 1999**)**. This conversion is actually favored by the sandy nature of our soil, the irrigation technique that prevents water logging of the soil and also by climatic conditions, ie high temperature, 30-35 ° C (Marot-Gaudry ., 1997). This rapid conversion of ammonium to nitrate, often leads to an accumulation of nitrate ions in the soil (Page et al, 1998). Similarly, Vazquez-Montiel et al. (1996) reported that the concentration of ammonium in soils irrigated with treated wastewater is generally low. Berdai et al. (1998) find that the maximum levels of ammonia nitrogen are achieved at 48 hours after irrigation. After 48 hours, the nitric and ammonia nitrogen content decreases due to losses through gaseous channels, leaching and absorption by the plan

The addition of 120kgN/ha results in an enrichment of the soil profile with nitrate more important on the surface of the soil and with the TWW irrigation. This enrichment is, on the one hand, the result of an intense microbial activity in the rhizosphere and, on the other hand, the product of an acceleration of the nitrification of the organic nitrogen in the soil under the effect of nitrogen fertilizer. Recently, Belligno et al. (2000), by incubating soil in the presence of treated wastewater and clear water plus the same amount of N, find that the amount of nitric nitrogen in the soil is higher in irrigation with treated wastewater.

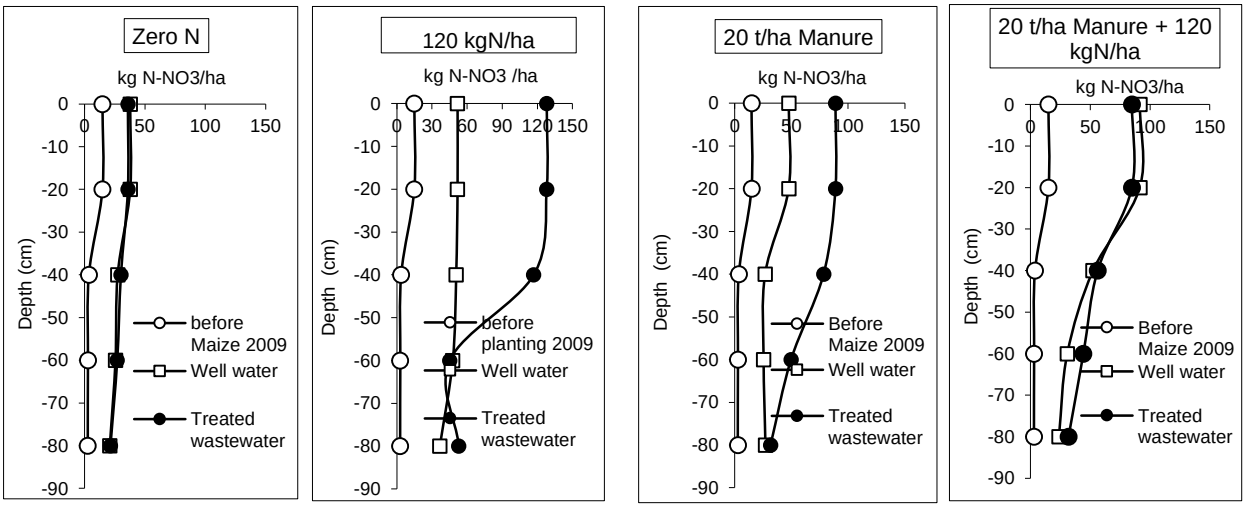

Fig.4: Nitrate remaining in the soil after the first harvest in 2009

The same authors suggest that the addition of nitrogen fertilizer in irrigation with treated wastewater accelerates nitrification in the soil. Papadopoulos and Styliano (1988), using a treated wastewater with 30 mg N/l and receiving 60 mg N/l as ammonium nitrate fertilizer and a well water with the same total amount of N (90 mg/l), find that nitrate migration below the root zone is higher in the treatment with treated wastewater. Nashikkar (1993) showed that the organic matter load explained by the high value of the BOD can also accelerate the conversion of NH_4 to NO_3 due to the anoxic conditions that it favors. The retention of nitrate nitrogen in the upper zone of the soil when TWW is used for irrigation reflects the intensity of microbial activity in this part of the ground. However, in the absence of uptake by crops, this nitrogen will migrate into the water table with the first autumn rainfall.

In irrigation with well water the addition of 120 N slightly increases the amount of nitrate in the soil profile compared to the control "WW-0N" while keeping the same aspect of the profile. This flat profile indicates that a large amount of N is lost only by nitrate leaching. This loss in fact explains the absence of the response of the DM yields to nitrogenous fertilizers.

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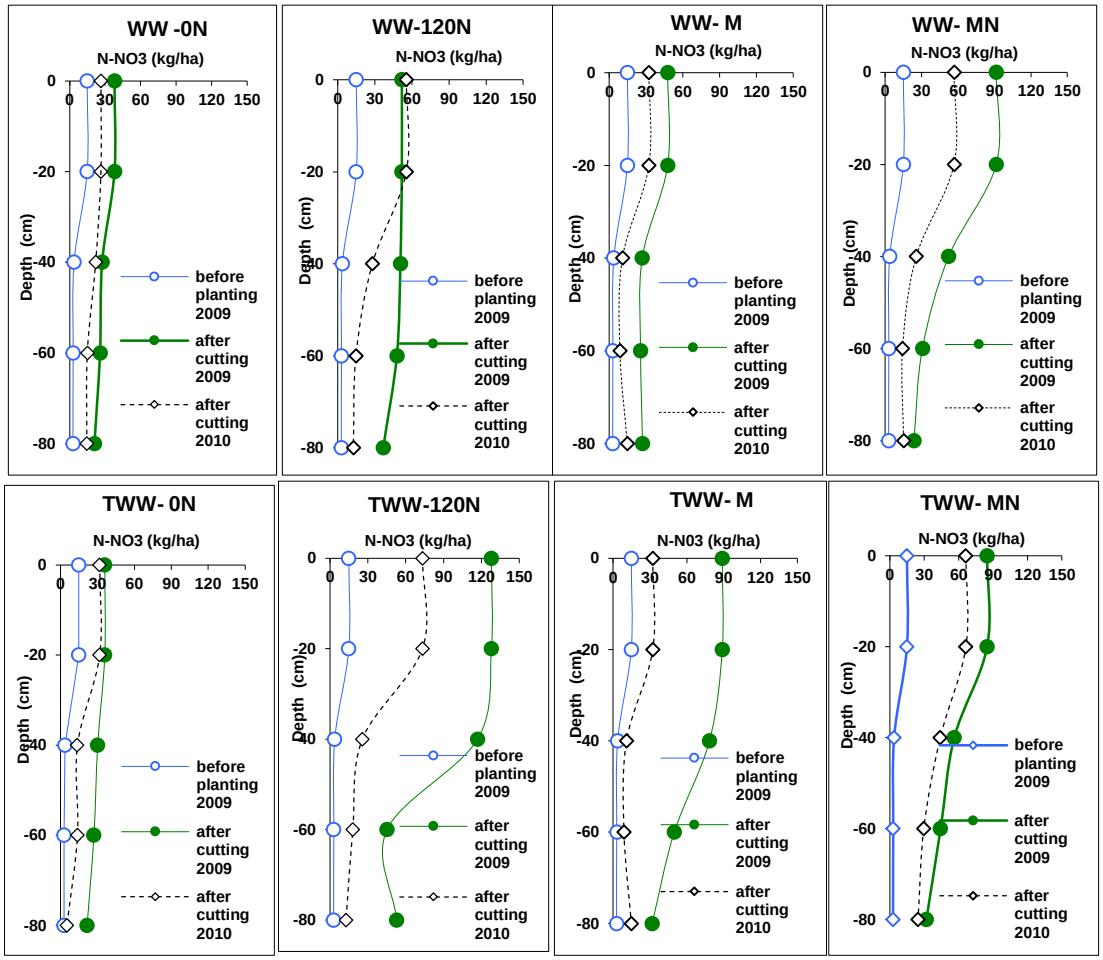

Fig.5: comparison of residual nitrate nitrogen in the soil after harvest in 2009 and 2010

Moreover, in the presence of manure we note an accumulation of nitrates in the upper part of the soil. This nitrogen has two origins, depending on the type of water used. In WW irrigation, these nitrates certainly come from irrigation water. Thus, in this case, the manure has to help to retain this nitrogen and prevent it from going to the water table, at least temporarily before covering the soil with a nitrate trap crop (NTC). However, in TWW irrigation, the residual nitrogen was greater than in WW irrigation, this may be the result of significant mineralization of manure organic matter due to high microbial activity sustained by the supply of a carbonaceous substrate by the treated wastewater (HCO_3 = 345 mg/l). The comparison of the nitrate profile between control and manure treatments supports this hypothesis. In fact, in irrigation with WW the nitric profile is similar for the control and the treatment with manure. While in TWW irrigation, the nitric profile is more enriched in the presence of manure. Only in the presence of fertilizer and manure that an enrichment of the nitric nitrogen profile has been noticed especially on the soil surface in WW irrigation,. This significant increase in nitrate nitrogen at the soil surface is probably the result of active mineralization of the organic matter of the manure under the effect of the mineral fertilizer. By cons, On the other hand, in irrigation with TWW, the nitric profile is generally more enriched in nitric nitrogen than in irrigation with WW. The comparison of the nitric profile between the two years shows that, in general, the shape of the nitric profiles was practically similar between the two campaigns (fig 5). However, the profile appears to be less loaded with nitric nitrogen after the second season (2010). It is therefore assumed that the nitrogen retention and its storage in the soil are achieved with the high total nitrogen dose brought in 2009, especially in TWW irrigation with manure and fertilizer. Jordan et al. (1997) reported that if the soil can be used for storage of nitrogen contributed by TWW during the first year of irrigation (of 1-46 micromol/l before irrigation to 30-71 mmol/l after irrigation), in the second year retention capacity decreases and a significant increase in nitrate leaching occurs. This leaching can be minimized by using plant species that could effectively remove nitrogen from the soil as forage crops (Gant et al, 1982; FAO, 2003). In

irrigation with WW loaded with nitrate, the situation is different. Residual nitric nitrogen at the end of cultivation is practically low and does not vary between the two years especially for the treatment zero N but it increases with the total N added. This in turn increases leaching losses of nitrates, especially in the presence of manure and nitrogen fertilizer. Hook and Burton (1979) suggest using crops that can undergo several cuts to reduce leaching.

IV. CONCLUSION

Irrigation with treated wastewater has significantly improved the DM production and maize grain yield compared to well water despite the richness of well water by nitrate nitrogen. We believe that the form of nitrogen contributed by water played a decisive role in the development of yields. In contrast to ammonium provided by treated wastewater, that can be fixed on the clay-humus complex of the soil and it's released slowly according to culture needs; the nitrates are mobile enough and because they are provided throughout the vegetative cycle by the well water, are rather exposed to losses by leaching. The observation of nitric profile end of the culture supports this hypothesis. Irrigation with WW, where nitrogen is mainly nitric form, causes a rapid migration of nitrogen to the soil depth. However, irrigation with TWW, where nitrogen is mainly ammoniacal, results in a lower nitrate distribution in the soil depth and greater in the soil surface, notably in 2010. These yield differences between the two types of water are probably the result of the higher fertilizer value (P, K, Ca, Mg and S) in the treated wastewater.

If the yields were stable in the two years in irrigation with WW, a drop in yields with TWW irrigation and especially with supplementary fertilization was noticed. Indeed, the TWW and fertilizer contributions seem to favor a situation of excess N which has led to a depressing effect on yields. However, according to other studies (Khelil et al., 2005), a reasoned starter fertilizer taking into account the N content in water and soil could be recommended. This fertilizer should't exceeds 30% of the dose usually given.

In addition, additional mineral or even organic fertilization enriches the soil profile with nitric nitrogen. These nitric reserves may be very different for similar yields. In 2009, the profile was more loaded with nitric nitrogen than in 2010 and especially in the TWW treatment with fertilization. So, if the soil can be used as storage for the nitrogen contained in TWW in the first growing season from 24 kg N-NO_3/ha before irrigation to 115 kg N-NO_3/ha after irrigation. At the end of the second growing season, storage capacity dropped to 64 kg N-NO_3/ha, which suggests that a considerable increase in nitrate leaching occurred. Therefore, in contrast to manure, which seems to enrich the upper part of the soil with nitrogen, at least during the first year of growth, unreasoned mineral fertilization leads to a more rapid migration of nitrogen at depth.

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Effect of *Trichoderma* Fortified Compost on Disease Suppression, Growth and Yield of Chickpea

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Abstract- *Trichoderma species are commonly used as effective biological control agents against phytopathogens especially the soil-borne fungi while some isolates are able to ameliorate plant growth. In the present study, Trichoderma fortified compost with different substrates were evaluated to reduce the pre-emergence and post-emergence seedling mortality, diseases of stem and root of chickpea caused by several soil-borne fungal pathogens, including Fusarium oxysporum, Rhizoctonia solani and Sclerotium rolfsii at different growth stages in the field under natural epiphytotic conditions. Among the twenty isolates of T. harzianum, Co-7 showed the most effective antagonist against the test pathogens in dual culture method. In field experiment, subsequently it was used for inoculum preparation with colonized wheat grain and mixed with well-matured decomposed composting materials like, saw dust, cow dung, tea waste, water hyacinth and poultry manure. Trichoderma fortified compost with poultry manure was found significantly effective in reducing pre-emergence and post emergence seedling mortality, disease incidence and disease severity of chickpea in the field. Interestingly, all the treatments significantly increased but Trichoderma fortified compost with poultry manure was the best to boost seed yield and quality.*

Keywords— biocontrol, Cicer arietinum, seedling diseases, soil-borne fungi, yield.

I. INTRODUCTION

Chickpea (*Cicer arietinum*) is the world's fourth most important pulse crop after soybeans, beans and peas (FAO, 2012). Diseases are one of the main constrains for the low production of this crop (Godhani *et al*., 2010). Chickpea crop is attacked by wide range of pathogens including fungi, viruses, bacteria, nematodes and mycoplasma (Nene and Sheila, 1996). Some of the serious pathogens usually known as soil-borne pathogens in order of their importance including *Fusarium oxysporum* f. sp. *ciceri*, *Rhizoctonia solani*, and *Sclerotium rolfsii* are the most devastating in both seedling and mature stage. Under favourable conditions, outbreaks of these pathogens it cause yield and quality deterioration or, even there is a total crop failure and result in a huge economic losses.

Many attempts have been made to control *F. oxysporum* f. sp. *ciceri*, *R. solani*, and *S. rolfsii* including cultural or chemical methods (Jaacov, 2000) but neither cultural nor chemical measures alone were found to be effective against these pathogens. Controlling of these soil-borne diseases with fungicides is uneconomical and difficult to achieve because of the soil and seed-borne nature of the pathogen (Ahmad *et al*., 2010). Moreover, the application of fungicides causes groundwater pollution, killing of non-target beneficial flora and evolving fungicidal resistance variants of the pathogen. The antagonistic *Trichoderma*, a cosmopolitan soil and compost-borne saprophytic fungus can be used to suppress soil-borne pathogens that cause diseases such as damping-off, root rots, stem rots, and wilting in many vegetables. In addition to its effect as a natural enemy of plant pathogens, *Trichoderma* has also a positive impact on plant growth as it produces different kinds of secondary metabolites which are important for plant growth regulation (Vinale *et al.*, 2009) and improves the soil fertility by acting as decomposer. Composts or compost extracts used as an organic fertilizer have beneficial effects on plant growth and considered as a valuable soil amendment (Gharib *et al*., 2008). Recently *Trichoderma* fortified compost have been used in many countries and appeared very effective in controlling different soil-borne

pathogen as well as increasing growth and yield of many crops (Kaewchai *et al*., 2009, Rahman, 2013). However, scanty published reports on disease suppression and improvement of growth and yield of this pulse crop are available in Bangladesh utilizing *Trichoderma* fortified compost. Therefore, this study was undertaken to select the most effective isolates of *Trichoderma* species against different soil-borne fungi of chickpea identified under lab condition and to assess the potential of *Trichoderma*-fortified compost in controlling fungal diseases and enhancing growth and yield of chickpea in the field.

II. MATERIALS AND METHODS

2.1 Collection, isolation and preservation of *Trichoderma* spp. isolates

A total of 20 isolates of *Trichoderma* spp. were collected from soils of different crop fields of Chandina Upazilla of Comilla districts of Bangladesh. Soils samples were collected from rhizophere soil of carrot, radish, tomato, potato, brinjal and chilli. Fungi were isolated from individual samples following the soil dilution plate technique (Mian, I995). Briefly, a total of 10 gm of soil from a sample was mixed with 90 ml of sterilized water in a sterile conical flask while suspension was in motion. The initial soil sample was diluted through serial dilutions in order to achieve a small number of colonies on each plate. Then 5 ml of each dilution was incorporated into a plate with PDA (potato, 250 g; dextrose, 20 g; agar, 20 g; distilled water upto 1L) amended by 100 ppm streptomycin sulfate. The Petri dishes were incubated for 3-5 days at room temperature (25±2°C). Fungus was purified on PDA following hyphal tip culture technique (Tuite, 1969). A total of 20 fungal isolates were identified as *T. harzianum* on the basis of growth, colony and morphological characters following the standard key (Barnett and Hunter, l998). The other isolated fungi were discarded.

2.2 Testing the antagonistic activity of *T. harzianum* isolates *in vitro*

Twenty isolates of *T. harzianum* were tested against *F. oxysporum, R. solani* and *S. rolfsii* on potato dextrose agar (PDA) medium f dual culture technique by placing 5 mm diameter young mycelium disc of pathogen at one end and that of the biocontrol agent at the other end of a 9 cm diameter Petri dish (Dhingra and Sinclair, 1985). The selected virulent isolates of test pathogens were collected from the stock culture of the laboratory of the Department of Plant Pathology, BSMRAU. All plates were incubated in the dark at 25°C until the mycelium of *F. oxysporum, R. solani* and *S. rolfsii* covered the whole area of the control plate. Inhibition percentage of the radial growth of *F. oxysporum, R. solani* and *S. rolfsii* were calculated following the formula as suggested by Sundar *et al.* (1995):

$$\% \text{ Inhibition of growth} = \frac{X - Y}{X} \times 100 \quad \text{(equation 1)}$$

Where, X = mycelial growth of pathogen in absence of *T. harzianum* (control), Y= mycelial growth of pathogen in presence of *T. harzianum*).

2.3 Preparation of *Trichoderma* fortified compost with different substrates

Before setting the experiment in the field, a total of five compost pits (1.0 m x 1.0 m x 1.5 m) were prepared separately where each compost pit contained 40 kg each of saw dust, tea waste, poultry manure, water hyacinth and cow dung. After 45 days of decomposition wheat grain colonized *Trichoderma* inoculum @2.5 kg was mixed in each compost pit. Then it was left for 90 days for decomposition and degradation following the procedure of the preparation of standard quality compost as described by James (2008). On the basis of screening test against the studied virulent isolates of *F. oxysporum, R. solani* and *S. rolfsii*, the highly antagonist isolate of *T. harzianum* Co-7 was selected for the field experiment

2.4 Application *Trichoderma* fortified compost with different substrates for controlling major diseases of chickpea in field condition

A field experiment was conducted to evaluate the most effective composting substrate mixed with *T. harzianum* isolate Co-7 for controlling major soil borne diseases of chickpea as well as its impact on growth promotion and increasing the yield of chickpea. Prepared *Trichoderma* fortified compost @ 5 kg plot^{-1} (8.33 t ha^{-1}) with individual substrates as described earlier were used in the field as treatments. Seeds of chickpea (*Cicer arietanum*) variety BARI Chola-5 collected from Bangladesh Agricultural Research Institute, Joydebpur, Gazipur were grown for this purpose in a non-sterile field soil. This experiment included following seven treatments:

- T_1=sowing of seeds only in field soil (Control 1)
- T_2= Wheat bran colonized *Trichoderma* without compost (Control 2)
- T_3=Colonized *Trichoderma* with saw dust
- T_4=Colonized *Trichoderma* with cow dung
- T_5=Colonized *Trichoderma* with tea waste
- T_6=Colonized *Trichoderma* with water hyacinth
- T_7=Colonized *Trichoderma* with poultry manure

2.5 Monitoring of disease development

Chickpea plants were observed regularly after sowing to record the incidence of pre-emergence and post-emergence seedling mortality, infection both on plant organs and pods at different growth stages. The causal agents of the recorded diseases were identified on

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isolation of the pathogen from the infected roots, stems and pod. The disease incidence was recorded continuously at 3 days interval from transplanting to final harvest. Observations were made by selecting five plants randomly from each plot. Disease severity of wilt, wet root rot and collar rot caused by *F. oxysporum, R. solani* and *S. rolfsii*, respectively was rated as 0-4 scale in which 0= no symptoms, 1=1-25%, 2=26-50%, 3=51-75% and 4=76-100% of chickpea organ covered with lesions (Morid *et al*., 2012). Diseases of the crop were expressed as percentage using the following formulae:

- Disease incidence (%) $= \frac{\text{Number of infected plants}}{\text{Total number of plant observed}}$ x 100 (Equation 2)
- Percent disease index (PDI) = $\frac{\text{Summation of all rating}}{\text{Number of plant observed} \times \text{Maximum rating}}$ x 100 (Equation 3)

2.6 Observation of growth promoting factors at maturity and yield

Growth promoting factors including plant height, no. of branches plant^{-1} and numbers of pods plant^{-1} were recorded randomly five plants from each replicated plots of all the treatments attain after certain maturity. Pods plant^{-1} was harvested and the total dry weight of seed was also measured.

2.7 Design of experiment and data analyses

The field experiment was laid out in the Randomized Complete Block Design (RCBD) with four replications. Data recorded on various diseases and yield components were analyzed statistically using the STATISTIX 10 computer program after proper transformation whenever necessary and the means were compared following DMRT.

III. RESULTS

3.1 Screening of *T. harzianum* isolates against *F. oxysporum* f. sp. *ciceri, R. solani,* and *S. rolfsii*

All the 20 isolates of *T. harzianum* showed more than 60% inhibition of radial growth of the tested pathogens as compared to untreated control except the isolate Co-6 where the growth inhibition against *R. solani* was 58.8%. Among the screened isolates of *T. harzianum* only four isolates namely Co-5, Co-7, Co-12 and Co-20 showed above 70% growth inhibition against all the tested pathogens (Table 1). The same isolate of *T. harzianum* had highly varied antagonistic ability and it ranged from 60 to 80% depending on the test pathogen. However, the tested isolates Co-7 showed the highest inhibition of the radial growth among all the tested pathogens (Table 1 and Fig. 1).

3.2 Assessment of *Trichoderma* fortified composts applied in the chickpea field in controlling soil-borne diseases

3.2.1 Effect on seedling mortality

Immediately after sowing of chickpea seed, pre-emergence and post-emergence seedling mortality caused by *F. oxysporum, R. solani* and *S. rolfsii* were recorded up to five weeks of the plant growth. The highest reduction of total seedling mortality over control (69.31%) was recorded in plants receiving a combined treatment with the *Trichoderma* fortified compost mixed with poultry manure as substrate (T_7). However, the similar result with T_7 was also observed in treatment T_4 and T_5 where the field soil received cow dung and tea waste with *T. harzianum* isolate, respectively (Table 2). Other treatments (T_6 and T_3) showed statistically similar effect on seedling mortality in comparison to the untreated control where only chemical fertilizers were mixed without *Trichoderma* fortified compost.

3.2.1 Effect on disease development

All the *Trichoderma* fortified composts reduced superiorly diseases of chickpea in comparison to untreated control. Among the diseases, wet root rot caused by *R. solani* was the most prevalent followed by collar rot caused by *Sclerotium rolfsii* and Fusarium wilt caused by *F. oxysporum* (Table 3). Significantly the highest reductions of the marked three diseases over control were achieved with *Trichoderma* fortified compost with poultry manure (T_7) followed by the treatment containing mixtures of *Trichoderma* and cow dung (Table 3). Additionally, the highest reduction of PDI over control was also observed in the treatment T_7 (64.9% of *F. oxysporum*, 67.8% of *S. rolfsii* & 65.7% of *R. solani*) in case of all the diseases but those of treatments T_4 and T_5 were numerically almost similar followed by T_6 in case of collar rot and wilt (Table 4). However, in case of the reduction of PDI caused *R. solani* treatment T_7 was highest (65.7%) but T_4 and T_5 were almost numerically identical to T_7.

3.3 Performance of *Trichoderma* fortified compost on growth promoting components and yield of chickpea in the field

Trichoderma fortified compost increased the growth promoting components including the number of branch plant^{-1} and plant height compared to those of untreated control (T_1). In case of pods plant^{-1} treatments T_2, T_3, T_5, T_6 were statistically identical but significantly lower to those of T_7 treatment where *Trichoderma* were mixed with poultry manure (Table 5). The treatments containing mixtures of colonized *Trichoderma* with cow dung and *Trichoderma* with poultry refuses were found statistically similar in increasing all growth promoting characters. Similarly significantly the highest seed yield (1.54 t ha^{-1}) was recorded in the plot with *Trichoderma* compost using poultry manure (T_7) followed by treatment T_4 using cow dung as compost. On the other hand, identical seed yield

and seed weight were observed in T_5 and T_6 treated plot but they were significantly lower than that of T_7 in seed yield (Table 5).

IV. DISCUSSION

Soil-borne pathogens such as *F. oxysporum* f. sp. *ciceri*, *R. solani* and *S. rolfsii* are primarily known as devastating pathogens to cause seedling diseases in chick pea cultivation. These pathogens are mostly difficult to control as they often reside alive for many years as sclerotia in soil or as mycelia in soil under several environmental conditions and subsequently attack the crops resulting poor yield. Now a-days, *Trichoderma* is widely considered as a potential cost-effective means against several pathogens attacking vegetables, fruits, field and industrial crops (Tran, 2010). In our study, we got many isolates of *Trichoderma harzianum* from rhizophere soil of different vegetables in Bangladesh. *In vitro* assay clearly showed that antagonists *T. harzianum* halted the radial mycelium growth of highly virulent isolate *F. oxysporum*, *R. solani* and *S. rolfsii*, with varying levels of antagonism. Similar antagonistic effect of *T. harzianum* against these soil-borne pathogens infecting many other crops was also observed by several other investigators (Sundar *et al*., 1995, Bhuiyan *et al*., 2007, Nitu et al., 2016). The variation among the different isolates of *T. harzianum* may be come about due to their genetic makeup for the antagonistic activity (Shanmugam *et al.*, 2008, Kumar *et al.*, 2011), production of virulence factor such as metabolites (Shentu *et al.*, 2014), trichodene (Malmierca *et al*., 2015) etc. Moreover, a variety of extracellular lytic enzymes such as high chitinase and β-(1,3)-glucanase have been reported to be produced by *T. harzianum* (Kumar *et al.*, 2012), and there may be relationship between the production of these enzymes and the ability to inhibit the pathogen (Elad et al., 1982, Sivan and Chet, 1989). Results in this study also revealed that the radial mycelial growth of the pathogens was interfered by *T. harzianum* within the contact area or interface zone.

Under field conditions, we have observed seedling mortality caused by *F. oxysporum*, *S. rolfsii*, and *R. solani.* Some reports accounted these soil-borne pathogens causing diseases in different vegetable crops including chick pea (Prashad *et al*. 2014, Akhter *et al.*, 2015). As these warm-dependent pathogens are mostly common in Bangladesh soil, soil amendments using composted agricultural wastes fortified with biocontrol agents could be acceptable approaches in this regard. A number of investigations have also showed that the organic amendments of soil with various origins were potential biological control agents in suppressing soil-borne plant pathogens (Litterick *et al*. 2004). Result of this study is also consistent with the consequence of a variety of soil amendments (Noble and Coventry 2010, Akter *et al.*, 2016). The highest disease occurrence in the untreated control plot (T_1) in case of all diseases followed by treatment T_2 where wheat bran colonized *Trichoderma* without compost was used indicated that the soil-borne pathogens were the most prevalent in the natural field condition even without artificial inoculation of the pathogens. Consequently, organic amendments with *Trichoderma* fortified with poultry manure yielded the lowest disease incidence as well as disease severity in this study. Moreover, crop treated with *T. harzianum* grown better and had higher yields to compare with the one without application. Different mechanisms have been suggested as being responsible for the action of individual bio-agents and composts. Disease suppressive effect of *Trichoderma* compost might be due to increase in microbial biomass of *Trichoderma* with the ideal food base compost, it aids in their introduction and establishment into the soil for sustained biocontrol activities of soil micro biota as stated by Hoitink and Boehm (1999). An antagonist parasitizes the pathogens, and poultry manure might be improved soil nutrients status and enhanced the efficacy of antagonist. The superior inhibitory effect of poultry manure is suggested to be related with the release of antifungal compounds from it. Poultry compost contains NH4-N and NO3-N while Brady (1974) claimed that nitrate-nitrogen can be readily taken by plants but where carbon-based organic residue are available, resident soil micro organisms tend to exploit NH4+ more quickly than plants. Considerable evidence has piled up to support the idea that ammonia liberated following application of high nitrogen amendments contributes to kill soil-borne pathogens (Shiau *et al*., 1999). Additionally, the bio-control activities of *T. harzianum* against *F. oxysporum* f. sp. *ciceri*, *R. solani* modulate the induced plant resistance and enhance the plant growth (Sivan, 1989, Malik *et al*, 2005, Saxena *et al*., 2015). These results support the present findings of controlling soil-borne diseases as well as increasing growth promoting parameters and seed yield of chick pea by *Trichoderma* fortified composting.

V. CONCLUSION

Results from this study showed that poultry manure was appeared to be excellent and promising substrates for the preparation of *Trichoderma* fortified compost in controlling soil-borne diseases of chickpea. *Trichoderma* fortified compost with different substrates had also better effect in increasing different growth parameters as well as yield of chickpea than the untreated control where *Trichoderma* was not mixed. Farmers can adopt eco-friendly control measures of different soil borne diseases

of vegetables by using *Trichoderma* fortified compost with the lower cost in comparison to chemical pesticides which is a key to profitable organic farming. Overall, results obtained from this study clearly indicated that *Trichoderma* fortified compost was the most effective not only control the pathogenic diseases but also achieved directly through storage of compost carbon, and indirectly through enhanced plant growth and biological control which in turn contributes to increased soil carbon levels. However, the experiment should be repeated to standardize the ratio and composition of the compost substrate to prepare the most effective *Trichoderma* fortified compost.

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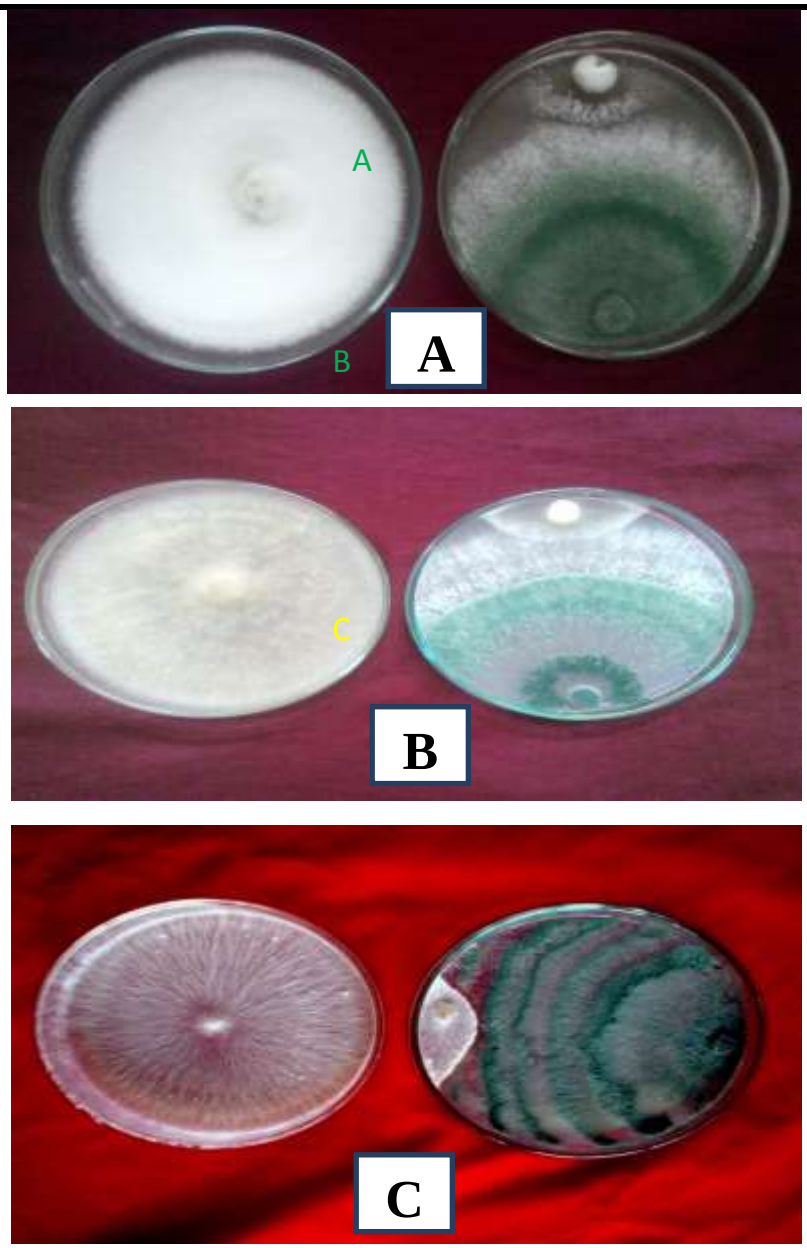

Fig. 1: Antagonism of T. harzianum isolate Co-7 in dual culture PDA plate against A. Trichoderma and Fusarium oxysporum, B. Trichoderma and Rhizoctonia solani, C. Trichoderma and Sclerotium rolfsii [right]; and control plates [left] along with dual culture.

Table 1. Screening of Trichodermaharzianum isolates against Fusarium oxysporum, Rhizoctonia solani and Sclerotium rolfsii by dual culture technique on PDA plates.

Isolates of *T. harzianum*	% inhibition of radial growth over control		
	F. oxysporum	*R. solani*	*S. rolfsii*
Co-1	70.00	67.7	77.7
Co-2	61.1	66.6	73.3
Co-3	71.1	64.4	68.8
Co-4	60.0	72.2	65.5
Co-5	76.6	73.3	77.7
Co-6	62.2	58.8	67.7
Co-7	82.2	78.8	83.3
Co-8	72.2	74.4	63.3

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Co-9	64.4	62.2	66.6
Co-10	68.8	65.5	80.0
Co-11	62.2	81.1	71.1
Co-12	78.8	77.7	75.5
Co-13	71.1	80	67.7
Co-14	66.6	62.2	64.4
Co-15	76.6	68.8	72.2
Co-16	66.6	70.0	78.8
Co-17	73.3	65.5	61.1
Co-18	74.4	64.4	76.6
Co-19	72.2	63.3	66.6
Co-20	71.1	78.8	73.3

Table 2. Effect of Trichoderma fortified compost in controlling seedling mortality of chickpea in open field.

Treatments	% Mortality*			% Reduction of total mortality
	Pre-emergence	Post-emergence	Total	
T1= Untreated control	10.5 a	27.4 a	37.8 a	-
T_2= Wheat bran Colonized *Trichoderma* without compost	5.5 b	13.2 b	18.7 b	50.52
T_3= Colonized *Trichoderma* with saw dust	5.2 bc	12.0 b	17.3 b	54.23
T_4= Colonized *Trichoderma* with cow dung	4.1 d	8.9 d	13.0 d	65.6
T_5= Colonized *Trichoderma* with tea waste	4.5 bc	9.1 cd	13.6 cd	64.02
T_6= Colonized *Trichoderma* with water hyacinth	5.0 bc	11.6 bc	16.1 bc	57.4
T_7= Colonized *Trichoderma* with poultry manure	3.2 d	8.4 d	11.6 d	69.31

**Means in a column followed by the same letters does not differ significantly (p= 0.05) according to DMRT test.*

Table 3. Effect of Trichoderma fortified compost on incidence of chickpea diseases in the field.

Treatments	Fusarium wilt (*F. oxysporum*)		Collar rot (*S. rolfsii*)		Wet root rot (*R. solani*)	
	Disease incidence	% reduction over control	Disease incidence	% reduction over control	Disease incidence	% reduction over control
T1= Untreated control	36.4 a	-	35.9 a	-	25.7 a	-
T_2= Wheat bran Colonized *Trichoderma*without compost	26.6 b	26.9	25.2 b	29.8	20.9 b	18.7
T_3= Colonized *Trichoderma* with saw dust	22.7 c	37.6	21.8 c	39.3	16.2 c	36.9
T_4= Colonized *Trichoderma* with cow dung	17.3 e	52.5	16.8 e	53.2	12.9 e	49.8
T_5= Colonized *Trichoderma* with tea waste	19.5 de	46.4	19.5 de	45.7	14.6 de	43.2
T_6= Colonized *Trichoderma* with water hyacinth	22.5 c	38.2	21.4 c	40.4	15.2 cd	40.9
T_7= Colonized *Trichoderma* with poultry manure	13.2 f	63.7	12.9 f	64.1	12.5 f	51.4

Means within same column followed by common letter(s) are not significantly different (P=0.05) by DMRT.

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Table 4. Effect of Trichoderma fortified compost on severity of chickpea diseases in field.

Treatments	Fusarium wilt (*F. oxysporum*)		Collar rot (*S. rolfsii*)		Wet root rot (*R. solani*)	
	PDI	% reduction over control	PDI	% reduction over control	PDI	% reduction over control
T_1= Untreated control	38.5 a	-	32.3 a	-	36.4 a	-
T_2= Wheat bran Colonized *Trichoderma*without compost	30.3 b	21.3	28.1 b	13.0	27.1 b	25.5
T_3= Colonized *Trichoderma* with saw dust	25.0 c	35.1	22.9 c	29.1	23.9 b	34.3
T_4= Colonized *Trichoderma* with cow dung	15.7 ef	59.2	12.5 de	61.3	14.6 c	59.9
T_5= Colonized *Trichoderma* with tea waste	18.8 de	51.2	15.7 d	51.4	16.7 c	54.1
T_6= Colonized *Trichoderma* with water hyacinth	22.9 cd	40.5	20.8 c	35.6	22.9 b	37.1
T_7= Colonized *Trichoderma* with poultry manure	13.5 f	64.9	10.4 e	67.8	12.5 c	65.7

Means within same column followed by common letter(s) are not significantly different (P=0.05) by DMRT.

Table 5. Effect of Trichoderma fortified compost on growth promoting and yield components of chickpea

Treatments	No. of branch plant^{-1}	No. of pods plant^{-1}	Plant height (cm)	1000 seed wt. (g)	Seed yield (t ha^{-1})
T_1= Untreated control	3.0 e	32.8 c	42.1 d	140.2 d	1.02 f
T_2= Wheat bran Colonized *Trichoderma* without compost	3.2 de	35.5 c	46.1 c	140.9 c	1.13 e
T_3= Colonized *Trichoderma* with saw dust	3.5 cd	37.0 c	47.1 c	150.1 c	1.19 de
T_4= Colonized *Trichoderma* with cow dung	4.2 ab	43.0 ab	52.1 a	160.5 a	1.42 b
T_5= Colonized *Trichoderma* with tea waste	4.1 b	38.0 bc	51.5 ab	150.7 b	1.30 c
T_6= Colonized *Trichoderma* with water hyacinth	3.6 c	37.2 bc	47.5 bc	150.3 bc	1.27 cd
T_7= Colonized *Trichoderma* with poultry manure	4.5 a	46.8 a	55.2 a	160.9 a	1.54 a

Means within same column followed by common letter(s) are not significantly different (P=0.05) by DMRT.

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Endemic Fluorosis and Occurrence Gastrointestinal Disorders in Prakasam District A.P.

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Abstract— *Fluoride has been known to cause significant effect on human health through drinking water. Excessive ingestion of fluorides not only causes dental and skeletal fluorosis but also leads to gastro intestinal disturbances. Prakasam district of Andhra Pradesh, India is having severe fluorosis. In the present study epidemiological survey was conducted in five villages of Prakasam district regarding the incidence of Gastrointestinal symptoms such as loss of appetite, indigestion, nausea, vomiting, bloody vomiting, Pain in the upper part of the abdomen and blotting in the upper abdomen after eating. The results showed that nausea was found in 23 % of population. Loss of appetite was found in 18 % of villagers. Indigestion, bloody vomit was very low (6%) Experimental results show that male albino rats with ingestion of 4mg/L of fluorides for 120 days caused damage to the intestinal mucosa and sub mucosa. The possible reasons for the gastrointestinal problems and mechanism of action of fluorides on gastrointestinal tract was discussed.*

Keywords— Endemic fluorosis, gastrointestinal problems, damage of sub mucosal layers.

I. INTRODUCTION

Fluoride occurs naturally in public water systems as a result of runoff from weathering of fluoride-containing rocks such as fluorspar-CaF2,Cryolite-Na3AlF6 and fluorapatite-Ca10F2(PO4)6. Lower concentrations of Fluorides are having beneficial effect on teeth by preventing and reducing the risk of tooth decay. In fact, concentrations lower than 0.5mg/L intensify risk of tooth decay. However, higher concentration (more than 1.5 – 2mg/L) fluoride becomes quite detrimental to health. Fluoride is potentially toxic at high doses or with prolonged lower-level exposure. It may cause dental fluorosis, osteomalacia, ligament calcification, hypocalcemia, arrhythmias, neurotoxicity, headaches, vertigo, thyroid dysfunction and anaemia (McNeely et al., 1979). Recent studies indicate that fluoride exposure from fluoridated water correlates with increased risk of bone cancer in young boys, and hip fracture in the elderly. Major sources of internal exposure of individuals to fluorides are the diet (food, water, beverages) and fluoride-containing dental products (toothpaste, fluoride supplements). Internal exposure to fluorides also can occur from inhalation (cigarette smoke, industrial emissions). . Fluorides are known to disturb enzymes and interfere with the intermediary metabolism (Barbier et al., 2010).They inhibit growth and development of animals by controlling the cellular respiration and ATP Synthesis(Mendoza-Shulz et al. 2009).

Prakasam district of Andhra Pradesh, India is having serious endemic fluorosis (Raghava Rao 2016, Sudhakar et. al. 2015). Fluorides not only affects the human beings but also other animal population. No specific studies are there on the influence of fluorosis on intestinal disorders. In the present study an epidemiological survey was made on the influence of fluorosis on gastrointestinal tract disturbance in Prakasam district. Apart from this the histological studies were made on the experimental rats to prove the damage of Intestinal mucosa by fluorides. A cross sectional survey was conducted in the fluorosis infested villages of Prakasam district of Andhra Pradesh. The aim of the study was to find out the prevalence of gastrointestinal disturbances among the population and to assess the relation between drinking water fluoride level and prevalence of fluorosis.

II. MATERIAL AND METHODS

2.1. STUDY AREA

Prakasam District occupies an area of 17626 km2 .It is the largest in area among the coastal districts of Andhra Pradesh. This district lies between 140 50’ 27.725” to 160 17’ 21.168” north latitude and 780 31’ 1.298” to 80 30’ 22.62” east longitude. The average elevation is 10m (30ft). It has a population of above 3054940 as per 2001 census. Many areas in this district depends on ground

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water for drinking and other purposes. The base map representing the boundaries of 56 mandals are collected from collector office, Ongole. The collected map has been digitized by Arc Map 9.2 software. Map of the study area are shown in the following figure 1.

Taking these things into consideration a study has been conducted in selected villages of Prakasam district of Andhra Pradesh to understand the intensity of the problem.

2.2 METHODS

The study has been carried out in Giddalur, Kanigiri, and Chimakurthy, Kondapi, and Kandhukur mandals of Prakasam district in the year of 2016-17. From each mandal one village was selected and questioner was prepared and Household survey was made from 300 subjects from 120 households in each village where the fluoride incidence is high. Prior to starting the survey, information sharing and community consent was obtained by talking with Sarpanch and a large number of villagers. The nature and purpose of study was explained and oral consent was taken from the community. A cross sectional survey was made among the male population five villages from five mandals. In each village 60 males in the age group of 40-60 years were selected. Prevalence of symptoms related to the Gastro intestinal problems were recorded from them. The collected data was edited for completeness, accuracy and consistency.

2.3. Animal trails:

20 Wistar strain male albino rats were taken for the study. They were divided into two groups consisting of 10 animals in each group. First group was treated as control group without any treatments. Rest of the 10 animals were treated as treatment group. They were given 4mg/l sodium fluoride treated for 60 days. After 60 days all the animals were sacrificed and autopsy was made and histological sections were made from intestine.

III. RESULTS

The data regarding the incidence of gastro intestinal disorders in the fluorosis infested villages is presented in Table.

Majority of villagers are above 40 years are suffering from nausea (22%). Blotting and anorexia was noted in 18% population. Indigestion and occasional vomiting were recorded in 12-14% Population. All these symptoms indicate the prevalence of gastrointestinal disorders in the villagers.

Table: Incidence of symptoms of gastrointestinal problems. No of cases is out of 300 subjects surveyed in five villages of Prakasam District.

No	Symptom	Total subjects surveyed	No. of cases with symptom	% incidence
1	Loss of appetite	300	54	18
2	Indigestion	300	36	12
4	Nausea	300	66	22
5	Vomiting	300	42	14
6	Blood vomiting	300	18	6
7	Pain in the upper part of the abdomen	300	48	16
8	Blotting in upper abdomen	300	54	18

3.2. Histopathological studies

The c.s of the gastric region of control and fluoride treated rats were given below.

Figure1:

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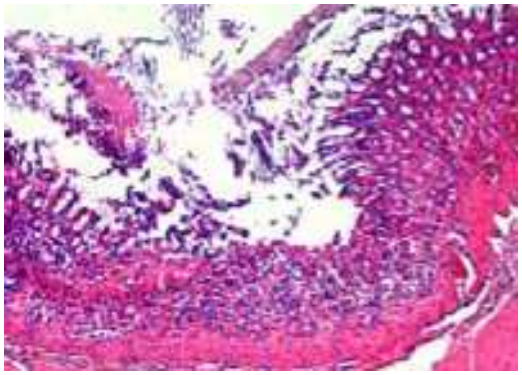
C.S. of gastric region of Control Rats



C.S.of Gastric region of fluoride treated rats

IV. DISCUSSION

Fluoride occurs in drinking water primarily as free fluoride. When ingested, some From the above figures we can conclude that there is a severe damage of gastric region of Flurried treated rats. In them mucosa and sub mucosa were severely damaged leading to cause gastro intestinal symptoms.

Fluorides combine with hydrogen ions to form hydrogen fluoride (HF), depending on the pH of the contents of the stomach (2.4% HF at pH 5; 96% HF at pH 2). HF easily crosses the gastric epithelium, and is the major form in which fluoride is absorbed from the stomach upon entering the interstitial fluid in the mucosa where the pH approaches neutrality; HF dissociates to release fluoride and hydrogen ions which can cause tissue damage. Whether damage occurs depends on the concentrations of these ions in the tissue. Single high doses of ingested fluoride are known to elicit acute GI symptoms, such as nausea and vomiting, but whether chronic exposure to drinking water with fluoride at 4 mg/L can elicit the same symptoms has not been documented well. Hence in the present study an attempt is made to record the chronic effect of fluorides on gastric symptoms.

Fluoride can stimulate secretion of acid in the stomach (Assem and Wan 1982; Shayiq et al. 1984), reduce blood flow away from the stomach lining, dilate blood vessels, increase redness of the stomach lining (Fujii and Tamura 1989; Whitford et al. 1997), and cause cell death and desquamation of the GI tract epithelium (Easmann et al. 1984; Pashley et al. 1984; Susheela and Das 1988; Kertesz et al. 1989; Shashi 2002).

V. CONCLUSIONS

The survey findings revealed that the prevalence of gastro intestinal symptoms in Fluoride polluted villages of Prakasam district. It was more in elders then the youngsters. Our experimental results proved the damage of gastrointestinal mucosa due fluoride ions in rats on chronic treatment. Fluoride disrupts enzyme activity by binding to functional amino acid groups that surround the active centre of an enzyme. This includes the inhibition of enzymes of the glycolytic pathway and the Krebs cycle (Barbier et al., 2010). Studies by Mendoza-Shulz et al. (2009) indicate that fluoride at micromolar concentrations can act as an anabolic agent and promote cell proliferation, whereas at mill molar concentrations it acts as an enzyme inhibitor on e.g. phosphatases, which play an important role in the ATP (cellular energy) production cycle and cellular respiration.

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Influence of Human Urine on Rice Grain Yield (*Orzya sativa L.*) and Selected Soil Properties in Abakaliki Southeastern Nigeria

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***Abstract*—** *An experiment was carried out at Abakaliki Southeastern Nigeria to study the influence of human urine on rice grain yield, selected soil physical and chemical properties in Abakaliki southeastern Nigeria in 2014 and 2015. The experiment was arranged in randomized complete block designed (RCBD) with human urine applied in the following rates: A = Control (no application of treatment); B = 2 kilolitres/ha; C = 4 kilolitres/ha and D = 6 kilolitres/ha. Treatments were not applied in 2015 to test the residual effect. In general, human urine improved rice grain yield, bulk density, total porosity, hydraulic conductivity, moisture content, organic carbon, total nitrogen, C/N ratio, pH, available phosphorus and exchangeable bases in 2014 it was applied and the following year as residual effect. An increase in the rate of urine application also resulted to an increase in rice grain yield and higher improvement in soil properties studied.*

Keywords* — *Effects, faeces, sewage, rice grain, urine

I. INTRODUCTION

Use of human urine as alternative to synthetic fertilizer has not been put into usage at Abakaliki, the study area. The reason is that the method of collection is very difficult as most human beings urinate on land directly or discharge mixture of urine and faeces to tanks and pit latrine resulting to sewage thereby making collection of pure urine difficult. According to [1], more than 95% of sewage in developing countries do not undergo any form of treatment as to use them as alternative to synthetic fertilizer without adverse effect. Also, the use of synthetic fertilizer as amendments in most developing countries for crop productions can no longer be relied upon since it is too costly, unavailable when needed by farmers [2] and leads to soil degradation on continuous usage. Thus, there is a need to consider alternative sources of synthetic fertilizer such as human urine due to the fact that it could be readily available and cheap.

Unlike faeces, human urine from a healthy person is generally sterile and can be used as a fertilizer without recourse to any further purification [3]. However, even sterile human urine can get contaminated from faeces during collection due to dysfunctional collection systems or improper use of urine diversion toilets. It is therefore recommended to sanitize human urine before applying it to crops [4]. According to [5] storage periods up to 6 and 3 months at about 4 °C and above 20 °C, respectively are necessary for a safe handling of human urine. Human urine contains most of the nutrients of human excreta, and it can yield considerable amounts of N, P, K, S, Ca, and Mg [6].

The objective of the study was to determine the influence of human urine on rice grain yield, selected soil physical and chemical properties in Abakaliki southeastern Nigeria.

II. MATERIALS AND METHODS

2.1 Experimental site

The study was conducted at Abakaliki Southeastern Nigeria in 2014 and 2015 cropping seasons. Abakaliki coordinates at latitude 06º2'N and longitude 08º05'E at an altitude of 447.2 m above mean sea level in the derived savannah of the southeast agro-ecological zone of Nigeria. The mean annual minimum rainfall is 1800 mm while the mean annual maximum rainfall is 2000 mm distributed between April and early November. There is short spell in August referred to as "August break". At onset of rainfall, it is violet and often torrential lasting for 1 – 2 hours. The minimum temperature is 27ºC while maximum is 31ºC. The relative humidity is highest during rainy season (80%) and declines to 60% in dry season especially at harmattan period. The bedrock geology is shale residuum due to successive marine deposit. The soils belong to the order Ultisol classified as Typic Haplustult [7].

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2.2 Sources of materials, land preparation and experimental design

Three containers were provided for the urine collection at one of the Primary Schools in Abakaliki, Ebonyi State, Nigeria. Teachers and pupils in the school were advised to urinate into any of the three containers for 2 weeks. At the end of each day, urine in the three containers were collected, mixed together and stored in air-tight plastic container at the temperature of 25^{o}C. At the expiration of the 2 weeks of collection, the urine was allowed for 6 months before application in the field. Faro 52 (the test crop) was bought from Ebonyi State Agricultural Development Programme.

The experiment was laid out in Randomized Complete Block Design (RCBD) while the 20 plots, each 3 X 4 m were used. Each plot and block was separated by 0.5 and 1.0 m, respectively. Four treatments replicated five times were used for the study. Treatment details are – A = Control (no application of amendment); B = 2.4 litres/plot equivalent to 2 kilolitres/ha; C = 4.8 litres/plot equivalent to 4 kilolitres/ha and D = 7.2 litres/plot equivalent to 6 kilolitres/ha.

Treatments were applied three weeks after planting of rice seeds.

Four rice seeds were planted per hill three weeks before treatment application at the inter and intra row spacing of 25 cm and 20 cm, respectively whereas the planting depth was 1.5 cm. Hand weeding was used to control weeds throughout the period of the experiment. The experiment was rain fed and neither pesticides nor synthetic fertilizers were applied. The same procedure was repeated in the second year of the experiment but without the application of treatment to test the residual effect.

2.3 Sampling and laboratory analysis

Undisturbed core soil samples of 157 cm^3 and auger soil samples were collected from all the plots at 90 days after planting (DAP) from four observational points each cropping season and used for the determination of the physical and chemical properties of the soil in the laboratory. Auger soil samples were collected at 0 – 20 cm soil depth. Proximate analysis of urine and initial soil analysis were also carried out and the results are as presented on Table 1. Bulk density (Bd) was determined using the method described by [8]. Total porosity (Tp) was determined using the formular –

Tp = 100(1 – Bd/Pd) where Pd = particle density assumed to be 2.65 gcm^{-3}. Hydraulic conductivity (Hc) was determined as described by [9]. Moisture content (Mc) was determined by calculation as outlined by Obi [10]. Particle size distribution was determined using Bouyoucous hydrometer method as described by [11]. Soil pH was determined using a suspension of soil and distilled water in the ratio of 2:5 – soil: water [12].

Table.1: Proximate analysis of urine and initial soil properties

Test parameter	Urine	Soil
Sand	-	680 gkg^{-1}
Silt	-	210 gkg^{-1}
Clay	-	110 gkg^{-1}
Bulk density	-	1.66 gcm^{-3}
Texture	-	Sandy loam
Total porosity	-	37.36%
Hydraulic conductivity	-	19.58$cmhr^{-1}$
Moisture content	-	26.16%
pH	9.3	6.15
Total N	8.6 gL^{-1}	0.08%
Total Carbon	8.4 gL^{-1}	0.85%
C/N ratio	0.98	10.63
Available P	0.09 gL^{-1}	18.23 $mgkg^{-1}$
Ca	0.4 gL^{-1}	2.1 Cmol(+)kg^{-1}
Mg	0.13 gL^{-1}	0.8 Cmol(+)kg^{-1}
K	1.3 gL^{-1}	0.2 Cmol(+)kg^{-1}
Na	1.8 gL^{-1}	0.02 Cmol(+)kg^{-1}

Total nitrogen was determined using modified kjeldahl digestion procedure [13]. Available phosphorus was determined by Bray 11 method [14]. Organic carbon was determined by the method of [15]. Exchangeable bases were determined using [16] method. At maturity, 10 rice plants per plot were selected and tagged [17]. The grain yields from the tagged plants were harvested, dried to 11 % moisture content. Grains/plot was weighed and then converted to its hectare equivalent. Statistical analysis of the data was carried out using the General Linear Model of SAS software for Randomized Complete Block Design [18] while treatment means were separated using the Duncan's Multiple Range Test (DMRT).

III. RESULTS

3.1 Soil physical properties

The influence of urine on soil bulk density and total porosity is as presented on Table 2. The application of urine in 2014 at B, C and D significantly decreased bulk density and increased total porosity in 2014 and 2015 when compared to control. The higher the increase the lower the decreased in bulk density and higher the increased in total porosity. Each treatment recorded higher bulk density and lower total porosity in 2015 than 2014.

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Table.2: Influence of urine on soil bulk density and total porosity

Treatment	Bulk density (gcm^{-3})		Total porosity (%)	
	2014	2015	2014	2015
A	1.67^{a}	1.69^{a}	36.98^{d}	36.23^{d}
B	1.62^{a}	1.63^{b}	38.87^{c}	38.49^{c}
C	1.58^{bc}	1.60^{c}	40.38^{b}	39.62^{b}
D	1.54^{c}	1.56^{d}	41.89^{a}	41.13^{a}

Note: Means in the same column with the same letter do not differ significantly at $P < 0.05$.

A = Control (no application of amendment); B = 2 kilolitres/ha; C = 4 kilolitres/ha and D = 6 kilolitres/ha

Table 3 shows the influence of urine application on soil hydraulic conductivity and moisture content. Hydraulic conductivity was significantly increased with an increase in the application of urine in 2014 cropping season. Similarly, the residual effect in hydraulic conductivity was significantly higher with those plots treated with higher rates of urine. Also, all the urine treated plots had higher hydraulic conductivity in 2014 than the residual year. Lowest moisture content of 25.45 % was observed in control while moisture content in urine treated plots ranged between 27.08 – 28.46 %. The order of increase in moisture content in residual year was D > C > B > A.

Table.3: Influence of urine on soil hydraulic conductivity and moisture content

Treatment	Hydraulic conductivity ($cmhr^{-3}$)		Moisture content (%)	
	2014	2015	2014	2015
A	17.32^{d}	19.23^{c}	25.45^{d}	24.86^{cd}
B	20.68^{c}	19.98^{c}	27.08^{c}	26.08^{bcd}
C	23.56^{b}	22.36^{b}	28.13^{ab}	27.34^{abc}
D	31.21^{a}	24.01^{a}	28.46^{a}	27.98^{ab}

Note: Means in the same column with the same letter do not differ significantly at $P < 0.05$.

A = Control (no application of amendment); B = 2 kilolitres/ha; C = 4 kilolitres/ha and D = 6 kilolitres/ha

3.2 Soil chemical properties

The influence of urine on pH and available P is as shown on Table 4. The Table also, show significant ($p < 0.05$) changes in pH and available P among the treatments studied. Soil pH and available P increased with an increase in urine applied. Also, urine recorded lower effect on residual year than the year in which urine was applied.

Table.4: Influence of urine on pH and available phosphorus

Treatment	pH		Available phosphorus ($mgkg^{-1}$)	
	2014	2015	2014	2015
A	6.01^{bc}	5.98^{ab}	15.23^{d}	13.23^{d}
B	6.23^{abc}	6.18^{a}	22.36^{c}	15.96^{c}
C	6.25^{a}	6.21^{a}	25.01^{b}	17.28^{b}
D	6.25^{a}	6.23^{a}	27.36^{a}	21.23^{a}

Note: Means in the same column with the same letter do not differ significantly at $P < 0.05$.

A = Control (no application of amendment); B = 2 kilolitres/ha; C = 4 kilolitres/ha and D = 6 kilolitres/ha

Table 5 shows influence of urine on organic carbon, total nitrogen and C/N ratio. There was non-significant ($p < 0.05$) changes among the treatment with regard to organic carbon in both 2014 and 2015 with the values of organic carbon observed residual year lower than the organic carbon observed in the year of treatment application.

Urine application significantly increased the total N in both the two years of the study. Also, the increase in the urine application resulted to an increase in total N in both 2014 and 2015 with higher total N in all the treatments in 2014. The order of increase in C/N ratio in both year of treatment application and residual year was D > C > B > A. Unlike other parameters in this study, C/N ratios were higher in the residual year than the year of treatment application.

Table.5: Influence of urine on organic C (%), total N (%) and C/N ratio

Treatment	Organic C		Total N		C/N ratio	
	2014	2015	2014	2015	2014	2015
A	0.79^{a}	0.75^{a}	0.06^{c}	0.04^{d}	13.17^{a}	18.75^{a}
B	0.80^{a}	0.77^{a}	0.12^{b}	0.10^{c}	6.67^{b}	7.70^{b}
C	0.79^{a}	0.76^{a}	0.14^{a}	0.12^{abc}	5.64^{c}	6.33^{c}
D	0.79^{a}	0.78^{a}	0.15^{a}	0.14^{a}	5.27^{cd}	5.57^{d}

Note: Means in the same column with the same letter do not differ significantly at $P < 0.05$.

A = Control (no application of amendment); B = 2 kilolitres/ha; C = 4 kilolitres/ha and D = 6 kilolitres/ha

The influence of urine on exchangeable bases is presented on Table 6. There was a significant increase in Ca with an increase in urine application in the two years of the experiment with 2015 recording the lower values of Ca when compared to 2014. Increase in urine application resulted to significant increase in Mg in both years of the experiment with lower values observed in the residual experiment. The order of K increase in both 2014 and 2015 was D > C > B > A while values were higher in 2014 than 2015. Control had the lowest Na value of 0.02 and 0.01 $Cmol(+)kg^{-1}$ in 2014 and 2015, respectively while Na in plots treated with urine ranged between 0.03 – 0.04 $Cmol(+)kg^{-1}$ in 2014 and 0.02 $Cmol(+)kg^{-1}$ in 2015.

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Table.6: Influence of urine on exchangeable bases (Cmol(+)kg^{-1})

Treatment	Ca		Mg		K		Na
	2014	2015	2014	2015	2014	2015	2014
2015							
A	1.8^{d}	1.2^{d}	0.6^{c}	0.4^{c}	0.16^{c}	0.12^{d}	0.02^{b}
0.01^{ab}							
B	2.2^{c}	2.0^{c}	0.9^{b}	0.7^{b}	0.22^{b}	0.18^{b}	0.03^{ab}
0.02^{a}							
C	2.6^{b}	2.4^{b}	1.0^{ab}	0.9^{a}	0.22^{b}	0.16^{c}	0.03^{ab}
0.02^{a}							
D	3.1^{a}	2.8^{a}	1.1^{a}	0.9^{a}	0.26^{a}	0.20^{a}	0.04^{a}
0.02^{a}							

Note: Means in the same column with the same letter do not differ significantly at P < 0.05.
A = Control (no application of amendment); B = 2 kilolitres/ha; C = 4 kilolitres/ha and D = 6 kilolitres/ha

3.3 Rice grain yield

Table 7 shows the influence of urine on rice grain yield. Increase in the application of urine resulted to a significant increase in rice grain yield harvested in the year of treatment application and the residual year. In the year of treatment application, control plot recorded rice grain yield of 2.56 t ha^{-1} while rice grain yield observed in urine treated plots ranged between 4.78 – 6.08 t ha $^{-1}$. Whereas in the residual year, control had rice grain yield of 2.32 t ha^{-1} and rice grain yield in urine treated plots ranged between 3.43 – 4.24 t ha^{-1}.

Table.7: Influence of urine on rice grain yield (t ha^{-1})

Treatment	2014	2015
A	2.56^{d}	2.32^{c}
B	4.78^{c}	3.43^{b}
C	5.18^{b}	4.12^{a}
D	6.08^{a}	4.24^{a}

Note: Means in the same column with the same letter do not differ significantly at P < 0.05.
A = Control (no application of amendment); B = 2 kilolitres/ha; C = 4 kilolitres/ha and
D = 6 kilolitres/ha

IV. DISCUSSION

Results of initial soil properties (Table 1) showed that the soil studied was a sandy loam. Sandy loam is highly permeable and allows large quantities of nutrients to pass through it [19]. As a result of this high permeability, soil of this texture contains poor plant nutrients and, hence, inorganic or organic amendment is necessary for good crop production. Initial soil pH was slightly acidic with a pH of 6.15. This slightly acidic nature could be attributed to low rainfall and high cropping intensity [20]. According to [21] organic carbon was low (0.85 %). This might be attributed to low natural organic matter returns and other human factors such as bush burning and crop removal. The total nitrogen was very low with the value of 0.08 %. This very low nitrogen content was a reflection of the organic carbon content in the soils [22]. Similarly, according to [21] exchangeable Mg and Ca were moderate with the values of 0.8 and 2.1 Cmol(+)kg^{-1}, respectively. The exchangeable K was very low with value of 0.2 Cmol(+)kg^{-1} which was equal to 0.20 Cmol(+)kg^{-1} regarded as the critical limit of exchangeable K in the soils [23]. The exchangeable Na was also low with the value of 0.02 Cmol(+)kg^{-1}.

Similarly, Table 1 showed that the various nutrients contained in urine were of higher concentration than that of soil, hence the need to use urine as soil treatment. [6] showed that human urine contained considerable amounts of primary crop nutrients such as N, P and K; and secondary nutrients such as S, Ca and Mg; and that urine application as an organic fertilizer in small-scale agricultural plots have shown the potential to match the crop yield quantity and quality commonly achieved with mineral fertilizers. [24] in his study of effect of different urine sources on soil chemical properties and maize yield in Abakaliki, Southeastern Nigeria observed significant higher effect of different sources of urine on to N, available P, exchangeable Ca and Mg when compared to the control. According to [25] human excreta improves maize crop production and water productivity in rain-fed agriculture. [24] also obtained significantly higher maize grain yield in plots treated with urine sources than the control. Also, [26] and Njoku and [17] showed that organic amendments improve soil physical properties such as bulk density, hydraulic conductivity, total porosity and moisture content which results to better crop yield. [27] on their study of effects of animal faeces and their extracts on maize yield in an Ultisol of eastern Nigeria showed that animal faeces and their extracts significantly increased the soil organic matter, exchangeable bases, cation exchange capacity and the available phosphorus and with the increase of soil nutrients following the application of the organic wastes, all amendments increased maize performance over the control.

V. CONCLUSION

The study showed that nutrients content of the urine have higher magnitude than the nutrients in the soils hence, the need to use urine as soil amendment. The application of urine in different rates improved rice grain yield and soil properties in this study. Unlike faeces, urine from a healthy person is generally sterile and can be used as a fertilizer without recourse to any further purification. However, urine can get contaminated from faeces during

collection due to failure of the collection systems or improper use of urine diversion toilets. It is therefore recommended to sanitize urine before applying to crops.

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Changes in Selected Soil Physical Properties and Maize Yields as Affected by Animal Wastes Application in Abakaliki Southeastern Nigeria

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Abstract— *The study was conducted at Abakaliki to determine the changes in selected soil physical properties and maize yields as affected by animal wastes application in Abakaliki southeastern Nigeria in 2014 and 2015 cropping seasons. The experiment was laid out in Randomized Complete Block Design with four treatment replicated five times. The treatments were poultry droppings at 5 tha^{-1} (PD), cow dung at 5 tha^{-1} (CD), mixture of PD + CD at 5 tha^{-1} and control (C) – non application of amendment. Bulk density, total porosity, moisture content, aggregate stability and mean weight diameter were determine in the laboratory using appropriate procedure while plant height, leaf area index and grain yield were also, measured in the field using recommended methods. The results showed positive changes in selected soil physical properties and maize yield in the two cropping seasons with the application of animal wastes. Also, improvement in soil physical properties and maize yields were higher in the second cropping season when compared to the first cropping season. Poultry dropping is recommended for farmers to use as fertilizer in maize production because plots treated with poultry droppings recorded the highest maize grain yield in the two cropping season than other treatments.*

Keywords — Animal wastes, improvement, physical properties, treatment, yield.

I. INTRODUCTION

Turning agricultural wastes into organic fertilizers is one of the waste recycling technologies. Organic fertilizers are used as a supplement particularly in some parts of Africa where nutrients availability in the soil is low and is a serious challenge for production of food [1]. The use of agricultural wastes as soil amendment can be used to improve soil productivity and increase crop yield thereby ensuring food security [2]; Njoku and [3]. Application of animal wastes on soil as amendments reduces the accumulation of the waste in the environment, reduces odour, bulk density and increase total porosity [4]. Using poultry droppings and cow dung as soil amendment have been reported by many researchers to give significant improvement in crop growth and yield. Parameters such as leaf area index, plant height, grain yield etc increased with the application of animal wastes. According to [5] and [6] poultry dropping improved soil properties which translated to higher crop yield. [7] observed that there was significant increase in yield of corn grains under the treatments of ploughing with composts as compared to the treatment of ploughing only, regardless the level of ploughing. Addition of individual residues with manures had resulted in higher dry matter weight of fodder sorghum compared to the control treatment [1]. In order to achieve a global trend towards organic farming we have to use poultry manure as a substitute for inorganic fertilizer [8]. Waste utilization in agriculture is a common phenomenon; it is a means of enhancing soil quality, creating livelihood for farmers and providing nutrients for plants [9]. Recent studies have shown that waste utilization in crop production has a positive effect on social, economic and environment.

The objective of the study was to determine the changes in selected soil physical properties and maize yields as affected by animal wastes application in Abakaliki southeastern Nigeria.

II. MATERIALS AND METHODS

2.1 Study area

The experiment was carried out at Abakaliki southeastern Nigeria. Abakaliki lies in latitude and longitude of $04^{0}6^{'}$ N and $08^{0}65'$ E, respectively in the derived savannah of the Southeastern agro-ecological zone of Nigeria. The yearly rainfall ranges between 1700 -2000 mm. The rainfall pattern is bimodal which normally start at April – July and September – November and there is short break in

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August generally referred to as August break. January – March were normally known as dry season while the minimum and maximum temperatures of the area were 27°C and 31°C, respectively [10]. The relative humidity of the area during the dry season and rainy season are 55 - 60% and 75 - 80%, respectively. The soil of the area belongs to the order Ultisol.

2.2 Sources of materials

Animal wastes and maize (Oba super II) were purchased from animal unit of Ebonyi State University and Ebonyi State Agric Development Programme, respectively.

2.3 Land preparation and experimental design

The experiment was laid out in Randomized Complete Block Design with four treatment replicated five times. Cutlass was used in clearing the vegetation and debris was removed and the beds were made using hoe. Treatments were incorporated into the plots immediately after cultivation using hoe. Two maize seed were planted per hole. The maize seed was planted at the depth was 3 cm while spacing of 75 cm between rows and 25 cm within rows were also used. Two weeks after germination (WAG) the young plant was thinned down to one plant per stand and lost stands were replaced. The crop population was 48 seedlings per plot. Weeding was done manually at three weeks interval till harvest period. The same procedure was repeated in 2015 cropping season. Treatments used for the experiments are as follows:

i. C – 0 t ha^{-1} (Control)
ii. PD – 5t ha^{-1} of Poultry droppings = (4.5kg/plot)
iii. CD – 5t ha^{-1} of Cow dung = (4.5kg/plot)
iv. MX– 5 t ha^{-1} of Mixture (2.5t $ha^{-1}$of Poultry droppings + 2.5t ha^{-1} of Cow dung)

2.4 Soil Sampling

Initial auger soil samples and core soil samples of 170.9cm^{-3} were collected from 5 different places from the site before cultivation and used for the determination of initial soil properties. Also, undisturbed core soil samples of 170.9 cm^{-3} and auger soil samples were collected from each plot at 90 days after planting (DAP).

2.5 Laboratory Analyses

The following soil physical properties were determined:

Bulk density and total porosity were determined as described by [11]. Moisture content was determined using the procedure outlined by [12]. Aggregate stability and mean weight diameter were also determined using the method described by [13]. Bouyoucous hydrometer method was used to determine particle size distribution as described by [14]. Textural triangle was used to determine textural class of the soil.

2.6 Crop Parameters Determined

At 90 DAP, ten maize plants per plot were selected and tagged [3]. The tagged plants were used for the determination of the following crop parameters:

i. Plant height: Plant height was measured from the ground surface to the tip of the plant using a meter rule.
ii. Leaf area index: Leaf area index was determined by calculation – using the formular: Length X Width X 0.905 – where 0.905 is a correction factor.
iii. Grain yield: Grain yield was determined by shelling the cobs of harvested plants and dried to 14 % moisture content. Dried grain yield per plot was weighed and then converted to its hectare equivalent.

2.7 Data Analyses

Statistical analysis of the data was carried out using the General Linear Model of SAS software for Randomized Complete Block Design [15] while differences between treatments means were determined using the Fisher's Least Significant Difference (F-LSD)

III. RESULTS

3.1 Initial properties of the soil

Table 1 shows the initial properties of the soil studies. The soil studied was a sandy loam recording the values of sand, silt and clay of 480gkg^{-1}, 402gkg^{-1} and 118gkg^{-1}, respectively. Similarly, the bulk density, total porosity, moisture content, aggregate stability and mean weight diameter of the soil before planting were 1.2gcm^{-3}, 51.32%, 11.98%. 6.8% and 1.34mm, respectively.

Table.1: Initial Properties of the Soil Studied

Parameters	**Value**
Sand	480 gkg^{-1}
Silt	402 gkg^{-1}
Clay	118 gkg^{-1}
Textural	Sandy loam
Bulk Density1.2gcm^{-3}	
Total Porosity	51.32%
Moisture Content	11.98%
Aggregate Stability	6.8%
Mean weight diameter	1.34mm

3.2 The Effect of Animal Waste on Soil Physical Properties

The effect of animal waste on soil physical properties is shown in Table 2. There was a significant ($P<0.05$) differences in the values of bulk density, total porosity, moisture content and mean weight diameter observed in all the plots in the two cropping seasons. Higher bulk

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density of 1.28gcm^{-3} was recorded by control in 2014 cropping season. This observed bulk density in control was higher than bulk density in PD, CD, and MX by 2%, 1%, and 1%, respectively. Similarly, in 2015 cropping season higher bulk density of 1.36 gcm-3 in control while that of animal waste treated plots ranged between 1.23 – 1.25 gcm^{-3}. The order of total porosity increase in 2014 cropping season was MX>PD=CD>C while the order of total porosity increase in 2015 cropping season was CD>PD>MX>C. The lowest moisture content of 11.23% was observed in control in 2014 cropping season moisture content animal wastes amended plots ranged between 12.94 – 14.38%. Also, in 2015 cropping season lowest moisture content of 9.68% was recorded in control and moisture content in plots treated with animal wastes ranged between 13.24 – 14.26%. The order of increase in aggregate stability in 2014 cropping season was PD>MX>CD>C whereas the order of aggregate stability increase in 2015 cropping season was CD>MX>PD>C. In 2014 cropping season, the lowest mean weight diameter value of 0.95mm was observed in control. This observed mean weight diameter value in control was lower than mean weight diameter in PD, CD and MX by 75%, 56% and 68%, respectively. Whereas, in 2015 cropping season the lowest mean weight diameter of 0.86 mm was observed in control while that of animal wastes treated plots ranged 1.51 – 1.68 mm.

Table.2: Effect of Animal Waste on Soil Physical Properties

Treatments	BD (gcm^{-3})		TP (%)		MC (%)		AS (%)		MWD (mm)	
	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
C	1.28	1.36	51.70	48.68	11.23	9.68	8.00	6.38	0.95	0.86
PD	1.26	1.24	52.08	53.21	13.93	14.26	10.64	11.31	1.66	1.68
CD	1.27	1.23	52.18	53.59	12.94	13.24	9.23	12.41	1.48	1.51
MX	1.27	1.25	52.45	52.83	14.38	13.56	10.45	12.01	1.60	1.54
F–LSD	0.02	0.11	0.03	0.25	0.02	0.36	0.03	0.28	0.03	0.10

Where C = Control (Non-application of amendment; PD = 5tha^{-1} of Poultry droppings; CD = 5tha^{-1} of Cow dung; MX = 2.5t ha^{-1} of Poultry droppings + 2.5tha^{-1} of Cow dung; BD = Bulk density; TP = Total porosity; MC = Moisture content; AS = Aggregate stability and MWD = Mean weight diameter

3.3 Effect of Animal Wastes on Agronomic Parameters

The effect of animal wastes on plant growth, leaf area index and maize grain yield is shown in Table 3. There was a significant ($p < 0.05$) differences in the values of leaf area index, plant growth, and grain yield in all the plots studied. The lowest value of plant height of 100.96cm was observed in control and the highest value of 118.40cm was recorded in PD in 2014 cropping season. Similarly, in 2015 cropping season control recorded the lowest plant height of 98.68 cm while that of animal wastes treated plots ranged between 110.24 cm – 135.65 cm. The lowest leaf area index of 203.31 cm was recorded in the control in 2014 cropping season. This observed leaf area index in control in 2014 cropping season was lower than that of PD, CD and MX by 10, 6 and 2%, respectively. In 2015 cropping season, lowest leaf area index of 198.23 was observed in control and leaf area index recorded in animal wastes treated plots ranged between 211.36 in MX – 228.51 in PD. The order of increase in maize grain yield in 2014 and 2015 cropping season was PD>CD>MX>C.

Table.3: Effect of Animal Waste on Plant Growth, Leaf Area Index and Maize Grain Yield

Treatments	Plant height (cm)		Leaf area index		Grain yield (t ha^{-1})	
	2014	2015	2014	2015	2014	2015
C	100.96	98.68	203.31	198.23	2.10	1.86
PD	118.40	120.63	224.73	228.51	2.87	3.01
CD	108.60	110.24	216.95	221.13	2.44	2.98
MX	133.59	135.65	207.42	211.36	2.24	2.56
F–LSD	2.809	3.697	0.032	0.086	0.276	0.309

Where C = Control (Non-application of amendment; PD = 5tha^{-1} of Poultry droppings;
CD = 5tha^{-1} of Cow dung; MX = 2.5t ha-1 of Poultry droppings + 2.5tha^{-1} of Cow dung

IV. DISCUSSION

4.1 Soil Physical Properties

The results indicated that the bulk density in plots treated with animal wastes reduced more than the bulk density in control during the two cropping seasons studied. In the second season of the experiment the bulk density in control increased when compared to first season of the experiment. On the other hand, total porosity was higher in plots treated with animal wastes than control. Total porosity unlike bulk density decreased in control and increased in treated plots in second season of the experiment more than first season of the experiment. This indicated that the application of animal wastes in soils reduce soil bulk density and increase total porosity. This agrees with the work of [5] who noted that addition of

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animal wastes to soil reduced soil bulk density and increased total porosity. They also recorded that annual addition of poultry wastes to soil had beneficial value on soil physical properties. [9] also observed reduction in soil bulk density and increase in total porosity with increase in different levels of poultry droppings application.

Plots treated with animal wastes gives higher moisture content when compared with control in the two cropping seasons. Control had lower moisture content in second cropping season than first cropping season whereas plots treated with animal wastes recorded higher moisture content in second cropping season than control. This showed that animal wastes have the capacity to increase the water content of the soil. The positive effect in soil water accumulation could be ascribed to greater quantity of poultry manure added to the soil. This is in supports of the earlier work of [16] who reported that addition of poultry manure to soil enhance organic matter content of the soil thereby improved the moisture absorption in the soil. The improvement in the moisture absorption of soil might be due to the positive development in the soil structure which was associated with poultry manure application [17]. [9] reported that poultry additions of poultry manure up to 50 tha^{-1} increases moisture content of the soil. Moisture content therefore can be improved by adding animal wastes which helps to improve soil quality.

Application of animal wastes to soil improved the aggregate stability in the two cropping season when compared to control. Aggregate stability in control reduced in the second cropping season while aggregate stability in the animal wastes treated plots increased in the second cropping season more than the aggregate stability observed in the first cropping season. Plots treated with animal wastes recorded higher mean weight diameter than control in the two cropping seasons when compared with control. Also, in the second year of the experiment control and animal wastes treated plots recorded lower and higher mean weight diameter, respectively than the first year of the experiment. [18] reported that the beneficial effects on physico-chemical properties of soil were as a result of compost added to the soil which is in support of this study.

4.2 Effect of Animal Wastes on Maize Yield

Plant heights were significantly higher in animal wastes treated plots than control in the two cropping seasons. Animal wastes treated plots had higher plant height in the second cropping season than first cropping season while that was lower in the second cropping season than first cropping season. Similarly, leaf area index was higher in animal wastes treated plots than control in the two cropping seasons. Leaf area index decreased in control and increased in animal wastes treated plots in the second cropping season than first cropping season. [6] showed that poultry droppings are made up of important elements that are related with high photosynthetic actions which enhance vegetative and roots growth. [19] reported earlier that development of maize leaf, vigorous and healthy growth of the crop is as a result of poultry droppings added to the soil. [8] noted that poultry manure helps in vigorous growth, physiological activities and also increased meristematic tissues in the plant due to the content of plant nutrient in the soil. Also, grain yields were higher in animal wastes treated plots than control in the two cropping seasons. Plots treated with poultry droppings recorded the highest maize grain yield in both cropping seasons. Maize grain yield in animal wastes treated plots were higher in the second cropping season while maize grain yield in the control decreased in the second cropping season. This showed that animal waste treatment is important for sustainable maize production. [6] in their own view also reported that poultry manure had effects positively on the physical attributes of water melon which they said could be due to the fact that poultry manure contained important elements that is good for high photosynthetic activities that promote vegetative growth and prolific root. [19] reported earlier that, different types of manure application rates leads to significant response in grain yield which are in the support of this study.

V. CONCLUSION

The results indicated that animal wastes added to the soil at 5tha^{-1} improved soil physical properties and maize yield. The improvement was higher in the second cropping season than that of first cropping season which proved that animal wastes application to soil promotes sustainability. Poultry dropping is recommended for farmers to use as fertilizer in maize production because plots treated with poultry droppings recorded the highest maize grain yield in the two cropping season than other treatments.

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Trees Lose Their Leaves Later in Agroforestry Systems

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***Abstract*—** *In Brazilianagroforestry systems (AFS), Cordia oncocalyx trees, a native species of Caatinga, lose their leaves late in relation to the trees of the same species occurring in secondary forest. Our hypothesis is that, due to environmental features, the trees of the AFS maintain better water status. This work aims to present environmental humidity (rainfall, soil moisture and air relative humidity) and trees (photosynthesis, stomatal conductance and transpiration) data to explain the late loss of leaves in anagrosilvopastoral system (AGP) in the Brazilian semiarid region compared to a secondary forest (SF).Meteorological data were obtained from two weather stations installed in the AGP and SF areas. The physiological traits were measured using an infrared gas analyzer. There was a correlation between physiological processes (transpiration and stomatal conductance) and soil water content in plants of AGP, but not in SF, showing some independence of the plants of this system to variations in soil moisture. This indicates that AGP plants may have developed the physiological and anatomical features that enable to them to keep photosynthesis even when climatic conditions are more severe. Although the most inhospitable environmental conditions in the AGP system, the lower density of plants, and therefore less competition for water, favoring photosynthesis longer, causing the leaves to fall later.*

Keywords*— *Cordia oncocalyx, tree density, gas exchange, semiarid, secondary forest.

I. INTRODUCTION

The development of plants depends on both intrinsic (inherent to the plants themselves) and extrinsic (environmental) factors. Plant can respond to changes in the environment with both morphological and physiologicalmodifications and adaptations. In high temperature situations, high incidence of solar radiation or water shortage, i.e., may decrease the photosynthesis by closing or reducing the number of stomata [1], modifying hormone levels [2] or modifying the storage of proline, soluble sugars and amino acids [1].

Not only the abiotic factors (extrinsic) from the environment act on the growth and development of plants [3], these are also affected by biotic factors such as population density and intra and interspecific competition, which, in turn, are also affected by extrinsic factors, such as soil conditions [3]. For example, trees in stands with higher densities are thinner and absorb more superficial water, by which they start to compete, in view of the smaller amount of available water in this horizon of the ground. While trees in lower density have thicker stem and the roots can capture deeper water [4].

In agroforestry systems, which are production systems that combine crops and trees, the number of trees is reduced relative to that of the forest. Due to this reduction in density, greater intensity of solar radiation reaches the treetops and the ground. Thus, in semi-arid environments, higher temperatures are expected to be recorded in the soil, higher soil water evaporation and lower relative humidity. While, in relation to plants, a reduction in tree height and higher biomass can be recorded [5].

Agroforestry systems have provided an alternative to conventional cropping systems by including larger plants (shrubs and trees). Some studies show the benefits of agroforestry in comparison to monoculture [6, 7], despite the potential for water, light and nutrient competition between cultivated plants and trees [8, 9]. The agrosilvopastoralsystems (AGP) is a kind of agroforestry system where both agricultural and animal production are developed together and the trees remain. In this way, another component - animal - emerges as an influencer of tree development.

A more in-depth analysis of water cycling patterns between environmental pools and plants (including transpiration, water use efficiency and water absorption zone) is needed to better understand differential responses in the water absorption patterns of plant species and communities. For example, plants with low water use

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efficiency consume more water, depleting the resource in order to maintain stomatal conductance and photosynthesis. Water competition between species through interference or exploration is thus intensified, especially when root distribution patterns overlap. On the other hand, with lower tree density, competition for water resources could be lower.

Authors [5] observed, in a Brazilian agroforestry system, that the trees of *Cordia oncocalyx*Allemão, a species native to the *Caatinga*, lose leaves late in relation to trees of the same species that occur in a forest area. The investigation of this fact started from the hypothesis that the AGP trees maintain a better water state at the beginning of the dry period. This work aims to present environmental and physiological data of the trees to explain the late loss of leaves in an agrosilvopastoral system (AGP) in the Brazilian semiarid compared to a secondary forest (SF).

II. MATERIAL AND METHODS

Study area

The study took place at Crioula farm, which belongs to the National Center for Research on Sheep and Goats of Brazil's Agricultural Research Corporation (EMBRAPA). The farm is located in the municipality of Sobral (3º41' S, 40º20' W) in the State of Ceará. Mean annual temperature and precipitation are respectively 30ºC and 821.6 mm [10]. The dry season lasts for seven to eight months (June to January), and the wet season is shorter (January to May). The climate is semiarid, classified as BSw'h' according to Köppen: very hot and with most rainfall occurring during the fall. Typical Chromic OrthicLuvisols are the predominant soil type in the study area [11]. The predominant vegetation in the region is a type of woody savannah [12], locally known as *Caatinga*. It is composed mainly of deciduous species which lose their leaves during the dry season [13].

A long-term experiment was established in 1997 on this farm to evaluate traditional cropping systems (slash-and-burn) and alternative AFS (AGP and silvopastoral). An area was also left under native vegetation (secondary forest) and is used as a control. For this work, we selected two of these experimental areas, and their main characteristics are:

- AGP: area covering 1.6 ha, where rows of *Leucaenaleucocephala* (Lam.) de Wit were established. In the 3-m wide alleys maize (*Zea mays* L.) and/or sorghum (*Sorghum bicolor* L. Moench) are grown, with 1 m between plants. Tree density in this plot is approximately 200 trees ha^{-1}, which corresponds to 22% soil cover. No fertilizers are applied and all cropping operations are completed manually. After crop harvest, sheep and goats are allowed to graze the area.
- SF: area covering 1.6 ha under tree-dominated *Caatinga*, which represents approximately 50 year old secondary vegetation.

The AGP and SF plots contained, respectively, approximately 360 and 2,600 plants with height ≥ 1 m and with a stem diameter ≥ 3 cm at soil level, per hectare. Nine tree species were represented, and the four most common are *Cordia oncocalyx*Allemão, *Mimosa caesalpiniifolia*Benth.,*Poincianellabracteosa* (Tul.) L.P. Queiroz and *Bauhinia cheilantha* (Bong.) Steud [14].

Meteorological data

Meteorological data was obtained from two weather stations (Campbell Scientific, INC, Utah, USA) installed in the AGP and SF plots. Relative humidity of the air (RH) and soil volumetric moisture content at depths of 30 and 50 cm (Vw30 and Vw50) were measured. Weather stations collected data on these parameters every 30 seconds, and means over 15-minutes increments were stored in dataloggers. Data was collected between 1st May and 30th September 2011. Each day, therefore, 96 readings were recorded.

One rain gauge, connected to the weather stations, was installed in each experimental area, and it was located among the trees in SF and 3 m away from a *Cordia oncocalyx* tree in AGP. Amounts of rainfall over 15 minutes increments were stored in the dataloggers. Rainfall data shown here represent the total of all rain events over each month, between May and September.

Physiology in *Cordia oncocalix*

Cordia oncocalyx is a member of the Boraginaceae. It is abundant in the State of Ceará and dominant in the study area, with a frequency of 49% in SF and 50% in AGP [15, 16]. The SF and AGP plots contain 670 and 80 *C. oncocalyx* adult individuals per hectare, respectively.

Net photosynthesis (A), transpiration (E) and stomatal conductance (gs) were measured using an infrared gas analyzer (IRGA, LI-6400XT, LI-COR Biosciences, USA). Measurements were made on three sun-exposed leaves on three trees in each of the land-use systems. The trees where measurements were made were selected based on similarities in stem diameter. Scaffolds were erected to a height similar to that of the trees (between 8 and 9 m above the ground). Measurements were taken once a month between noon and 1pm, in May, June, July, August and September. Water use efficiency (WUE) was calculated as the ratio of photosynthesis to transpiration.

Rooting depth in *C. oncocalix*

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Main lateral root length of *C. oncocalyx* was measured by excavation in July for three trees in AGP and three in SF. Soil was removed from the surface until main lateral roots were found, and these were followed away from base of tree. Rooting depth was determined by cutting trees and completely removing roots from the soil.

Isotope analyzes

C. oncocalyx roots samples were collected, along with soil and rainwater to analyze stable oxygen isotopes ($\delta^{18}O$). Cylinders (5.0 cm high and 0.5 cm diameter) from the stalks of roots were taken. These samples were placed in plastic bottles, capped and sealed with semitransparent, flexible and watertight plastic film. As the stalk cylinders from each of the one tree in each treatment were composited placed in a single sample per treatment for isotopic analysis, no statistical analysis was possible.

Soil samples were collected at depth increments of 0-20, 20-40 and 40-60 cm under the crown of trees, and processed similarly to roots. All samples for isotopic analysis were placed on ice to further minimize evaporation during transport to the laboratory where they were stored, refrigerated, until water extraction by vacuum distillation. *C. oncocalyx* roots and soil were collected on 19th May 2011.

Approximately 1 µL of water extracted from root and soil samples, along with rainwater, were used to measure ratios between concentrations of water molecules with different combinations of H and O isotopes ($HD^{16}O$, $H_2{}^{16}O$ and $H_2{}^{18}O$), using a liquid water isotope analyzer (DLT-100, LGR, CA, USA). The ratios, corrected using a calibration curve and working standards, were expressed as δ in parts per mil (‰) as relative deviations from the V-SMOW international standard, as calculated by equation $\delta = ([\{R_{sample}/R_{standard}\}-1] \times 1000)$, where R is the ratio $^{18}O/_{16}O$ of the sample and the standard.

Data analysis

The significance of the differences between means of photosynthesis, water use efficiency and air relative humidity for the two land use systems was assessed using the Student t test, $\alpha=0.05$. Correlations were made between the trees physiological parameters (stomatal conductance and transpiration) and soil volumetric water. The graphs were constructed using Microsoft Excel and the statistical program MicrocalOrigin™.

III. RESULTS AND DISCUSSION

In the period from May to the end of the first half of July the rainfall was 302.7 mm in the AGP. No rainfall was recorded in the months of August and September. The volumetric soil water content in the depth of 30 cm was greater than 50 cm throughout the study period and declined progressively from July, reaching the lowest values at the end of September, when the average was bit above 0.2 cm^3 cm^{-3} (Figure 1). Researches [17] also show a trend of higher soil moisture near the surface and a decrease in depths greater than 30 cm. In the shallower soil (30 cm depth), the oscillations in the volumetric content notably accompanied the rainfall, rather than in deeper layers, a fact also recorded [18].

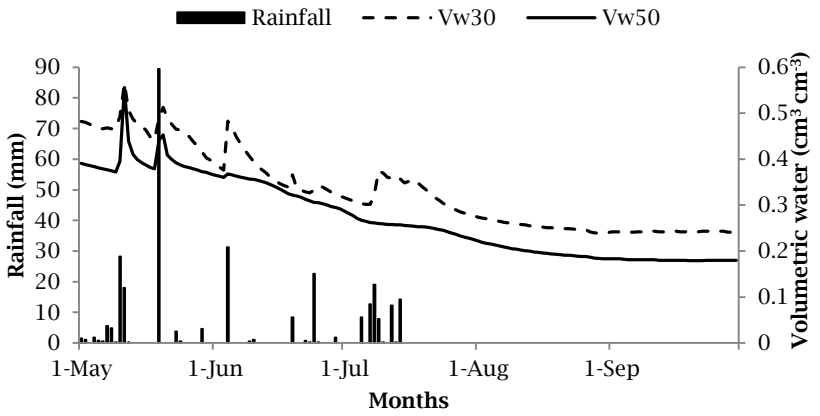

Fig.1: Rainfall and soil volumetric water to 30cm (Vw30) and 50cm (Vw50) depth in agrosilvopastoral system (AGP)

In secondary forest (SF), rainfall recorded in the months of May to July was only 187.7 mm. This value, 115 mm lower than that of the AGP, reflects the interception by the crown of the trees, which are in greater density in SF. This difference in recorded rainfall, however, did not lead to differences in soil moisture at 30 cm depth between systems. This is because only a small part of the water intercepted by the trees is absorbed by the leaves, most of it flows down the trunk and reaches the ground. The interception of rainwater by the canopy can vary from 13

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to 22% of the precipitated total [19] and for precipitations of less than 11.0 mm the intercepted water does not even reach the soil, due to its rapid evaporation [20].

From the second half of July, when there are no more rainfall, the Vw30 in SF starts to reduce gradually, reaching the lowest values at the end of September (0.24 cm^3 cm^{-3}) (Figure 2), which are equivalent to the values found at the same depth in the AGP (Figure 3). The soil volumetric water content in SF was higher at depth of 50 cm and peaks were recorded on rainy days, although less notable than at 30 cm. The biggest difference between the systems in the Vw is in the depth of 50 cm. In the AGP, in September, the mean values fluctuated around 0.18 cm^3 cm^{-3}, while in SF the averages were slightly above 0.3 cm^3 cm^{-3}. There are biotic and abiotic factors that can justify this difference, such as differences in ground cover and soil characteristics, which may induce variations in soil moisture in depth [21, 22].

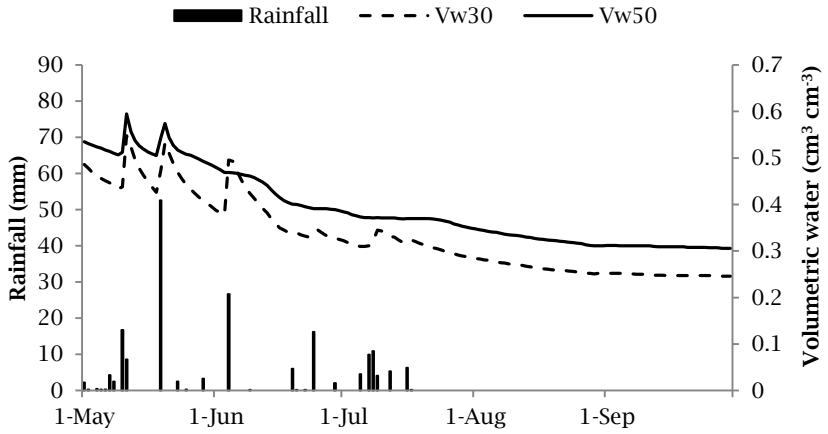

Fig.2: Rainfall and soil volumetric water to 30cm (Vw30) and 50cm (Vw50) depth in secondary forest (SF)

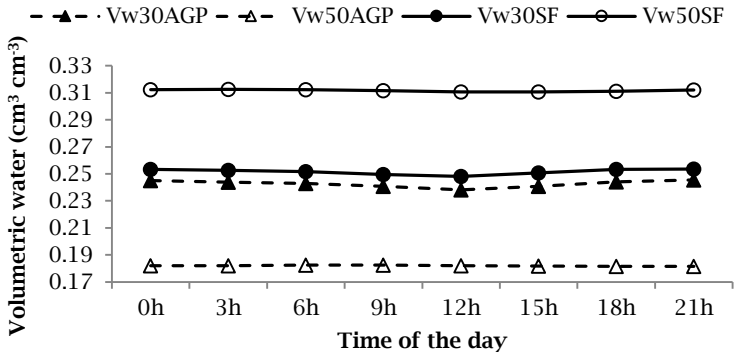

Fig.3: Soil water volumetric content at 30 cm (Vw30) and 50 cm (Vw50) depth throughout the day in agrosilvopastoral system (AGP) and secondary forest (SF) in the dry period (mean values recorded in the month of September)

In September, the driest of the observed months, AGP and SF presented the same value of Vw30; however, the SF presented higher soil moisture throughout the day in 50 cm depth and lower in the depth of 30 cm and the opposite was recorded in the AGP (Figure 3). As recorded by other authors [23, 24], soil moisture usually increases in depth, as in the case of SF. Among the factors that alter the moisture along the soil profile are evapotranspiration and infiltration. The conditions for the occurrence of higher evaporation can be better recorded for the AGP due to the direct contact of the sun's rays with the soil, whereas in SF, a more closed canopy favors the permanence of water in the soil; this explains, in general, why there is more water in SF soil than in 50 cm depth AGP soil. On the other hand, higher moisture values were recorded at a lower depth (30 cm) than at 50 cm in the AGP. In order to explain the differences in the behavior of soil moisture between systems it is important to evaluate the water absorption pattern of the plant species of the systems. In this sense, it was evaluated, through the isotopic constitution of soil water, rainfall and roots, the preferred source of water absorption. The Figure 4 shows the results.

Soil excavations demonstrated that *C. oncocalyx* does not have a taproot, but numerous similar roots which reach 20 to 80 cm in depth, which one varies with the size of the plant, in both land use systems. The roots extend laterally up to 3 m in SF and 4 m in AGP. This shows that all water absorbed by the root comes from the soil profile less than 100 cm. In addition, this large lateral extension of the roots allows it to explore more water at the horizon of the soil than at depth.

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Isotopic analyzes showed similar values between *C. oncocalyx* root sap water and the deeper soil (40-60 cm) in the AGP and with the more superficial soil (0-20 cm) in the SF (Figure 4). This point to preferential water absorption at these depths. Such evidence could explain the differences in soil moisture levels recorded throughout the study period for these two areas: lower water content at 30 cm depth in SF and lower at 50 cm depth in AGP. However, the source of water taken up by plants can change depending on the season [25] or according to water availability and rooting depth [26]. When the water requirement of the plant exceeds the supply, it must find other sources of water or use it more conservatively to minimize water stress and maintain metabolic functions. Indeed, plants can take up more superficial water during rainy periods and deeper water during dry periods [27]. Such a capacity to rapidly to change the source water from different regions of the soil can give an advantage to plants, when water competition occurs within the ecosystem.

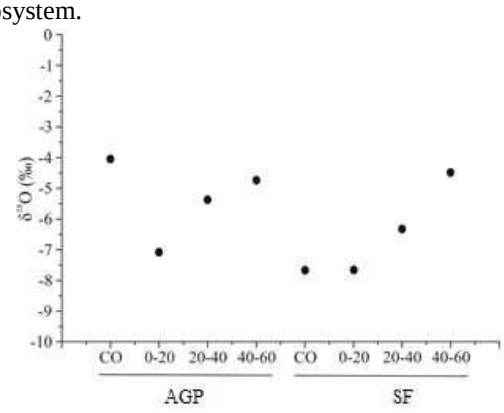

Fig.4: Oxygen isotope ratios ($\delta^{18}O$) of C. oncocalyx xylem root sap (CO) and soil water at the 0-20, 20-40 and 40-60 cm depth increments under an agrosilvopastoral system (AGP) and secondary forest (SF)

Photosynthesis (*A*), in general, decreased from the rainy season to the dry season, following reductions in RH, both in AGP and SF (Figure 5). In the months of May to June (rainy season) the photosynthesis was similar between the two systems, ranging from 10 to 12 μmol m^{-2} s^{-1}, when higher RH values were recorded in SF. This could indicate a good acceptability of *C. oncocalyx* to maintain the rates of photosynthesis under HR of 80 to 88%. However, in spite of the still high levels of RH in July (81% and 86%, for AGP and SF, respectively), the *A* reduced in the plants of both systems to 7.43 (AGP) and 8.51 μmol m^{-2} s^{-1} (SF). In the dry months (August and September) the photosynthesis was higher in the AGP plants, when RH was similar between the two systems. These observations indicate that other physical factors of the environment, besides RH, are influencing this physiological process in the trees. Among these factors, Larcher [28] points out the air temperature, winds, incidence of solar radiation, soil nutrients and soil moisture. In view of these findings, it is necessary to evaluate the influence of other parameters, which is done by checking the effect of soil moisture on stomatal conductance (*gs*) and transpiration (*E*).

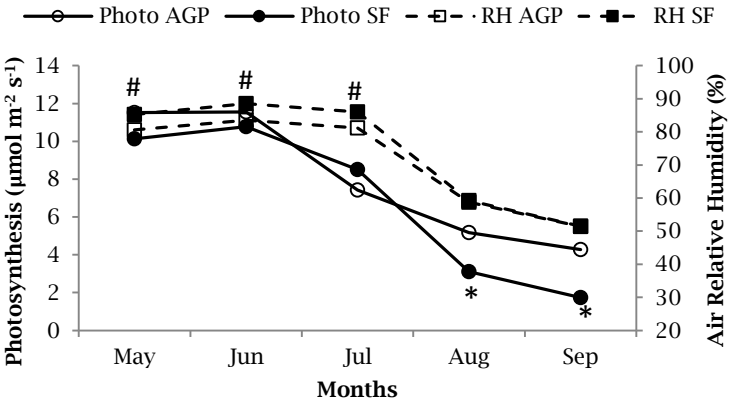

*Fig.5: Photosynthesis (Photo) in leaves of Cordia oncocalyx and air relative humidity (RH) in agrosilvopastoral system (AGP) and secondary forest (SF). * Photosynthetic averages are different between the two systems, p <0.05 by the t test (n = 9). # Relative humidity averages are different between the two systems, p <0.05 by the t test. RH values represent the average of the photosynthesis recording day (n = 96)*

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The variation in transpiration values was higher in SF (from 2.56 to 6.0 mmol m^{-2} s^{-1}) than in AGP (from 3.79 to 5.75 mmol m^{-2} s^{-1}). Similar to stomatal conductance (0.08 to 0.29 mol m^{-2} s^{-1} in AGP and 0.04 to 0.31 mol m^{-2} s^{-1} in SF). This higher variance in the physiological parameters of the trees in SF indicates a greater variation in the physical parameters of the environment, since the response of the plants is proportional to the variations in the environment [29], to some extent. Solar radiation may be one of the factors that explain these variations. The solar radiation that surpasses the canopy in a forest is quite variable and of inferior quality that arrives at the canopy [28]. This variable amount and quality of the radiation reaching the lower branches and leaves leads to greater oscillations in photosynthetic processes, such as stomatal conductance and transpiration, when compared to plants growing in more open areas whose entire canopy can receive similar levels of radiation, leading to lower oscillations in the rates of these processes.

Statistical analysis showed correlation of soil water content at both 30 cm and 50 cm with transpiration and stomatal conductance in *C. oncocalyx* plants in AGP (Figure 6A and B). Lower values of transpiration and stomatal conductance were observed in lower levels of water in the soil, showing the dependence of this environmental parameter on the trees to keep their stomata open and thus to continue photosynthesizing. However, there was no such correlation for SF trees (Figure 7A and B), pointing to a certain independence of these physiological processes in relation to soil moisture. The physiological processes linked to photosynthesis occur in response not only to an isolated environmental factor, but to the whole of them, one influencing the sensitivity of the plant in relation to another factor. Shen et al. [30] observed that the sensitivity of the canopy conductance process in response to the air vapor pressure deficit decreased when the soil moisture content was reduced. Thus, there are possibly other factors influencing the response of stomatal conductance and transpiration to soil moisture in SF. It is known that physiological processes in plants respond to environmental conditions [29]. On the other hand, it is also known that many species anatomically modify to adapt to stressful environmental conditions or alter biochemical processes to tolerate environmental stresses [31]. Investigations in this sense may help to understand these differences in the physiology of trees between the two different land use systems.

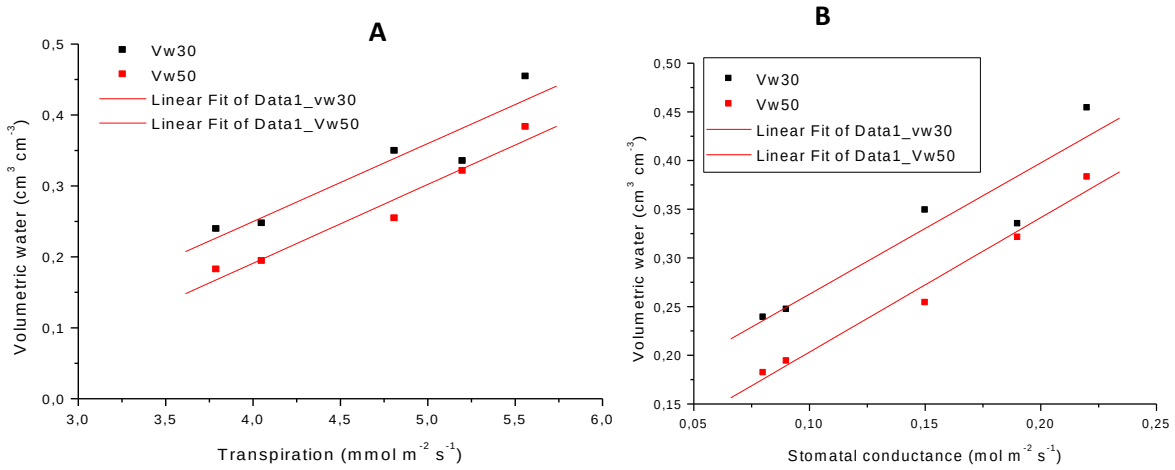

Fig.6: Correlation between soil water volumetric content at 30cm and 50 cm depth (Vw30 and Vw50) and transpiration (A) and stomatal conductance (B) in Cordia oncocalyx in agrosilvopastoral system. E: n = 9; Vw: n = 96 (readings along the day of photosynthesis recording). Each point represents the averages of one day per month. (A) Vw30xE: p = 0.017; R = 0.94. Vw50xE: p = 0.004; R = 0.97. (B) Vw30xgs: p = 0.01; R = 0.93. Vw50xgs: p = 0.001; R = 0.98. E – transpiration; gs – stomatal conductance

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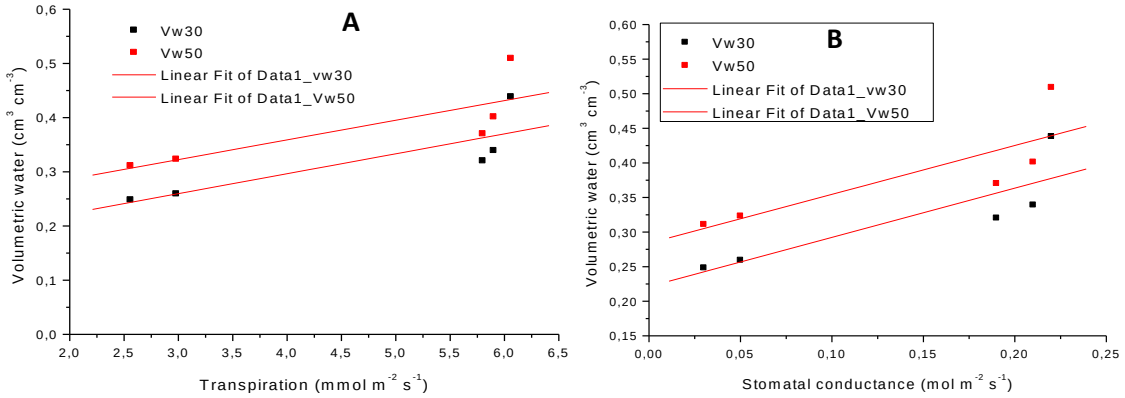

Fig.7: Correlation between soil volumetric water content at 30cm and 50 cm depth (Vw30 and Vw50) and transpiration (A) and stomatal conductance (B) in Cordia oncocalyx in agrosilvopastoral system. E: n = 9; Vw: n = 96 (readings along the day of photosynthesis recording). Each point represents the averages of one day per month. (A) Vw30xE: p = 0,07, R = 0,83; Vw50xE: p = 0,10, R = 0,79. (B) Vw30xgs: p = 0,06, R = 0,86; Vw50xgs: p = 0,08, R = 0,82. E – transpiration; gs – stomatalconductance

There was a trend towards lower water use efficiency (WUE) in the SF trees throughout the study period, although there was an effective difference ($p < 0.05$) only in the months of May and September (Figure 8). As the WUE represents how much carbon is being fixed for each unit of water used, this result points to a higher water expenditure per unit of carbon fixed in SF trees, which is only possible for *Caatinga* plants if there is a water supply. However, the AGP plants showed lower water expenditure in the photosynthetic process, which becomes fundamental in the period of water scarcity, considering that the soil and air are drier, and, in the absence of physiological or anatomical strategies for survival at climate more arid, the plants may have their development impaired. In addition, low water use efficiency situations indicate the need for more water to make photosynthesis compared to those with high WUE; so when there is water shortage in the soil, tree low WUE are the first to present reductions in photosynthesis.

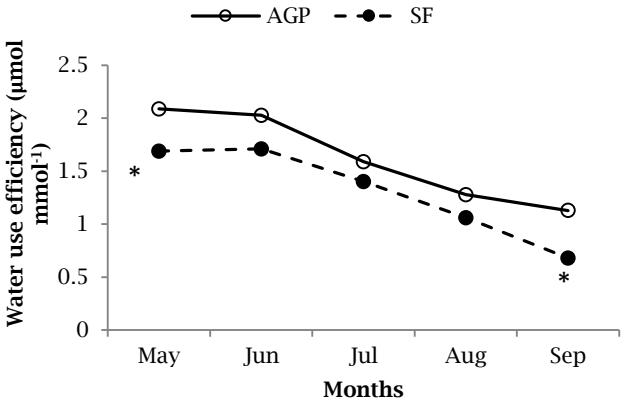

*Fig.8: Water use efficiency (WUE) in C. oncocalyx trees in agrosilvopastoral system (AGP) and secondary forest (SF). * WUE means are different between the two systems, p <0.05, by t test (n = 9)*

IV. CONCLUSION

Although the most inhospitable environmental conditions in the AGP system, the lower density of plants, and therefore less competition for water, besides greater independence of water variations in soil and greater water use efficiency, favoring photosynthesis longer, causing the leaves to fall later. In view of the above, it's possible that AGP plants have developed physiological and anatomical features that enable to them to keep photosynthesis even when climatic conditions are more severe.

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Annual Biomass Production, Chemical Composition and *In- sacco* Degradability of Different Cultivars of *Moringa oleifera*

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Abstract**—** *Types of plant cultivars and seasons often affect production and productivity of fodder biomass and nutritional quality to animals. Selection of suitable cultivars and better understanding of year round biomass production are indispensable for improving quality feed supply to animals. Black Seed Moringa (BSM-L) and White Seed Moringa (WSM), the two local cultivars and Black Seed Moringa (BSM-T) cultivar of Thailand origin of Moringa oleifera were cultivated in the fodder research field of the Bangladesh Livestock Research Institute (BLRI) during the period of 19 August 2014 to 23 December 2015. An agronomical trial was conducted to determine the biomass yield of the three cultivars in different seasons of a year under common agronomical practices. The effect of the cultivars on the daily relative growth rate (RGR), chemical composition and in-sacco dry matter (DM) degradability were also evaluated. The cultivar response to biomass production performances, chemical composition and nutritional values were analyzed in an ANOVA of a Randomized Block Design (RBD), while the differences in the rate and extent of the DM degradability in-sacco determined using three rumen cannulated bulls were analyzed in an ANOVA of 3x3 Latin Square Design. The annual biomass yield of BSM-L tops (114.5 t/ha fresh; 22.7 t/ha DM) was significantly higher than that of WSM (29.0 t/ha fresh; 5.80 t/ha DM) or BSM-T (83.5 t/ha fresh; 16.0 t/ha DM). No significant difference in chemical composition (224.9, 222.4 & 223.8 g.kg^{-1} DM of crude protein (CP), respectively, and 450.9, 455.3 & 435.4 g.kg^{-1} DM of neutral detergent fiber, respectively) or nutritional value (47.4, 46.7 & 45.3% of potential, and 62.8, 64.2 and 63.6% of effective degradability of dry matter) was found for the cultivars. BSM-L had a significantly higher survivability (97.2%), prune number per plant (3.50) and RGR (15.6 mg DM/day) than WSM (25.0%, 2.30 & 4.20 mg DM/day) or BSM-T (55.6%, 3.10 & 10.8 mg DM/day) respectively. The hot and dry, and hot and humid climate having a Heat Index (HI) range of 25^o to 35^o F and monthly total rainfall of 130 mm to 332 mm were suitable for cultivation of all the Moringa cultivars. It was concluded that considering biomass production and its quality in terms of chemical composition and nutritional values, Black Seed Moringa (Moringa oleifera) may be cultivated as a plant fodder crop for the production of feed for ruminant animals.*

Keywords— Moringa cultivars, Biomass, Season, Nutritional values, Chemical composition.

I. INTRODUCTION

Demand and supply gaps of feeds and fodders [1] and seasonal and regional variations in biomass availability [1] often limit ruminant production and productivity in many developing countries including Bangladesh. Besides, the gradual transformation of subsistence animal farming to input-supported systems is intensifying farmers' demand for high biomass yielding and quality feeds and fodders. Fodder production against the backdrop of the decreasing cultivable land and growing competitions for land use, especially for cereal crop production, is undoubtedly a daunting task in most developing countries. A fodder crop, if at all is competitive to the existing cereal crops considering its biomass production, nutritive value, feeding response to animals, and profitability, may be introduced into existing cropping systems in some selected regions of the country, especially where livestock production is being intensified[1]. Moringa (*Moringa oliefera*) a native plant that grows fast round-the-year [2] produces biomass of high nutritional attributes [3] boosts milk and meat production of cattle (30 to 40%) [4, 5] improves product quality [6-8] and supports animal health [9-12] may be considered as one of the fodder crops for cultivation. It produces high quality

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biomass, has higher pruning efficiencies and lower defoliations [13, 14]. Shajna or Bajna, local titles of available Moringa cultivars are used for production of drumsticks, but their comparative production performances of biomass, both in terms of quantity and quality, in variable climatic conditions in a year is not known yet. Moreover, conventional local Moringa cultivars, especially used for harvesting drumsticks, may not be suitable for repeated lopping of branch tops and leaves, for using as feed/fodder. Thus, selection of cultivar(s) of Moringa plants, having comparatively high productivity of biomass throughout the year with chemical compositions and nutritional values suitable for feeding of animals, is of utmost important for the introduction of Moringa plant as a fodder crop.

The present study, thus, was undertaken to identify Moringa cultivar that may be cultivated in different seasons of a year as a plant fodder crop for the production of biomass of high nutritional values for the feeding of ruminant animals.

II. MATERIALS AND METHODS

2.1. Location and agro-climate of the experimental site:

The agronomical trial was conducted at the Cattle Research Station of the Bangladesh Livestock Research Institute (BLRI) from 19 August 2014 to 23 December 2015. The station was located at 23°42'0" N, 90°22'30" E at an altitude of 4 m above the sea level. The clayey textured soil of the station is strongly acidic (pH 4.5-5.7) containing a very little (<1.5%) organic matter and it belongs to the Madhupur Tract Agro-ecological Zone (AEZ-28) of Bangladesh. During the experimental period, the day temperature ranged from 21^0C to 35^0C and humidity ranged from 50% to 75%.

2.2. Preparation of experimental plots:

Three different cultivars of Moringa were used in the present programme. The seeds of the two cultivars, White Seed Moringa (WSM)) and Black Seed Moringa (BSM-L) were collected from selected local sources. The third cultivar having black seeds was collected from Thailand (BSM-T). The three cultivars of Moringa (taxonomical identification not completed yet) are entitled according to their seed color, considered as a major phenotypic difference. The seeds of three cultivars were tested for determining the rate of germination and it ranged from 65.0 to 75.0%. Two seeds in each polythene pouch containing sandy alluvial soil were sown, and saplings were raised up to an age of five weeks. The saplings were transplanted in predesigned experimental plots. Before transplantation, the soil of the plots was ploughed and fertilized with a basal dose of cattle dung at the rate of 3.0 t/ha and a mixture of TSP (Triple Super Phosphate) and MP (Murate of Potash) of a ratio of 30:15 kg per hectare. The urea N at the rate of 90kg/ha was top dressed when the plants were initially established in the research field and it was repeated at each harvest. All other agronomical practices e.g. weeding, irrigation etc were common for all cultivars.

2.3. Experimental layout design and treatment:

A uniformly plain land area of 97.2 m^2 was divided into four blocks, each of 24.3 m^2 separated by 1.0 meter wide walking alleys. Each block was again divided into three experimental plots, each of 8.1 m^2 for the planting of 90 saplings at a space of 0.3 m x 0.3 m per sapling. The blocks and plots were arranged in a Randomized Block Design (RBD) to determine the production responses of the three cultivars of Moringa.

2.4. Yield determination and sample collection:

After a post-transplantation growth period of 90 days, branch tops with leaves were harvested at a 60 days interval keeping an average stem height from the ground of 40 cm. The plants were allowed to grow after each cut and fertilized accordingly. A total of six cuts were given. The biomass yield of each of the three cultivars in six(6) different cuts of a year (Dec-Jan, Feb-Mar, Apr-May, Jun-July, Aug-Sep and Oct-Nov) was added to determine the annual yield of biomass production. Survival rate (% of saplings grew after transplantation), the number of prunes per plant, defoliation rate (% of total leaf biomass defoliated), and the growth rate of biomass were determined at different harvesting times. Fresh tops were harvested avoiding any surface water on plants and weighed on a top loading balance and the fresh yield per plot was recorded. Fresh yield (kg or ton) was converted to DM yield $plot^{-1}$ ha^{-1} according to the equation of DM yield $plot^{-1}$ = Weight of fresh material × (%) DM.

2.5. Chemical analysis:

The tops were manually separated into stems and leaves to determine stem to leaf ratio and weighed accordingly. Representative samples of tops, stem or leaves were taken to determine fresh dry matter, total ash, crude protein (CP) and ether extract (EE) according to AOAC [15]; and neutral detergent Fiber (NDF) or acid detergent Fiber (ADF) and acid detergent lignin (ADL) according to Van Soest [16]. All the analyses were done in the animal nutrition laboratory of the BLRI. The tops and stems were chopped

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manually at a range of 0.03 m to 0.05 m, dried in the sun, and milled for chemical analyses of the biomass of different harvests.

2.6. The determination of Relative Growth Rate (RGR) and HI (Heat Index):

The data on rainfall, temperature and humidity were collected during the period of 14 September 2014 to 23 September 2015. The relative growth rate (RGR) of different cultivars was calculated using the equation of $RGR = \frac{lnW_2 - lnW_1}{t_2 - t_1}$ described by Hoffmann and Poorter [17]; where, ln = natural logarithm, t_1 = time one (in days), t_2 = time two (in days), W_1= Dry weight of plant at time one (in grams), W_2= Dry weight of plant at time two (in grams). Heat Index, a measure of how hot it really feels when relative humidity is factored in with the actual air temperature, was calculated from the HI chart of National Weather Service of the US Department of Commerce [18].

2.7. The rumen kinetic parameters of Moringa tops:

Three local growing bulls of an average live weight of 225 kg fitted with rumen cannulae (14 cm diameter & 9 cm length) were used to determine rumen degradability *in-sacco* of dry matter of Moringa tops. The animals were fed Napier and German grass mix *ad libitum* and the roughages were supplemented with locally mixed concentrate mix of wheat bran, sesame oil cake, Kheshari (*Lathyrus sativus*) bran, di-alcium phosphate (DCP) and common salt at the rate of 1.0% of the live weight. Having the animals adjusted to the diet for at least three weeks Dacron bags (7x16 cm, pore size 45 µm) containing the samples (2 g each) of three Moringa tops (oven dried, milled and passed through a sieve of 1.0 mm size) were incubated in the rumen following the method described by Ørskov[19]. Considering animals and three periods as replication the samples of Moringa tops of three cultivars were incubated at 0, 8, 16, 24, 48 and 72 hrs in the rumen. Each hour of incubation of a sample of each cultivar was repeated in three animals in a period, and the incubations were repeated in three different periods. The degradation kinetics of DM were determined by fitting the disappearance values to the equation P= a+ b (1-e^{-ct}) Ørskov and McDonald [20], where P represents the disappearance after time t. Least-squares analyses were used for the estimation of rapidly degradable fraction (a), slowly degradable fraction (b) and the rate of degradation (c). The effective degradability (ED) of Moringa tops were estimated using the equation of McDonald [21], where ED= a+bc/(c+k), and 0.05 rate constant (k) was considered.

2.8. Statistical analysis:

Considering the three Moringa cultivars as treatment, their responses to biomass production performances (yield, growth rate, pruning efficiency and ratio of botanical fractions), and nutrient yield and contents (DM, CP, ADF, NDF or ADL) were analyzed in an ANOVA of a Randomized Block Design (RBD) using general linear model of SPSS-17.0 statistical software program in a computer. Any significant differences in the rate and extent of the DM degradability *in-sacco* of Moringa tops of different cultivars were analyzed using an ANOVA of 3x3 Latin Square Design.

2.9. Seasonal effect on Moringa production:

The average HI was 20, 23, 31, 30, 29 and 29°F, and monthly total rainfall was reported as 12, 86.5, 332, 364, 130 and 28 mm during December-January, February-March, April-May, June-July, August-September, and October-November harvesting periods, respectively (Fig1), and the fresh tops yield (t/ha/cut) varied according to the variations in HI and rainfall in a year. The yield was the lowest (average 0.76 t/ha/harvest) during the dry (monthly total rainfall 12.0 mm) and cool (HI 20^0F) months (December to January) of a year. The yield peaked during the dry and hot period, from April to May (average 23.4 t/ha/harvest) with the rise of HI (31^0F) and rainfall (332 mm). A further increase in rainfall affected peak productions (17.2 to 22.4 t/ha/cut) during the hot and humid months of the year.

III. RESULTS

The effect of different cultivar of Moringa on survival rate (%), the number of prunes per plant, growth rate (kg/ha/day) and defoliation rate (%) are shown in Table 1. The survivability of BSM-L was the highest (97.2%) followed that of BSM-T (55.6%) and WSM (25.0%); and the difference between the cultivars were significant (P<0.001). A similar trend was found in the RGR of the cultivars (15.6, 10.7 & 4.2 mg/day, respectively, P<0.001). Both the black seed cultivars having a significantly higher average number of prunes (3.5 vs. 3.1 prunes/plant) were bushier than WSM. The average defoliation rate of all the cultivars varied from 2.4 to 4.0% and it did not differ significantly (P<0.46).

Table 1.

3.1. Biomass yield:

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The effect of three different cultivars on fresh or dry matter (DM) yield of tops, leaf and stem fractions of Moringa and their leaf to stem ratios are shown in Table 2. The annual fresh (114.51 t/ha) and dry matter (22.73 t/ha) yield of BSM-L tops were significantly (P<0.001) higher than that of BSM-T (83.52 t/ha and 16.03 t/ha) or WSM (29.01 t/ha and 5.79 t/ha). Similarly, the annual fresh or dry matter yield of stem (75.82 t/ha and13.19 t/ha) was the highest for BSM-L, followed by BSM-T (51.61 t/ha and 9.07 t/ha) and WSM (18.24 t/ha and 3.75 t/ha) and the difference was significant (P<0.001). The annual fresh or dry matter yield of leaves was 38.7 t/ha and 8.50 t/ha for BSM-L and 31.9 t/ha and 7.62 t/ha for BSM-T and differed significantly (P<0.05) between the two cultivars. Both BSM-T and BSM-L had a significantly (P<0,001) higher fresh or DM yield of leaves than that of WSM (10.6 t/ha and 2.3 t/ha). The average leaf to stem ratio of BSM-L was 0.45 and it reflects that almost a half of the whole tops dry matter was shared by leaves. The ratio varied from 0.56 to 0.58 for WSM and BSM-T. Nevertheless, the variation in the leaf to stem ratio among the cultivars was not significant (P<0.42) (Table 2).

Table 2.

Table 3 shows the chemical composition of different biological fractions of the three Moringa cultivars. BSM-L had a significantly (P<0.05) higher fresh dry matter of tops (206.3 g kg^{-1}) than BSM-T (191.9 g kg^{-1}) and lower (P<0.05) fresh leaf dry matter (222.5 g kg^{-1}) than the latter (235.0 g kg^{-1}) or WSM (233.5.0 g kg^{-1}). The ash content of BSM-L (66.8 g kg^{-1}) was significantly (P<0.01) lower than BSM-T (83.4 & 77.4 g kg^{-1}, respectively). All other chemical components (CP, ADF, NDF, EE and ADL) in the tops, stem or leaves of three cultivars did not differ significantly (P>0.05). Their average contents for the three cultivars were 223.7, 419.8, 442.8, 277.3 & 208.2 g kg^{-1}, respectively for tops; 124.8, 632.5, 711.5, 87.4 and 248.2 g kg^{-1}, respectively, in stem; and 299.4, 215.2, 343.3, 106.2 and 326.8 g kg^{-1}, respectively in leaves (Table 3).

Table 3.

3.2. Degradation kinetics:

Table 4 shows that the calculated soluble fraction (a) was significantly higher for BSM-L tops (23.0%) than BSM-T (20.9%) and WSM (21.53%); while the rate constant (c=0.08) of BSM-L was significantly (P<0.001) lower than that of the later two cultivars (0.12 and 0.13, respectively). The rate of rumen dry matter degradation of WSM tops was the highest (0.13, P<0.01) followed by 0.12 of BSM-T and 0.08 of BSM-L. The potential (b) or effective degradability of tops of the three cultivars ranged from 45.3 to 47.4 %, and 62.8 to 64.2 % at a rate constant of 0.05 passage rate and their differences among the cultivars was not significant (P>0.05).

Table 4.

3.3. Seasonal effect on Moringa production:

The average HI was 20, 23, 31, 30, 29 and 29^{o}F, and monthly total rainfall was reported as 12, 86.5, 332, 364, 130 and 28 mm during December-January, February-March, April-May, June-July, August-September, and October-November harvesting periods, respectively (Fig1), and the fresh tops yield (t/ha/cut) varied according to the variations in HI and rainfall in a year. The yield was the lowest (average 0.76 t/ha/harvest) during the dry (monthly total rainfall 12.0 mm) and cool (HI 20^{0}F) months (December to January) of a year. The yield peaked during the dry and hot period, from April to May (average 23.4 t/ha/harvest) with the rise of HI (31^{0}F) and rainfall (332 mm). A further increase in rainfall affected peak productions (17.2 to 22.4 t/ha/cut) during the hot and humid months of the year.

Fig1. And Fig2.

The daily RGR of all three cultivars was affected by seasons, and it varied from 0.61 mg to 2.88 mg in dry & cool months and rose to daily 8.86 mg to 13.26 mg in dry and hot months (Fig2). With the rise of HI and rainfall, the RGR of BSM-L was the highest (0.61 to 10.47) followed by BSM-T (1.03 to 8.86) and WSM (2.88 to 13.26).

IV. DISCUSSION

Identification of locally and regionally available best cultivar(s) and a better understanding of trade-offs and synergies of production performances between climatic variations are indispensable for Moringa fodder production. The motivation for using Moringa fodder is that it has the potential for being an alternate crop to cereals as well as soybean. Except for the rate of defoliation, a genotypic characteristic of Moringa, both the Black Seed Moringa cultivars (BSM-L) performed better in terms of survivability, the number of prunes/plant and daily biomass growth. Having a higher survivability of saplings (97.2 vs. 55.6%) and similar pruning ability to that of BSM-T (3.5 vs. 3.1 prunes/plant), BSM-L had the highest daily biomass growth (72.9 kg/ha vs. 51.2 kg/ha). The yield of fresh or DM of the tops or stem of BSM-L was the highest. It was leafier (stem: leaf; 0.45 vs. 0.58 or 0.56 of BSM-T and WSM) than the other cultivars (Table 2). Nevertheless, the leaves of BSM-L had a comparatively lower DM content

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(222 g kg^{-1} vs. 235 g kg^{-1} in BSM-T and 233 g kg^{-1} in WSM). It decreases differences between the leaf DM yield of BSM-L and BSM-T and make the difference non-significant (P>0.05) (Table 2). A higher survivability of *M. oleifera* and its growth have also been reported [2, 3, 7, 22, 23].

Hot and dry and hot and humid seasons compared to dry and cool months were suitable for Moringa fodder production. The HI above 23^0 F and monthly total rainfall at a range of 86.5 to 332 mm favored growth (Fig 2) and the production of Moringa fodder. A continuous downpour, even at a monthly rainfall range of 332 to 364 mm, may reduce growth rate and biomass production. Nouman [13] reported a suitable ambient temperature range of 27^0C to 35^0C in Nicargua. Moreover, Moringa can grow on a wide range of soils [24] and may not compete with floodplain arable fertile land used mostly for staple food crop production.

Table 5 shows the comparative production performances of DM and CP of BSM-L with other conventional and unconventional feeds and fodders. BSM-L produces a higher amount of DM (23.6 t) and CP (5.31 t) per hectare per year compared to other available conventional (12.7 t and 1.26 t of *Lathyrus sativus* and 6.60 t and 0.69 t of *Vigna mongu* per ha and per year*)* or unconventional (15.6 t & 1.64 t of *Vigna unguiculata*, 10.7 t & 2.15 t of *Leuchena leucocephala* and 17.3 t &1.75 t of *Sesbania sesban* per ha and per year) fodder crops in Bangladesh. The CP yield per hectare per year of BSM-L is about six times higher than that of soybean meal (0.93 t) produced on hectare of land (Table 5).

The average CP content of Moringa leaves of three cultivars was 299.4 g kg^{-1} DM. Similar CP levels between 290 and 320 g.kg^{-1} was reported by Al-Mashri [25] and Soliva [26]. The CP of stem did not vary significantly among the cultivars and the average content was 124.8 g kg^{-1}. The CP content of even Moringa stem of different cultivars was higher than that of Napier or Guinea grass (109.0 g kg^{-1} DM and 91.7 g kg^{-1} DM, respectively; [27, 28]. The CP contents of Moringa tops reported in this study are within the range of 193.0 to 264.0 g kg^{-1} DM, reported earlier [25, 29, 30, 31, 32, 33, 34, 35].

The CP of Moringa tops containing leaf to stem ratio of 0.53:1 of the present study was 22.4% (Table 3). When it was compared with other feed sources (Table 5) it was found that except Soybean meal (51.8%) the CP content of others varied from 10.1% in Sesbania to 20.2% in *Leucaena leucocephala.* The average ADF (419.8 g kg^{-1}), NDF (442.8 g kg^{-1}) or ADL (208.2 g kg^{-1}) content of the tops of three different cultivars were similar to those reported by Makkar and Becker [29]; Foidle [31]; Aregheore [32] and Al-Mashri [25]. However, different stem to leaf ratio of a harvest affected the level of different cell wall components in different cultivars of Moringa.

Table 5.

The soluble biomass (a) of Moringa tops in the rumen was 21.8% and its potential degradable (b) fraction was 46.1%, and the extent of rumen DM degradability (a+b) was 67.8%. The extent of rumen degradability of CP of similar type of Moringa feed (stem+leaf) was 69.8% [36] (Table 6) compared to 85.3% of Berseem. It was even lower than the extent of rumen N degradability of Soybean meal, Leucaena and Alfalfa hay (94.2%, 80.9% and 92.8%, respectively; Table 6). It also shows that the effective degradability of Moringa CP in the rumen was only 55.1% and it was lower than the CP degradability of Berseem (67.7%) or the effective N degradability of Soybean meal, Leuceana and Alfalfa hay (65%, 45.0% and 79.0%, respectively) in the rumen. The total tract digestibility of CP of similar Moringa tops was 74.4% in cattle [37]. Thus, it may be estimated that at least 19.3% (differences in CP degradability in the rumen and digestibility in the total tract) of Moringa CP was digested in the lower gut and escaped microbial degradation.

The methionine and lysine content in the CP of Moringa feed was 0.66% and 7.69% and their contents were similar to those in other feed resources (Table 6). However, the methionine content in the CP of Moringa leaf was reported as 1.5% [30]. Makkar and Becker [29] stated that its leaf protein has the amino acid profile comparable to that of the WHO/FAO/UNO standard protein for growing children. Foidl [38]; Sanchez-Machado [39] and Moyo[40] reported that Moringa contains high quality protein, due to the presence of high levels of essential amino acids.

Table 6.

Thus, considering biomass production and its quality in terms of chemical composition and nutritional values to animals Black Seed Moringa may be cultivated as a plant fodder crop for the production of feed for ruminant animals.

V. CONCLUSION

The local cultivar, Black Seed Moringa (*Moringa oleifera*) had the highest production of high quality biomass; and hot and dry, and hot and humid seasons are the best period for Moringa fodder production. Other agronomical practices like cutting height, weeding, irrigation, cropping density etc may affect Moringa biomass production and their impacts under local conditions need to be evaluated through further research.

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Table.1: Performances of survivability and growth of different cultivars of Moringa oleifera (means±SE; n=4)

Parameters	Moringa cultivars			Significance		
	BSM-T	WSM	BSM-L	Overall mean	Overall SE	Level
Survival rate (%)	$55.6^{b} \pm 10.1$	$25.0^{c} \pm 5.3$	$97.2^{a} \pm 2.7$	59.3	9.6	P<0.00
No of prunes/plant	$3.1^{a} \pm 0.2$	$2.3^{b} \pm 0.2$	$3.5^{a} \pm 0.1$	2.9	0.2	P<0.01
Annual RGR (mg/day)	$10.8^{a} \pm 1.8$	$4.2^{b} \pm 3.0$	$15.6^{a} \pm 1.2$	7.4	2.76	P<0.00
Defoliation rate (%)	2.6 ± 0.14	3.7 ± 0.9	3.1 ± 0.6	3.1	0.4	P<0.46

BSM-T= Black Seed Moringa of Thailand; WSM= White Seed Moringa; BSM-L= Black Seed Moringa of local origin

Table.2: Biomass production and composition of botanical fractions of different Moringa oleifera cultivars

Parameters	Moringa cultivars			Significance		
	BSM-T	WSM	BSM-L	Overall mean	Overall SE	Level
			Fresh yield(t $ha^{-1}year^{-1}$)			
Tops	83.5^{b}±12.0	29.0^{c}±6.7	114.5^{a}±4.7	75.7	11.5	P<0.00
Stem	51.6^{b}±9.7	18.2^{c}±4.9	75.8^{a}±4.5	48.5	7.9	P<0.00
Leaf	31.9^{b}±2.5	10.6^{c}±1.7	38.7^{a}±1.3	27.1	3.7	P<0.00
			Dry matter yield(t $ha^{-1}year^{-1}$)			
Tops	16.0^{b}±2.3	5.8^{c}±1.3	23.6^{a}±1.1	14.9	2.3	P<0.00
Stem	9.1^{b}±1.7	3.8^{c}±1.0	13.2^{a}±0.8	8.7	1.3	P<0.01
Leaf	7.6^{a}±0.6	2.3^{b}±0.4	8.5^{a}±0.3	6.1	0.8	P<0.00
Leaf: Stem	0.58±0.10	0.56±0.06	0.45±0.02	0.53	0.04	P<0.42

BSM-T= Black Seed Moringa from Thailand; WSM= White Seed Moringa of local origin; BSM-L= Black Seed Moringa of local origin

Table.3: Chemical composition of different Moringa cultivars and their botanical fractions

Parameters	Moringa cultivars			Significance		
	BSM-T	WSM	BSM-L	Overall mean	Overall SE	Level
			DM(g kg^{-1})			
Tops	191.9^{b}± 2.3	198.6^{ab} ± 5.51	206.3^{a} ± 1.1	198.9	2.54	P<0.05
Stem	161.5± 3.5	162.9± 5.3	165.7 ± 4.3	163.4	2.36	P<0.80
Leaf	235.02^{a} ± 2.7	233.50^{a} ± 2.4	222.4 ± 2.20^{b}	230.3	2.13	P<0.01
			Ash(g kg^{-1})			
Tops	83.4^{a}± 0.6	77.4^{b} ± 1.4	66.8^{c}± 1.7	75.8	2.1	P<0.01
Stem	70.0± 3.8	67.8± 2.2	63.3 ± 3.3	67.1	1.86	P<0.35
Leaf	86.6 ± 2.8	79.6 ± 1.1	80.7 ± 2.5	82.3	1.51	P<0.11
			CP(g kg^{-1} DM)			
Tops	223.8±1.7	222.4±1.9	224.9±2.3	223.7	1.1	P<0.68
Stem	126.1±4.5	122.2±2.6	126.2±5.2	124.8	2.31	P<0.75
Leaf	305.1±3.4	296.8±4.0	296.3±2.8	299.4	2.17	P<0.32
			ADF(g kg^{-1} DM)			
Tops	422.6±51.1	422.1±7.8	414.7±0.4	419.8	13.43	P<0.97

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Stem	619.6±5.4	656.1±17.7	622.0±10.2	632.5	9.23	P<0.20
Leaf	212.65±2.5	217.75±7.3	215.08±4.9	215.2	2.56	P<0.31
			NDF(g kg^{-1} DM)			
Tops	435.4^{b}±0.75	455.3^{a}±1.12	450.9^{a}±3.40	447.2	6.58	P<0.934
Stem	724.7±6.2	707.2±9.1	702.7±7.4	711.5	5.44	P<0.24
Leaf	351.3±3.8	339.9±4.2	338.6±1.4	343.3	2.97	P<0.13
			EE(g kg^{-1} DM)			
Tops	277.1±1.9	277.8±0.3	277.1±1.9	277.3	0.72	P<0.92
Stem	87.7±0.4	86.5±1.5	87.9±0.7	87.4	0.51	P<0.37
Leaf	106.3±0.8	105.8±0.5	106.3±0.8	106.2	0.35	P<0.83
			ADL(g kg^{-1} DM)			
Tops	205.5±1.4	209.1±0.9	209.8±0.7	208.2	0.96	P<0.11
Stem	232.8±1.30	231.8±1.3	231.6±0.1	248.2	0.53	P<0.48
Leaf	332.6±3.4	328.7±0.5	319.1±8.8	326.8	3.52	P<0.43

BSM-T= Black Seed Moringa of Thailand; WSM= White Seed Moringa; BSM-L= Black Seed Moringa of local origin;
DM, Dry Matter; CP, Crude Protein; ADF, Acid Detergent Fiber; NDF, Neutral Detergent Fiber; EE, Ether Extract; ADL, Acid Detergent Lignin;

Table.4: Rumen degradation kinetics of different Moringa cultivars

Parameters	**Moringa cultivars**			**Significance**		
	BSM-T	WSM	BSM-L	Overall mean	Overall SE	Level
a	20.9^{b}±0.7	21.5^{ab}±0.2	23.0^{a}±0.2	21.81	0.37	P<0.03
b	45.3±1.7	46.7±1.3	47.4±0.6	46.1	0.73	P<0.50
c	0.12^{a}±0.01	0.13^{a}±0.05	0.08^{b}±0.07	0.11	0.08	P<0.01
Effective degradability (%)	63.6±0.4	64.2±1.1	62.8±0.52	63.5	0.51	P<0.14
RSD	2.9±0.9	2.4±0.7	4.4±0.5	3.27	0.49	P<0.23

BSM-T= Black Seed Moringa of Thailand; WSM= White Seed Moringa of local origin; BSM-L= Black Seed Moringa of local origin;

Table.5: Biomass yield and crude protein content cultivated fodder in Bangladesh

Feeds & Fodders	Harvest composition	DM (t /yr/ha)	CP content & yield		Sources
			g.kg^{-1} DM	t/yr/ha	
Lathyrus sativus	Whole plant with soft pods	12.7	152.0	1.96	Rahman *et al* (2015)
Vigna mongu	Whole plant with soft pods	6.6	105	0.69	
Vigna unguiculata	Whole plant	15.6	105	1.64	**Unpublished data, BLRI 1995*
Sesbania sesban	Tops with stem &leaves	17.3	101	1.75	
Leuchena leucocephala	Intermittently cut tops with stem & leaves	10.7	202	2.15	
Soybean meal	Oil extracted grain biomass	1.8	518	0.93	*Feedipedia; http://www.feedipedia.org*

**KS Huque, SA Chowdhury & ME Hoque "Study on the productive and nutritional characteristics of Maize intercropped with different varieties of legumes" BLRI report, 1995, PP: 575-588*

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Table.6: Rumen digestion kinetics of protein and amino acid composition of Moringa and other feed sources

Nutrients	*(Feedipedia; http://www.feedipedia.org)*			*Khalel et at (2014)*	
	Soybean meal	*Lleucocephala*	Alfalfa hay	Moringa feed	Berseem
In sacco degradability in the rumen		Nitrogen, %		Crude protein, %	
Soluble, a	15.3	18.5	55.8	22.1	25.9
Potential degradable, b	78.9	62.4	37	47.7	59.4
Effective degradability % at 0.06	65.0	45.0	79.0	55.1	67.7
		Amino acids, % CP			
Methionine	1.4	1.3	1.2	0.66	0.74
Lysine	6.3	5.5	4.7	7.69	4.92

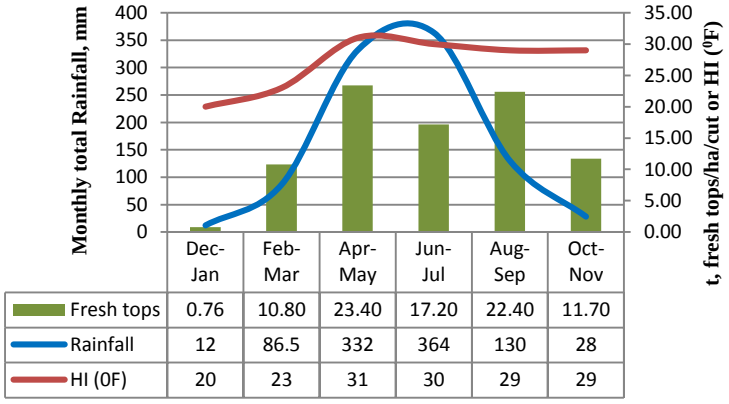

Fig.1: Seasonal impacts on annual Moringa production

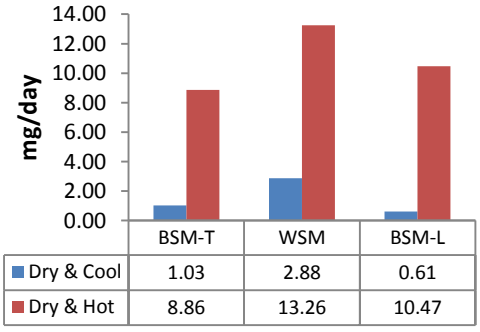

Fig.2: Seasonal impact on Relative Growth Rate of Moringa

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Thiamethoxam in Papaya (*Carica papaya* Linnaeus) Agroecosystems

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Abstract— *Papaya (Carica papaya L.) is a profitable fruit of economic and food importance in Mexico and Central America. Veracruz is the state in Mexico with the highest cultivable area, eventhough its production presents numerous phytosanitary problems, which are being faced with the use of the pesticide thiamethoxam. The aim of this study was to make a diagnosis of the use and management of thiamethoxam in papaya agroecosystems in the municipality of Cotaxtla, Veracruz. Two surveys were applied, one to a 30% of the total number of producers organized by an association dedicated to papaya culture, and the other survey was through key informants, both surveys were designed using the snowball sampling, a non-probability sampling technique. The results indicate that 6% of papaya producers use mainly the pesticide thiamethoxam, which belongs to the chemical group of neonicotinoids. It was found out that for five years there have been records of thiamethoxam use in vertisoils. During the cycle of papaya cultivation the producers use a maximum dose of 3 L/ha and a minimum dose of 250 ml/ha per crop cycle. One hundred per cent of those who apply thiamethoxam are not aware of its use and efficient management, nor of the damage they are doing or have caused to agroecosystems.*

Keywords— Thiamethoxam, Carica papaya L., neonicotinoids.

I. INTRODUCTION

Tropical agroecosystems are ecological systems modified by man that have the job of generating a good or service, and are obtained from the processes of agricultural production, with the purpose of satisfying the needs of the population, such as economic, moral, social or spiritual type [22]. *Carica papaya* L. agroecosystem, is a dynamic production system integrated by short cycle crops that allow having an income several times a year, unfortunately affected by the economic loss that is generated by the pests and diseases that occur in the crops. Papaya is a profitable fruit tree in Mexico and Central America, its cultivation is carried out with a minimum investment of one hundred thousand Mexican pesos per hectare [25], most of this investment is focused in the acquisition of agrochemicals to combat pests and diseases. In 2015, Veracruz became the main papaya producer state with a cultivated area of 18% of the total area of papaya established in Mexico, and the municipality of Cotaxtla contributes with 19% of the total agricultural land of papaya in Veracruz [24]. Aphids are the most economically important pests in Mexico, among them the red spider (*Tetranychus cinnabarinus*) and the white mite (*Polyphagotarsonemus latus*) stand out; a characteristic of these aphids is that they damage the foliage of papaya plants. Another important pest in Mexico and in other countries like India is the mealybug (*Planococcus sp*.) that acts as a vector of viral diseases. Of the minor pests are the parakeet (*Acanophora projecta*) and the papaya fly (*Toxotrypana curvicauda*). For that reason cultural management in the control of pests is fundamental to obtain fruit of commercial quality. It is necessary to respond to the sustainable development policies of the General Assembly of the United Nations (UN), where the use of organic products, entomopathogenic fungi and plant barriers is recommended to minimize the damage caused by pests and at the same time maintain an ecological balance of biodiversity [14, 28]. This effort to use organic products does not respond to the demands and requirements of the agricultural sector, therefore producers use pesticides massively in papaya crops. Training is also considered necessary for proper management culture of chemical waste that is the result of pesticides used at plot level. This chemical waste is one of the major factors in soil and groundwater contamination through leaching, percolation and entrainment of chemical molecules towards the aquifer. Currently, farmers use organophosphorus products for the management of the "Maradol" papaya crop, one of the disadvantages of handling pesticides such as parathion, diazinon and malathion, is the harm caused in humans by intoxication known as cholinergic syndrome. However,

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this chemical group is not the only one that has caused damage, there are also the neonicotinoids, which are a chemical group that causes ecological damages, such as death of birds that eat tropical fruits [7, 9 and 8], mainly in the flowering stage until reaching the production one that begins three or four months after flowering [4]. The use of chemicals in pest control is of global interest due to its motility ability within plant organs, besides having the capacity to accumulate in certain specific organs [27]. Currently of the authorized agrochemicals in Mexico, the group of neonicotinoids such as imidacloprid, acetamiprid, nitenpyram, thiamethoxam, thiacloprid, clothianidim and dinotefuram [26] are highly required by producers for their effectiveness in the control of sucking insects. From this chemical group, thiamethoxam is used in the control of Spodoptera frugiperda, *Bemisia tabaci*, aphids and triocides, sucking insects that transmit diseases in papaya crops [5]. Thiamethoxam is a systemic neonicotinoid pesticide that is characterized by its rapid effect, active by ingestion and contact, besides being an inactivator of the nicotinic receptors of the acetylcholine of the insects nervous system, causing paralysis and then death, these pesticides are effective in controlling lepidoptera [10, 11, 2]. Thiamethoxam can last 3 years in soil, it is soluble in water and its use in crops can cause bee hives collapsing at concentrations of 0.1 to 0.5 µg/hive [15], whereby the Environmental Protection Agency (EPA) and the European Food Safety Authority (EFSA) set the maximum permissible limits in fruit trees of 0.4 and 0.05 πg/L. In addition, the damage caused to ecology and human health is innumerable. Therefore the aim of this study was to make a diagnosis of the use and management of thiamethoxam in a papaya agroecosystem, in the municipality of Cotaxtla, Veracruz in Mexico.

II. MATERIALS AND METHODS

The municipality of Cotaxtla, Veracruz México is located at the geographical coordinates 18° 44'-18° 59' north latitude and 96° 11'- 96° 32' longitude west at an altitude of 10 to 200 meters above the sea level and is formed by papaya producers of 216 localities (Figure 1), among the most important are La Tinaja, La Capilla, Cotaxtla, Colonia Ejidal and Mata tejón. The municipality of Cotaxtla has a population of 18, 821 inhabitants.

Fig.1: Location of the study area in the municipality of Cotaxtla, Veracruz México. [INEGI, 2005].

This study was carried out by means of a design and application of two surveys, one applied first to 30% of the total of papaya producers of the localities of Las Lomitas, Mata Espino, Mata Tambor, Loma de los Hoyos, Los Bajos del Tlachiconal and the municipal seat of Cotaxtla. This group of producers is constituted in an organized manner by an "Agricultural Grouping" which facilitated to carry out the surveys. The first survey was applied with an open-ended questionnaire in order to know: which groups of agrochemicals are currently used for the management of papaya crops; which are the main phytosanitary problems that they have during crops. And also if there is, as a consequence of these, a low yield of the crops that generates a negative impact to the agroecosystem. A second survey was conducted to determine the use and management of the systemic pesticide thiamethoxam. It was designed through the non-probabilistic qualitative snowball sampling technique, and the interview was applied to key informants in order to obtain the following information: 1. If they knew the chemical characteristics of the thiamethoxam. 2. Who the main leading papaya producers are. 3. To have knowledge about thiamethoxam's distribution and sale in a papaya

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agricultural area. 4. Locate the major suppliers of thiamethoxam.

As a study strategy, the main commercial centers for agrochemicals in the municipality of Cotaxtla and Piedras Negras in the municipality of Tlalixcoyan, Veracruz, were identified; and key informants were interviewed (Figure 1). The data obtained were analyzed with the program statistica version 7, along with a nonparametric and parametric analysis of Kruskal Wallis.

III. RESULTS AND DISCUSSION

In this research the key informants were interviewed, of which 56% of them used to grow papaya "Maradol". Among pests present in papaya crops, the producers mention that the main pest species that cause severe damage to papaya plantations are the red spider 44%, mites 33% and aphids 11% (Table 1). For producers to learn to know main species, their classification and control, it is necessary to transfer technology on integrated management of pests and diseases. It is important to consider that the producers that are devoted to this activity are among 30 to 70 years old [16], so it is necessary to anticipate and train groups of young producers with attitude and aptitude for food production between 18 to 30 years old. And in doing so, to identify and form innovative leaders in the agricultural sector. Currently, 35% of agricultural producers are using organophosphorus pesticides in their crops, in order to achieve control of mites; 23% use those of the macrocyclic lactate chemical group; 12% use pyrethroids, and 6% use those of the chemical group neonicotinoids. Of the latter group 6% of the producers apply the pesticide thiamethoxam (Table 1). The massive use of pesticides in crops, mainly thiamethoxam due to its high solubility chemical characteristics, is causing an impact on the ecosystem. Aquatic systems by runoff are being affected, besides its use also represents a potential risk for productive activities that use water as a resource. Aquaculture is a productive activity in the Gulf of Mexico that is being negatively impacted by the presence of this pesticide in surface and groundwater. In addition, it is important to consider that the presence of these chemicals affects public health and provoke damages to the environment [19,24].

Table.1: Chemical groups and active ingredients most used by producers in papaya agroecosystems and main pests that attack the crops.

Chemical Groups	Producers who use it (%)	Active Ingredient	Producers who use it (%)	Pests	Population (%)
Nitroguanidines	6	Clotianidin	6	Nematodes	6
Carbamates	6	Oxamyl	6	Red spider	44
Macrocyclic lactone	23	Abamectin	23	Mites	33
Organophosphates	35	Parathion	11	Wire worm	6
Pyrethrins	12	Cypermethrin	12	Aphids	11
Neonicotinoids	6	Dimethoate	12		
Chloronicotinyls	6	Methamidophos	6		
Saccharopolyspora spinosa	6	Thiamethoxam	6		
		Imidacloprid	6		
		Malathion	6		
		Spinosad	6		

Nowadays, it has become a must the use and management of new pesticides in agriculture to combat pests and diseases in crops of commercial importance. This has favored the use of the systemic pesticide thiamethoxam, which can be persistent in soil for 90 days, in addition it degrades and percola itself settling in groundwater in which it is highly soluble (4.95 X 10^{-11} ha 25° C). Its hydrolytic degradation is in a range of pH of 5 to 9, reason why it is necessary to carry out studies of dissipation and motility in water, as well as learning how to handle and apply it in papaya crops, since producers use it due to its systemic and contact activity, that becomes suitable for an efficient control of sucking insects such as aphids [21, 13]. Producers from the study area of the municipality of Cotaxtla, have mentioned that mite has caused severe damage to papaya agroecosystems (Table 1). The main species present in papaya crops is the white mite (*Polyphagotarsonemus latus*), this causes reduction and deformation of young leaves, buds, flowers and fruits. It presents a symptomatology called “monkey

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hand", which is associated with the presence of the Papaya Annular Blight Virus (PRSV-P), this is due to the fact that the adoption of technology has been limited by the producer himself and the presence of hoarders generates that papaya producers look for alternatives for the control of pests. In addition, it is considered that the crop requires sufficient inputs for the integral management of papaya crops, the leading producers seek to share their tacit knowledge to other adoptive producers in order to improve their production systems [1 and 6].

The distribution of thiamethoxam is currently carried out in the town of Piedras Negras in the municipality of Tlalixcoya, Veracruz, under the trade name Engeo (Thiamethoxam + Lambda cyalotrina) and Actara 25WG (thiamethoxam) distributed in the municipality of Cotaxtla, Veracruz, with the name Unikum (thiamethoxam), this means that there is a probability of a trend towards the use of thiamethoxam in the agricultural area of the municipality of Cotaxtla, mainly for the cultivation of papaya and main vegetables in the region (Table 2).

Rotation and association of papaya crops with watermelon cultivation and the proximity of papaya crops to farms where watermelon and tomato are planted or, where appropriate, the location with old papaya orchards has caused crops to be affected negatively by the presence of the virus. This phenomenon is associated with migration of major insect vectors to new papaya plantations [23].

Table.2: Characteristics of thiamethoxam distribution in the main papaya production areas in the center of the state of Veracruz, México

Variables	Municipalty of Tlalixcoyan	Municipality of Cotaxtla	Commercial brand of thiamethoxam	Sale percentage (%)	Pests controlled (%)
Thiamethoxam suppliers (%)	67	33	Engeo	60	36 Whitefly
Thiamethoxam sale (L/year)	140	6	Actara	20	18 Trips
Use of thiamethoxam in crops	Papaya, vegetables, sugar cane	Papaya, lemon, watermelon	Unikum	20	45 Aphids

The survey showed that 45% of the producers are dedicated to the production of papaya and watermelon, 27% are only cultivating papaya, while the rest is focused on other types of crops (Table 3). There are records of the use of thiamethoxam for 5 years in crops with vertisol soils, the maximum dose used is 3 L/ha^{-1} during papaya cultivation cycle on sandy loam soils (Table 4). According to studies carried out with other pesticides, thiamethoxam could become an important non-point source of groundwater contamination, due to its high soil motility [3].

Studies reported in Venezuela show that thiamethoxam applications have been made in clay-loam and clay soils, which predominate at the foot of high savannas that are vulnerable to intermittent flooding [32]. Land losses caused by conventional farming practices are considered to cause severe ecological damage, it is estimated that worldwide soil erosion is 3 million hectares and 2 million hectares are desertified on agricultural land [20], this degradation will contribute to the vulnerability of surface water and groundwater by the presence of the pesticide. Among these pesticides of the chemical group of the neonicotinoids, thiamethoxam due to its systemic characteristics and high motility, is highly effective in papaya cultivation, mainly in the combat of sucking insects that transmit diseases. Its efficacy can be observed 7 days after its application [12]. Although damage to the agroecosystem could be a high risk, it has been reported that thiamethoxam in concentrations of 1 to 100 ppb in flowers and fruit plants causes death in bees; 10 ppb in water causes death in aquatic species, and also 10 ppb in agricultural layer damages macrofauna and soil microflora [31].

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Table.3: Predominant cultures in papaya agroecosystems and its relation to thiamethoxam.

Crops	Presence in agroecosystem (%)	Thiamethoxam use (Years)
Papaya-Vegetables	9	4
Papaya-Corn-Watermelon	9	0
Papaya	27	2
Papaya-Corn	9	0
Papaya-Watermelon	45	2.5

Table.4: Applications of thiamethoxam in different types of soil and crop cycle in the municipality of Cotaxtla Veracruz, México.

Soil types	Application of thiamethoxam (Years)	During the growing cycle (ml/ha)
Clay	3.8	700
Sandy-clay	1.5	250
Sandy	0	0
Loamy sand	2	3000
Vertisol	5	600

In the locality of Loma de los Hoyos Cotaxtla the maximum dose used in crops is 3 L/ha, unlike the producers of the locality of Lomitas who mentioned that they do not use thiamethoxam in papaya crops. The group of producers of Los Bajos de Tlachiconal used a dose lower than 500 ml/ha in the cycle of papaya cultivation, which represents a significant difference with respect to the group of producers of the locality of Mata Espino, who apply a dose of 1 l/ha (Figure 2). Currently in countries like India, in relation to the control of *Paracoccus marginatus* in papaya cultivation, they found out that profenophos 50 EC (0.05%) and acephate 75 SP (0.075%), are more efficient in 90% and 80% with reference to 78% of the mortality index of the pesticide thiamethoxam, although papaya producers are using biopesticides to control this pest [18, 12]. Thiamethoxam is efficient in the control of *Empoasca fabae* Harris with a protection of 31 to 38 days on the crop and of *Bemisia* spp. as a major vector of papaya ringspot virus, reducing the adult population to 97% after 14 days of foliar treatment [2]. In addition, it controls the pests of the coleopteran, hemiptera and lepidoctera families, mainly to *Tagosodes orizicolus* in a period of 21 days, these species may be responsible for the presence of papaya virus [32]. An alternative for the control of aphids in papaya cultivation is the use of reflective and black plastic mulch, these plastic covers reduce insect-pest populations and contribute to reduce the presence of virus, it is also important to consider the use of biodegradable plastics since it provides better development of plants [30; 29]. Biological control may be an alternative for tropical papaya agroecosystems that are negatively impacted by the excessive use of pesticides, although the management of neonicotinoids such as thiamethoxam has regained importance because of its nicotine-like effect by blocking acetylcholine receptors of the central nervous system of the insect. The use of organic products and live barriers such as Maize can be an alternative for the control of pests and diseases of the crop, as well as for the production of innocuous foods [10, 23].

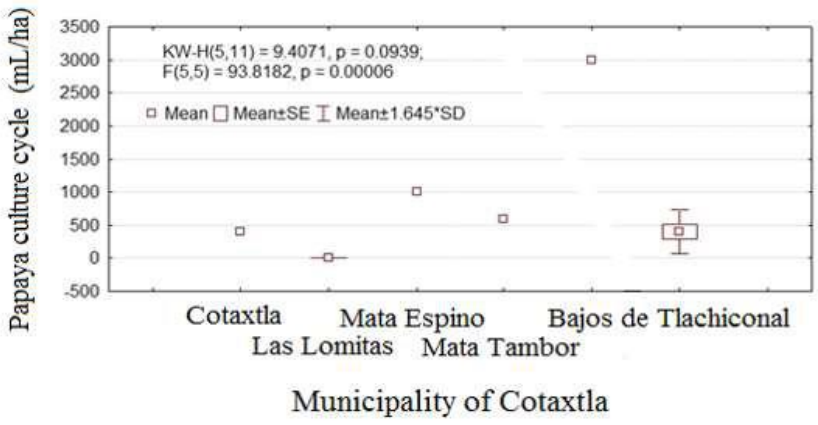

Fig.2: Thiamethoxam use in papaya culture cycle in the municipality of Cotaxtla Veracruz, México.

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IV. CONCLUSIONS

Producers prefer ENGEO® (Thiamethoxam + Lambda-cyhalothrin), which is most frequently used in papaya cultivation. One hundred per cent of those applying this product are unaware of its use and efficient handling, and damage that may cause to agroecosystems. It is necessary to carry out scientific research to know the concentrations of thiamethoxam in soil, water and plant and to know if it does not exceed the permissible limits established by EPA and EFSA. Thiamethoxam tends to be used more by papaya producers because of its efficiency in combating crop pests, but it is necessary to validate the product in the field to use the effective dose in crops and it is also important to evaluate it with organic insecticides and chemical products recommended for each crop.

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Impact of the Mixed Consortium of Indigenous Arbuscular Mycorrhizal Fungi (AMF) on the Growth and Yield of Rice (*ORYZA SATIVA* L.) under the system of Rice Intensification (SRI)

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Abstract*—The effect of inoculation of indigenousarbuscular mycorrhizal fungi (AMF) co-inoculated with Azospirillumlipoferum(strain Az204) and phosphobacteria (Bacillus megaterium - strain PB2) on the growth and yield of rice under the System of Rice Intensification (SRI) in the nursery and field was studied by conducting a field trial at Agricultural College & Research Institute, Madurai. The indigenous AMF was isolated from rice fields of this Institute and were identified as Glomus sp., Gigasporasp. and Acaulospora sp. These AMF were mass multiplied in maize plants using vermiculite as substrate and used as mixed consortium AMF. The mat nursery was prepared and AMF inoculated at the rate of 100g/m^2. Also treatment was done using Azospirillum and phosphobacteria on treatment wise. At the time of transplanting seedling dip was done for the 8-day old rice seedlings using the same microbial inoculants. In the main field seed also application of mixed consortium AMF along with Azospirillum and phosphobacteria was carried out based on the treatment schedule. The results of the field trial revealed that the seedlings in the nursery showed vigorous growth and AMF colonization and spore count were recorded the maximum in the treatment with AMF, Azospirillumand 75% RDF of N and P. In the main field also there was increased growth and yield of rice plant in the same treatment due to the inoculation of mixed consortium AMF co-inoculated with Azospirillum on rice variety, ADT43 in the presence of 75% N and P. The yield of rice in this treatment recorded 11.8% higher than with 100% NP alone, besides saving 25% NP. We conclude that the mixed consortium ofindigenousAMF inoculation at the nursery and main field under SRI increased growth and grain yield of rice.*

Keywords*—arbuscularmycorrhizal fungi, Azospirillum, phosphobacteria, rice, growth, yield, System of Rice Intensification.

I. INTRODUCTION

Rice in India is cultivated as an irrigated enterprise in which farmers face major constraints that can adversely affect production levels. The yields of rice (*Oryza sativa*) in India are low because of a gradual decline in soil fertility. In order to tackle the situation farmers have intensified their tillage and cropping practices without making the necessaryorganic inputs to restore and maintain soil fertility. The use of AMF that maintain a type of mutualistic association between crop and fungus may contribute to reducing chemical fertilizer inputs and sustaining crop productivity. In contrast to other crop species, there is little experimental evidence about the role of mycorrhizal colonization in rice plants (Purakayastha and Chhonkar, 2001; Gao *et al*., 2007). It was reported that rice plants readily form mycorrhizal associations under upland conditions, but under submerged conditions infection is rare due to the anoxic environment (Ilag*et al.,* 1987). Barea (1991) reported, however, that AMF can survive in waterlogged conditions, and this is supported by the fact that *Glomus etunicatum*, showed fairly high colonization in rice roots and best survival under submerged conditions (Purakayastha and Chhonkar, 2001). In a work on six aerobic rice genotypes, relatively high colonization of roots, 28-57% depending on genotypes was observed (Gao *et al.,* 2007). However, there is a paucity of information available on the involvement of AMF in rice particularly under waterlogged conditions.

Arbuscular mycorrhizal fungi have their greatest effect when a host plant associated with them is of deficient in phosphorus (Koide, 1992). It is a fact that mycorrihzal fungus is able to increase growth in a number of agricultural crops (Mosse, 1973; Gredemenn, 1975; Tinker, 1975). Sanni (1976) demonstrated the increase in the growth of rice plants after inoculation with *Giasporagigantia*. Some studies under pot culture conditions revealed that AMF increased grain and straw yields of wetland rice (Sivaprasad*et al.,* 1990) and increased the grain yield and P and Zn content in rice

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(Secilia and Bagyaraj, 1994a and 1994b). Gupta and Ali (1993) reported a significant increase in the grain yield by AMF colonization in wetland rice under both pot and field conditions. The inoculation of AMF directly into the flooded soil was not effective for wetland rice (Solaiman and Hirata,1995). However, inoculation of seedlings under dry nursery conditions was effective for promotion of wetland rice growth and nutrient acquisition (Solaiman and Hirata,1996). The present investigation was, therefore, undertaken to study whether the growth and yield of rice grown under the SRI could be enhanced through inoculation with AMF at the nursery and field conditions.

II. MATERIALS AND METHODS

2.1. Experimental Setup

The experiment was conducted in a field at Agricultural College & Research Institute, Madurai, Tamil Nadu, India with 8 treatments and 3 replications. The indigenous AMF cultures isolated from rice fields of this Institute and identified as *Glomus sp.*, *Gigasporasp.* and *Acaulospora sp.* were used as mixed consortium of AMF inoculants. The nitrogen fixer, *Azospirillumlipoferum*(Az204)andphosphobacteria (*Bacillus megaterium*- PB2) were also included in the treatments as biological inputs for N and P.The replications were made in a random throughout the plot. Also the recommended dose of N:P:K(120:38:38 kg/ha) at various levels was added to the treatments. The Statistical Design adopted was RBD. The various treatments were as follows

T1 *Azospirillum* + Concentrated AMF + 75% N and P
T2 *Azospirillum*+ Normal AMF + 75% N and P
T3 *Azospirillum* + Concentrated AMF + 100% N and P
T4 *Azospirillum* + Normal AMF + 100% N and P
T5 *Azospirillum* + phosphobacteria + 75% N and P
T6 *Azospirillum* + phosphobacteria + 100% N and P
T7 75% N and P
T8 100% N and P (120:38:38 kg/ha)

2.2. Arbuscular mycorrhizal fungi (AMF) isolation and multiplication

Soil samples were collected from different locations of rice fields of Agricultural College & Research Institute, Madurai and were examined for the presence of AMF spores by wet sieving and decanting technique (Gerdemann and Nicolson, 1963) and examined under a stereozoom microscope for their shape, colour and the hyphal attachment to spores. Based on the taxonomic keys of Schenck and Perez (1988) and through INVAM web based identification (http://invam.caf.wvu.edu/cultures/cultsearch.htm), the AMF isolates from rice field soil were identified and mass multiplied in maize plants using vermiculite as substrate and used as mixed consortium AMF. The mycorrhizal inoculum consisting of spores in vermiculite substrate, and infected root fragments is Normal AMF. The AMF colonized maize root bits alone is Concentrated AMF.

2.3. SRI rice nursery preparation and transplantation to main field

Raised nursery bed is formed and mixed consortium of indigenous arbuscular mycorrhizal fungi is inoculated @100g / $m^2$and is spread in the nursery bed to a depth of 2-3 cm. Also rice seeds were treated with *Azospirillum* (*A*z.204) and phosphobacteria (*Bacillus megaterium*PB2) as per the treatment and germinated in the dark. Germinated seeds were sown in the nursery treatment wise. At the time of transplanting seedling dip was done for the 8-day old rice seedlings using the same microbial inoculants. In the main field also application of mixed consortium AMF along with *Azospirillum* and phosphobacteria was carried out based on the treatment schedule.

2.4. Mycorrhizal assays

Spores of AM fungi in the soil were estimated by the wet-sieving and decanting method described by Daniels and Skipper (1984). The roots in each treatment plot were washed free of soil particles and organic debris on a 2 mm sieve under a jet of tap water. A 0.5 g sample of fresh roots at the nursery stage or a 1.0g sample of the roots was excised from each hill from field samples, to assess the percentage of AMF colonization. The root samples were preserved in formalin-aceto-alcohol (FAA) to fix the roots and the standard procedures for clearing and staining of roots as modified from Kormanik and McGraw (1984) were used. Percentage colonization of roots was estimated by visual observations of stained root segments mounted in lactoglycerol, counting the number of root bits colonized to that of the total number of root bits observed (Giovannetti and Mosse, 1980).

2.5. Dry matter production

Three plants were randomly selected from each treatment, washed and dried in an oven at 80°C till constant weight was observed. The plants were weighed and dry weight was expressed in g/plant during transplanting, tillering, flowering and harvest stages.

2.6. Grain yield

The grains harvested from each treatment plots were weighed and the mean value was expressed in tones /acre.

2.7. Statistical analysis

The experimental results were statistically analyzed in randomized block design (RBD) and in Duncan's multiple

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range test (DMRT) as per the procedure described by Gomez and Gomez (1984).

III. RESULTS AND DISCUSSION

3.1. Arbuscular mycorrhizal fungi (AMF) isolation and multiplication

AMF spores isolated from rice fields were examined under a stereozoom microscope for their shape, colour and the hyphal attachment to spores. Based on the taxonomic keys of Schenck and Perez (1988) and through INVAM web based identification (http://invam.caf.wvu.edu/cultures/cultsearch.htm), the AMF isolates from rice field soil were identified as *Glomus sp., Gigasporasp.* and *Acaulosporasp*(Fig.1). These AMF were mass multiplied in maize plants using vermiculite as substrate and used as mixed consortium AMF.

3.2. AMF colonization and sporulation

Colonization of rice roots increased from nursery to tillering stage and decreased after the flowering stage. Colonization was negligible in rice roots when the soil was not inoculated with AMF. Sporulation also increased till tillering stage and decreased thereafter.AMF colonization and sporulation was maximum in the T2 treatment, *Azospirillum* + Normal AMF + 75% N and P (Table.1;Fig.2). A unique characteristic of rice roots that overcomes these reduced conditions in soil is the presence of large air spaces in mature roots (Yoshida,1975; Velu*et al.*,2009). Thus, the aerated region around the rice roots may provide a suitable environment for rhizosphere microorganisms including mycorrhizal fungi.

3.3. Dry matter production

Dry weight of the rice plant was significantly higher in the T2 treatment, *Azospirillum* + Normal AMF + 75% N and P followed by T1 in all the stages of sampling (Table.1). There are few reports to elucidate the essential role of AMF on rice plants at the nursery-stage and its function after transplanting to the field. The inoculated seedlings had a higher total biomass than uninoculated seedlings at transplanting to the field. This indicates that seedlings benefited from mycorrhizal colonization prior to transplanting as already reported (Dhillion and Ampornpan, 1992; Solaiman and Hirata, 1996).

3.4. Grain yield

The grain yield after harvest, at 110th day was recorded the highest in T2 treatment, *Azospirillum* + Normal AMF + 75% N and P of 2.18 t/acre and it is 11.8% increase over the control, T8 tratment with 100% N and P (Table.1). Mycorrhizal inoculation with *Glomus fasciculatum*in dry nursery-stage seedlings increased grain and straw yields (Sivaprasad*et al.,* 1990; Chinnusamy*et al.*,2006**;** Bhuiyan*et al,* 2006**;** Ashok Kumar., 2011; Maiti*et al.,* 2011; Shukla *et al.,* 2013). Secilia and Bagyaraj (1992) evaluated 18 different inoculants of AMF on nursery seedlings for 15 days of growth under dry and then up to 28 days of growth under wet conditions. *Acaulospora*sp., *Glomus etunicatum*and *Scutellospora*sp. exhibited stimulation of wetland rice growth.In our experiment, the indigenous AMF isolated are *Glomus sp., Gigasporasp.* and *Acaulosporasp*. which were significantly effective for increasing the yield when inoculated at the nursery stage and also applied in the main field.

IV. CONCLUSION

The effect of inoculation of arbuscular mycorrhizal fungi (AMF) along with other microbial inoculants viz., *Azospirillum* and phosphobacteria on the growth and yield of rice under the System of Rice Intensification (SRI) in the nursery and field was studied by conducting a field trial at AC & RI, Madurai. The indigenous AMF was isolated from rice fields of Agricultural College & Research Institute, Madurai and were identified as *Glomus sp., Gigasporasp.* and *Acaulospora sp.* These AMF were mass multiplied in maize plants using vermiculite as substrate. The mat nursery was prepared and AMF inoculated at the rate of 100g/m^2. Also seed treatment was done using *Azospirillum* and phosphobacteria on treatment wise. At the time of transplanting seedling dip was done for the 8-day old rice seedlings using the same microbial inoculants. In the main field also application of AMF along with *Azospirillum* and phosphobacteria was carried out based on the treatment schedule. The results of the field trial revealed that the seedlings in the nursery showed vigorous growth and the AMF colonization and spore count were recorded the maximum in the treatment with AMF, Azospirillum and 75% RDF of N and P. In the main field also there was increased growth and yield of rice plant in the same treatment. The increase in yield was 11.8% in the treatment with AMF, *Azospirillum*and 75% RDF over the uninoculated.

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***Glomus* sp.**

***Gigaspora*sp**

***Acaulospora*sp**

Fig.1: AM fungal spores isolated from the rhizosphere soil of rice.

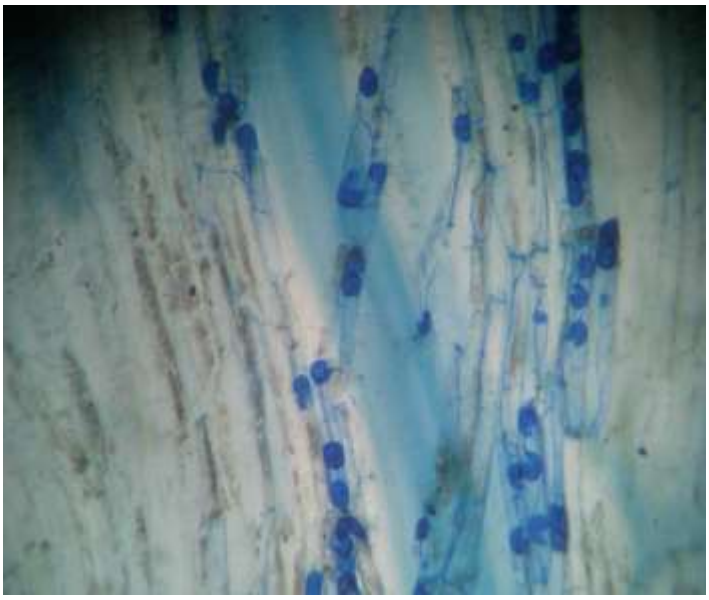

Fig.2: AMF root colonization in the rice field

Table.1:AMF colonization (%), Spore count (nos./100g), Dry weight (g/plant) and Yield of rice under SRI

Treatments	**AMF colonization (%)**			**Spore count (nos./100g)**			**Dry weight (g/plant)**			**Yield of rice plants at harvest (110days)**	
	Transplanting stage	Tillering stage	Flowering stage	Transplanting stage	Tillering stage	Flowering stage	Transplanting stage	Tillering stage	Flowering stage	Yield (t/acre)	
T1	18.6	26.0	26.3	5.5	12.3	10.2	9.26	15.7	66.9	2.12	**8.7 %**
T2	21.3	31.3	30.0	8.2	15.9	12.5	9.80	18.1	70.2	2.18	**11.8 %**
T3	18.0	23.6	23.3	5.3	11.5	9.4	8.96	12.3	62.6	2.05	**5.1 %**
T4	16.6	26.0	23.0	4.7	10.0	8.6	8.96	11.0	55.5	2.02	**3.6 %**
T5	3.3	2.3	2.3	1.0	2.2	2.0	9.16	12.0	62.3	2.06	**5.6 %**
T6	4.0	2.3	3.3	1.0	2.5	2.0	8.03	10.8	54.7	1.99	**2.1 %**
T7	2.6	2.6	2.3	0.0	1.5	1.2	7.36	7.8	38.5	1.92	**-1.5 %**
T8	1.3	2.6	2.6	0.0	1.0	1.0	8.06	9.2	45.4	1.95	**8.7 %**
CD(P=0.05)	**3.21**	**3.74**	**2.99**	**2.12**	**2.50**	**1.90**	**0.55**	**0.84**	**3.26**	**0.05**	

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Perception towards Family Planning and its Implication to Environmental Sustainability: The Case of two Selected Kebeles in Aroresa Woreda in Sidama Zone, Ethiopia

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Abstract— *This study was conducted aiming at to assess perception of couples towards family planning and its relevance to environmental sustainability and to identify factors hindering family planning practice. Sample respondents for study comprises 90 couples in rural and 28 in urban. Data was collected by using random systematic and simple random sampling methods. The instruments used for data collection were interview and focus group discussion. The research found that there is promising level of awareness on family planning and main source of information were health extension agents.*

Reported reasons in sought of large number of children were old age support, son or daughter preference, considering children as a wealth, and labor support, religious prohibition. Nearly half of respondent couples approve contraceptives with more approval of women in both settings. Most of the respondents showed positive attitude towards family planning. Furthermore, half of the rural and nearly less than half of the urban respondents approve the importance of family planning for environmental sustainability. During the study period 23.2% of urban and 18.9% of rural couples were using some method of family planning; among which 21.4 of urban and 16.7% of rural women were using modern methods, hence, awareness level and practice in family planning showed a gap in both urban and rural. In general, urban showed more favorable attitude and practice than rural couples in family planning. Despite of their lower practice in family planning, rural respondents likely showed more favorable attitudes towards relevance of family planning for environmental sustainability.

Keywords—Environment, Family planning, Perception, Population.

I. INTRODUCTION

Fertility is the most important component of population dynamics and plays a major role in changing the structure of the population of a given area. And this high fertility is main factor for rapid population growth in developing countries. Population increase exerts pressure on land recourses and contributes to the loss of its productive capacity. The degradation of the natural resources of the environment has serious economic and environmental consequences (Bekure and Singh, 1996). An increasing population density leads to a greater depletion of rural community resources like fire wood, water and soil… furthermore, poverty, environmental degradation and high fertility drive one another in a vicious cycle (Wright, 2008).

There have been the two contrasting views concerning rapid population growth on one side and food supply, resource use, economic development and environmental quality issues on another. The two contrasting arguments on these issues have long history, 18th century, age of Thomas Robert Malthus and thereafter by the opponent of him was Ester Boserup. The main contends of Malthus in "Limit to growth" were that the growth of world population at the time at 2% per annum was rapidly endangering man kinds ability to feed itself, that some of the world's non renewable resources were about to be exhausted, and that even if we managed to avoid these two dangers, we would pollute choke to death. Assuring the problem of rapid population increase, he suggested some solutions to reduce population growth that are people would have to die whether starvation, or of pestilence, or of war as positive check. Another alternative he expressed were moral restraints such as postponing marriage and absistences as negative checks (Neurath, 1994). On the other hand, Boserup 1965, believed that people have the resources of knowledge and technology to increase food supplies for ever growing population. She advocates that population growth (pressure) led to innovation and more production in agricultural activity rather than being cause of problem.

However, beyond her suggestions of satisfying food supply, there are also environmental implications of rapidly growing population and in the application of such technologies. In her view, population growth might have a

positive influence on the productivity of the environments as population increase demands for food increase, follow periods shorten and crop production increases, but, this situation cannot be free of environmental degradation since rate of exploitation of the natural environment is intensifying. According to Malthusians view, the environment is likely a gas bottle, with a fixed ability to absorb human activity. This fixed ability is termed as carrying capacity of a resource, pushed beyond that point of ability, it degrades very rapidly, similar to a container buckling or exploding under pressure. Contrastingly, Boserupians conception is that the environment is more like a balloon- its shape and capacity is actually changed as pressure builds up. Carrying capacity is, therefore, not fixed but can be influenced or shaped by the application of labor and technology (Sarre and Blunden, 1995).

But, today in most of developing agrarian countries, rapid population growth along with unsustainable use of resources, have remain one of the hindering factors for economic development, led to poor social conditions and environmental degradation. Todaro has stated features of developing countries asdeveloping countries denote those areas having low and slowly growing per capita income, poor health, condition, high rate of illiteracy, pronounced income disparities, Substantial dependency on small scale agriculture and primary product (Todaro, 1981). Hence, Most of the sub-Saharan African countries can be cited as prime examples of this phenomenon. Being a developing and one of sub Saharan countries, Ethiopia is characterized by such rapid population growth, small scale agriculture, low per capita income, and food insecurity.

The emergence of families having no farmland in typical rural areas, of Ethiopia, was a clear sign of the ever increasing imbalance between available farmland and the population (Getu, 2009). Consequently, recognizing the associated problems of rapid population growth on the socio-economic development and environment, the government of Ethiopia has recently begun giving due consideration integrating population variables in the development planning processes.

In with this line, Family planning is considered as one of the important means in controlling undesirable population growth particularly in such conditions of imbalances between population and available resources and population growth induced environmental degradation. Strengthening family planning facilities and promoting the information dissemination and population issue education are essential instruments in regulating rapidly growing population of the country.

Concerning researches progress on issues of family planning, family size and quality of life, and relation of fertility and environmental degradation, some researches have been conducted. For instance, Joekes, S (1994) entitled "Children as a Resource: Environmental Degradation and Fertility" through several case studies in developing countries, who concluded that Environmental pressures are just one of several economic factors that may influence reproductive decisions… Sometimes the counteracting facts which lead women on the one hand to desire more children and on the other to desire fewer, are so balanced as to cancel each other out, and result in no actual influence on desired fertility levels; Haq, A etal(2010) "Perception, Environmental Degradation and Family Size Preference: a Context of Developing Countries" who concluded that People perception to environment and family size is very important to use contraception. People who perceive their immediate environment is declining will use contraceptive than those who do not perceive environment as declining. If people perceive that their environment is degrading for excessive access into the natural resources, they may adopt to use contraception and reduce family size.

Giving the above assertion on the problem of rapid population growth, there is needed to investigate perception of couples towards family planning as it is the way to optimize population growth. The problem of imbalance between rapidly growing population on one side and economic development, natural resource degradation, food insecurity on the other side, and the absence of research conducted in particular to the current study area in relation to family planning and environment, are deriving forces to conduct this research.

II. METHODOLOGY OF THE STUDY

2.1 Study Population and design

The design of this study is cross sectional so as to collect data at a point of time. Study population is residents of two selected kebeles (local smallest administrative unit in Ethiopia) namely Saddeka kebele, and Girja town (Girja kebele includes urban and rural parts), in Aroressa Woreda (district), SNNPR. The Couples, men and women, in terms of household units are the target population of this study in the above mentioned kebeles. The source of population data obtained from documents of respective kebele offices. Based on this information, currently, residents of household units are 895 for Sadeka, and 281 for Girja town.

2.2 Sampling technique and sample size

The study kebeles were purposively selected because of proximity of rural and urban areas for data collection process and they are among those environmentally suffered kebeles. Before get into process of data collection, the study areas were divided into geographical strata with due consideration of the number of housing units, this was done to avoid possibility of exclusion and/or to enhance

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chance proportionally for all areas of the study settings. Based on this, rural kebele was divided into 10 geographical strata whereas urban area into 4 strata. From each of the study kebele, 10 percent of couples were made participated for interview, since method itself is time and budget consuming. In addition to this, assuming that, most probably the study population in each of their respective settings has homogeneous socioeconomic characteristics. To select the subjects or couples by their housing units, random systematic sampling method was employed, and by doing this every 10th house was selected starting from edge of already subdivided stratum. The quota was already assigned for each stratum. Hence, 90 couples from rural areas and 28 couples from urban were selected and participated in the interview. The subjects of the study were couples (women and men), if either of the selected or both of them are not volunteer, interviewer pick next house within the stratum, until desired number of subjects would achieved. In case of polygamy, men with their younger women (if reluctant with the elder one) since younger one has more reproductive span, were interviewed.For group discussion, 4 sessions for each study settings were arranged and scheduled. Each group consists of 8 members for rural (32 individuals) and 4 members for urban (16 individuals) with equal involvement of women and men. Participants of discussion were selected by simple random sampling or lottery method and proportionally from all areas of the study and discussion within group averagely took about 2 hours.

The age of women who participated in the study range from 15 to 49, as it is fertility life span for women.

2.3 Instrument of data collection

In order to gather realistic information for stated objectives of the study, two types of data collection instruments were employed. Structured questionnaire for interview and guidelines for group discussion, health extension workers and for health offices interview, were prepared. And local language, Sidama, version of the questionnaire was used for data collection. Some of the questionnaires are adapted from 2000 Ethiopian Demographic and Health Survey, and some of the questionnaire were prepared by researcher himself based on the reviewed literature. In order to get correction on interview and discussion results the tape recorders were used.

2.4 Data collection procedures

The Author has recruited 5 nurse diploma new graduates of private colleges, 2 males and 3 females, who are residing in the study areas and this enables easily data collection as collectors are familiar with the areas. Including the author totally 6 data collecting persons. They were chosen because of their familiarity with the concept of family planning. During interview male and female data collectors interviewed their respective gender, this was done so as to avoid fear for respondents and to enhance free expression of their idea. Data collectors were given appropriate training for two days on the objectives of the study and on the detailed issue of the each part of the questionnaire. In specifically, training consists of objective of the study, introduction of questionnaire format, procedure of interviewing, issue of privacy and consents of respondents.

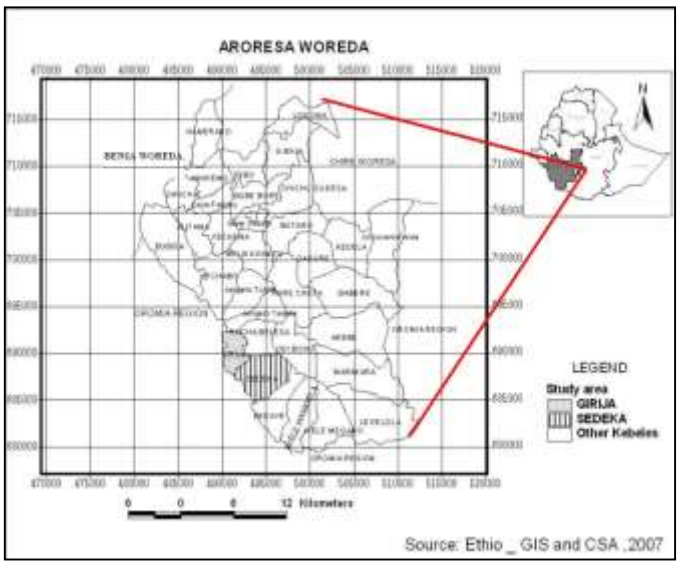
Group discussion sessions were undertaken among women and men in their respective gender grouping, and every group was assisted by their respective gender of data collectors. This was done so as to promote free expression of ideas for participants. Researcher did an interview with health extension worker, woreda health office and Girja health center to get supplementary data.

2. 5 Methods of data analysis

The data was collected through interview and focus group discussion. Moreover, it was analyzed by using both quantitative and qualitative methods. Frequencies, percentage distribution and mean were applied to summarize the results.

III. LOCATION OF THE STUDY AREA

Aroresa Woreda is found in SNNPR and southern part of the Sidama zone. The study kebeles, particularly, located in the western part of the Woreda. According to WIDS,2007, the absolute location of the woreda is about between 6^0 4'45"N and 6^0 26'30"N, and 38^0 54'50"E and 39^0 12'30 "E



3.2 Health services in the Woreda

According to the Woreda health office, the government health facilities in Aroresa Woreda includes 4 health centers(Girja, Mejo, Kinkamo and Wele magado), 25

health posts in 22 peasant associations (some of them are not functioning currently), 8 private drug shops.

The study kebeles that are Girja and Sadeka are found adjacent to each other. As mentioned above Girja kebele which includes urban and rural parts has one health center and four private drug shops whereas Sadeka kebele has one non-functioning health post. But each of these study kebeles have two health extension workers who have been playing important role in different areas of health extension programs in which family planning is one of the areas of the services.

3.3Environmental conditions

According to WIDS (2007) document, in the Woreda there is great threat resulting in loss of natural vegetation cover that occurred by is ever existing demand for farm land and human encroachment. Wildlife of the Woreda is limited to few common wild animals; the sustainability of the resource is threatened by deforestation, settlement encroachment and illegal hunting. Furthermore, land degradation has occurred that resulted from deforestation, agricultural depletion and settlement conversion. This all are mostly consequences of increasing in population density. Although, no particularly written document about study kebeles, from my experience by living long, about 15 years, in the area it important to say something about study areas. Being situated in the drought prone areas of the Woreda these kebeles reflect more sever features what is stated above. Forests are cleared for agricultural activities and soil erosion is become increasing.

IV. PRESENTATION AND DISCUSSION OF RESULTS

The data for this study was collected both from rural and urban areas of two selected kebeles. From one rural kebele, the data gathered from 90 Couples (90 women and 90 men) and from urban area 28 couples were selected and participated for interview. In addition focus group discussion among couples also undertaken, to obtain supplementary data health center office and health extension workers were interviewed. The information gathered comprises some of the socio- Economic and demographic characteristics, awareness about family planning, attitudes towards family planning and its implication to environmental sustainability, and practices in family planning.

4.1 Socio economic and Demographic Characteristics of Respondents

The data collected in this study reveals that most of respondent women have married in rural and urban below 18 years with mean of 16.5 and 17.5 respectively while mean age at marriage for rural men is 24 and for the urban 25 years. In both urban and rural the age at marriage for women was founded low, however, the urban late by one year. Regarding age at first birth in rural area, 79 (87.8%) of respondent women have bore their first child at the mean age of 18 years while majority of men, 44 (48.9%), bore their first child at the mean age of 25 years. Whereas in urban, 24 (85.7%), of women reported that they have bear their first child at the mean age of 19 years. And 15 (53.6%) of men bear first child in the mean age of 26. Many studies in developing countries revealed that early marriage is one of the major factors that contribute to bear many children in reproductive life span particularly for woman. Average number of living children varies for rural and urban settings and at the same time also it differs for women and men. Mean number of children is 4.8 for women and 6 for men in rural while 4 for women and 5 for men in urban. Men have more average number of children; this is evident that the study areas are known by polygamy that enables men to have more children than their women.

The mean age of respondent women is 28 for rural and 29 for urban. By having these sizes of children at this age level, in their reproductive life span they possibly could bear large number of children.

In terms of educational attainment, nearly less than half of the rural respondent women cannot read and write though most of general couples reported lower level of education that is primary and below which constitutes 88.3% for rural and 64.2% for urban couples. Consequently, low level of education may negatively affect the perception of couples towards family planning. Haq, A etal(2010) stated that since educational process makes more information available to women and may expose them to increase understanding about the family size, environmental degradation, their children's health, further educational opportunities and contraceptive use. Usually educated women may prefer to get married educated men who will be more positive to contraceptive use and lower family size.

Concerning couples occupation in rural, 85 (94.4%) of women and 83 (92.2%) of men were housewives and farmers respectively. In urban, most of women again, 19 (67.8%), were housewives and 13 (46.4%) of men were merchants. If women are busy with work out of home or employed worker they give less priority to bear many children otherwise being homemaker perhaps most women are encouraged to have large number of children.

Regarding religion most of, 85 (94.4%), women and 86 (95.6%) of men were protestants Christians in rural settings. In urban also the majority, 23 (82.2%), of both women and men were protestant Christians. Religion has its implication towards family planning, in case of Christianity most of the time followers believe in the command of God (that says multiply in the earth) means

no need of controlling birth and against this is considered as disobey to God.

4.2 Awareness of family planning

According to data obtained from survey, most of the respondent couples, 90.5% of rural and 98.2% of urban, were informed about family planning. Traditional methods in rural and pills in urban are the most known methods, however, pills, injectables and traditional methods are nearly equally known. Besides, in group discussion, 96% of urban and 89% of rural couples aware of family planning. The major sources of information in both rural and urban are health extension agents (workers) which accounts 73.6% for rural and 63% for urban spouses that followed by friends. The most reported sources of family planning services were also health extension agents, 81.6% for rural and 76.3% for urban couples. This implies that HEA are playing important role in family planning activities than other service providing centers. Majority of the respondent couples, 81.6% of rural and 61.8% of urban, responded that child spacing help for health of mother and children. Furthermore, though an a awareness and/or knowledge of family planning methods is prerequisite before practicing of family planning, it is impossible to say all those who know these methods can adopt them. And aware of it means not mean that an individual know how to use it. Therefore, based on the information obtain it can be concluded that spouses in the study areas are well aware of family planning and its methods.

4.3 Attitudes towards family planning

Currently, Eight-four percent of rural and eighty percent of urban spouses want more children than they have now. The mean desired number of children was 9 for rural and 6.7 for urban couples. In both settings, men have more level of fertility desire than women do. This is possibly because of children serve as labour force especially in rural areas. Consequently, higher level of desired number of children is driving force for couples to produce large number of children until they achieve targeted size in their reproductive life span.

The reported reasons of wanting many children for most of the respondents is old age support, which followed by having few children and want sons. Group discussion participants also responded similar reasons adding that children help them in labor in rural settings. Contrastingly, in both settings, a few of the respondents not want more children and their reasons were because of economic problem, for seek of family welfare, to promote the health of mother and children.

Sixty eight percent of urban and sixty two percent of rural spouses approve preventing pregnancy. And 62.5% of urban and 53.9% of rural also approve modern contraceptives, with more approval of women than men, however, when results presented in terms couples, it does not indicate that both men and women of the same couple do approves, but, it shows either of the spouses or both of them approving because it is average total of the women-men results. Here women more approve controlling birth and using contraceptives than men. This may because of women being victims of birth risks and they have more access to information of family planning through health extension agents. Besides, it is also important to identify whether spousal partners know approval of contraceptives by their spouses. 36% of the rural and 50% of urban couples reported that they know that their spousal partners approve using modern contraceptives while 53.9% of rural and 41.1% of urban spouses do not know whether their partners approve using contraceptives. This indicates that there may no further communication about family planning between women and men though urban couples showed better result than their counterparts.

Unsurprisingly, 31.1% of rural men believe that using contraceptives causes loss of loyalty between women and men while only 10% of women supporting the same view. 35.7% of men and 17.8% of women in urban also have the same view, with higher percentages of men than women supporting the idea.

Most of respondents in both sittings showed favorable attitudes on the advantages or benefits of child spacing, family planning for all members of a family and for health of mother and children. Majority of them also believe that discussion about family planning between spousal partners is important, and both wife and husband have responsibility when deciding family planning so as to fixing size of family. But a few of women do not accept the importance of discussion about family planning with men, this is because afraid of disapproval by their men if they discuss on the issue.

Concerning large family and its economic implication to family members, 81.7% of rural and 83.9% of urban couples agreed that larger family face more difficulty managing for food, clothing, schooling and health care than those smaller one. About 84% of urban and 80% of rural couples also in discussion have the same view. This implies that most of the couples were aware of the burden of large family despite socioeconomic factors enforce them to have many children. Regarding the issue of large family, several studies showed that larger family will result inability to function well and fulfill necessities for family members. For instance, Arthur (2009) wrote that households with smaller family size enjoy better social and economic life compared to those with relatively larger family size. However, 33.9% of urban and 42.8% of rural couples consider large number of children as a wealth.

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Some of the of respondent couples in group discussion also reported that many children may accompany with several lucks and they consider many children not only burden but also support for parents in different socio economic activities. This is also possibly related to several factors that some researchers have founded, for example, Goldwell 1982, quoted in Binyam, 2007, wrote that in sub Saharan Africa children are viewed as an investment in the future economic security. Having many children is considered as gains of prestige and status for the family in community.

The results of interview with health center and health extension workers showed that in the early years of their services, women's attitude towards family planning is not as such positive, most of them disapprove contraceptives by religious and traditional believes. But later on, through time attitudes of the most of women become favorable. As the information obtained from their women, most of the time aged men disapprove using contraceptives because of traditional believes and they need large number of children.

Moreover, 62.2% of rural and 76.7% of urban spouses have intension to use family planning in the future. In addition, nearly more than half of rural and three-fourth of urban spouses in group discussion reported they have desire to use family planning in the future with having more interest by women. This possibly shows that most of the couples have interest to use family planning and their attitudes are becoming favorable though actual use of family planning methods is low.

4. 4Attitudes towards relevance of family planning to sustainability of natural resources and environment

This research includes assessing attitudes of men and women towards relevance of family planning to environmental sustainability. Regarding this attitude, about eighty percent of the respondents in both settings agree that rapid population increase can lead to over utilization of natural resources and large family may contributes to environmental degradation. Majority of respondents, 71.7% of rural and 71.4% of urban, also believe that family planning and/or contraceptive use can reduce family size and further pressure on the natural environment. Moreover, 52% of rural and 46.4% of urban spouses believe that people should use family planning to reduce family size for seek of sustainability of natural resources and environment. This indicates that most of the respondent couples were aware of the problems of rapid population growth to natural environment even if their use of family planning is founded as low, since existing socio cultural factor influence them to have large number of children.

Study in Kenya by Joekes, 1994, revealed that influence on fertility levels of Environmental pressure is mixed; pressure affects the general productivity of a dependent livelihood systems. Thus, the prevailing change in perception of the values of children is towards appreciation of their future income contribution. Therefore, environmental pressures have indirect effect of influencing women and men to desire a smaller family size.

The issues of population, environment and development are inter related and should be integrated as emphasized by the united nation strategies of environment, population and development. By considering the importance of empowering women for the sustainable development and environmental sustainability, the world Bank (1994) stated that investing proportionally more women than men- in education, health, family planning, access to land, inputs, extensions, is an important part of development that contribute environmentally sustainable development. It produces significant social gains lower fertility, better household nutrition, and reduced infant, child and maternal mortality. On the other hand, environmental degradation increases women's burden, as they trek long distances to fetch fuel wood, and water. Hence, empowering women promotes family planning and then family planning offers meaningful contribution to environmental sustainability by reducing rapid population increase thereby minimizing pressure on a natural environment particularity in developing countries. Because in developed countries the development of industries rather than population pressure, enables people to highly exploit natural environment and degrade it.

4.5 Family planning practices

Family planning practices, 54.4% of rural and 60.7% urban spouses means either of them or both of women and men have ever used some methods of family planning with highest percentage of traditional methods in both settings which followed by the two contraceptives that are pills and injectables, the least used method among three modern contraceptives was condom. However, majority of group discussion participants believe that contraceptives are more secure than traditional methods. About half of discussion participants, likely more in urban, reported that they have ever used some methods of family planning. Here it is necessary to note that except condom no other modern male methods of family planning so that whether the method is traditional or modern, men have reported methods as used that either they or their women used methods. Among purposes of using family planning child spacing is the most common one in both settings.

Some of the users of those methods have discontinued. Common reasons for discontinuation were want to have more children, religions prohibition, fear of side effects of

contraceptives and spousal disapproval in order of highest to lowest. Similar reasons were founded from discussion in both urban and rural. Wanting more children is again major reason for never using family planning. In addition, some of respondents believe that children are gift from God and preventing pregnancy is considered as against will of God. Spousal communication about family planning is important tool to make joint decision between spouses. Couples discussion is better in urban (39.3%) than in rural (27.8) since last 12 months. However, majority of participants in group discussion believe importance of discussion between spousal partners, few of the women, more in rural, afraid of disapproval of men.

Concerning relations between awareness and practice of family planning, 90.5% rural and 98% of urban couples reported that they are aware of family planning but current use of family planning methods showed gap when compared to that of awareness. Hence, 23.2% of urban and 18.9% of rural couples were using some methods of family planning.

Still practice of family planning is low in the study areas though factors traced to variety of sources. In relation to schools' influence through students' education, it may be weak. It is obvious that today most parents send their children to school and these students have to learn issues of population and family planning. Probably, so it is believed that those students may have influence their parents to have few children if they would equipped with appropriate knowledge on the concepts of family planning and problems of rapid population increase.

Current prevalence of modern contraceptives for women is 16.7% for rural and 21.4% of urban. But average of the two settings is 19.0%, most of them using injectable and pills. This is likely better than pervious national survey, 2005, EDHS, in which 15% of women were using some methods of family planning with majority relying on modern contraceptives such as injectabel and pills. This may be due to time difference and the efforts made by government through health extension programs. However, contraceptive prevalence among couples is not such appreciable.

V. CONCLUSION

The major findings of the study are concluded as follows:

There is a promising awareness level of family planning in both urban and rural settings. And the main source of information for family planning was health extension agents. This implies that HEA are playing important role in family planning activities than other service providing centers.

The mean desired number of children is 11 for men and 7 for women in rural while 7.4 for men and 6 for women in urban. So the higher level of desired number of children is driving force for couples to produce large number of children until they achieve targeted size in their reproductive life span. The reasons for wanting many children are old age support, sex preference, labor support and considering children as sources of wealth. In other words, these are the socio cultural factors that hindering family planning practices. In addition, religious prohibition as common one and, others influence also included.

Regarding approval of contraceptives, 62.5% of urban and 53.9% of rural couples approve modern contraceptives with more approval by women than men in both settings. Women more approve controlling birth and using contraceptives than men. This may because of women being victims of birth risks and they have more access to information of family planning through health extension agents. However, some of the respondents, more men and few women believe that using contraceptive cause loss of loyalty between wife and husband.

In terms of relation between family size and family wellbeing, about eighty percent of both rural and urban couples believe that large family face more challenge than smaller one for their facility, even if other factors encourage them to desire large number of children. In general attitude items towards family planning, most of the respondents showed positive attitude, however, urban couples likely showed more favorable attitude than the rural one.

Regarding the views to relevance of family planning for environmental sustainability, about 71% of both urban and rural couples believe that use of family planning reduce population growth and further pressure on the natural environment. And nearly more than half of rural and nearly less than half of urban respondents believe the importance of family planning to reduce family size for seek of sustainability of natural resources and environment. In their attitude towards natural environment, rural couples likely have more favorable attitudes than their counterparts.

Concerning practices of family planning, 60.7% of urban and 54.4% of rural spouses have ever used some methods of family planning with highest percentage of traditional method. Pills, injectables and condom are the three contraceptives among ever used methods in their order from highest. Currently, 16.7% of rural and 21.4% of urban respondent women are using modern contraceptives in the study areas. However, nearly more than half of the respondents approve contraceptives. Therefore, there is a gap between awareness and practices of family planning, so all concerned bodies work to fill the gap as family planning useful not only for family welfare but also wellbeing of environment and ecosystem.

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Influence of Irrigation Regimes on Quality Attributes of Olive Oils from Two Varieties Growing in Lebanon

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***Abstract*—** *An increasing interest on supplemental irrigation is observed in modern olive orchards because of its effect in increasing yield. In this study, the effect of three irrigation regimes (0, 60 and 100% ETC) on quality and chemical composition of olive oil is assessed in Baladi and Edlbi varieties planted in Lebanon. Significant differences ($p<0.05$) between varieties were observed for the majority of studied traits. Meanwhile, the response to irrigation regimes was strongly different between varieties. In Baladi variety, irrigation regimes resulted in increasing fresh fruit weight together with slight effects on oil yield, quality and composition. Only oleacein content showed significant decrease with irrigation (50.35 mg/kg for 0% ETC, 28.25 for 60% and 34.60 for 100%). On the contrary, in Edlbi variety, irrigation resulted in a strong decrease of total phenols (509.91 mg GAE/kg for 0% ETC, 385.87 for 60% and 365.74 for 100%) and chlorophylls (20.83 mg/kg for 0% ETC, 14.54 for 60% and 14.81 for 100%). Curiously, 60% ETC showed high content of the majority of individual phenols, including higher than 0% ETC.*

***Keywords*— Fatty acid profile, Monovarietal olive oil, Olea europaea L., Oxidative stability, Olive oil quality, Phenolic compounds, Supplemental irrigation.**

I. INTRODUCTION

The olive tree (*Olea europaea* L.) has a long history in Mediterranean countries where there is evidence of human cultivation from as far back as 5,000–6,000 years ago [1]. It is considered as one of the best adapted species to the Mediterranean climate characterized by limited water availability and high evaporative demand [2]. Therefore, it has been traditionally cultivated under dry-land conditions where trees were spaced widely to take full advantage of the stored soil water from winter rains for spring and summer growth. More recently, olive cultivation is progressively expanding in many countries in response to increased oil consumption, and many agricultural practices have been changed for improving production, productivity and quality of olive oil [3–5]. One of the most important changes in olive cultivation is the expansion of high-density irrigated olive orchards.

The substantial increase in olive oil and table olives consumption is due not only to its nutritional properties and potential role in protecting against cardiovascular and neurodegenerative diseases [6]; but also to its organoleptic attributes [7]. These outstanding properties are attributed to the high oleic acid percentage of olive oil and to the presence of several minor components endowed with antioxidant activity such as phenolic compounds, pigments, tocopherols, etc. However, this peculiar chemical composition is the result, besides the fundamental genetic basis, of a complex interaction between several factors clustered into four main groups: environmental (soil, climate), agronomic (irrigation, fertilization), cultivation (ripeness harvesting) and technological factors (fruit storage, extraction procedures) [8].

Among these factors, irrigation can have large impact on both the production of the olive tree and the composition of the olive fruits [4], even with small amounts of water [9]. In fact, several studies showed increased fruit and oil yield with irrigation, but conflicting results were observed regarding relative oil content of the fruits. For instance, Lavee and Wodner [10] showed a decrease in relative oil content as a function of increased amounts of applied water. However, Moriana et al. [4] indicated an increase in oil content with irrigation; and, Costagli et al. [11] reported no effect. In addition, it is relevant to state that any irrigation regime employed in the olive orchard must take into

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account the ultimate effect on olive oil as several researches have shown variations in oil quality and composition between rainfed and different irrigation managements. Indeed, Stefanoudaki et al. [12] revealed that the irrigation regimes had little or no effect on free acidity, peroxide value, or fatty acid composition of olive oil. Conversely, Tognetti et al. [13] and Berenguer et al. [14] stated that irrigation influences the oil quality parameters, fatty acid composition, phenolic content, volatile profile and sensorial characteristics of the olive oil. It is worth mentioning that, an inverse relationship between the amount of water applied to the olive trees and the phenolic content, the oxidative stability and the bitterness of the oil has been previously reported [5, 15–17].

In Lebanon, olive growing is widespread from the coast until around 1000 m a.s.l. It represents around 23.43% of the total cultivated land with more than 58000 ha [18]. The Lebanese groves are dominated by the variety Baladi; although, other varieties are also present such as Souri, Ayrouni, Edlbi, Nabali, Manzanilla de Sevilla, Fantoio and Picholine. Only 8% of the olive cultivated areas are irrigated, the rest is rainfed. This is mainly due to the lack of information on the performance of local varieties under different irrigation regimes. On the other hand, the Lebanese olive oil industry consists of around 544 mills among which 85% still traditional [19]. The main obstacle facing the modernization of the Lebanese olive oil industry is the fact that the modern continuous systems provide oils with a bitter taste not preferred by local consumers who are familiar with sweetie oil tastes generated by traditional systems. Thus, irrigation may provide a solution for decreasing bitterness attribute of olive oil obtained by continuous systems. Therefore, the main goal of this study was to investigate the effect of different irrigation regimes on fresh fruit weight, oil yield, quality and composition of olive oil from Baladi and Edlbi varieties planted in Lebanon.

II. MATERIALS AND METHODS

2.1 Experimental site and irrigation regimes

The study was carried out during 2012-2013 olive crop season in an experimental field maintained by the Lebanese Agricultural Research Institute (LARI), and located in Jezzine city (South of Lebanon) at 351 a.s.l, 33°32'33'' N and 35°27'11'' E. The annual precipitation recorded in Jezzine in 2012 was 810 mm, the minimum registered temperature was 3.1 °C below zero and the maximum was 34.9 °C. Olive trees from the two varieties Baladi and Edlbi were planted since 1996 at a distance of 4 m between trees. The orchard is characterized by a calcareous soil to which 3 kg per tree of 17-17-17 NPK complex were added in December of each year.

The irrigation was done by using a localized irrigation system consisting of 2 mini sprinklers of 32 L/hour/tree. Three irrigation regimes were adopted: I0: without irrigation (rainfed), I60: 60% of crop ETC, and I100: 100% of crop ETC calculated from climatic data registered in the weather station located close to the orchard (for calculating reference evapotranspiration, ET0) and using the Penman–Monteith–FAO method [20], with an estimated crop coefficient (Kc=0.75 in spring and 0.55 in summer) [21], and a coverage coefficient (Kr = 0.5) [22] where:

$$ETc = Kr \times Kc \times ET0 \quad (1)$$

2.2 Plant material and olive oil extraction

For each variety and each irrigation regime, four fruit samples of 700 g each were collected from four trees at 6 harvesting times. The ripening index (RI) was calculated according to the fruit skin color as proposed by Frías et al. [23]. The weight of the 100 fruits from each sample was also recorded. Then, oils were extracted using an Abencor system (Mc2 Ingenieria y Sistemas, Seville, Spain) equipped with a hammer mill, a thermobeater and a centrifuge. After crushing the olives, the paste underwent malaxation at 28 °C for 30 min, and then centrifuged at 3500 rpm for 2 min. When stopped, the oil will separate from the paste and will be collected in graduated cylinders. After decantation, the oil samples were separated from the vegetable water, transferred into glass bottles, and stored in the dark at -20 °C until analysis. The oil yield was calculated according to the following formula:

$$\text{Oil yield} = \text{Volume of extracted oil} \times 0.915 \times 100 / \text{Mass of the olive paste} \quad (2)$$ [24]

2.3 Analytical determination in olive oil

2.3.1 Quality indices

Free acidity, peroxide value and UV absorbance at 232 and 270 nm (conjugated dienes (K_{232}) and conjugated trienes (K_{270}), respectively) were determined following the methods described in the European Union Commission Regulation EEC No 2568/91 [25]. All parameters were performed in triplicate for each sample.

2.3.2 Fatty acid composition

Fatty acid methyl esters (FAMEs) were prepared by vigorously shaking, for 1 min, 0.1 g of oil dissolved in 2 mL of *n*-hexane with 200 µL of a methanolic solution of KOH (2 M). After settling for 5 min, an aliquot of 975 µL of the upper phase containing *n*-hexane and FAMEs were transferred to a test tube containing 25 µL of C19:0 as external standard [26]. The resulting mixture was injected by duplicate into a Shimadzu GC-2010 Plus coupled to a

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flame ionization detector (FID) (280 °C), and equipped with a fused silica capillary column (DB-wax; Agilent Technologies, Wilmington, DE; 30 m length x 0.25 mm i.d. and 0.25 µm of film thickness). Nitrogen was used as carrier gas at 1.69 mL/min with split injector system (Split ratio: 1:50, 250 °C). The initial oven temperature program was kept at 165 °C for 15 min, then increased from 165 °C to 200 °C at a rate of 5 °C/min, and maintained at 200 °C for 2 min, then raised from 200 °C to 240 °C at a rate of 5 °C/min, and finally held at 240 °C for 5 min. Peak identification was achieved by reference to authentic commercial standards. The concentrations of EFAs were expressed as relative percent of total area.

2.3.3 Phenolic profile

The phenolic compounds were isolated by double extraction of a solution of oil (3 g) in *n*-hexane (2 mL) with a methanol-water mixture (60:40, v/v) [27]. A solution of the internal standard (250 µL of 15 mg/kg of syringic acid in methanol) with 1.75 mL of methanol-water mixture was used for the first extraction and 2 mL of methanol-water mixture for the second one. The extracts from both extractions were combined and placed in the dark at -20 °C for further determinations.

Total phenols content was determined calorimetrically using the Folin-Ciocalteu method [28]. The absorbance was measured at 765 nm by a jenway UV/Vis spectrophotometer (Staffordshire, ST15 OSA, UK). The results were expressed as mg gallic acid equivalent (GAE)/kg of oil.

The extracted phenolic fraction was analyzed in triplicate by a Shimadzu HPLC equipped with an automatic injector, a column oven and a diode array UV detector (using 280 nm as quantification wavelength). Separation of individual phenols was achieved on a Microsorb-MV 100 C18 column (250 × 4.6 id mm, 5µ particle size), maintained at 40 °C. The injection volume was 20 µL and the flow rate 1.0 mL/min. Mobile phases were 0.2 % *o*-phosphoric acid in water (mobile phase A) and a mixture methanol-acetonitrile (50:50, v/v) (B). The initial concentrations were 96% of A and 4% of B and the gradient was changed as follows: the concentration of B was increased to 50% in 40 min, increased to 60% in 5 min, and to 100% in 15 min, and maintained for 10 min. Initial conditions were reached in 7 min. The identification of olive phenols was performed on the basis of their maximum absorption and retention times compared to those of commercial standard compounds. Standards of oleocanthal and oleacein were acquired from Prof. P. Magiatis (University of Athens). Results were elaborated by Shimadzu LabSolution software. Phenolic compounds quantification was achieved using syringic acid as internal standard and 9 points calibration curves of authentic standards. Results were expressed as mg of the target analyte per kg of oil.

2.3.4 Pigments

Total chlorophyll, chlorophyll a and chlorophyll b were determined according to the Official Methods of Analysis [29]. The method consists of measuring the absorbance of a solution of oil (0.5 g) in *n*-hexane (10 mL) at 642.5 ($A_{642.5}$) and 660 nm (A_{660}) using a UV/Vis spectrophotometer (Jenway Scientific Instruments, Staffordshire, ST15 OSA, UK). The results are given by the following formulas:

$$\text{Total chlorophyll} = 7.12 \times A_{660} + 16.8 \times A_{642.5} \quad (3)$$

$$\text{Chlorophyll a} = 993 \times A_{660} - 0.777 \times A_{642.5} \quad (4)$$

$$\text{Chlorophyll b} = 17.6 \times A_{642.5} - 2.81 \times A_{660} \quad (5)$$

The concentration of *β*-carotene was obtained using 6 points calibration curve of corresponding commercial standard and the readings were achieved at 436 nm.

The results of chlorophylls and *β*-carotene were expressed in mg/kg of oil

2.3.5 Oil oxidative stability

Oil oxidative stability was evaluated by the Rancimat method [30]. Stability was expressed as the induction time (h) measured with the Rancimat 892 model (Metrohm SA, Herisau, Switzerland). An oil sample of 3 g was warmed to 120 °C under a constant air flow of 20 L/h. The analytical determinations were carried out in duplicate and the results were expressed in hours.

2.4 Statistical analysis

Analysis of variance (ANOVA) was performed for testing differences in olive oil quality and composition between varieties and irrigation regimes. Tukey´s test ($p<0.05$) was used to discriminate among the mean values. Multivariate analysis based on principal component analysis (PCA) was also performed from a set of 32 studied variables (fatty acids: 11, sums and ratios of fatty acids: 6, total and individual phenols: 10, chlorophylls: 3, *β*-carotene and induction time) after standardization. Statistical analyses were carried out using Statistix 8.0 (Analytical Software, Tallahasse, FL, USA) and Unscrambler (CAMO A/S, Trodheim, Norway) statistical packages.

III. RESULTS AND DISCUSSION

3.1 Harvesting time and ripening index (RI)

Analysis of variance revealed significant differences in RI between varieties ($p=0.0000$). As Fig. 1 shows, the evolution of ripening process in Edlbi variety was faster than in Baladi variety. On the other hand, the difference

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between irrigation regimes was not significant in both varieties, although it was remarkable that the trees under I0 regime showed higher RI (1.44 in Baladi and 2.88 in Edlbi variety). A positive relationship between RI and the amount of water applied was observed as I100 regime provided olives with higher RI than I60 regime (1.21 in Baladi and 2.75 in Edlbi variety for I100, and 1.13 in Baladi and 2.42 in Edlbi variety for I60). These results are partially in agreement with those described by Berenguer et al. [14] who indicated higher RI in rainfed trees, but a negative relationship between RI and the amount of applied water.

3.2 Fruit fresh weight

The mean fruit fresh weight ranged from 1.43 to 3.20 g in Baladi variety and from 2.22 to 5.23 g in Edlbi variety with statistically significant difference between varieties (p=0.0000). Besides, irrigation in both varieties yielded higher fruit fresh weight than under rainfed conditions, although the difference was only significant in Baladi variety. These results are in agreement with previous studies in which irrigation has been shown to increase fruit size, especially in dry years [5, 31].

3.3 Oil yield

Oil yield obtained through Abencor system showed highly significant differences between varieties (p<0.0001). The oil yielded by Edlbi variety (197.1 g/kg) was significantly higher than Baladi variety (176.4 g/kg). Apart, oil yield was not significantly affected by irrigation regimes in both varieties (p=0.7218 for Baladi and p=0.1581 for Edlbi), albeit the highest oil yield was recorded for I0 (183.8 g/kg for Baladi and 214.0 g/kg for Edlbi variety), followed by I60 (177.6 g/kg for Baladi and 194.7 g/kg for Edlbi variety) and I100 (167.8 g/kg for Baladi and 182.7 g/kg for Edlbi variety). The low extractability of irrigated olives could belong to the higher water content accumulated in the fruits of the irrigated regimes [31].

3.4 Quality indices

The free acidity was highly significantly affected by the variety (p=0.0000), where Edlbi showed higher values than Baladi variety (Table 1). It is agreed that oil quality indices are less affected by the olive variety; however, in this study, Edlbi variety recorded very high free acidity levels in the last two harvesting (21 October and 04 November) as it ripened earlier than Baladi variety. A positive relationship between ripening and free acidity was previously recorded [32]. In opposite, no statistically significant differences were obtained between studied varieties for peroxide value, K_{232} and K_{270}.

Regarding the influence of different irrigation regimes on quality indices, only K_{232} and K_{270} showed significant differences in both varieties, with higher values in oils obtained from I0. Similar results were obtained by Gómez-Rico et al. [5] who reported slight effects of irrigation on free acidity and peroxide value, and a decrease in K_{232} and K_{270} with increasing irrigation amounts. According to the same authors, this decrease could result from the interference of phenolic compounds (higher content in rainfed), which absorbs in the UV region in these analytical determinations. Opposite results were found by Dabbou et al. [33] who indicated significant effects of irrigation regimes on free acidity, peroxide value and K_{232} of oils from Coratina variety. This discrepancy is probably due to the interaction between the experimental conditions and the genetic characteristics of each variety that might induce different effects on the oxidative reactions of the oil during the extraction process.

3.5 Fatty acid composition

Great differences were found between Baladi and Edlbi varieties with regard to their fatty acid composition (Table 2). While Baladi variety recorded significantly higher percentages of C16:0, C18:0, C18:1, C18:3, C20:0, C20:1, C22:0 and C24:0, Edlbi variety presented significantly higher C14:0, C16:0 and C18:2. Moreover, the studied fatty acid sums and ratios revealed highly significant differences between varieties with Baladi exhibiting higher SFA, MUFA, MUFA/PUFA and C18:1/C18:2 and Edlbi higher PUFA and UFA/SFA.

The fatty acid composition was strongly affected by irrigation regimes (Table 3). In both varieties, C14:0, C16:0, C16:1, C18:1, C18:3, C24:0, SFA, MUFA and UFA/SFA were the most affected. Besides, C18:0, C20:0 and C22:0 presented inconsistent differences between varieties. Regarding the major fatty acids, significant differences were observed mainly between rainfed and irrigated trees. In fact, the percentages of C16:0 and C18:3 in oils from irrigated trees were significantly higher than in oils from rainfed ones. However, the percentage of C18:1 was higher in oils from rainfed trees than in oils from irrigated ones. Conversely, the percentage of C18:2 didn't show any significant difference with irrigation regimes. Regarding C18:0, significant difference between irrigation regimes was observed only in Edlbi variety with higher percentages in oils obtained from rainfed trees. The results of this study are partially in agreement with those obtained by Gómez-Rico et al. [5] and Dabbou et al. [34] who reported higher C18:1 in rainfed trees, but also higher C16:0 and C18:2 in irrigated trees. Conflicting results

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indicating slight effects of irrigation on fatty acid composition are also present [17].

In addition, irrigation increased significantly the sum of saturated fatty acids (SFA) while decreasing the sum of monounsaturated fatty acids (MUFA). In fact, it is well known, that a higher percentage of MUFA is considered as one of the main characteristics of a good quality olive oil. Moreover, the ratio of unsaturated to saturated fatty acids was influenced by different irrigation regimes with higher ratios in oils from rainfed trees.

It is worth to note that the differences in fatty acid profiles were significant but at slight levels of difference. These observations are in accordance with the results of Gómez-Rico et al. [5] who reported higher ratios of MUFA in oils from rainfed trees but at difference levels that don't have any nutritional relevance.

3.6 Total and individual phenols

Statistical analysis showed that individual phenols, except tyrosol and vanillic acid, revealed significant differences between Baladi and Edlbi varieties. Higher contents of *p*-coumaric acid, oleocanthal and luteolin were found in oils from Baladi variety, and of hydorxytyrosol, oleacein and apigenin in oils from Edlbi variety (Table 4). It was relevant that *o*-coumaric acid was only detected in oils from Edlbi variety. The genetic variability of the phenolic profile has been widely described in national and international olive collections, with most varieties displaying a particular phenolic composition [35].

As shown in Table 5, the influence of irrigation regimes on oil phenolic composition appears to be strongly dependent on the olive variety. Regarding total phenols, the content was significantly higher only in oils form Edlbi variety in rainfed conditions. This behavior is consistent with several varieties showing reduction of total phenols with irrigation [36, 37]. In contrast, Baladi variety showed a prominent behavior with no significant reduction in total phenols with irrigation. This is very relevant since the phenolic compounds contribute to nutritional and organoleptic characteristics of olive oil [38]. As per individual phenols, the effect of irrigation was also more pronounced in Edlbi than in Baladi variety. In fact, the contents of hydroxytyrosol, tyrosol, vanillic acid, *p*-coumaric acid, *o*-coumaric acid, oleacein and luteolin in oils from Edlbi variety showed the same trend with higher amounts observed in I60 irrigation regime. Higher irrigation level negatively affected these compounds. However, oleocanthal and apigenin showed higher amounts in oils from Edlbi variety under rainfed conditions; although, for oleocanthal the difference between I0 and I60 was negligible. These results are partially in agreement with those obtained by Patumi et al. [39] and Tovar et al. [40] who reported higher concentrations of phenols in oils from olive trees exposed to a certain level of water deficit. These higher concentrations can be attributed to changes in the activity of the enzymes responsible of the biosynthesis of phenolic compounds, such as L-phenylalanine ammonia-lyase whose activity is greater under water stress conditions Tovar et al. [41]. Regarding Baladi variety, the effect of irrigation on individual phenols was only significant for oleacein content. Previous studies stated that the content of oleacein and oleocanthal significantly increases in oils from the most stressed irrigation regimes [17]. In the present study, this was observed only for oleacein content of oils from Baladi variety, and for oleocanthal content of oils from Edlbi variety. It is worth mentioning that oleacein is a compound that plays an important role in the intensity of the oil bitterness [33]. Accordingly, the irrigation of Baladi olives may induce a reduction in the bitterness of the oil without significant effect on the contents of total and other individual phenols.

3.7 Pigments

Oils from Baladi variety showed significantly higher contents of total chlorophyll, chlorophyll a and *β*-carotene than oils from Edlbi variety (Table 4). These pigments are relevant for the consumers because they play a key role as hedonistic factor and affect the sensorial acceptability of the oil [42].

Similarly to phenolic compounds, the effect of irrigation regimes on pigments was more marked in Edlbi varieties. In fact, the contents of total chlorophyll, chlorophyll a and chlorophyll b in oils from Edlbi variety decreased significantly with irrigation (Table 5) as previously described by Romero et al. [43]. Conversely, Gómez-Rico et al. [5] reported that pigments are not influenced by irrigation, in concordance with results obtained in this study for oils from Baladi variety.

3.8 Oil oxidative stability

The oil oxidative stability expressed as induction time presented significantly higher values for oils from Edlbi (11.89 h) than from Baladi variety (9.46 h) (Table 4). In fact, strong correlation was previously reported between oil oxidative stability and oleacein content. The latter (higher content in oils from Edlbi variety) has been shown to extend the storage time of the oil due to its antioxidant activity [44].

In both varieties, the oils obtained from rainfed trees showed the highest oxidative stability, although the difference was only significant in Edlbi variety (Table 5).

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The significant decrease of oxidative stability with irrigation can be explained by the parallel decrease in total phenols, in accordance with findings of previous studies [45, 46].

3.9 Multivariate analysis

Principal components analysis (PCA) revealed that the first two principal components explained 59% of total variance and showing groupings and subgroupings. The first PC accounted for 40% of total variance, with a high positive correlation with C18:0, C20:0, C20:1, luteolin, *p*-coumaric acid and vanillic acid, and negative with C16:1, tyrosol and hydroxytyrosol. The second PC accounted for 19% of total variance and was positively correlated with total phenols, induction time and chlorophyll b. Along the PC2, oil samples were differentiated in two groups: the first one belong to Baladi variety and the second one to Edlbi variety, independently from the irrigation regimes. In addition, PCA allowed the discrimination of the oil samples from irrigated and rainfed trees in each variety as oils from rainfed trees showed a considerable grouping (Fig. 2).

IV. CONCLUSIONS

This work is the first evaluation of the performance of olive varieties growing in Lebanon under different irrigation regimes. The response of olive trees to different irrigation regimes showed strong varietal differences, mainly between rainfed and irrigated trees. The most relevant is that in Baladi variety, irrigation increased fruit fresh weight with limited effect on oil content, fatty acid composition, total and individual phenols, pigments and oil oxidative stability. The only significant decrease in individual phenols was in oleacein content that was described to have a positive relationship with olive oil bitterness attribute. A premise of this work was that Baladi variety may provide high production, high quality and less bitter oil, if small amounts of water are applied during the dry season. This issue may increase the local demand for oils from Baladi variety grown under deficit irrigation conditions and processed with continuous system characterized by strong bitter oils. Interestingly, in Edlbi variety, deficit irrigation of olive trees with 60% ETC appears to be beneficial as it increased fruit fresh weight, maintained high oil content, showed the highest content of all individual phenols except apigenin; nevertheless, it decreased total phenols and oil oxidative stability. Finally, more work is necessary in order to confirm the results obtained for Baladi variety, and further experiments with more severe deficit irrigation strategies (lower than 60% ETC) must be explored in case of Edlbi variety to improve the quality of the olive oil produced from this variety.

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FIGURES

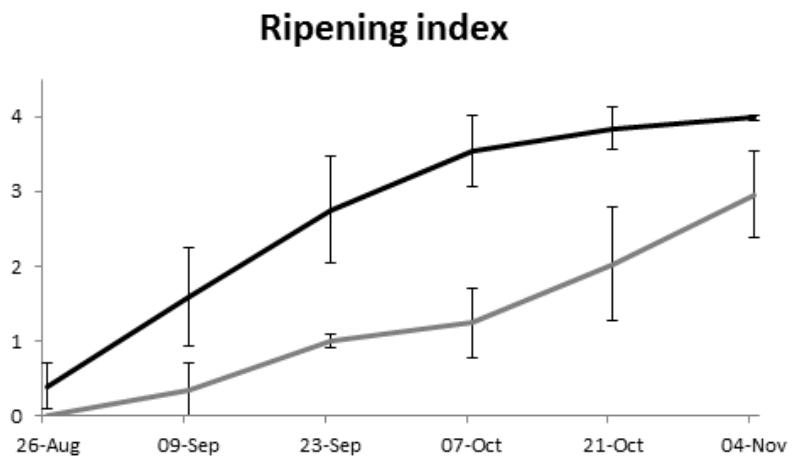

Fig.1: *Evolution along harvesting dates of ripening indexes in 'Baladi' (grey) and 'Edlbi' (Black) varieties. Error bars represent means ± Standard deviation.*

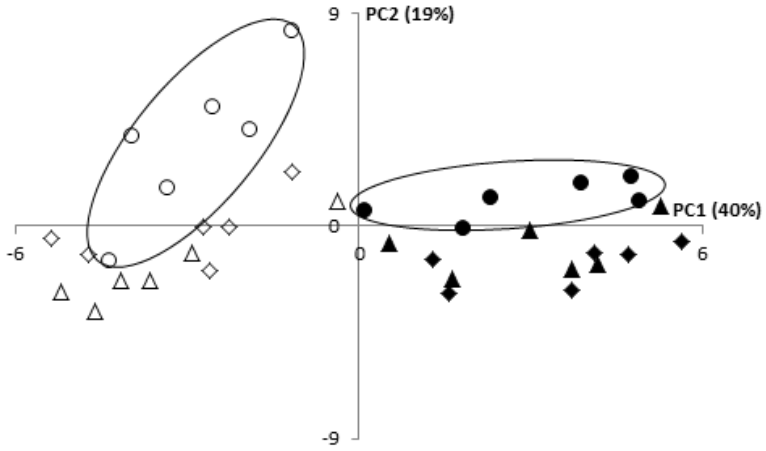

Fig.2: *Distribution of the olive oil samples in the axes of the first and the second principal components. Baladi I0: ●, Baladi I60: ◆, Baladi I100: ▲, Edlbi I0: ○, Edlbi I60: ◇, Edlbi I100: △.*

TABLES

Table.1: Quality indices of olive oil from 'Baladi' and 'Edlbi' varieties, as affected by irrigation regimes.

	'Baladi'				'Edlbi'			
	I0	**I60**	**I100**	**Sig.**	**I0**	**I60**	**I100**	**Sig.**
Free acidity (g/kg of oleic acid)	2.8a	3.0a	2.4a	NS	7.3x	4.5x	6.3x	NS
Peroxide value (meq O_2/kg of oil)	8.06a	7.93a	7.57a	NS	9.42x	7.47x	8.30x	NS
K_{232}	1.52a	1.40b	1.33b	***	1.52x	1.30y	1.39xy	***
K_{270}	0.10a	0.09ab	0.08b	*	0.10x	0.07y	0.08y	***

I0: rainfed; I60: irrigated with 60% ETC; I100: irrigated with 100% ETC; Sig.: significance; RI: ripening index; FFW: fresh fruit weight; NS: statistically not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0$. Means followed by different letters within the same row and variety (a–b or x–y, resp.) differ significantly by Tukey's test ($p<0.05$) with respect to the irrigation level.

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Table.2: Fatty acid composition (g/kg) of olive oil from 'Baladi' and 'Edlbi' varieties.

	'Baladi'	'Edlbi'	Significance
C14:0	0.114b	0.133a	***
C16:0	130.2a	126.6b	*
C16:1	4.7b	6.9a	***
C18:0	35.3a	31.7b	***
C18:1	718.5a	700.2b	***
C18:2	93.2b	119.0a	***
C18:3	6.9a	6.0b	***
C20:0	5.4a	4.8b	***
C20:1	3.3a	2.6b	***
C22:0	1.4a	1.3b	***
C24:0	0.9a	0.8b	*
SFA	173.5a	165.3b	***
MUFA	726.5a	709.7b	***
PUFA	100.1b	125.0a	***
UFA/SFA	47.7b	50.6a	***
MUFA/PUFA	74.8a	58.7b	***
C18:1/C18:2	80.0a	61.1b	***

*: $p < 0.05$; ***: $p < 0.001$. Means followed by different letters within the same row (a–b) differ significantly by Tukey's test ($p<0.05$).

Table.3: Fatty acid profile (g/kg) of olive oil from 'Baladi' and 'Edlbi' varieties, as affected by irrigation regimes.

	'Baladi'				'Edlbi'			
	I0	**I60**	**I100**	**Sig.**	**I0**	**I60**	**I100**	**Sig.**
C14:0	0.106b	0.123a	0.114ab	***	0.128xy	0.126y	0.144x	*
C16:0	125.7b	133.7a	131.2a	***	118.3y	129.7x	131.6x	***
C16:1	4.1b	5.1a	4.9a	***	5.8y	7.2x	7.8x	***
C18:0	35.8a	35.6a	34.7a	NS	35.8x	29.6y	29.7y	***
C18:1	726.2a	710.8b	719.1ab	**	712.1x	700.0xy	688.4y	**
C18:2	91.0a	96.8a	91.8a	NS	113.0x	117.7x	126.3x	NS
C18:3	6.2b	7.4a	7.1a	***	5.4y	6.2x	6.4x	***
C20:0	5.3b	5.6a	5.4ab	*	5.0x	4.7x	4.8x	NS
C20:1	3.3a	3.3a	3.3a	NS	2.6x	2.6x	2.6x	NS
C22:0	1.40b	1.49a	1.45ab	*	1.25x	1.27x	1.27x	NS
C24:0	0.8b	1.0a	0.9ab	*	0.7y	0.9x	0.9x	**
SFA	169.2c	177.4a	173.8b	***	161.1y	166.3x	168.4x	***
MUFA	733.6a	718.5b	727.3ab	*	720.5x	709.8xy	698.9y	**
PUFA	97.2a	104.2a	98.9a	NS	118.4y	123.9xy	132.7x	NS
UFA/SFA	49.1a	46.5b	47.6b	***	52.1x	50.2y	49.4y	***
MUFA/PUFA	77.0a	71.3a	76.2a	NS	62.3x	59.3x	54.5x	NS
C18:1/C18:2	81.8a	76.3a	81.8a	NS	64.7x	62.0x	56.7x	NS

I0: rainfed; I60: irrigated with 60% ETC; I100: irrigated with 100% ETC; Sig.: significance; NS: statistically not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Means followed by different letters within the same row and variety (a–c or x–y, resp.) differ significantly by Tukey's test ($p<0.05$) with respect to the irrigation level.

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Table.4: Phenolic profile, pigments and oxidative stability of olive oils from 'Baladi' and 'Edlbi' varieties.

	'Baladi'	'Edlbi'	Significance
Hydroxytyrosol (mg/kg)	6.59b	9.78a	*
Tyrosol (mg/kg)	3.10a	3.29a	NS
Vanillic acid (mg/kg)	2.46a	2.24a	NS
***p*-coumaric acid (mg/kg)**	3.47a	1.43b	***
***o*-coumaric acid (mg/kg)**	N.D	1.13	-
Oleacein (mg/kg)	37.74b	71.26a	***
Oleocanthal (mg/kg)	36.98a	29.11b	*
Luteolin (mg/kg)	27.42a	2.09b	***
Apigenin (mg/kg)	4.24b	9.88a	***
Total phenols (mg GAE/kg)	411.95a	420.51a	NS
Total chlorophyll (mg/kg)	18.84a	16.72b	*
Chlorophyll A (mg/kg)	9.70a	7.54b	***
Chlorophyll B (mg/kg)	9.16a	9.20a	NS
***B*-carotene (mg/kg)**	16.13a	10.45b	***
Induction time (hours)	9.46b	11.89a	***

N.D: not detected; NS: statistically not significant; *: $p < 0.05$; ***: $p < 0.001$. Means followed by different letters within the same row (a–b) differ significantly by Tukey's test ($p<0.05$)

Table.5: Phenolic profile, pigments and oxidative stability of olive oils from 'Baladi' and 'Edlbi' varieties, as affected by irrigation regimes.

	'Baladi'				'Edlbi'			
	I0	**I60**	**I100**	**Sig.**	**I0**	**I60**	**I100**	**Sig.**
Hydroxytyrosol (mg/kg)	7.22a	5.19a	7.35a	NS	8.25y	14.33x	6.77y	**
Tyrosol (mg/kg)	2.82a	2.65a	3.83a	NS	2.90xy	4.40x	2.57y	*
Vanillic acid (mg/kg)	2.24a	2.41a	2.72a	NS	1.56z	3.03x	2.14y	***
***p*-coumaric acid (mg/kg)**	3.46a	3.35a	3.60a	NS	1.11y	1.87x	1.31y	**
***o*-coumaric acid (mg/kg)**	N.D	N.D	N.D	-	1.03y	1.42x	0.95y	**
Oleacein (mg/kg)	50.35a	28.25b	34.60ab	*	72.48xy	85.68x	55.61y	NS
Oleocanthal (mg/kg)	42.46a	35.51a	32.97a	NS	33.22x	33.11x	21.01x	*
Luteolin (mg/kg)	31.72a	23.83a	26.70a	NS	1.52y	2.80x	1.98y	***
Apigenin (mg/kg)	5.62a	3.42a	3.67a	NS	14.788x	8.66y	6.20y	***
Total phenols (mg GAE/kg)	454.81a	376.94a	404.09a	NS	509.91x	385.87y	365.74y	**
Total chlorophyll (mg/kg)	19.18a	19.99a	17.38a	NS	20.83x	14.54y	14.81y	**
Chlorophyll A (mg/kg)	10.09a	10.17a	8.83a	NS	9.15x	6.88y	6.60y	**
Chlorophyll B (mg/kg)	9.08a	9.83a	8.56a	NS	11.70x	7.68y	8.23y	***
***B*-carotene (mg/kg)**	16.29a	17.43a	14.68a	NS	11.39x	10.90x	9.07x	NS
Induction time (hours)	10.18a	9.22a	8.97a	NS	15.28x	10.43y	9.99y	***

I0: rainfed; I60: irrigated with 60% ETC; I100: irrigated with 100% ETC; N.D: not detected; Sig.: significance; NS: statistically not significant; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$. Means followed by different letters within the same row and variety (a–b or x–z, resp.) differ significantly by Tukey's test ($p<0.05$) with respect to the irrigation level.

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Environmental Assessment of Vehicular Emission in Port-Harcourt City, Nigeria

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Abstract—*Port Harcourt is a coastal city located in the Niger Delta region of Nigeria, with very short dry season and long heavy rainy season periods. The objective of this study was to assess air pollution level from vehicular emission during the rainy season period. Three locations in the city noted for high traffic congestion were selected for the study. Air sampling in these locations were carried out for 11 days, covering peak and off peak periods. The following air pollutants were measured namely; nitrogen oxides (NO_x), sulphur oxides (SO_x), carbon monoxide (CO) and unburnt hydrocarbons (C_xH_y), as well as climatic elements – ambient temperature and relative humidity. The air pollutant levels obtained were compared with local and international standards. CO complied with international standard, but exceeded local standard. There is need for effective air pollution monitoring and control, this will go a long way to reduce the health risk associated with air pollution in the city.*

Keywords— ambient, air pollution, coastal city, congestion, exposure.

I. INTRODUTION

Environmental problems constitute one of the key challenges of the 21st century, and urban air pollution is a major health hazard worldwide (Avogbe *et al.*, 2011). Air pollution results from four main sources namely; industrialization, tobacco smoking, domestic cooking and vehicular or machinery fuel combustion (Tanimowo, 2000). However, the level of air pollution depends on a country's technology and pollution control. All over Africa, studies have shown that air pollution from the four sources adversely affect people's respiratory health (Theron *et al*., 1994; Tanimowo, 1995; Mohammed *et al.*, 1995; Hsairi *et al*. ,1996; *Nriagu et al*., 1996; Shamssain and Shamissian 1997; Mengasha and Bekele 1998; Uko *et al*., 1998; Tanimowo, 1998).

The composition of the ambient air is complex and depends on the equality of fuel, the type of engine, and engine maintenance (leong *et al*., 2002). Motor vehicles in developing countries cause serious air pollution because they are concentrated in a few large cities, besides, many are in poor mechanical conditions, and few if any emission standards exist. Vehicular emissions continue to be a major threat to environmental health, which is expected to increase reasonably as automobile ownership increase globally (Abam and Unachukwu, 2009). Vehicle exhaust generally contains poly cyclic aromatic hydrocarbons (PAHs), particles, carbon monoxide (CO), nitrogen oxides (NO_x) and volatile organic compounds (VOCs) such as benzene. Diesel-powered engines are the major source of particle, whereas two stroke motor bikes and petrol powered cars emit high levels of VOCs.

CO is different from most other air pollutants in its acute health effects. Benzene has been classified by the International Agency for Research on Cancer (IARC) as a human carcinogen (IARC,1997). In addition, exposure to benzene has been associated with health effects including hematopoietic disorders such as bone marrow deficiency and acute myetogenous leukemia in both rodents and humans (Hayes *et al*., 2001).

Port Harcourt City has acquired national and international prominence, because it is the pivot of Nigeria's oil and gas activities, and a state capital. It is therefore not surprising that there has been a great influx of people into the city in recent years (Nkwocha *et al*., 2008; Nkwocha *et al.*, 2010), with attendant increase in population. In Nigeria, much attention is given to general industrial pollution and pollution in oil industries, with little reference to damage of pollution caused by mobile transportation sources of air

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pollution (Faboye, 1997; Iyoha, 2001; Magbagbeola, 2001). Limited road network, and increase in per capita vehicle ownership as a result of economic growth, has led to high traffic congestion on city roads and increased the risk of pollution from mobile transportation sources. The situation is worsened by the fact that most of the vehicles are older models, imported used vehicles, and there is no exhaust emission control (Mustapha, 2011). The consequence is increased health risk on human population.

Port Harcourt is a coastal city located in the Niger Delta region of Nigeria, with very short dry season and long heavy rainy season periods. The peak rainfall occurs in the month of September. The objective of this study was to assess vehicular pollution levels in the city during the rainy season period.

II. MATERIALS AND METHOD

The research involved the collection of data on the following air quality parameters; CO, NO_x, SO_x, and C_xH_y. The climatic elements sampled were the ambient temperature (^oC), and relative humility (%). Three highly notorious high traffic density junctions in the city were selected for the study namely;

- Rumuokoro Round - about (location A)
- First Artillery Junction, along Aba Road express way (location B)
- Nkpogu Junction, along Trans-Amadi Road (location C).

Measurement of the selected pollutants at the locations was carried out in three (3) time periods, for the duration of three working days of the week. They are as follows:

- 7.30 am - 9.00am (Morning peak hours)
- 1.00 - 3.00pm (Off peak)
- 5.00 pm - 7.00pm (Evening peaks hours).

The peak hours are periods of high traffic congestion. This occurs mainly in the morning, and evening hours, while the off peak is period of low traffic, which occurs in the afternoon. Measurement of air quality parameters was carried out using the Testo 350XL Emission Analyzer. The climactic elements - temperature and relative humidity were obtained using Thermo- hydrometer- IT202 .The study was conducted over a period of 11 days, between 20th August and 5th September, 2013.

III. RESULTS AND DISCUSSION

The average emission estimate and climatic elements for the different locations and periods are presented in Table 1, while Figs 1-4 compare graphically the concentration of the air quality parameters for the different locations. As can be observed, all the air quality parameters investigated where generally detected. However, in a previous study by Utang and Peterside (2011), SO_x were generally not detected. Location B experienced higher concentrations of NO_x and CO at evening peak periods and lower concentration of C_xH_y at morning periods, while the highest and lowest concentrations of SO_x were detected at location C during off peak and evening peak periods respectively

On the other hand, high concentrations of NO_x, CO, and C_xH_y were prominent at evening peak periods. From Table 1, the NO_x concentrations for all the locations were above the limit of 0.04-0.06ppm set by local (Nigeria) standard (FEPA,1991)and 0.053ppm set by International standards, for all the locations and periods, except location C during peak periods. The level of CO was within local standard (10-20ppm) for the off peak period, but exceeded at peak periods in some locations namely - location B at morning and evening peak, locations A and C at evening peak. However, it is noteworthy that the CO levels were within international standard of 35ppm.

Climatic elements and variations in pollutant concentration

As the relative humidity of sampled locations rose, temperature decreased, and the concentration of the pollutants decreased across each location. The highest variation of relative humidity and temperature occurred during morning and off peak periods respectively. All temperatures measured were within the maximum temperature range for the city.

Generally, the air pollutants exhibited the highest variation in concentration across location during the morning period and the lowest during off-peak periods .While CO varied most in concentration across locations and periods (standard deviation 4.48-6.62), SO_x generally did not show much variation. This is in agreement with an earlier study (Utang and Peterside, 2011). Thus, CO and SO_x accounted for the highest and lowest variations respectively, in air quality parameter concentration during all periods in all the locations.

General assessment of pollutant level

From the results obtained the variations in the concentration of pollutants can be attributed to weather conditions, mode of operation of traffic, quality of vehicle (that is, age and maintenance routine), and fuel type vehicle (that is, diesel or petrol) (Faize and Stum, 2000; Rouphail *et al.*, 2001; Udeozor and Nzeakor, 2012). Activities common to the different location studied include, intersection of road, traffic lights, queues, cars packing due to engine being temporarily switched off and driving that involves repeated acceleration, idling, and deceleration cycles.

The high concentrations of NO_x, CO and C_xH_y observed in the evening peak periods could be attributed to constantly

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high ambient temperatures and traffic congestion. It has been reported that high ambient temperatures are associated with increase in exhaust emissions, which are further influenced by air conditioner usage, causing high engine load (Utang and Peterside, 2011). This, coupled with the high temperature of the vehicle combustion chambers, NO_x could be high as indicated by the result. Similarly, at peak periods characterized by deceleration, there is excess fuel in the combustion chamber, so C_xH_y are increased (Colls, 2002; Abhisheck and Colls, 2010). Hydrocarbon emissions can be influenced by ambient temperatures. The high concentration experienced in the afternoon off peak periods could be as a result of evaporative emissions from running losses and diurnal ways (Heywood, 1997). CO air concentrations are generally high in areas with heavy traffic congestion, which characterize the peak periods. The generally low levels of SO_x in all the locations attest to the fact that the fuels used are low in sulphur. However, the isolated case of high concentration observed in location C at off peak, could be due to other industrial activities taking place near the area.

The adverse health effects associated with the high concentration of these pollutants are great. NO_x is responsible for immune system impartment, exacerbation of asthma and chronic respiratory diseases, reduced lung function and cardiovascular disease (Schwela, 2000); while CO reduces oxygen flow in blood stream, exacerbates coronary heart diseases, and at low concentrations can impair concentration and neuro-behavioural function (Udeozor and Nzeakor, 2012).

IV. CONCLUSION

The study revealed that the concentration of NO_x in all the locations did not comply with local and international standards. Though, the concentration of CO was within international standard, it did not meet local standard. In view of the health effects of these pollutants, it implies that the health of roadside artisans, street hawkers, traffic workers, traders and others who are stationed near and around these location, and off course, city dwellers are at risk. There is need for effective air pollution monitoring and control programmes for mobile and stationary emission sources. In addition, improved road net work and traffic control will ease congestion and associated air pollution problems.

Table.1: Average concentration of ambient air pollutants at various locations and periods

Period	**Location**	**NOx(ppm)**	**SOx(ppm)**	**CO(ppm)**	**CxHy(LEL)**	**RH (%)**	**Temp (^{0}C)**
Morning Period	A	0.89	0.012	13.37	0.41	72.7	30.2
	B	1.16	0.011	21.8	0.24	69.1	32.6
	C	0.53	0.009	11.43	0.36	70.6	32.5
	Mean	**1.05**	**0.012**	**17.46**	**0.37**	**70.6**	**32.5**
	Std Dev	**0.14**	**0.00**	**6.62**	**0.10**	**0.87**	**0.36**
Off Period	A	1.1	0.01	14.43	0.26	72.9	30.4
	B	1.2	0.01	18.07	0.41	69.6	32.1
	C	0.31	0.08	16.07	0.4	70.8	31.5
	Mean	**0.42**	**0.011**	**16.84**	**0.38**	**71.1**	**31.33**
	Std Dev	**0.07**	**0.00**	**4.48**	**0.17**	**0.25**	**0.50**
Evening Period	A	1.17	0.013	24.57	0.44	74.3	29.7
	B	1.29	0.011	24.57	0.44	74.3	29.7
	C	0.42	0.007	23.03	0.51	70.3	32.00
	Mean	1.22	0.008	22.02	0.42	71.13	31.5
	Std Dev	**0.10**	**0.00**	**6.06**	**0.08**	**0.40**	**0.36**
	Grand Mean	**0.97**	**0.01**	**18.61**	**0.40**	**71.00**	**31.50**
	Grand Std Dev	**0.27**	**0.00**	**5.73**	**2.27**	**1.89**	**1.15**
FEPA LIMIT		**NA**	**0.01**	**10.00**	**NA**	**NA**	**NA**

LEL= Lower emissible limit ppm = parts per million FEPA = Federal Environmental Protection Agency
NA= not available

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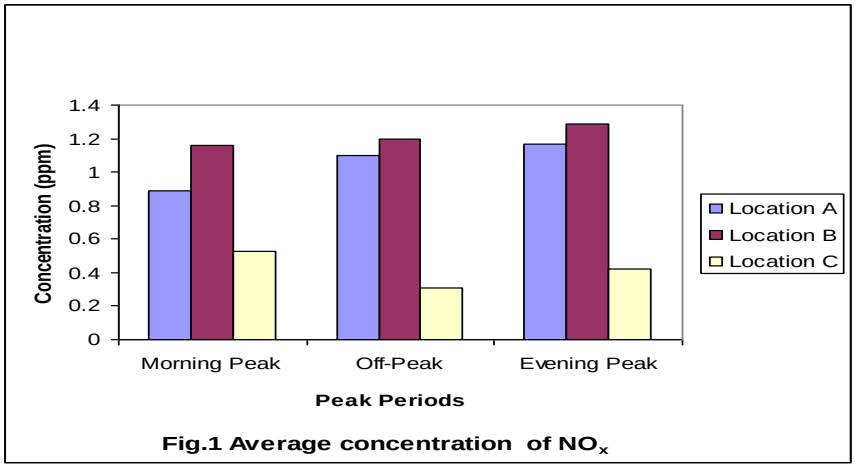

Fig.1 Average concentration of NO_x

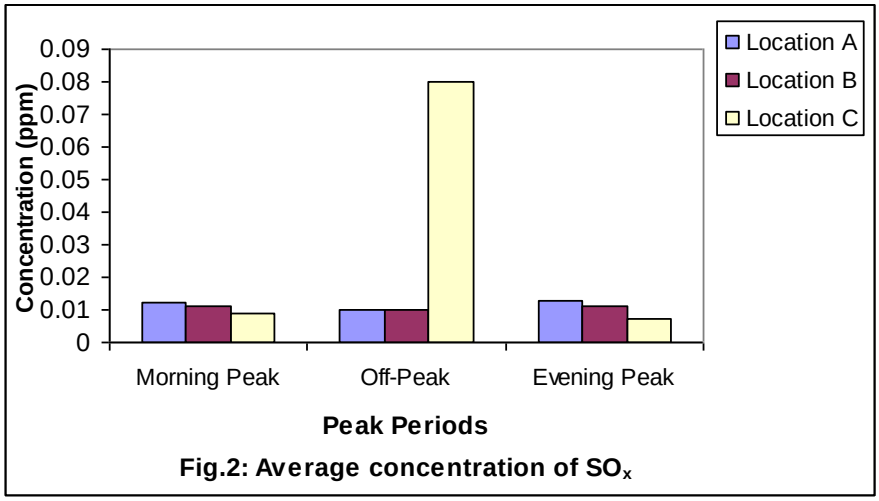

Fig.2: Average concentration of SO_x

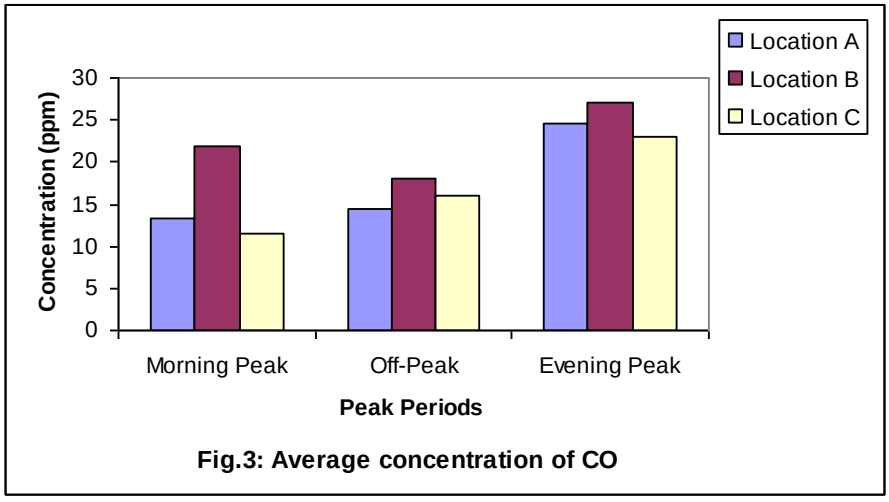

Fig.3: Average concentration of CO

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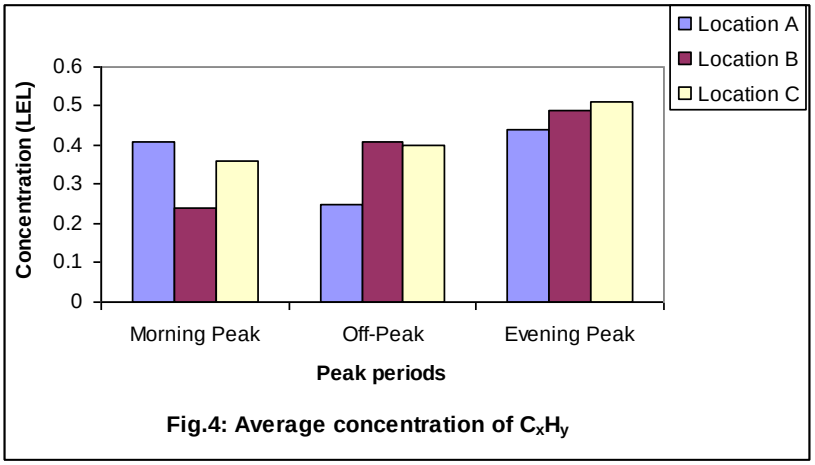

Fig.4: Average concentration of C_xH_y

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Non- Chemical Management of Apple Scab- A Global Perspective

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***Abstract*—** *Apple scab, caused by the fungus Venturia inaequalis (Cooke) G. Wint. is the most widespread disease in apple orchards worldwide. In order to manage apple scab and produce a marketable crop, growers across the globe have relied on 10 to 18 applications of synthetic chemicals at an annual cost of US$202 to $506 per hectare. Until recently, fungicidal control was perceived as the only economical control measure but this perception is changing because of the high costs of new molecules such as the strobilurine-based fungicides, increased fungicide resistance in populations of V. inaequalis, and increased appreciation of environmental costs and consumers negative perceptions of fungicide use. For all these reasons cited above, interest is increasing to develop alternative strategies to manage apple scab. These changes include re-designing orchards so that cultivars with differential susceptibility can be treated with fungicides based on different schedules and using post-harvest treatments, such as leaf shredding or application of biological control agents. New knowledge of the resistance mechanisms in Malus may also present new management options. Despite the increased complexity of integrated scab management, it can prove more sustainable as it involves the use of more than one method and reduces the risk of development of resistance to fungicides in the pathogen population. Ultimately, sustainability will depend on the cost effectiveness of integrated approaches as compared to total dependence on fungicides to control apple scab.*

***Keywords*—Apple scab, Malus, Strobilurine-based fungicide, Sustainability, Venturia inaequalis.**

I. INTRODUCTION

Apple scab, which is caused by the ascomycete *Venturia inaequalis* (Cke.) Wint. is the most important disease in all apple-growing districts with high spring and summer rainfall (Belfanti*et al*., 2004). In some circumstances, the losses from apple scab can be 70% or more of the total fruit value (Agrios, 2005). Floral buds are first exposed to ascosporous infection (Falk *et al*., 1995; Godec, 2004; Percival, 2008). Under dry weather conditions, the number of primary infections may be low and growers can use extended spray intervals (Rosenberger and Cox, 2010). Conidiophores, on which conidia (the asexual spores) are produced and cause secondary infections on all succulent plant parts throughout the growing season (Stensvand*et al*., 1998). These lesions produce infective conidial spores, and they may overwinter and not undergo a sexual reproduction cycle in warmer areas (Schwabe*et al*., 1984). Over the years, fungicides has become the sole means to control apple scab and there has been little effort to commercialize alternative strategies. Even in Integrated Pest Management systems, scab is currently controlled by up to 15–20 applications of protective and curative fungicides during the growing season, regardless of the presence of ascospores in the orchards (Demeyere and De Turck, 2002). Like other apple growing regions worldwide, scab too is currently being managed by fungicidal sprays from pink bud to harvest in Jammu and Kashmir state of India. (Padder *et al.*, 2013).Until recently, it was perceived as the only economical control measure. This perception is changing as a result of the high costs of new molecules such as the strobilurine-based fungicides, increased fungicide resistance in populations of *V. inaequalis*, and increased appreciation of environmental costs and consumers negative perceptions of fungicide use (Beresford and Manktelow, 1994). Fungicide use entails certain environmental risks that include disruption of pest and predator balances, such as the adverse effect on predacious mites, and health concerns for both farmers and consumers (Bower *et al.*, 1995; Schneider and Dickert, 1994). The pathogen has become increasingly resistant to some fungicides (e.g. dodine and benomyl) along with mounting concerns about resistance to the DMI fungicides (sterol demethylation inhibitors) (Braun and McRae, 1992; Carisse and Pelletier, 1994; Smith *et al*.,

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1991). In a survey of Ontario orchards, about 50% of the isolates of *V. inaequalis* were resistant to the eradicant fungicides currently used (Ontario Ministry of Agriculture and Food, 1993).

For all these reasons, interest is increasing to develop alternative strategies to manage apple scab based on non-fungicidal methods that include genetic resistance, physical destruction of the pathogen, and biological controls (Carisse*et al.*, 2000; Sutton *et al.*, 2000).

II. PHYICAL CONTROL

Strategies of physical control have included: (i) pruning to increase air circulation and thereby reduce leaf wetness duration; (ii) burning of leaf litter and; (iii) the use of earthworms to increase leaf decomposition. Orchard layouts that favour wind circulation, appropriate in-row and between-row spacing, and proper pruning have been shown to reduce severity of scab (Kolbe 1983).

2.1. Pruning

To discourage scab, it is advisable to keep the leaves as dry as possible, in other words, to avoid planting too close together, to ventilate the canopy by pruning and to avoid planting in wet, low-lying areas (Corroyer and Petit, 2002). Kolbe (1983) showed that orchards which promote circulation of air through the rows and between the rows by means of appropriate pruning have lower levels of scab in the long term. Holb (2005) compared three pruning models (intense, moderate and none) on two very susceptible cultivars (cv. Jonagold and cv. Mutsu), two susceptible cultivars (cv. Elstar and cv. Idared) and two resistant cultivars (cv. Liberty and cv. Prima) in an organic orchard. He concluded, notably, that intense pruning of susceptible cultivars results in significantly less scab on the leaves and fruit than in the other two models. Simon *et al*. (2006) showed the favourable effects of centrifugal training compared with conventional solaxetraining on scab control, interpreting these results as being due to better ventilation within the tree and, therefore, a microclimate which is unfavourable to scab.

2.2.Inoculum Reduction

Scab overwinters mainly on dead leaves that have fallen on the ground and these are therefore the main source of the primary inoculum that causes contamination the following spring (MacHardy*et al*., 2001). The two main ways of reducing the primary inoculum are (i) to reduce the mass of scabbed leaf litter and (ii) to prevent *V. inaequalis* developing in the litter that remains (MacHardy*et al*., 2001). Several studies have shown the effects of sanitary practices such as burning or burying leaves in the soil (Gomez *et al*., 2004), leaf shredding (Vincent *et al.*, 2004; Holb*et al*., 2006) and a combination of shredding and using urea (Sutton *et al*., 2000) on reducing scab inoculum.These studies showed an ascosporic inoculum reduction of between 40 and 95% and a correlated scab reduction of 45 to 85%.Collecting leaves from the ground in the inter-rows in autumn along with burying the leaves left along the row has a positive effect in reducing primary contamination (Gomez *etal*., 2004). Gomez *et al.* (2004) showed that for two consecutive years the practice of 'raking and ridging' reduced the severity of scab on the fruit by 68 to 74%, depending on the year. Burchill *et al*. (1965) first showed that application of 5% urea to English orchards in the autumn completely suppressed ascospore production the following spring.Burchill (1968) treated Bramley's Seedling trees at two sites in Kent with a postharvest, pre-leaf fall application of 5% urea; scab lesions on blossom-spur leaves were reduced by 59% and 46%, respectively, the following spring compared to the untreated control. Mitre*et al*. (2012) studied the effect of applications of urea 5% after harvest but before leaf-fall, as foliar application, in order to restrict perithecial production by *Venturia inaequalis* in a commercial super intensive apple orchard situated near Cluj-Napoca, Romania. The results (Table 1) showed large reductions in spore production, often as high as 70 to 80%, following application of 5% urea. Spraying the surface of the leaves on the ground with urea 5% reduced primary infection by about 60%.

Table.1: Attack degree(%) of apple scab on 'Golden Delicious' cultivar in the experience regarding effect of urea application in Romania (Mitreet al, 2012)

Variant	Attack degree (%)	Relative attack degree (%)
V_1(Untreated)	81.67	100.00
V_2 (sprayed in autumn with 5% urea)	21.33	26.12
V_3 (sprayed in autumn with 5% urea, followed by a second -pre-bud burst application at 2%)	11.00	13.47

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2.3. Fertilization

Professional fruit growing requires regular supplement of minerals to warrant fruit set and quality. Heavy nitrogen fertilization supports tree and fruit growth and therefore is a prominent controlling tool for yield. An enhanced vegetative growth of apple trees, however, is often correlated with an increasing susceptibility to pathogens such as *V.inaequalis* (Leser and Treutter, 2005). This may be due to the concomitant decrease of phenolic compounds by high nitrogen uptake (Leser and Treutter, 2005) indicating that environmental conditions favouring plant growth reduce investment of carbon for defence.Kumar and Gupta (1986) observed that a high level of potassium fertilizers increased resistance of apple tree to scab but a similar effect was not obtained with high levels of phosphorus fertilization.

2.4. Alternative Protectant Products

2.4.1. Botanicals

Gilliver (1947), tested plant extracts from 1915 different species for their effects on germination of conidia of *V. inaequalis*. Of all the plant extracts, 440 showed various levels of inhibition. In particular, extracts of watery ivy (*Hedera helix* L.) were the most effective.Bosshard (1992) tested the effect of watery ivy extracts and reported that a 1% ivy leaf extract diluted with water to 1:8 and even as low as 1:16 completely inhibited conidial germination on glass slides. On apple seedlings, the level of scab control was high, varying from 59.0 to 99.4% dependent on whether the extracts were applied 1 or 7 days before inoculation with *V. inaequalis* (Bosshard 1992). Northover and Schneider (1993) tested several plant oils against *V. inaequalis* and reported that soybean or canola oil emulsified with Agral 90 and applied at a rate of 1% every 7 to 10 days, reduced scab severity by 66 to 81%. Some russettingwas reported on Golden Delicious following the oil treatments.

2.4.2. Bicarbonate salts

The fungicidal properties of bicarbonates have long been known (Clayton *et al*., 1943) but have never been significantly exploited and used in agriculture. However, bicarbonate salts have experienced a revival of attention in recent years as alternatives for plant disease control (Tamm *et al.*, 2006). Bicarbonate of sodium, potassium and ammonium, in particular, are known to have fungicidal properties. A small body of research is currently available highlighting the effectiveness of bicarbonate salts in apple scab control (Schulze and Schonherr, 2003; Tamm *et al*., 2006). Ihlan*et al*., (2006) show the scab-reducing effect of 1% sodium bicarbonate treatments in orchards during the primary infection season. A new commercial formulation of potassium bicarbonate, called Armicarb, has recently been developed in the USA, especially for foliar applications (McGovern *et al.*, 2003).

Reluctance For Sanitation Practices

Sutton *et al*. (2000) observed that in general, growers do not use sanitation techniques, although they can result in significant reductions in ascospore load. Reluctance to use sanitation practices is through:

- the need for specialized equipment (shredder),
- the failure of sanitation to provide complete disease control, and
- lack of reliable relationships between sanitation measures and the degree to which fungicides can be reduced the following year.

III. GENETIC CONTROL

Breeding for resistance has been recognized as a viable technique to control apple scab since the beginning of the 20th century (Kellerhals 1989; Kumar and Sharma 1999). Resistance to *V. inaequalis*has been historically characterized in one of three manners: no visible symptoms from natural infection, reduced lesion number in comparison to another cultivar, and comparably smaller lesions with less severe symptoms that often includes reduced colonization of the sub-cuticular space, reduced sporulation, and necrotic or chlorotic flecks (MacHardy 1996). Several breeding programme started across Europe and North America, but the work during first half of 20th century remained affected due to World War II. One of the most prominent programs was started more than 50 years ago with the collaborative breeding effort of Purdue University, Rutgers University, and the University of Illinois. Known as the PRI program, it is responsible for the introduction of such resistant cultivars as Prima, Priscilla, and Jonafree. The PRI apple breeding program began in 1926 when crosses made from the crab apple, *Malus floribunda* 821, were found to show some resistance to apple scab. The V_f gene, while being the most frequently used over the last 50 years, is not the only qualitative resistance gene. However, there are other resistance genes such as the pit gene (Vm), from *M. micromalus* and *M. atrosanguinea*, the Vr gene that originated from *M. pumila* R12740-7A, Vbj from *M. baccatajackii*, Vb discovered in Hansen's baccata #2, and Va from Antonovka PI 172623 (Crosby *et al*., 1992).Until now, mostly the*Rvi6* gene previously known as Vf gene has been incorporated into

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commercially available cultivars for resistance. Table 2 depicts the list of R-genes imparting scab resistance.

Despite the many years of work on scab resistant of apple cultivars, they have yet to gain widespread popularity. Cultivars resistant to scab are reputed to have low fruit quality, poor storability, low yield, and lack of market acceptance (Crosby *et al*., 1992; MacHardy 1996). Producers are often hesitant to plant them, especially when they are relatively unknown by consumers (Crosby *et al*., 1992). Apples are one of the few horticultural crops that are purchased on the basis of recognition of the cultivar name. Therefore, when a cultivar is unknown to the public, sales tend to be low (Merwin*et al*., 1994). Hence, efforts to produce durable scab resistant cultivars with market acceptability should be given priority in breeding programmes. In order to devise such programmes with success, apple genotypes have to be screened for scab resistance under *in vitro* condition with fungus races present in particular region. Molecular techniques developed in early 1990's allowed for the identification of markers associated with the Vf gene. The benefits of marker identification are to speed and increase the accuracy of resistance screening of seedlings. Only the screening of those seedlings identified to have the markers in greenhouse or field would be necessary to confirm their resistance (Tartarini, 1996). Mapping of the Vf region began in the early 1990's. Initially isozymes were investigated because of the high allozyme polymorphism of the apple. With a bacterial artificial chromosome (BAC) library of the Vf carrying variety 'Florina', the feasibility to locate Vf with 'chromosome walking' was demonstrated by screening the library with a AL07 RAPD derived probe (Vinatzer*et al*., 1998). Of the other resistance genes, only Vmhas been extensively mapped. One marker, OBP12, was identified at the relatively long distance of 6 cM from the Vm gene. It was found only in cultivars and species closely related to M. micromalus. To test for resistance type, some accessions that carry Vmwere inoculated and all exhibited the pit-type resistance reaction (Cheng *et al*., 1998).The isolates of *V. inaequalis* are hypervariable and exhibit differential pathogenicity on apple cultivars (known as differential hosts). Based upon such differences, the pathogen has been categorized into eight physiological races (Bus *et al*., 2005; MacHardy, 1996). This is one of the good reason for producers and breeders to be concerned. The salient features of these races are summarized in Table 3. In India, particularly Kashmir valley, there is little information on the susceptibility of apple cultivars to scab races, including the cultivars such as Lal Ambri, Gulshan, Shreen, Firdous, Akbar, Shalimar 1 and Shalimar 2 which have been bred in the J&K state

Table.2: List of apple R-genes imparting scab resistance

S.No	R-Gene		Source /host	Linkage group	Reference
	Old name	New name			
1	V_a	Rvi10	Antonovka Type PI 172623 Differential host: h10	LG-1	Gessler *et al*., 2006
2	V_b	Rvi12	Hansen's baccata #2 Differential host: h12	LG-12 (Distal end)	Gessler *et al*., 2006
3	Vbj	Rvi11	*Malus baccata jackii* Differential host: h11	LG-2 (Distal end)	Gessler *et al*., 2006
4	V_d	Rvi13	Durello di Forli Differential host: h13	LG-10 (Proximal end)	Gessler *et al*., 2006
5	Vd_3		1980-015-025	LG1	Soriano *et al*., 2009
6	Vdg	Rvi9	J34; Differential host: h9	—	Patocchi*et al*., 2009
7	—	Rvi14	DülmenerRosenapfel Differential host: h14	LG-6 (Proximal end)	Soufflet-Freslon*et al*., 2008
8	V_f	Rvi6	"Priscilla" Differential host: h6	LG-1 (Distal end)	Gessler *et al*., 2006
9	V_{fh}	Rvi7	*Malus floribunda* 821 Differential host: h7	LG-8	Durel, 2006
10	V_g	Rvi1	Golden Delicious Differential host: h1	LG-12 (Distal end)	Gessler *et al*., 2006
11	V_{h2}	Rvi2	*Malus pumila* R12740-7A (TSR34T15) Differential host: h2	LG-2 (Distal end)	Gessler *et al*., 2006
12	Vh3.1	Rvi3	Q71; Differential host: h3	—	Patocchi*et al*., 2009
13	V_{h4}/V_{r1}	Rvi4	*Malus pumila* R12740-7A (TSR33T239)	LG-2 (Distal end)	Gessler *et al*., 2006

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			Differential host: 4		
14	V_{h8}	Rvi8	*Malus sieversii* W193B Differential host: 8	LG-2 (Distal end)	Gessler *et al*., 2006
15	V_m	Rvi5	*Malus micromalus* 245-38, *Malus atrosanguinea* 840 Differential host: h5	LG-17 (Distal end)	Cheng *et al*., 1998
16	V_{r2}	Rvi15	GMAL 2473 Differential host: h15	LG-2 (Proximal end)	Gessler *et al*., 2006

Table.3: Physiological races of V. inaequalis (Bus et al., 2005; MacHardy, 1996)

Races	Pathological characteristics on apple cultivars
Race1	Non sporulating lesion on Dolgo, R 12740-7A (a Russian cultivar) and Geneva
Race 2	Sporulating lesions on Dolgo, Geneva and some progenies of R 12740-7A
Race 3	Sporulating lesions on Geneva, and non sporulating lesion on Dolgo, R 12740-7A
Race 4	Non sporulating lesion on Dolgo, Geneva and sporulating lesion on those progenies of R12740-7A on which race 2 isolates cannot sporulate
Race 5	Sporulating lesions on Vm R gene containing cultivars
Race 6	Sporulating lesions on Vf hybrids but cannot infect Malus floribunda 821 containing Vfh R gene
Race 7	Can infect cultivars having Vf and Vfh R gene but cannot infect Golden delicious which contains Vg gene
Race 8	Can infect Golden delicious, Royal gala, and cultivars containing Vh8 R gene

IV. MIXED PLANTING CULTIVARS

The monoculture of a genetically uniform crop is a major feature of modern agricultural systems. However, this genetic uniformity favours the rapid development of plant diseases and renders these agricultural systems dependent on high pesticide input. One strategy proposed for increasing the spatial diversification of host resistance is the use of multiline cultivars or cultivar mixtures (Mundt, 2002).Apple cultivars are differentially susceptible to *V. inaequalis* (Dewdney, 2000). It is a challenge to use differential susceptibility of cultivars and virulence of the pathogen isolates in orchard design to reduce fungicide because apple trees are perennial plantings. A computer simulation experiment was conducted to test the effects of different cultivar planting patterns on the reduction of lesions (Blaise and Gessler 1994). Combinations of no more than three cultivars were tested in solid blocks, homogenous rows, and mixed rows. The cultivars were differentially susceptible to the inoculum, primary or secondary and the 'pathotype' of the inoculum depended on the cultivar of origin. The conidia dispersion was assumed to follow a Gaussian distribution for splash dispersal with equal distribution in each direction. Expectedly, the greatest amount of disease predicted was in a solid cultivar block. The greatest reduction, 79%, was found with three cultivar mixes within the row. When the cultivars were in alternating rows, potential scab was reduced by 65% in the simulation compared to 67% reduction when only two cultivars were in alternate rows. In this simulation, substituting one susceptible cultivar by a resistant one had no effect in the alternate-row planting scenario.

Didelot*et al*. (2007) studied the effects of two mixtures of resistant and susceptible apple cultivars on the development of scab caused by *Venturiainaequalis* in an experimental orchard over four years, initially for two years without fungicides against scab, and subsequently for two years with a moderate fungicide schedule. The row-by-row and within-row mixtures included a susceptible cultivar and a resistant cultivar in equal proportions. Without fungicides, the results showed a significant reduction of disease incidence over both years (7·3 to 21·3%), and severity in the second year (35·4%) in the within-row mixtures, compared to the monoculture of the susceptible cultivar. The best results were obtained when the within-row mixture was associated with moderate fungicide treatments; in this case the reduction in disease incidence reached 75·1% on leaves and 69·7% on fruits during the growth phase. The characteristics of the *Venturia inaequalis*/*Malus*×

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*domestica*pathosystem and the results obtained in this experiment suggest a moderate but not negligible ability of cultivar mixtures for reducing epidemics of the disease. **Figure 1** depicts the apple scab severity over the years of trial. In the future, this kind of planting system, combined with other methods such as sanitation or biological control, could represent an interesting alternative to chemical control.

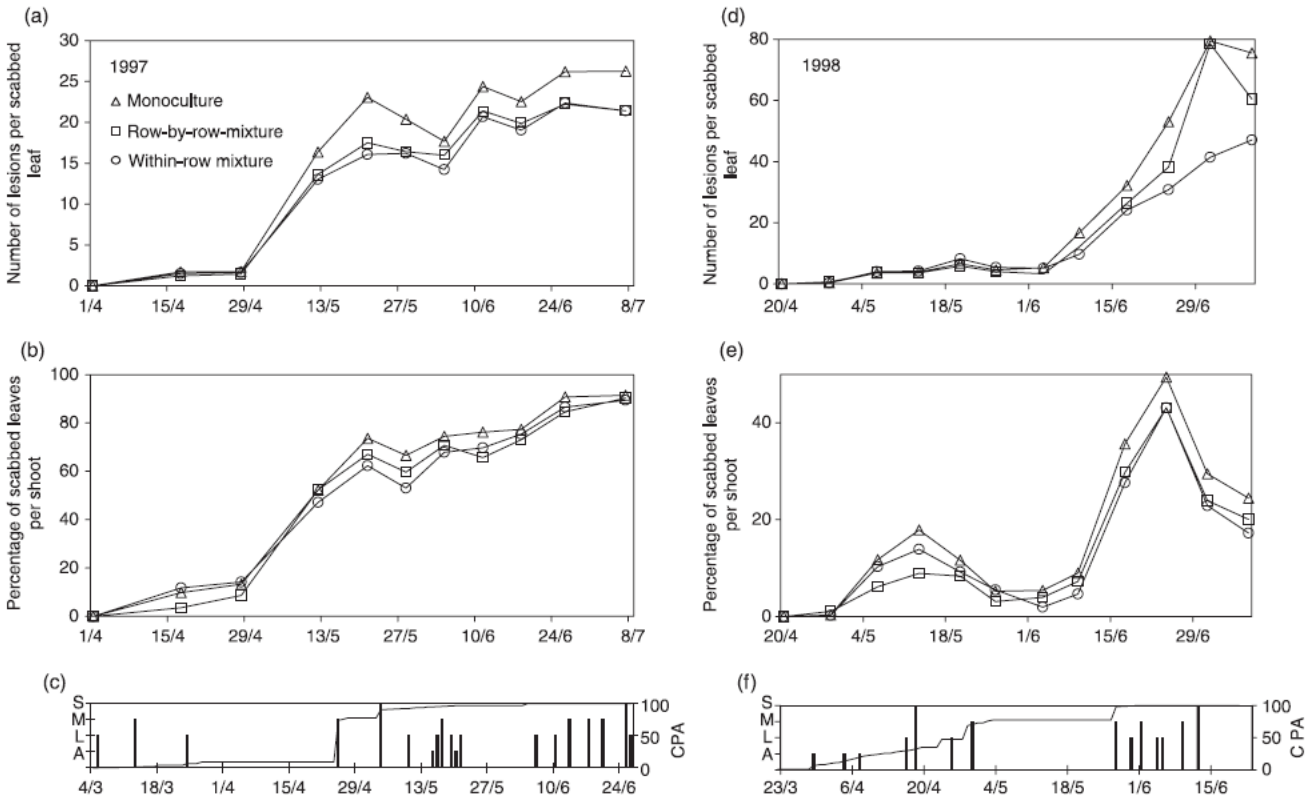

Fig.1: Evolution of (a, d) scab severity and (b, e) incidence on apple cv. Smoothee in monoculture plots and in mixed cultivar plots, and (c, f) infection periods (dark bars) and cumulative percentage of ejected ascospores (CPA, line without symbols), in (a–c) 1997 and (d–f) 1998. A, L, M and S are the different levels of the Mills and Olivier infection periods: Angers, Light, Moderate and Severe, respectively.

V. BIOLOGICAL CONTROL

Biological control can be adopted through either (i) the introduction of a microbial control agent or (ii) the manipulation of naturally occurring populations of microorganisms. The nature of life cycle of *V. inaequalis* has lent itself to studies that aim to interrupt overwintering of the perfect stage or else to control infection of leaves during the spring and summer. Cinq-Mars (1949) pioneered biological control of apple scab as the first scientist to isolate microorganisms from apple leaves with more than 25 different microorganisms, including fungi, bacteria, and yeasts.He showed that some of these organisms, mainly *Penicillium* species, produced antibiotics that inhibited mycelial growth of *V. inaequalis.*Hislop and Cox's (1969) work represented a turning point in the history of biological control of scab, as it was the first study to examine a possible integration of microbial and chemical control by investigating the effects of fungicides on the size and diversity of populations of microorganisms that lived on apple leaves. Cullen *et al.* (1984) evaluated the potential of *Chaetomiunglobosum* as a biofungicide against apple scab. A spore suspension of *C. globosum* applied every 1 to 2 week to apple trees in an orchard reduced scab severity by 20% as compared to untreated control.*Microsphaeropsisochracea*, a coelomycete isolated from dead apple leaves, recently has been identified as a biological control agent of apple scab (Carisse and Bernier, 2001). When *M. ochracea*was applied onto naturally infected leaves that were overwintered on the orchard floor, ascospore production the following spring was reduced by 76 to 84% and fall applications on the tree canopy (at 10% leaf fall) and on the ground (at 90% leaf fall) resulted in 75.5 and 62% fewer ascospores than control. (Carisse*et al.*, 2000).Foliar sprays of plant extracts, derived from

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Artemisia absinthium,Urticadioica and *Equisetum arvensae*, were combined with two antagonistic microorganisms, *Trichodermaasperellum* and *Pythium oligandrum*, and tested in organic apple orchards. The spray with only the microorganism *T. asperellum* showed the most efficacy during primary scab infection period and the level of scab was significantly different from the water control. During the secondary scab infection period, *T. asperellum* alone plus *T. asperellum* with each of the extracts and *P. oligandrum* alone showed significantly less apple scab when compared to the water control as shown in Table 4 (Kowalska*et al*., 2010).

Table.4: The incidence of apple scab on leaves in spring and autumn time (2009-2010) (Kowalskaet al., 2010)

No	Treatement	Mean % affected leaves during primary infection period (spring time)	Mean % affected leaves during secondary infection period (autumn time)
1	*T. asperellum*	1,47*	3,88
2	*T. asperellum + Urticadioica*	3,17	3,50
3	*T. asperellum + Artemisia absinthum*	4,75	14,88*
4	*T. asperellum + Equisetum arvensae*	3,17	17,38*
5	*P. oligandrum*	8,75	19,63*
6	*P. oligandrum + U. dioica*	4,20	47,63
7	*P. oligandrum + A. absinthum*	4,15	49,00
8	*P. oligandrum + A. arvensae*	4,42	72,25
9	Untreated (water)	2,24	31,65

* - statistically different from untreated trees

The potential of the antagonistic isolate *Cladosporiumcladosporioides* H39, originating from a sporulating colony of *V. inaequalis*, to control apple scab development was tested by Kohl *et al* .(2015) in eight trials during 2 years in orchards in Eperjeske (Hungary), Dabrowice (Poland), and Bavendorf (Germany) planted with different cultivars. Treatments were conducted as calendar sprays or after infection periods. Additional trials in an orchard in Randwijk (The Netherlands) focused on the effect of timing of antagonist application before or after infection periods. The overall results of the field trials consistently showed for the first time that stand-alone applications of the antagonist *C. cladosporioides* H39 can reduce apple scab in leaves and fruit. This was demonstrated in an organic growing system as well as in conventional orchards and the same control levels could be reached as with common fungicide schedules. Efficacies reached 42 to 98% on leaf scab incidence and 41 to 94% on fruit scab.

Despite the tremendous amount of research and number of publications on biological control of plant pathogens, there are only a few biofungicides registered in the world, most of them being commercialized for specific niches, such as high value crops for which there is a demand for pesticide-free products. Second, biofungicides are often made of a single strain of an antagonist and thus limits the use of the biofungicide against other diseases.

VI. CONCLUSION

- Apple scabis of major economic importance and if not managed, the disease can cause extensive losses following humid and cool weather conditions during the spring months. Direct losses result from fruit infections and indirect losses from defoliation, which can reduce tree vigor, winter hardiness, and subsequent yield
- Fungicidal control is generally consideredthe sole economically feasible control measure against apple scab. However, this may change due to the high costs of new fungicides, increased fungicide resistance in populations of *V. inaequalis*, and increasing concerns of environmental costs and consumers negative perceptions of fungicide use
- Repeated use of fungicides can interfere with pest and predator balance and have adverse effect on predacious mites and increasing health concerns for both farmers and consumers.
- For all of these reasons, interest in the development of alternative strategies such as genetic, physical, and biological approaches to manage apple scab and to reduce reliance on fungicides.
- Nevertheless, integrated management of apple scab may prove more sustainable on a long-term basis, mainly because it does not depend on the use of a

single method. Hence the risk of the development of fungicide resistance in the pathogen population is reduced.Ultimately, sustainability will depend on the cost effectiveness of integrated approaches as compared to total dependence on fungicides to control apple scab

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Characteristics Exploration of NIiCuZn Nano-Composite coated Permanent Magnets

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Abstract*—This paper presents the synthesis of*** $Ni_{0.5}\,Cu_{0.2}Zn_{0.3}\,Fe_2\,O_4$ ***compound using Citrate Precursor Sol- Gel Method and Ball millingfor grinding the compound. X-ray diffraction measurements (XRD) confirmed the formation of single-phase cubic spinel structure. The average crystallite size was calculated using XRD pattern and confirmed by Scanning Electron Microscope (SEM). The electromagnetic properties were investigated using Vector Network Analyzer (VNA) and molar magnetic susceptibility measurements. The magnetic measurements have proved that the entire preparation method has considerable effect in enhancing the magnetic properties of the system. And an application of PMBLDC machine design with ferrite coated permanent magnets having competitive power density and efficiency. The influence of temperature variation on the magnets on the electric machine performance is also observed.***

Keywords— ferrite coat; magnetic susceptibility; Citrate Precursor Sol- Gel Method; Ball Milling.

I. INTRODUCTION

At present surface-mounting devices have been developed for electronic applications, which are produced by coating ferrite and Ag electrode layers alternately and co-firing them. Low temperature sintered NiCuZn ferrites are the most universal ferrite materials to produce MLCIs because of their relatively low sintering temperature and high resistivity with good performances in the high frequency range [1–3].Typical NiCuZn ferrites with a regular particle size have sintering temperatures above 1000^0C. The usage of fine powder decreases the sintering temperature of ferrites. Fine powders can be prepared through various wet-chemical methods like co-precipitation [4],hydrothermal synthesis [5] and sol–gel processes [6]. Although theco-precipitation and sol–gel methods are the most popular, theyhave some disadvantages as most of them are highly pH sensitiveand require special attention for complex systems whereas the sol–gel technique requires expensive alkoxide precursor material and stringent process of gel product [7].Among the established synthetic methods, it is still critical tofind simple and cost-effective routes to synthesize nano-crystallineNiCuZn ferrites by using cheap, nontoxic and environmentally benign precursors. In addition to their high nutrition quality egg-white proteins are well known for their gelling, foaming and emulsifying characteristics [8-9]. NiCuZn ferrites of composition $Ni_{0.7-x}Cu_xZn_{0.3}Fe_2O_4$ (x = 0, 0.2, 0.4, 0.6) prepared by citrate precursor method, characteristics are investigated and reported[10]. X-ray diffraction(XRD) confirmed the formation of single-phase cubic spinel structure. The grain size, estimated by SEM micrograph, was found to increase with Cu content. The hysteresis data indicated that the maximum saturation magnetization was obtained for the composition with x = 0.2.Lima [11] synthesized $Ni_xCu_{0.5-x}Zn_{0.5}Fe_2O_4$ ferrite($0.2\leq x\leq 0.4$) nano-particles using the citrate precursor method.Vibrating sample magnetometer (VSM) showed that adding copperto NiZn ferrite decreases magnetization saturation and the calcining temperature. Ferrites with compositions of $Ni_{0.27}Zn_{0.64}Cu_xFe_{1.98O4}$ (x = 0.1,0.2) were prepared by conventional ceramic methods [12]. Theintergranular pores in the prepared ferrites were found to generate large demagnetizing fields, reduce the temperature dependence ofthe effective anisotropy field and thus decrease the temperature dependence of the relative initial permeability. Fine powders of $Ni_{0.6-x}Cu_xZn_{0.4}Fe_2O_4$, where $0\leq x\leq 0.4$ were prepared by the citrate precursor method [13]. XRD confirmed theformation of single-phase cubic spinel structure. The addition of copper was found to promote the grain growth, resulting in anincrease in the grain size. Curie temperature, however, was understandably lowered with the increase in Cu content. Ferrite with Cuconcentration of x = 0.4, showed the highest value of initial permeability.

In the present paper, synthesis of NiCuZn ferrites by a simple method uses Citrate Precursor Sol- Gel Method and Ball milling for grinding the compound. The synthesized nanocrystals have been characterized using thermal analysis techniques. Calcined nano-ferrite samples are characterized by X-ray diffraction (XRD) and Scanning Electron Microscopy (SEM). The magnetic properties of the ferrites

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were investigated using a Vector Network Analyzer (VNA) at room temperature and magnetic susceptibility measured at different magnetic fields and temperatures.

Permanent magnet technology is constantly developing. At present NdFeB magnets are being used in electrical machines. Due to their cost and low availability alternatives are being explored. An additional boost of the progress of permanent magnet synchronous machines (PMSMs) was got after establishing of the interior magnet rotor structure [14], [15] and with the development of tooth-coil winding approaches [16], [17]. [18] presents, At this power and speed areas conventional asynchronous machines should have similar performance characteristics as PMSMs with slightly smaller peak efficiency in the static efficiency map, less torque density and lower power factor [19]. After the rapid increase of the neodymium magnets' price in 2010, there appeared many companies and organizations searching for appropriate designs for so called "rare earth free" electric machines. The main purpose of the rare earth free electric machines is to reach almost the same torque density as in commercially available neodymium PMSMs, without efficiency deterioration. Major part of these attempts is done for hybrid electric vehicle applications [20-27]. Common measures in order to increase the power density of PMSMs are high angular speeds [28], increase the number of pole pairs and increasing the tangential stress [29].

This paper narrates the possibilities for improving the torque density with the use of ferrite coat on magnetic surface of PMBLDC machine. Permanent-magnet (PM) BLDC motor with rare-earth PMs is most popular,but rare-earth PMs have problems with high power low voltage applications, high cost and limited supply. Therefore, the electric motors with less or no rare-earth permanent magnets are available for numerous application.

II. SYNTHESIS OF NANO-COMPOUND

This section gives the details of synthesis of the nano compound $Ni_{0.5}\,Cu_{0.2}Zn_{0.3}\,Fe_2\,O_4$:

a. A magnetic spinel nano NiCuZn ferrite catalyst with composition $Ni_{0.5}\,Cu_{0.2}Zn_{0.3}\,Fe_2\,O_4$ was chosen for this study.
b. For the preparation of catalyst, aqueous solutions of stoichiometric amounts of Nickel nitrate, Copper nitrate and Zinc nitrate along with ferric citrate were reacted with citric acid in 1:1 molar ratio.
c. $p^H$of the solution was increased by the addition of ammonia to complete the reaction and ethane diol was added.
d. The solution was evaporated very slowly over a period of 24 hours to dryness. Viscosity and color were changed as the solution turned into puffy, porous dry gel. As soon as the solvent removal is completed, dried precursor goes under a self-ignition reaction to form a very fine powder known as synthesized powder.
e. The synthesized powder thus obtained was calcined in a muffle furnace at 600c for 2 hours to remove the residual carbon and furnace cooled. Then matter is subjected to Ball milling for 2 hours at speed of 450 rpm.

III. CHARACTERIZATION OF NANO COPPER FERRITE

a. X- RAY DIFFRACTION (XRD) ANALYSIS:

Fig1. shows typical XRD pattern for nano copper ferrite sample which was sintered at 600 degree celsius. The pattern shows all the characteristics peals of a spinal structure and confirms the phase formation indicating the absence of other impurity phases. The XRD parameters of various peals were compared with the standard data of the cubic copper ferrites and found to be in cubic phase. The particle size and other characteristics of the copper nano particles obtained from the XRD pattern using Scherer's formula was found to be 39nm and reported in table1. The peals can be indexed to (220),(311),(400),(422),(511) and (440) phases of a cubic unit cell are shown in fig 2.The X-ray diffraction pattern was studied in detail forthe determination of crystallite size by using the classical Scherrer equation [15]:

$$D = \frac{k\lambda}{\beta \cos\theta} \qquad (1)$$

Where, D is the average crystallite size, k is a constant equal to 0.89, λ is the X-ray wave length (0.1542 nm), θis the angle of diffraction and β is the full width at half maximum (FWHM) of the peak.

The average crystallite sizes of the powders were in the range 39 nm which indicates that the Cu substitution for Ni has no effect on the crystal size.

The lattice parameter (a) has been calculated from X-ray datausing the formula:

$$\frac{1}{d^2} = \frac{h^2 + k^2 + l^2}{a^2} \qquad (2)$$

Where, d is the lattice spacing and h, k and l are the miller indices ofthe plane.

The theoretical density or the X-ray density (D_x) was calculatedaccording to relation:

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$$D_X = \frac{ZM}{Na^3} \qquad (3)$$

Where, Z is the number of molecules per unit cell (Z = 8), M is themolecular weight, N is Avogadro's number and a3 is the volume ofunit cell.

The variation of the average crystallite size, lattice parameterand X-ray density, with copper content, are shown in Table 1.

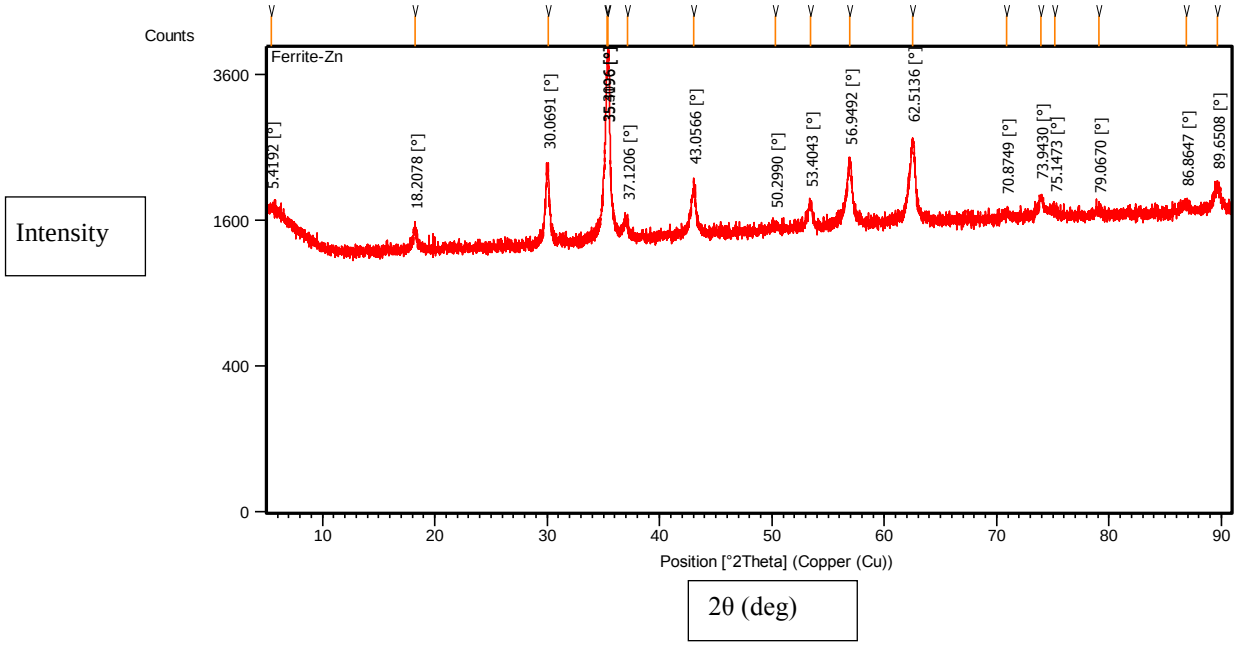

Fig.1: XRD Pattern of Nano-Composite $Ni_{0.5}\,Cu_{0.2}Zn_{0.3}\,Fe_2\,O_4$

b. Morphological and elemental analysis (SEM&EDS):

Fig3 shows the typical SEM image of the nano NiCuZn ferrite sintered at 600degree Celsius. The crystallite size calculated from XRD is in the range of below 30 nm which is in agreement with the SEM image. The structural composition and crystallinity of the NiCuZn ferrite nano particles was further examined by using SEM and TEM. The iron and copper ratio in the nano crystals as determined by EDX analysis was very much close to the atomic ratio in the formula NiCuZn ferrite.

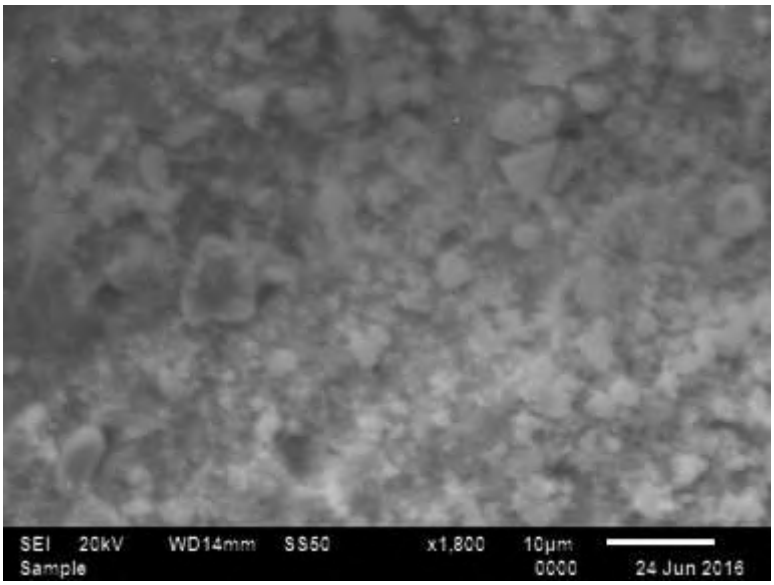

Fig.3: Obtained SEM image of NiCuZn ferrite

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Table.1: particle size and other characteristics of the nano $Ni_{0.5}Cu_{0.2}Zn_{0.3}Fe_2O_4$ *ferrite obtained from the XRD analysis.*

S. NO	Parameters	Values
1	Lattice parameter (a)	8. 379 A
2	Density (%)	94.2
3	X-ray density (D_x)	5.35g/m
4	FWHM	0.284
5	Grain size	1.42
6	Average crystallite size (D)	39 nm
7	Saturation magnetization (M_s)	47. 4 emu/g
8	Magnetic moment (η_B)	2.03 µB
9	Remnant magnetization, M_r	7.15 emu/g
10	Corecivity, H_c	67.8 Oe
11	Curie Temperature, T_c	690 ^{0}C
12	Effective magnetic moment (μ_{eff})	4. 65 µB

IV. RESULTS AND DISCUSSION

Electromagnetic properties measurement of $Ni_{0.5}Cu_{0.2}Zn_{0.3}Fe_2O_4$ system is using a Vector Network Analyzer(VNA). Fig. 3 **S**EM image of the sample with a field scan up to ±5.0 kOe at room temperature. The hysteresisloop of Ni0.5Cu0.2Zn0.3Fe2O4 is shown in fig.4. The sampleshows a ferromagnetic nature with &curves typical for soft-magneticmaterials.The values of saturation magnetization (M_S), remnant magnetization(Mr) and coercivity (H_C) areshown in Table 1. In ferrites,the magnetic moment arises mainly from the parallel uncompensatedelectron spin of individual ion. The intensity of magnetizationcan thus be explained by considering the metal ion distribution andantiparallel spin alignment of the two sub lattice sites as given byNeel's Model [19]. According to Neel's model, three types of interactionsAA, AB and BB are present with the intersub-lattice ABsuperexchange interaction is the strongest one of them. Since Zn^{2+}ions are non-magnetic, the contribution to the magnetization ismostly due to Ni^{2+}, $Cu^{2+}$and Fe^{3+} ions having magnetic momentsof 2.3, 1.3 and 5 µB respectively.

The experimental magnetic moment (η_B) is determined fromthe saturation magnetization data using the following formula [14]:

$$\eta_B = \frac{MW_X M_S}{5585} \qquad (4)$$

Where MW is the molecular weight of the sample

M_S is thesaturation magnetization in emu/g

The calculated values of the experimental magnetic moment (η_B) is presented in Table 1. The gradual decrease in the values of saturation magnetization and experimental magnetic moments with increasing copper content is accounted for the weakening of the AB interaction, which holds well with the decrease in theoretical values of magnetic moment. The decrease in coercivity (H_c) with increasing copper concentration may be attributed to lower magneto-crystalline anisotropy of Cu^{+2} ions as compared to Ni^{+2} that leads to lower coercivity according to the Stoner–Wolfforth model for coercivityof nano-particles [21].

Saturation magnetization value is obtained at room temperature is tabulated in Table 1 are relatively high especially at higher concentrations of copper content as compared with the results of Jadhav et al. [10]. On the other hand, the obtained coercivities show lower values as compared with the same results. This suggests that, the present method of synthesis used is Citrate Precursor Sol-Gel Method and Ball milling for grinding the compound, has an impact on improving the magnetic properties of the system. The temperature dependence of the molar magnetic susceptibility (χ), as a function of the magnetic field intensity is investigated for sample. The Curie temperatures and the effective magnetic moments are reported in Table 1.

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Table.2: Comparison of Permanent Magnet Materials:

Material	Remanence B_r(T)	Coercivity H_c (kA/m)	Curie temperature (^{0}C)	Comparisons
$Ni_{0.5}\,Cu_{0.2}Zn_{0.3}\,Fe_2\,O_4$	0.5….1.35	33.1…76.8	550….850	+ low Cost material +High magnetic properties +linear +Availability
SmCo	0.9…1.1	700…2400	500…850	+ High magnetic properties + linear + very High Cost
NdFeB	1.0…1.4	900…3200	310	+ High magnetic properties + linear -High temperature Coefficients -prone to corrosion

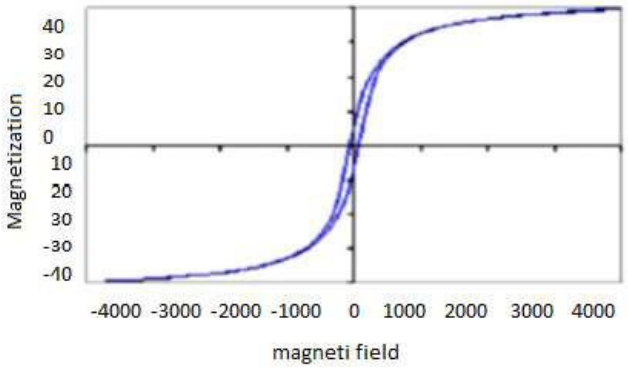

Fig.4: Magnetic hysteresis loops for $Ni_{0.5}\,Cu_{0.2}Zn_{0.3}\,Fe_2\,O_4$

For all the samples, the absence of any thermal stability of $\eta_M$with increasing temperature indicates that the thermal energy isquite sufficient to disturb the ordered spins even at lower temperatures.The measured Curie temperature decreases with increasing Cucontent (Table 1). This observed variation can be explained in termsof the magnetic super exchange interaction which has a direct relationwith Curie temperature [13,22]. Further, the strength of A–Binteraction, which is the interaction existing between the antiparallel uncompensated electron spin of A and B sub-lattices is the mostdominant [23]. This interaction is observed to decrease with Cusubstitution as indicated by the magnetization measurements thusaccounting for the fall in Curie temperature.

V. CONCLUSION

Nano-crystalline $Ni_{0.5}Cu_{0.2}Zn_{0.3}Fe_2O_4$wassuccessfully synthesized and prepared using Citrate Precursor Sol- Gel Method and Ball milling for grinding the compound. The obtained powders were characterized using TG,XRD, FT-IR and TEM techniques. The results indicate that, single phase cubic ferrites were obtained after calcining the precursors at 600^{0}C for 2hours. On investigation of characteristics and properties it is observed that, instead of the copper substitution has weak effect on the structural properties of the system, but it greatly affects the magnetic properties.PMBLDC machine with ferrite coated magnets is simulated and its performances have been evaluated.

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Effect of an Endomycorrhizal Inoculum on the Growth of Argan Tree

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Abstract*—The aim of this work is to study the effect of a composite endomycorrhizal inoculum on the growth of argan plants under nursery conditions. Analysis of the obtained results after ten months of inoculation showed a significant effect on the growth of the inoculated plants as compared to the controls. Indeed, the mean values of arial fresh weight (27.54 g) and root (23.64 g).The length (59.87 cm), the collar diameter (3.93 cm) and the number of branches (7.37) of the inoculated plants are superior to those observed in the control plants, 13.36 g, 13.43 g, 35.83 cm, 2.83 cm and 4.66 cm, respectively. In addition, frequency (100%), intensity (63.66%) and arbuscule contents (51.79%) and vesicles (25.52%) are very important. The roots of the control plants are not mycorrhizal. The mean number of spores formed in the rhizosphere of the inoculated plants is 246 spores per 100 g of soil. These spores are those of 29 endomycorrhizal species belonging to six different genera: Acaulospora, Scutllospora, Pacispora, Glomus, Entrophospora and Gigaspora. Representatives of the Glomus genus are the most dominant.*

***Keywords*— Argan tree (Argania spinosa), plants, nursery, inoculation, growth, mycorrhization parameters.**

I. INTRODUCTION

The argan tree (*Argania spinosa* L. Skeels), anendemic species to Morocco, is located in the south-west of the country and covers an area of 800.000 ha (Msanda *et al.*, 2005). It is ranked second among forest tree species in Morocco (Ayad, 1989).

Argan tree plays an important ecological role (Le Houérou, 1989) by creation of a favorable climate in the development of a high number of vegetal species participating in the protection against soil erosion especially in the accidental reliefs (Peltier, 1982; Msanda *et al.*, 2005; Achouri *et al.*, 2011). It plays an exceptional economic role (Benzyane, 1989; M'hirit *et al.*, 1998) by ensuring the subsistence of nearly 3 million riparian zones (Benzyane, 1995). Each part of the tree is usable and is a source of income or food. Wood is used as fuel, leaves and fruits as fodder for goats (M'hirit *et al.*, 1998; El Aich *et al.*, 2007). Argan oil, consumed almost exclusively in the region of production, is now widely exported to many countries (Europe, North America, Japan, etc.), as a luxury food product, appreciated for its nutritional and organoleptic qualities, or used in cosmetic products (Nouaïm *et al.*, 2007; Echairi *et al.*, 2008). Despite this exceptional value recognized by the users, the arganeraie has always been subjected to anthropozoic pressure. It regressed mainly because of clearing for crops and the extension of towns (Elyousfiand Benchekroun, 1992). Between 1969 and 1986, it lost nearly 9900 hectares (Benabid and Elyousfi, 1989). In parallel with the retreat of the argan tree, natural regeneration by sowing is very rare (Boudy, 1952) or absent. This absence is due to the excessive harvesting of the fruit, which the special legislation of the argan tree authorizes and which is part of the broad right of enjoyment granted to the users, it is also due to the grazing of the rare seedlings resulting from the germination of some remaining nuclei by livestock (Boudy, 1950). Climatic conditions are also not conducive to seed germination (Zahidi and Bani-Aameur, 1996).

The efforts of the Moroccan forestry services in the field of reforestation based on argan tree are hampered by the difficulty of resuming seedlings produced in nurseries (Ferradous *et al.*, 1997). According to these authors, several reasons can explain the observed failures: precipitation deficit, inadequate of the used plants. The improvement of plant production techniques at the nursery level is an unavoidable step and must imperatively be mastered (Lamhamedi *et al.*, 2000). Controlled mycorrhization of seedlings at nursery (Nouaïm and Chaussod, 1994), for example, could potentially increase the success of transplants and the initial growth of trees (Echairi *et al.*, 2008).

The argan tree has the ability to establish a symbiotic association with AM fungi (Achouri *et al.*, 2011; Nouaim and Chaussod, 1996). Arbuscula rmycorrhizae are found

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in more than 70% of vascular plant species (Fortin *et al*., 2008) and allow the extension of the absorption surface and the volume of the soil explored, well beyond the zone of depletion of the rhizosphere (Sylvia, 1986). This type of mycorrhizae also allows a better improvement of the assimilation of the nutrients in particular the P and N (Toro *et al.,* 1997; Haougui *et al.,* 2013), especially in arid and semi-arid environments, improved aggregation and soil stability (Rillig and Mummey, 2006) and protection against phytopathogens (Newsham *et al.,* 1995; Pozo *et al.*,1999 ; Dalpé, 2005; Tahat *et al.,* 2010). AMF also help plants to develop in arid and semi-arid areas via the reduction of drought stress (Augé, 2001; Herrera *et al.,* 1993; Roldan *et al.,* 1996b; Barea *et al.,* 2008; Honrubia, 2009), improvement of the physico-chemical and biological properties of soils (Carrillo-Garcci*aet al.,* 1999; Rillig and Mummey, 2006; Schmid *et al.,* 2008) and other environmental stresses (Barea *et al.,* 2007; Ouahmane, 2007; Martínez-García and Pugnaire, 2009; Martínez-García, 2010).

The mycorhization of argan plants is therefore an interesting way to explore for the restoration of degraded areas (Ammari *et al.,* 2006). The higher diversity of endomycorrhizal fungi at the rhizosphere of the argan tree growing in different areas of south west Morocco was been revealed (Sellal *et al*., 2016).

The present work aims to study the effect of a native composite endomycorrhizal inoculum on the growth and development of argan tree plants in nurseries.

II. MATERIALS AND METHODS

1- Vegetal Material

The used argan plants for inoculation are four months old. They were raised in a nursery on a substrate, disinfected Mamora's soil.

2- Inoculum production and multiplication:

The barley (*Hordeum vulgare*), mycotropic plant, was chosen for the production of a composite inoculum based on arbuscular mycorrhizal mushrooms belonging to six (6) genera: *Acaulospora*, *Glomus*, *Scutellospora*, *Entrophospora*, *Pascispora*, *Gigaspora*. Barley grains were disinfected with 5% sodium hypochlorite for 2 minutes and sprouted in plastic cups filled with a mixture of disinfected sand and soil of the argan tree rhizosphere. All the pots were placed in a greenhouse and watered regularly with distilled water.

After three months of culture, the frequency and intensity of barley mycorrhization were estimated using the method of Phillips and Hyman (1970).

3- Physico-chemical soil parameters

The used soil in all trials is that of the Mamora's forest, the characteristics of which are given in Table 1.

Table.1: Chemical characteristics of the Mamora's soil

Physico-chemical soil parameters	**pH**	**Organicmatter**	**Nitrogen(%)**	**Phosphorus P_2O5 (%)**	**Potassium K_2O (meq/100g)**	**Magnesium (Mg) (meq/100g)**	**Calcium (Ca) (meq/100g)**
Mamora's soil	7.53	0.7	0.05	0.239	0.15	0.20	7351.5

4- Inoculation

Argan plants, 4 months old, were transplanted into pots containing 50% of the soil of the disinfected Mamora and 50% of the inoculum (soil and mycorrhizal roots). The control plants were planted only on the sterilized soil of Mamora's forest. All plants are deposited in a greenhouse and irrigated every three days with distilled water for plants inoculated with AM fungi, either with tap water for other inoculated plants.

5- Evaluation of mycorrhization parameters

After ten (10) months of inoculation, colonization of the roots of the argan tree plants with the AMF was carried out using the root staining technique of Philips and Hayman (1970), modified by Koské and Gemma (1989).

The fine roots of the argan plants, recovered from the culture substrate, were washed with tap water and cut into fragments of 1 cm in length, immersed in a 10% KOH solution and placed in an oven at 90 ° C. for one hour. At the end of this period, the roots are rinsed with sterile distilled water and transferred to a solution of H_2O_2 (hydrogen peroxide) for 20 minutes at 90°C until the roots were bleached. The roots were then rinsed and then stained with 0.05% cresyl blue by submersion at 90°C for 15 min.

Thirty fragments, chosen at random for microscopic observation, were used to estimate mycorrhization parameters: Frequency of mycorrhization (F%), intensity of mycorrhization (M%) and arbuscular (A%) and vesicular (V%) contents, According to the mycorrhization index of Trouvelot *et al* (1986).

6- Spore extraction

The spores were extracted according to the wet sieving method described by Gerdemann and Nicolson (1963). In a 1 L beaker, 100 g of each composite soil sample is submerged in 0.5 L of running water and stirred for 1 min

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with a spatula. After 10 to 30 seconds of settling, the supernatant was passed through four superposed sieves with decreasing meshes (500, 200, 80 and 50 µm). This operation is repeated twice. The content retained by the 200, 80 and 50 µm sieves was distributed in two tubes and centrifuged for 4 min at 9000 rpm. The supernatant is discarded and a viscosity gradient was thus created by adding 20 ml of a 40% sucrose solution to each centrifuge tube (Walker *et al.*, 1983). The mixture was rapidly stirred and the tube returned to the centrifuge for 1 min at 9000 rpm.

The mixture is rapidly stirred and the tube returned to the centrifuge for 1 min at 9000 rpm, the obtained substrate is rinsed with distilled water to remove sucrose and then disinfected with an antibiotic solution (Streptomycin). The spores were then recovered with a little distilled water in an erlenmeyr.

7- Sporesidentification

The isolated spores were identified according to the morphological characteristics (color, shape, size and some characteristic structures, sporuloussaccule, germination shield, bulb and suspensor), referring to the determination key of Schenk and Perez (1990) and the INVAM website.

8- Evaluation of agronomic parameters

After ten (10) months of greenhouse cultivation, the measurements concerned the height of the stem from the collar to the apex, collar diameter, number of twigs, aerial and root biomass. The mycorrhization parameters were also measured on thirteen colored fine root samples.

9- Statical analysis.

Analysis of the variance and of the mean comparisons using the LSD test (p = 5%) were performed using the software STATISTICA program. (ANOVA1).

III. RESULTS

After ten months of greenhouse cultivation, the length of mycorrhizal plants reached 59.87 cm while that of the control plants was 35.83 cm (Table 2). The average diameter of the stems and the number of branches developed in the mycorrhizal plants far exceeded those of the control plants, respectively, 3.93 cm / 7.37 and 2.83 cm / 4.66. Similarly, for the fresh weight of the root system and that of the aerial part of the inoculated plants are greater than those noted in the control plants, the gains are respectively 10.21 g and 14.18 g.

Table.2: Effect of the inoculation on the growth of the argan plants.

Measured parameters	***Inoculated Plants***	***control***
Length of the aerial plant (cm)	59.87^{a}	35.83^{b}
Average diameter at the collar of the main axis (cm)	3.93^{a}	2.83^{b}
Biomass of the aerial part (g)	27.54^{a}	13.36^{b}
Rootbiomass (g)	23.64^{a}	13.43^{b}
Average number of twigs formed per plant	7.37^{a}	4.66^{b}

Two results, same line, accompanied by the same letter do not differ significantly at the 5%

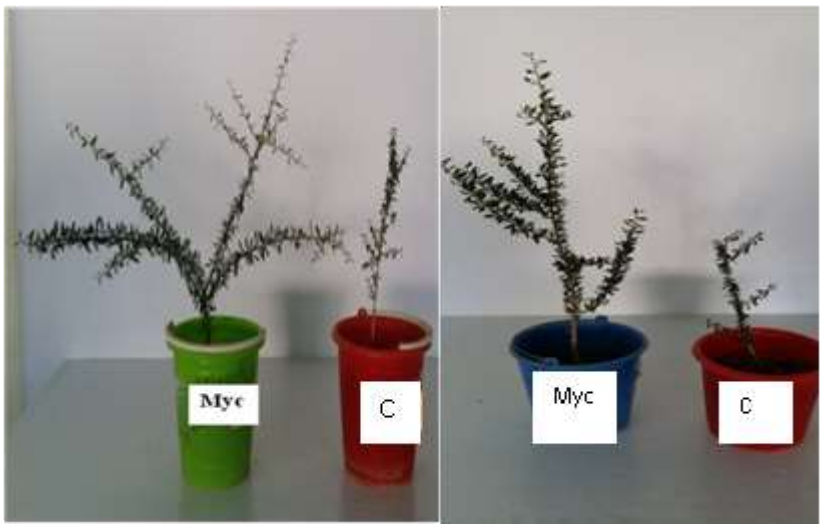

Fig.1: Effect of inoculation on thegrowth of the aerial part of the argan plant, 10 days after culture: Myc: mycorrhizal plants, C: controls.

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Fig.2: Effect of inoculation on the development of the root system of the argan plants: C: Control;Myc:mycorrhizal plant

Microscopic observation of the fragments of argan roots inoculated with mycorrhizae revealed the presence of structures of arbuscular endomycorrhizae: vesicles, arbuscules, intra and extracellular hyphae and spores **(figure 3)**. MA colonization was translated by a mycorrhizal frequency (F%) of 100% and a mycorrhizal intensity of 63.66%. While the roots of the control plants showed no mycorrhizal structure. The roots of the inoculated plants also showed a high level of arbuscules and vesicles of 51.79% and 25.52% **(figure 4).** Similarly, the mean number of spores in the rhizosphere of mycorrhizal plants is 246 spores / 100 g of soil. The identification of these isolated spores revealed the presence of 29 species belonging to six genera: *Glomus*, *Acaulospora*, *Scutellospora*, *Pacispora*, *Entrophospora* and *Gigaspora*. The *Glomus* genus was the dominate, it was represented by thirteen (13) species, namely: *Glomus aggregatum*, *G. ampisporum*, *G. clarum*, *G. claroideum*, *G. deserticola*, *Glomus* sp1, *G. etunicatum*, *G. geosporum*, *G. intraradices*, *G. macrocarpum*, *Glomus* sp2 , *Glomus versiforme*, *Glomus minitum*. The *Entrophospora* and *Acaulospora* genera were represented by five species which were respectively, *Entrophospora infenquens* and *Entrophospora nevadensis*, *Entrophospora* sp1, *Entrophospora* sp2 and *Entrophospora* sp3, *Acaulospora denticulata*, *Acaulospora reducta*, *Acaulospora* sp1 and *Acaulospora* sp2, *Acaulospora* sp3. As regards the genus Scutellospora, it is represented by four species: *Scutellospor acastanea*, *Scutellospora pellucida*, *Scutellospora* sp1 and *Scutellospora* sp2, the *Pacispora* and *Gigaspora* genera are represented by a single species respectively: *Pacispora* sp. and *Gigaspora.*

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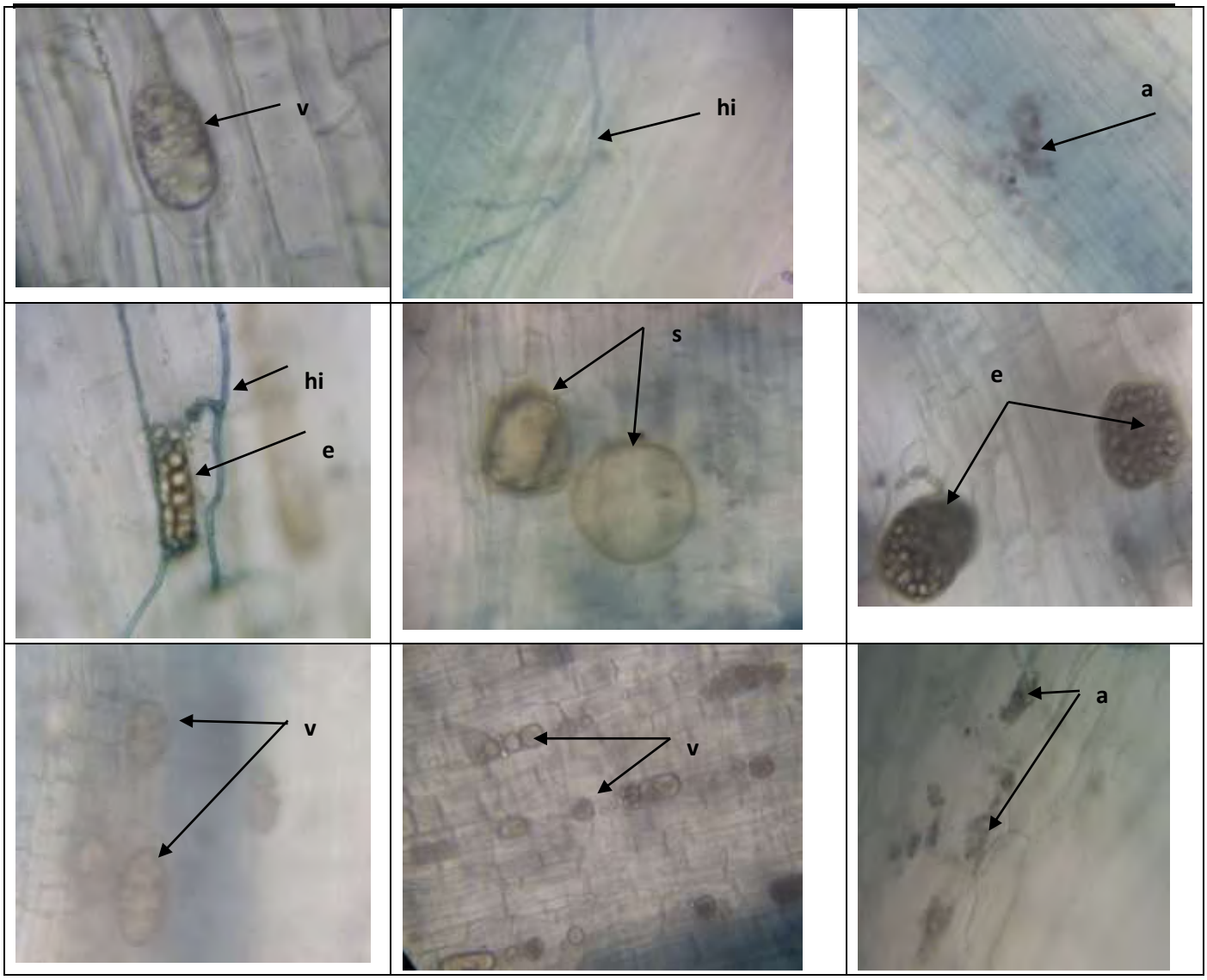

Fig.3: Different structures of arbuscular mycorrhizae in the roots of inoculated argan plants: a: arbuscule; e: endophyte; hi :internal hyphae; V: vesicule (G. ×400).

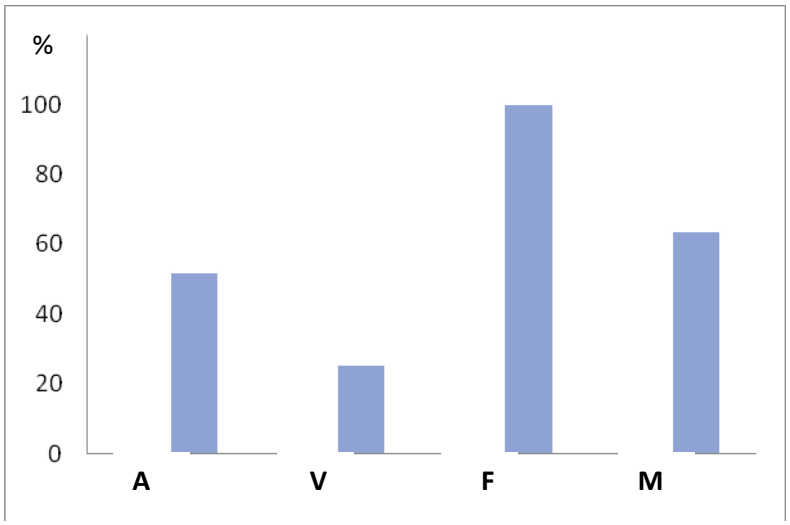

Fig.4 : Mycorrhization Parameters of the argan roots after ten (10) months of culture: mycorrhizal Frequency (F%) and Intensity (M%), Arbuscular (A%) and vesicular contents (V%).

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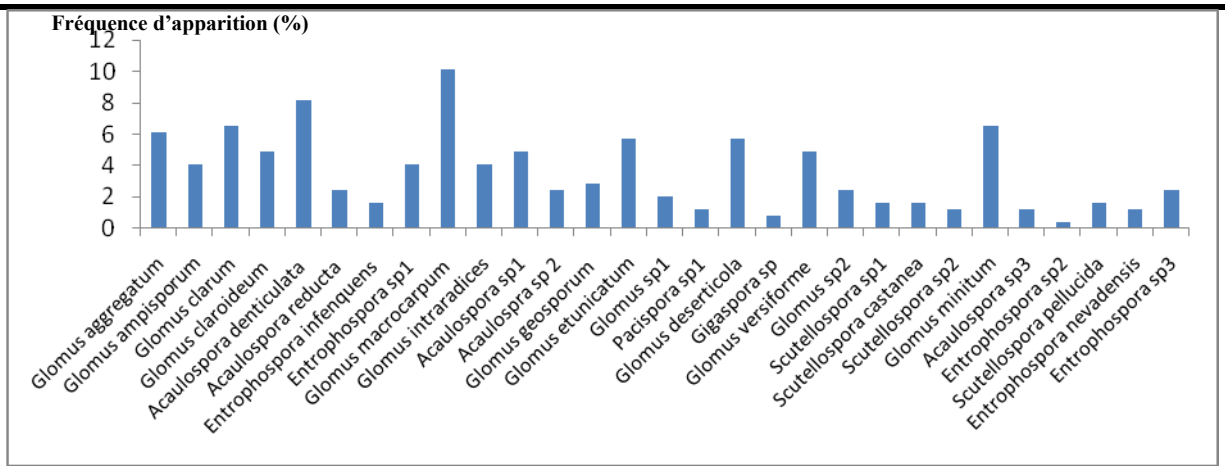
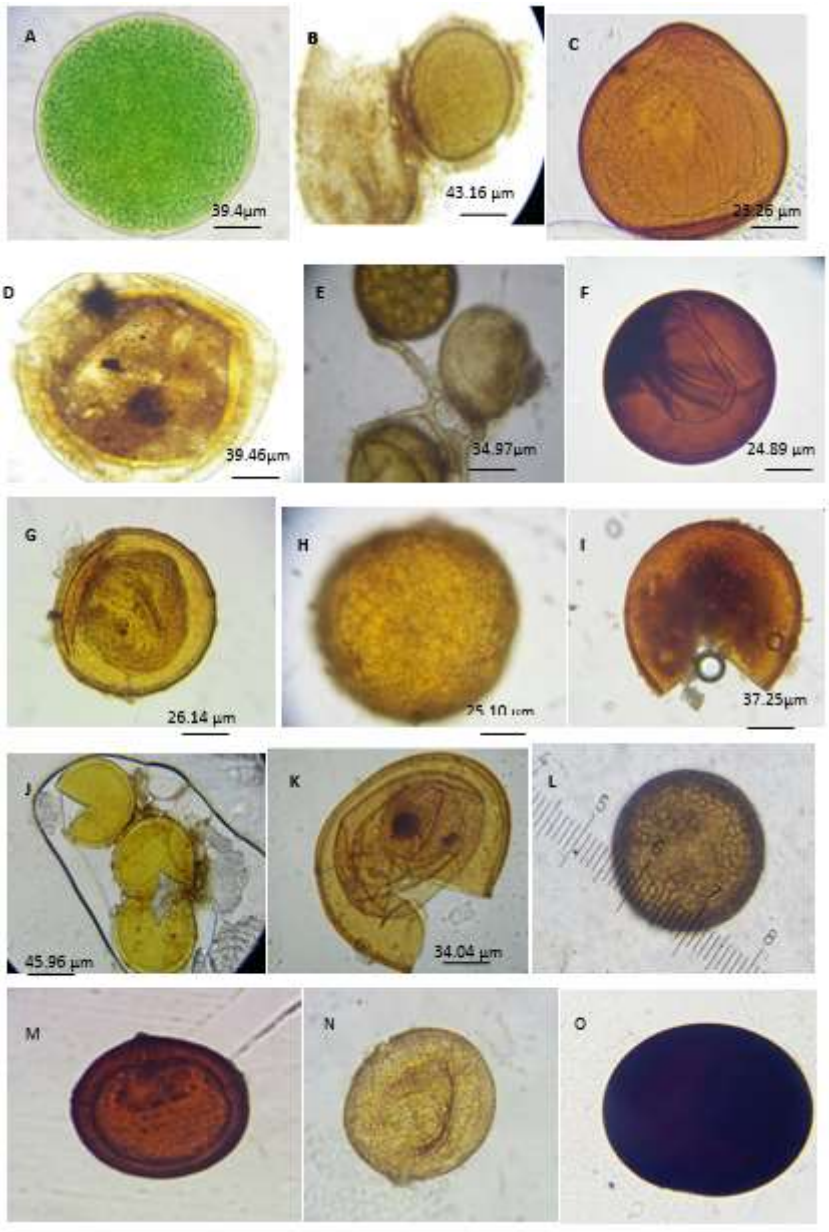


Fig.5:Isolation frequency of mycorrhizal species at the soil level of argan plants inoculated with mycorrhizae after ten (10) months of culture.



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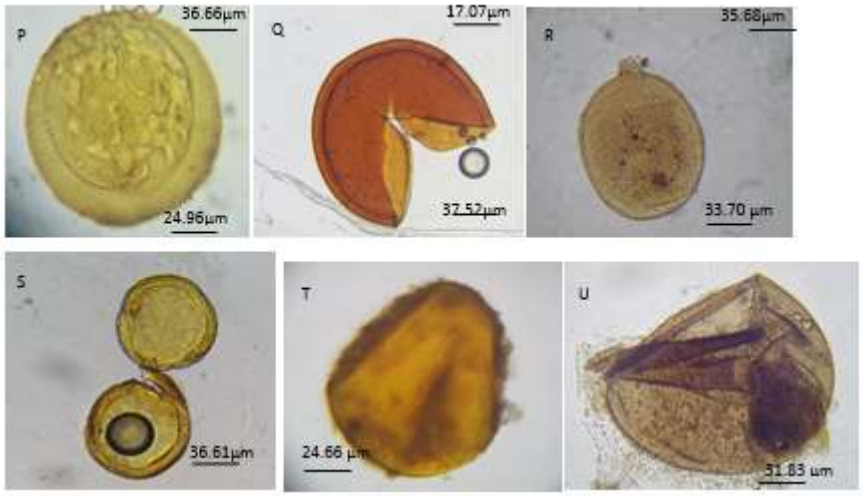

***Fig.6: Some species of endomycorrhizal fungi isolated from the argan tree rhizosphere: A** : Gigaspora sp. ; **B** : Entrophospora nevadensis; **C:** Scutellospora sp1 ; **D:** Entrophospora sp1; **E** : Glomus ampisporum ; **F** : Scutellospora biornata; ; **G** : Glomus etunicatum ; **H** : Acaulospora denticulata ; **I** : Scutellospora pellucida ; **J** : Glomus. macrocarpum ; **K** : Entrophospora sp2 ; **L** : Acaulospora reducta ; **M** : Glomus macrocarpum ; **N** : Acaulospora sp1 ; **O** : Glomus deserticola ; **P** :Glomus sp2;**Q:** Glomus aggregatum ; **R** : Glomus sp3 ; **S** : Glomus intraradices ; **T** : Entrophospora sp 3; **U** : Acaulospora sp2 .*

IV. DISCUSSION AND CONCLUSION

The present study shows that the growth of the argan plants has been improved by the presence of endomycorrhizal fungi at the level of the culture substrate. Other authors (Nouaim and Chaussod, 2002; Bousselmane *et al.,* 2002; Echairi *et al.,* 2008) reported the beneficial effect of mycorrhizal inoculation on the growth of argan tree plants from cuttings or seedlings. Thus, the response to mycorrhization of seedlings from seedlings has already been observed by other authors (Bâ *et al.,* 2001; Turjaman *et al.,* 2006).

These results are consistent with those of Laminou (2010) who show that mycorrhizal inoculation stimulates the growth of sorghum (*Sorghum bicolor* L. Moench) and cowpea (*Vigna unguiculata* (L.) Walp). Contrary to the work of Plenchette *et al.* (2000), who observed that mycorrhization of millet by *Glomus aggregatum* did not stimulate its growth.

The results on biomass showed that the productions of fresh matter are improved by inoculation, with significant differences compared to the controls. Similar responses were reported in the case of *Leucaena* inoculated with *Glomus* sp., of clover inoculated with *G. mosseae* and, in the case of *Acacia nilotica* and *Acacia senegal* inoculated with a mycorrhizal complex of native strains (Dixon *et al.,* 1993; Laaziza *et al.,* 2003; Laminou *et al.,* 2009).

The mycorrhizal argan plants showed a well-developed and highly branched root system. This important root branching in mycorrhizal plants has also been reported in other plant species: *Oleaeuropea* (Citernesi *et al.,* 1998; Chliyeh *et al.,* 2014), *Prunus cerasifera* (Berta *et al.,* 1995), *Vitis vinifera* (Schellenbaum *et al.*,1991), date palm (Sghir *et al.,* 2014), carob tree (Talbi *et al.,* 2016) and *Fragaria ananassa* (Norman *et al.,* 1996). Caravaca *et al.* (2003) showed that the root mass of *Dorycnium pentaphyllum* plants inoculated with *Glomus intraradices* increased by 116% compared to that observed in non-mycorrhizal plants. Nouaim and Chausoud (2002) showed that the inoculation of argan tree plants by *Glomus intraradices* resulted in a better efficiency of the root system. Indeed, this stimulation of root growth has been able to improve the absorption of water and mineral nutrition (Fidelibus *et al.,* 2000; Fester *et al,* 2002; Derkowska *et al.,* 2008; Stavros *et al.,* 2011), which resulted in a good development of the vegetative mass. El Mrabet *et al.* (2014) showed the effect of inoculation of argan plants by endomycorrhizae on the biomass of argan plants. At the end of the growth period, mycorrhization resulted in a 169% gain in aerial biomass compared to the control plants.

Through various physiological mechanisms (Augé, 2001), the mycorrhizal symbiosis can help the young plants to face the difficult conditions of arid zones (Nouaîm and Chaussod, 1996). The ability of the inoculum to adapt to its edaphic environment, its extra-root development and its competitiveness with the indigenous microflora are important parameters (Caravaca *et al.,* 2003). Duponnois *et al.* (2005) showed that the more inoculated plants are robust compared to non-inoculated plants, the more they could survive by showing a high capacity to resist environmental conditions.

In plantation trials, several authors have obtained significant improvements in the recovery rates, in very unfavorable environments, of many forest species such as chestnut (Strullu *et al.*, 1986), oack (Boutekrabt *et al.*, 1990), pine and hazel (Strullu and Plenchette, 1991). The contribution of fungi symbiotes improves the assimilation of water and nutrients by the plants and consequently contributes to an improvement in their recovery rate especially during the first months following their establishment in natural conditions (Nouaim, 1994).

The establishment and multiplication of endomycorrhizal fungi in the roots of the argan plants are probably at the origin of root and vegetative mass development. The frequency and intensity of mycorhization of argan plants root after ten months of cultivation were 100% and 63.66%. The work of Nouaim *et al.* (1994) showed that the frequency and intensity of mycorrhization in argan plants multiplied *in vitro* by micro-propagation were respectively 95% and 60%.

Elmrabet *et al*. (2014) showed that the effect of inoculation on the biomass of argan tree plants was positively correlated with root colonization by AM fungi. Studies by Bousselmane *et al.* (2002) showed that the argan tree showed a high rate of mycorrhization of the order of 70%, three (3) months after inoculation with a *Glomus* sp1 strain and one (1) month later by the *Glomus* sp2 strain. According to the same authors, the delay noted in the infection by the *Glomus* sp2 strain can be attributed to its weak infectivity. According to Plenchette & Fardeau (1988), the rate and duration of infection depend on three factors, namely the host used, the infectivity of the mycorrhizogenic fungus and the culture substrate.

The presence of a large number of spores (246 spores per 100 g of soil) in the rhizosphere of the inoculated plants is indicative of an important activity of the endomycorrhizal symbiosis. The species identified are of the order of 29 endomycorrhizal species belonging to six different genera: *Acaulospora, Scutllospora, Pacispora, Glomus, Entrophospora* and *Gigaspora*. The *Glomus* genus is the most dominant. According to Stutz *et al*. (2000), these representatives are the most adapted to fluctuations in environmental conditions.

The study revealed that indigenous AM fungi, based on an indigenous composite endomycorrhizal inoculum, could be considered as a preferential inoculation tool to ensure the re-establishment of native shrub species in degraded soils in semi-arid areas, the case of the argan tree.

The mycorrhization of this plants species in the nursery before the transfer to the perimeter of plantation, as demonstrated in this study, should be a mandatory step in any reforestation or silviculture program.

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Some new observations on the *Volvariella* genus Speg. 1898

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Abstract*—Three fungal species of the Volvariella genus were described in this study. Volvariella bombycina and Volvaria speciosa were harvested at the level of the Mamora forest. V. media was collected from one garden grass in the city of Kenitra, this species is new to the Moroccan fungal flora.*

Keywords— Morocco, Mamora, Volvariella, fungal flora.

I. INTRODUCTION

The Volvariella Speg genus includes about 50 species worldwide (Kirk *et al.*, 2008). It has been positioned in the family of Amanitaceae (Lee *et al.*, 1959), then in the Agaricaceae family (Lee, 1973). According to Kirk *et al*., 2008, the *Volvariella* genus belongs to the Pluteaceae family (Agaricales, Hymenomycetidae, Eubasidiomycetes, Basidiomycotina, Eumycota) (Kirk *et al.*, 2008), but recent molecular research has challenged its monophyletic and taxonomic position into the Agaricales (Moncalvo *et al.*, 2002, Matheny *et al.*, 2006). Most species of *Volvariella* are characterized by a stape with a volva at the base, absence of ring, free and spaced lamellae and pink spores with a more or less thick wall (Imai, 1938; Singer, 1986; Kühner & Romagnesi 1956; Courtecuisse et Duhem, 2000 and Roux, 2006). Monographic studies of this genus have been mainly used in Europe (Kühner & Romagnesi 1956; Orton 1974, 1986; Boekhout 1990) north America (Shaffer 1957) and in Africa (Heinemann, 1975 and Pegler, 1977). Six species of Pluteaceae belonging to the *Volvariella* genus have been reported in the flora of the upper mushrooms of Morocco (*Volvaria bombycina; Volvaria murinella; Volvaria parvula; Volvaria plumulosa; Volvaria pusilla; Volvaria speciosa*) (Malençon & Bertault, 1975).

In this work, three species of the *Volvariella* genus, encountered in the Mamora forest, were studied: *Volvariella bombycina*, *V. speciosa* and *V. media.*

II. MATERIALS AND METHODS

Surveys were carried out in the cork oak forest of Mamora (North-West of Morocco) between 2009 and 2014 allowed us to study the fungal flora of this region. Specimens of the *Volvariella* Speg. genus were collected and returned to the laboratory. The macroscopic descriptions were based on morphological characters (Shape, color, size, appearance,…) as well as other particularities of the cap and stipe (odor, flavor,…). This study was supplemented by a microscopic description of the spores and sections in the hymenium, cuticle, flesh and stipe. The dimensions of spores, cystidia, basidia and sometimes sterigmata are measured within a large-field micrometric eyepiece 10× (18mm) scale of 10 mm divided into 100 graduations (0.1mm). Microscopic observations were made using an optical microscope (magnification × 400). The mounting liquid is tap water. The forms of the basidiospores are obtained from the calculation of the quotient of Bas (Q= L/l, L and l are respectively the length and the width of the spore in µm) (Bas, 1969).

Identification of the species was carried out by consulting the references of Malençon and Bertault (1970), Courtecuisse and Duhem (2000) and Roux (2006).

III. RESULTS

Three species of the *Volvariella* genus have been described in this study (*Volvariella bombycina*, *Volvaria speciosa* and *Volvariella media*), of which *Volvariella media* is new for the fungal flora of Morocco.

Volvariella bombycina (*Volvaria bombycina*) (Schaeff.) P. Kumm. 1871.

Lignicolous species harvested on 02/03/2009 and 12/08/2014 in the hollow trunks of *Quercus suber* in the forest of Mamora.

The cap (9-12 x 1 cm) is fluffy, silky, convex to plano-convex and white to pinkish to pale yellowish (Figure 49, A and B). **The flesh** is thick in the center, thin at the edges and whitish. The margin is inflected. The stipe (7-13 x 0.6-1 cm) is central, cylindrical, solid, firm, almost glabrous, thick and whitish cream colored. **The volva** is broad, black and spotted with brown. **The lamellae** are tight, wide, free, uneven and white to pink darker as they age.

The basidia (20 x 8 microns) are calviformes, hyaline and tetrasporic. **The sterigmata** are 6 to 8 µm (Figure 49,

D). **The basidiospores (**6.6-8.5 x 4.5-5 µm) are elliptic and pink in color (1.3 <Q <1.7). **The pleurocystidia** (63 x 10 µm) are fusiform and hyaline (Figure 1).

Volvaria speciosa (*Volvopluteus gloiocephalus*) (Fr.) P. Kumm. 1871

Lignicolous species harvested on 20/03/2009 and 08/12/2014 on the living trunks of Quercus suber in the forest of Mamora.

The cap (8 to 13 cm) is parabolic then flared raised center with a rounded nipple and color: yellowish gray, pale yellow to white. **The flesh** is thin, elastic and concolorated to the cap. **The margin** is somewhat inflected. **The stipe** (19.5-20 x 1.5-2 cm) is robust, flared under the gills, full, calviform towards the base and white or cream colored. **The volva** is short, fairly firm and whitish. **The lamellae** are tight, free, uneven and white and then pink to the pink-ocher end.

The basidia (40 x 13 microns) are calviformes sub-hymenium very long, hyaline and tetrasporic. **The sterigmata** are 3.3 µm (Figure 50, C). **The basidiospores** (8-10 x 4.5-5 µm) are elliptic (1.3 <Q <1.7), amygdaliform, smooth and pink (Figure 50, D). **The pleurocystidia** (103 x 45 µm) are piriform to base more or less stretched and topped with a digiform expansion (Figure 2).

Volvariella media (*Volvaria media*) (Schumach.) Gillet 1876

The species was collected on 28-08-2013 from one garden grass of *Stenotaphrum secundatum* in the city of Kenitra.

The cap (4 to 6.5 cm) is parabolic then flattened (depressed), circular, smooth, viscous and creamy white. **The flesh** is thick in the center, thins towards the margin and is whitish in color. **The margin** is straight and striated. **The stipe** (8-9.5-20 x 0.5-0.6 cm) is cylindrical, solid, central, striped, glabrous, bulbous and white or cream colored. **The volva** is thin, fairly firm and whitish (Figure 51, C). **The lamellae** are loose, free, uneven and white in color and then pink to pink-briquetted. **The lamellar edge** is regular and whitish. **The basidia** (63.3 x 13.3 microns) are calviformes, sub-hymenium very long, hyaline and tetrasporic. **The sterigmata** are 4.5 µm. The basidiospores (11.6-13.3 x 8.5-10 µm) are elliptic (1.3 <Q <1.7), amygdaliform, smooth and pink. **The pleurocystidia** (76.6 x 13.3 µm) are cylindrical and hyaline (Figure 3).

IV. DISCUSSION

The *Volvaria* Fries (1821) genus is antedated by *Volvaria* de Candolle (1805), who designates a lichen, some modern authors substitute for it *Volvariella* Speggazini (1899), which has priority over *Volvariopsis* Murrill (1911) (Malençon and Bertault, 1970).

In Morocco six species of the genus *Volvaria* were encountered by Malençon and Bertault (1970), five of which are described (*Volvaria bombycina* (Schaeff.) Singer (1951), *V. murinella* Quél. (1883), *V. parvula* (Weinm.) P. Kumm. (1871), *V. pusilla* var. *biloba* Massee, ss. J. Lange et *V. speciosa* (Fr.) P. Kumm. (1871) and *Volvaria plumulosa* Lasch ex Quél. (1878), reported without specifying the substrate and the place.

Volvaria gloiocephala (DC.) Gillet (1876), was encountered in the forest of the Mamora (El Assfouri, 2006), near to the central plateau (Haimed, 2007) and under *Quercus rotundifolia* in the Middle Atlas (Larouz, 2007) and *V. gloiocephala* var. *speciosa* in the gardens of Kenitra (forest of the Mamora) (El Assfouri, 2006).

Volvaria bombycina was first described in 1774 by the German naturalist Jacob Christian Schäffer as *Agaricus bombycinus*. Throughout its taxonomic history, it has been redesigned to several genera, including *Pluteus* (Fries, 1836), *Volvaria* (Kummer, 1871) and *Volvariopsis* (Murrill, 1911). Whereas in 1951, it was placed in its current type *Volvariella* (Singer, 1951). This species is considered a rare and isolated generally believed mostly in autumn and winter. It occurs on *Quercus suber* and sometimes on *Quercus faginea* and on *Populus* (Malençon et Bertault, 1970). It has been reported in Europe, Africa, Asia, North and South America and Australia (Justo *et al.*, 2011) and prefers low and high altitudes (Heinmann 1975). However, this species is considered a very important edible mushroom with chemical and nutritional characteristics (Mallavadhani *et al.*, 2006), and has antioxidant, anti-tumor and hyporcholesterolemic effects (Badalyan & Suzanna, 2003). Jegardeesh *et al.* (2010) reported *V. bombycina* as an ideal edible food for health by its richness in protein and mineral salts and it contains dietary fiber that allows good digestion. This mushroom has chemical compounds that can be used as antibacterial agents in new medicines for infectious disease therapy caused by pathogens (Jegardeesh *et al.*,2010).

Volvaria speciosa, is an edible species, has long been considered poisonous by confusion with *Amanita phalloides* Secr. 1833 (Malençon and Bertault, 1970). Otherwise, *Volvariella gloiocephala* and *V. Speciosa* are currently considered to be co specified (Orton, 1974, Boekhout and Enderle, 1986; Boekhout, 1990). *Volvariella gloiocephala* was created from *V. speciosa* mainly by its grayish brown cap, while that of *V. speciosa* is whitish (Shaffer, 1957; Coutecuisse, 1984). However, the original description does not provide any arguments for this distinction since De Candolle (1815) described the cap of *Agaricus gloiocephalus* (DC.) like a gray white mouse, while Fries (1818) described the cap of *Amanita speciosa* (Fr.) like a white to gray center

(Boekhout & Enderle, 1996). In addition, the two colorations were experimentally obtained from the same mycelium (Herrmann, 1973).

Volvariella media (Schum.: Fr.) Singer ss. Quélet, is characterized by a cap of 3 to 6 cm in diameter, ivory, and whitish, a stipe (3-6 cm) with wholly volva and spores 11 to 16 µm in length and 7 to 8 µm in width. This species resembles *Volvariella gloiocephala*, but it is only a smaller and more slender form that develops on poor substrates (Orton, 1986; Gerault, 2005). However, Larouz (2007) and Haimed (2007) described *Volvariella gloiocephala*, but with macroscopic and microscopic characteristics that are very distinct from those observed in *Volvariella media*. To cope with these different characteristics, we have drawn up a comparative table between the descriptions of *Volvariella gloiocephala* described by Larouz (2007) and Haimed (2007) and those of *Volvariella media* presented in this study (Tableau 1).

Similarly *Volvariella media* has been described under the name of *Agaricus medius* (Schumacher, 1803). This species is very close to *Agarcicus speciosus*, but is distinguished by a small white cap and free lamellae (Fries, 1821; Lange, 1935-1940).

Table.1: Comparison between Volvariella gloiocephala described by Larouz (2007) and Haimed (2007) and Volvariella media and Volvariella gloiocephala described in this study.

	Volvariella gloiocepala described by Larouz (2007)	Volvariella gloiocepala described by Haimed (2007)	Volvariella media described in this study
Cap	(9 cm) greyish white, ovoid becomes campanulate then spreads out with age keeping a central nipple.	(6 to 11 cm) campanulate, yellow and gray to pinkish state with age, often hilly, viscous and furrowed margin short streaks	(4 to 6.5 cm), parabolic and then flattened (depressed), circular, smooth, viscous and creamy white.
Stipe	(7 × 1 cm) is white, firm and cylindrical thickening towards the base enclosed in a volva.	(11-17 × 1.5 cm) cylindrical, glabrous, whitish to light fawn to ample volva.	(8-9.5-20 x 0.5-0.6 cm) is cylindrical, solid, central, striped, glabrous, bulbous and of white color or cream with thin volva, fairly firm and whitish.
Lamellae	Free, fine, tight and white become pinkish at maturity.	Free, broad, clenched at first white and then pinkish.	Little tight, free, uneven and white in color and then pink to pink-briquetted.
Basidia	Clave and tetrasporic.		Claviform, sub-hymenium, hyaline and tetrasporic.
cystidia	In bulbs		(76.6 x 13.3 µm) are cylindrical and hyaline.
Basidiospres	16.6-18.3 × 6.7-8.3 µm.	18 × 8 µm.	(11.6-13.3 x 8.5-10 µm) are elliptical.

V. CONCLUSION

In this study, three species (*Volvariella bombycina*, *V. speciosa* and *V. media*) belong to the *Volvariella* genus, two of which (*Volvariella bombycina*, *V. speciosa*) have already been reported and described in Morocco by Malençon and Bertault (1970), El-Assfouri (2006), Haimed (2007) and Larouz (2007), while *Volvariella media* is newly described for the fungal flora of Morocco. However, a comparison between the latter species and *Volvariella glioicephala* allowed us to confirm the nomenclature of *Volvariella media*.

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Fig.1: Surface of the cap (A) and (B) insertion of the lamellae, stipe and volva (C), Basidia (C and D), basidiospores (E) and cheilocystidia (F) of Volvaria bombycina (×400).

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Fig.2: Cap surface (A) insertion of the lamellae, stipe and volva (B), Basidia (C), basidiospores (D) of Volvaria speciosa (×400)

Fig. 3: Cap surface (A), (B) insertion of the lamellae and stipe (B), volva (C), basidia(D), cheilocystidia (E) and basidiospores (F) of Volvaria media (×400).

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In Vitro Study on total Phenols, Flavonoids Content and DPPH Activity of *Withania* Species

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Abstract— *The escalating interest in appraisal of antioxidant power of herbal plant as medicine, the current study was carried out to explore the antioxidant potential of aqueous extracts of Withania somnifera root and Withania coagulan fruit in-vitro. Antioxidant activity; total phenol,total flavonoids and DPPH free radical scavenging assay of Withania somnifera root and Withania coagulans fruit aqueous extracts were determined by using reference standards gallic acid, quercetin and ascorbic acid, respectively. The highest total phenols content (mgGAE/g) and total flavonoids content (mgQE/g) was found to be 33.1±0.82 and 1.86±0.01 respectively in aqueous somnifera root extracts as compared to coagulans fruit extract . The DPPH radical scavenging activity of the both extracts was increased with the increasing concentration and was observed high in aqueous extract insomniferaroot (IC_{50}= 54) than coagulans fruit (69µg/ml) aqueous extract.Thus,Withania somnifera root* has potent antioxidant activity *and may serve as a good pharmacotherapeutic agent which could be explored to provide affordable medicines to masses.*

Keywords— DPPH (2, 2-diphenyl-1-picrylhydrazyl), Total Flavonoids Content, Total Phenol Content, Withania somnifera, Withania coagulans.

I. INTRODUCTION

The use of medicinal plants are increasing day by day in developing and developed countries due to their no side effects[1]. Traditional herbal medicines are new therapeutic contender because of their structural complexity, chemical diversity and wide variety of antimicrobial activity[2].The genus *Withania* (Family: Solanaceae) is a highly-acclaimedremedial plants in the Indian Ayurvedic system of medicine because of its valuable pharmaceutical and nutraceutical properties. It is a small group of herbs distributed from the Northern Africa to the South-west of Asia. Among the twenty-three-known species of *Withania*, only two (*Withania somnifera* and *Withania coagulans*) are economically significant medicinal plant[3].

Withania somnifera commonly known as "Ashwangandha" or "Indian Ginseng" and is an important plant in Indian traditional Ayurvedic system of medicines[4]. Ashwagandha improves energy and also memory by enhancing the brain and nervous function; shows anxiolytic effects, has hepatoprotective property, raises haemoglobin level and red blood cell count, improve energy level, improve the cell-mediated immunity; promotes vigour and vitality along with cheerful sexual life and reproductive equilibrium and act as powerful adaptogen[5].*Withania coagulans* also known as 'Indian cheese maker' or 'vegetable rennet'[6]. It is traditionally used as digestant, anti-flatulent, sedative, antihyperglycemic antimicrobial, anti-inflammatory, antitumor, hepatoprotective, cardiovascular, immune-suppressive, free radical scavenging and central nervous system depressant activities[7].

There are several plants used in Ayurvedic medicinal systems origin with potential therapeutic activity, which are widely used as Ayurvedic medicine[8]. Therefore, an endeavour has been made to investigate the antioxidant properties of *Withania somnifera* root and *Withania coagulans*fruit.

II. MATERIALS AND METHODS

Sample Collection

Withania somnifera roots and *Withania coagulan* fruits were collected from local market of Delhi and authenticated by scientist of National Institute of Ayurveda, Jaipur. Rajasthan. Roots and fruits were washed and dried in open air for 2-3 weeks at 35-40º C and then dried material was pulverized in an electric grinder and stored in plastic containers in refrigerator (5^0 C), until further analysis.

Aqueous Extract Preparation

20 g of powdered plant material was kept in 200ml conical flask and add 100 ml of distilled water. The mouth of the conical flask was covered with the aluminum foil and kept in a reciprocating shaker for 25 mins for continuous agitation at 150 rev/min for thorough mixing. Then extracts was filtered by using muslin cloth followed by Whatmann

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filter paper No. 42 (125mm). The content was filtered by using rotatoryvacuum evaporator with the water bath temperature of 65º C and finally the residue were collected and used for the analysis[9].

Withania coagulan

Withania somnifera

Determination of Total Phenols Content:

Total phenols were determined by Folin-Ciocalteu Reagent. A dilute extract of root and fruit (0.5 ml of 1:10g/ml) or Gallic acid (standard phenol compound) was mixed with Folin-Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) and 4 ml of aqueous sodium carbonate (1M). The mixtures were kept at dark ambient condition for 15 min and the total phenols were determined by spectrophotometer at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250mg/l solutions of Gallic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of Gallic acid equivalent (mg/g of dry mass), which is a common reference compound[10].

Determination of total flavonoids content:

Aluminum chloride colorimetric method was used for flavonoids determination. Root and fruit extracts (0.5 ml of 1:10 g/ ml) in aqueous were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a UV-Visible spectrophotometer. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 g/ml in aqueous[11]

DPPH radical scavenging activity:

The ability of the aqueous extracts to scavenge free radicals was determined against a very stable free radical DPPH (2, 2-diphenyl-1-picrylhydrazyl) determined Spectrometric method. Aliquots of the sample extract at different concentrations 20-200 µg/ml were added to 1 mm aqueous solutions of DPPH. Each mixture was vortexes vigorously and left for 30 min at room temperature in the dark. The absorbance was measured at 517 nm and activity was expressed as percentage. IC_{50} value was also determined by graph[12]. DPPH scavenging relative to control using the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \text{ X } 100$$

Statistical Analysis:

The results obtained were expressed as mean ±SD and student t-test of three determinations and also statistically analyzed to ascertain its significance. The significance was estimated at ($p \leq 0.05$level).

III. RESULTS AND DISCUSSION

Table.1: Antioxidant potential of Withania somnifera root and Withania Coagulans fruit aqueous extract

Antioxidants	*Withania Somnifera root*	*Withania Coagulans fruit*
TPC (mgGAE/ml)	33.1±0.82	14.5±0.78*
TFC (mgQE/ml)	1.86 ±0.01	1.08±1.7ns

(n=3) Values are expressed as means±SD.* significant, ns-non-significant when compared with *W.coagulan* aqueous extract at P ≤0.05. TPC-,Total Phenol Content TFC-Total Flavonoids Content

It has been documented that phenols as well as flavonoids show antioxidant activity and their effects on human nutrition and health through scavenging or chelating process[13]. In the present study, total phenols content of *Withania somnifera and Withania coagulans* aqueous

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extractexpressed in Gallic equivalents (GAE) were found to be 33.1±0.82 and 14.53±0.78 GAE mg/g respectively as shown in Table 1.The data showed that *somnifera* root powder was significantly increased by 56.19% from *coagulans* fruit aqueous extract at P ≤0.05 levels. The flavonoids content was measured by aluminum chloride technique in terms of quercetin equivalent and found low value in both extracts of *Withania somnifera* and *Withania coagulan* had 1.86.±0.01 and 1.08 ±1.7 (mgQE/ml) respectively. *W. somnifera* aqueous extract was insignificantly increased by at 22.54 % at p≤0.05 when compared to *W. coagualan.* According to study based on phytochemical analysis showed that *W. somnifera* had 180.80±0.01 mg/100mg gallic acid equivalent phenolic content whereas flavonoid content was 136.97 ±0.01 mg/100 mg quercetin equivalent in*Withania somnifera* root methanolic extract[14].

The medicinal effects described in the ayurveda that traditional plants have excellent phenolic acids and flavonoids content that are important ingredients to prevent against oxidative stress related disorders[15]. The results acquired in this study thus suggest the identified bioactive constituents in both *Withania* species is proving to be a valuable reservoir of bioactive compounds of substantial medicinal merit.

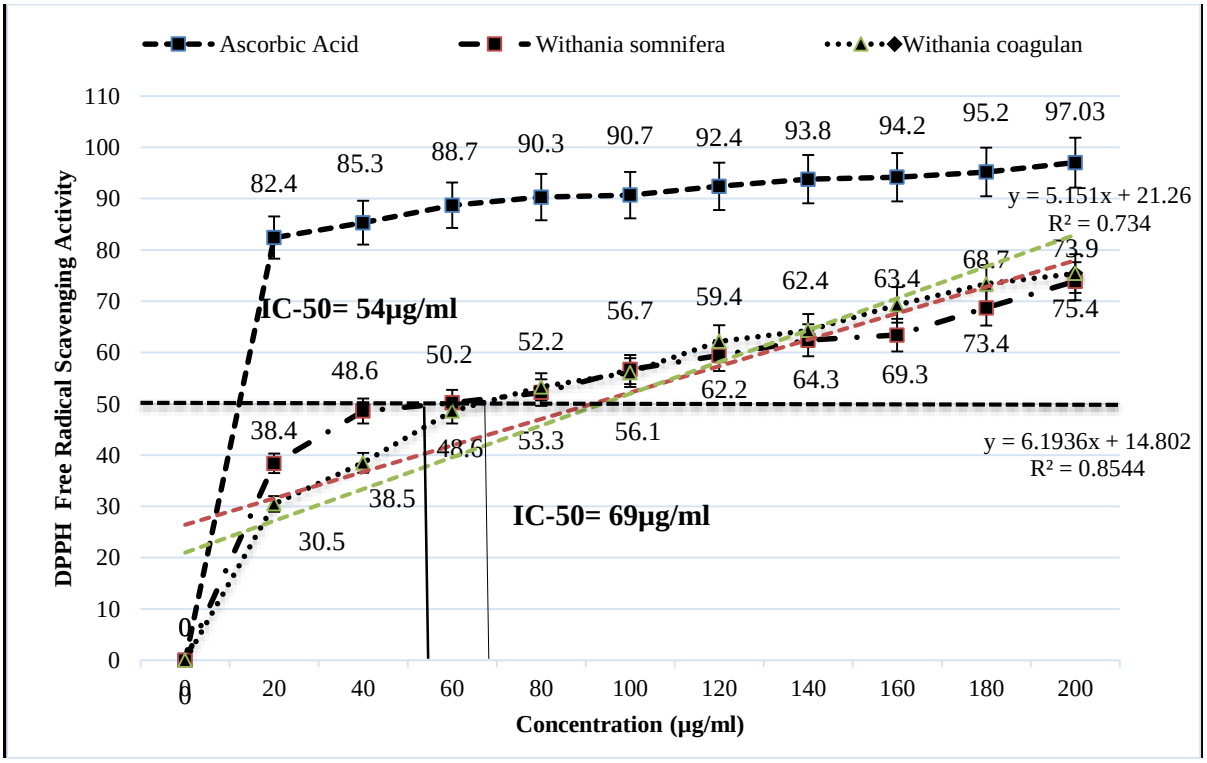

Fig.1: Scavenging effects of the aqueous extract of Withania somnifera and Withania Coagulans on DPPH at Different concentration.

DPPH free radical scavenging method is a sensitive way to determine the antioxidant activity of plant extracts. The reduction capability of free radicals was determined by decrease in its absorbance at 517nm[16]. Several concentrations ranging from 20–200 µg/ml of the aqueous extract of *Withania somnifera* and *Withania coagulan*were tested for their antioxidant activity in different in vitro models.IC_{50} value is defined as the concentration of substrate that causes 50% loss of the DPPH activity and was calculated by linear regression mentioned of plots of the percentage of antiradical activity against the concentration of the tested compounds[17]. The percentage scavenging at IC_{50} values were intended for both extracts were illustrated in Fig 1. It shows that there was a decrease in the concentration of DPPH radicals due to the scavenging ability of the soluble constituents in the aqueous extract of *W.somnifera and W.coagulans* and the standard ascorbic acid as a reference compound. As the concentration increases the free radical scavenging activity of plant extract also increases. The data depicts that IC_{50}value was 54µg/ml *for W. somnifera* and 69µg/ml *for W. coagulans* which was lower value according to data shown by Shariar[18] that methanol extract of *Withania somnifera* root showed IC_{50} of 267.818 µg/ml. Similar data stated by Nadia

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Alam[19] that the *Withania somnifera* extracts was 101.73-801.93 µg/ml at IC_{50} which indicated higher value from present data.

IV. CONCLUSION

Although both *Withania* species had potent antioxidant activity but from the study data indicated that *Withania somnifera* aqueous root extract showed higher bioactive compounds as well as DPPH radical scavenging activity when compared to *Withania coagulans* fruit aqueous extract. Thus,*W. somnife*ra root will definitely serve as a high-quality phytotherapeutic agent in various metabolic and degenerative diseases.

V. FUTURE PROSPECTIVE

The phytochemistry and pharmacology characteristics of both *Withania* species have been widely investigated but the studies on toxicology of the extracts in different solvents are very few. So,further exploration is needed for identification and isolation of the particular compound responsible for the specific activity.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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Study on Performance of Different Fodder Crops under Low Cost Green House Hydroponic Fodder Production System

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Abstract— *Hydroponics play most significant role in augmenting fodder shortage and helps for dairy production efficiently. A study was conducted to assess the performance and suitability of different crops under low cost green house hydroponic fodder production unit at SHE&CS Krishi Vigyan Kendra, Yagantipalle. Four varieties of cereals grains and four verities of Pulses were tested. One kilogram grain each of the variety was soaked for 12 hours in water for sprouting in air tight condition for 36 hours. The sprouted seed was spread in trays of size 2.5 ft X 1.5ft and kept in the Hydroponic Unit. Automatic sprinkling of water was managed by cyclic timer. Chemical fertilizer was not used. Data on sprouted seed weight and weight of biomass after 5 days was recorded using electronic weighing balance. The high biomass yield after 5days in cereals was recorded in Bajra followed by sorghum, Barley and Maize. Among pulses Pillipesara yielded highest weight followed by Cowpea, Lucerne and Horse gram. Highest plant height among cereals was recorded in Barley and cowpea in pulses. The difference among all the varieties in respect of biomass yield and plant height was found to be significant. Negative correlation was found between plant height and biomass yield.*

Keywords— Low cost hydroponics, Fodder yield, hydroponic fodder production system.

I. INTRODUCTION

Green fodder plays a vital role in production and reproduction performance of dairy animals. Unavailability of land, resources and labour requirement are the major constraints faced by dairy farmers. Non availability of quality fodder round the year is major limitation for sustainable dairy farming (Naik et al, 2015). Fodder shortages are continuously hindering the milk production in these parts of arid region. To overcome this problem hydroponics is an alternative for successful feeding of dairy animals. Hydroponic fodder can be produced in low cost green houses (Naik, 2015). Maize and barley are the most common fodders which can be successfully grown under hydroponics. The present experiment was conducted to assess the growth of different pulse and cereal fodder crops under low cost green house type hydroponic unit.

II. MATERIALS AND METHOD

Four varieties of cereal grains viz., Maize (T1), Sorghum (T2), Bajra (T3), Barley (T4), and four verities of Pulses viz., Cowpea (T5), Lucerne (T6), Horse Gram (T7), PilliPesara (T8) were tested. One kilogram grain in each of the variety was soaked for 12 hours in water for sprouting in air tight condition for 36 hours. The sprouted seed was spread in U.V. stabilized plastic trays of size 2.5 ft X 1.5ft and kept in the Hydroponic Unit. Automatic sprinkling of water was managed by cyclic timer. Chemical fertilizer was not used. Data on sprouted seed weight and weight of biomass after 7 days was recorded using electronic weighing balance and plant height was recorded using measuring scale. The data was statistically analyzed for its significance.

III. RESULTS AND DISCUSSION

Biomass yield:

The data recorded on the biomass yield of different cereal and pulse grains are given in table1. The data revealed that more weight of sprouted seed of cereals was observed in Bajra (2.17kg) followed by, sorghum (2.04kg), Barley (1.98kg), Maize (1.08Kg). In pulses high sprouted seed weight observed in Cowpea (3.45kg) followed by Pillipesara (3.26kg), Lucerne (3.22Kg) and Horse gram (2.88kg). Significant and positive correlation between sprout weight and biomass yield was observed among all.

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The high biomass yield after 5days in cereals was recorded in Bajra (6.37kg) followed by sorghum (6.1kg), Barley (5.06kg) and Maize (4.82kg). Among pulses Pillipesara (7.58kg) yielded highest weight followed by Cowpea (7.2kg), Lucerne (7.09kg) and Horse gram (5.85kg). The difference (P>0.05) among all the varieties in respect of biomass yield was found to be significant. Naik *et al* (2015) reported fresh yield of 8-10kg from one kg locally grown maize in 7-10 days under low cost devises. Brunean M.M. *et al* (2016) reported one kilogram of barley seeds provide 2.31 to 4.89 kg of green fodder.

Table.1: Showing day wise biomass yield (kg) of different varieties of cereal and pulses under low cost hydroponic fodder production unit.

Crop	Day wise biomass yield (kg)					
	Sprout weight	**One day**	**Two days**	**Three days**	**Four days**	**Five days**
Maize	1.08	1.46	2.07	2.36	4.1	4.82
Sorghum	2.04	2.87	3.57	4.57	5.285	6.1
Bajra	2.17	3.1	3.89	4.96	5.665	6.37
Barley	1.98	2.69	3.37	4.21	4.635	5.06
Cowpea	3.45	4.37	4.82	5.97	6.585	7.2
Lucerne	3.22	4.46	5.38	5.89	6.49	7.09
Horse gram	2.88	3.73	4.45	5.1	5.49	5.88
Pilli pesara	3.26	4.07	4.62	5.96	6.77	7.58
Mean	**2.323**	**3.158**	**3.850**	**4.66**	**5.46**	**6.09**
S.D.	0.824	1.465	1.925	2.319	2.748	3.18
S.E.	0.291	0.488	0.609	0.733	0.829	0.918

Plant height:

The data recorded on day wise height of the plants of different cereal and pulse grains are given in table2. The data revealed that the highest height of the plants after 5days in cereals was recorded in Barley (18.4cm) followed by Maize (18.1cm), sorghum (10.8cm) and bajra (7.9cm). Among pulses Cowpea (25.33cm) recorded highest height followed by pillipesara (11.16cm), Lucerne (8.56cm) and Horse gram (7.46cm). The difference (P>0.05) among all the varieties in respect of plant height was found to be significant. Negative correlation was observed among biomass yield and plant height after five days.

Table.2: Showing day wise plant height (cm) of different varieties of cereal and pulses under low cost hydroponic fodder production unit.

Crop	Day wise plant height (cm)				
	One day	**Two days**	**Three days**	**Four days**	**Five days**
Maize	1.33	3.03	7.8	11.33	18.1
Sorghum	0.53	1.76	2.26	4.36	10.8
Bajra	0.6	2.26	3.9	5.26	7.9
Barley	1.53	2.36	4.53	7.26	18.4
Cowpea	2.91	6.13	7.63	10.43	25.33
Lucerne	1.86	2.56	2.46	2.73	8.56
Horse gram	0.6	2.86	4.86	5.4	7.46
Pilli pesara	1.53	2.76	3.9	5.63	11.16
Mean	**1.36**	**2.96**	**4.66**	**6.55**	**13.46**
S.D.	0.807	1.253	2.087	2.967	6.438
S.E.	0.285	0.418	0.738	1.049	2.276

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IV. CONCLUSIONS

Low cost hydroponic fodder production technology play key role in intensive fodder production especially for land less dairy farmers. Cereals and Pulses can be successfully grown for fodder in low cost hydroponic system. Among the cereals Bajra and in pulses Pillipesara recorded highest bio mass within five days. Significant and positive correlation was found between sprout weight and bio mass yield among all the grains and difference among all the varieties in respect of biomass yield was found to be significant at 5% level. Among cereals Barley and pulses cowpea recorded highest plant height after five days. Negative relation was observed among biomass yield and plant height after five days.

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Influence of Growth Regulators on Shedding of Broad Bean, Growth, Yield and Seed Quality

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Abstract— *In order to study the effect of foliar spraying of growth regulators on growth,seed yield and seed quality, two field experiments were conducted at an extensive field during 2014/2015 and 2015/2016 seasons to determine following foliar spraying of Naphthalene Acetic Acid (NAA) concentrations, i.e. 0, 20, 40 and 60 ppm and Kinetin (Kin) concentrations, i.e. 0, 15, 30 and 45 ppm after 35 and 50 days from sown. Accumulative NAA levelsof to 60 ppm significantly increased total chlorophyll, plant height (cm),branches number/plant, number of shedding flowers, shedding %, pods and seeds number/plant,seedsnumber/pod, seed yield/plant, 100-seed weight (g), seed yield (ton/ha) and protein % in both seasons.Naphthalene Acetic Acid foliar spraying up to 60 ppm exceeded of total chlorophyll, plant height (cm), branchesnumber/plant, number of shedding flowers, podsnumber/plant, seedsnumber/pod, seedsnumber/plant, seed yield (g) /plant, 100-seed weight (g), seed yield (ton/ha) and protein % by 11.47, 23.92, 92.88, 20.53, 11.87, 23.48, 14.16, 24.91, 26.15 and 13.23%, respectively as the average of both seasons. But, reduced the shedding percentage by 11.91% as the average oftwo seasons. Kinetin (Kin) foliar spraying up to 45 ppm significantly increased total chlorophyll, plant height (cm), number of branches/plant, number of shedding flowers, shedding %, pods and seeds number/plant,number of seeds/pod, seed yield/plant, 100-seed weight (g), seed yield (ton/ha) and protein % in both seasons. It could be noticed that foliar spraying of Kinetin (Kin) concentrationsup to 45 ppm exceeded total chlorophyll, plant height (cm), branchesnumber/plant, number of shedding flowers, podsnumber/plant, seedsnumber/pod, seedsnumber/plant, seed yield /plant, 100-seed weight (g), seed yield (ton/ha) and protein % by 12.16, 19.39, 61.64, 5.60, 5.56, 6.96, 5.64, 18.75, 13.38 and 4.39%, respectively as average of both seasons. But, reduced the shedding % by 14.73 % as the average of both seasons.It could be recommended that foliar spraying of Naphthalene Acetic Acid up to 60 ppm and Kin of 45 ppm improved seed yield/ha by 38.2% compared without foliar application.*

Keywords— Naphthalene Acetic Acidand Kinetin levels, shedding percentage.

I. INTRODUCTION

Vicia faba L.is considered the most significant winter crops for human and animal consumption of the Middle East. The lack of adequate pollination and reduced seed set can be major constraints to yield. Flower drop and seed abortion plus pests are also major constraints to yield. Buds, flowers and immature pods abscission that fail to develop intofully mature pods offaba bean, isconsidered one of the greatestdifficultproblems of yield productivity.This study takes place to investigate the influence of spraying at different concentrations of Naphthalene Acetic Acid (NAA) and Kin on broad bean plants, to study its effects on abortion and drop of flowers, buds and pods, also the impact on vegetative growth and seed yields.Naphthalene Acetic Acid as hormones setthe physiological process of synthetic growth regulators mightimprove growth and development, thereby increased seed yield[1].Foliar spraying of Naphthalene acetic acid is a potential antifungal agent[2].Pant growth regulators are augmentedseedproduction by amassed biological yield. Naphthalene Acetic Acid belongs to synthetic forms of Auxins[3].Spraying of Naphthalene Acetic Acidimproved plant height, fruit set with increases in seed yield/ha[4].Sprayingtwiceof growth regulators improved the number of pods/plant, pod weigh/plant and increased seed yield by 17.7% compared without growth regulator foliar spraying[5].Application of Amcotone at 600 ppm (NA+NAA),decidedlyaugmented plant height, leaf area index, flowering set, seed yields and its attributes[6].The growth promoter NAA enhanced the mobilization of photo assimilatesinto filling seeds[7].Foliar spraying of Kinetin significantly enhanced plant growth and growth even grown under ecologicalworry. He added that foliar spraying of Kinetinenhanced flowering and delays leaves senescence[8].Foliar application of *Vicia faba*plants with indel-3-acetic or gibberellin increased the number of branches/plant and number of pods/plant [9].Plant growth

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substances are enhanced the source-sink relationship and increase the translocation ofphoto-assimilates to sink,formerlyenhanced flower formation, fruit and seed development and improving seed production[10].Foliar sprays with Oraset-x Naphthalene Acetic Acid significantly the superior treatment to produce plants with vigorous vegetative growth, i.e. plant height and number of branches/plant, earliness, highest fruit set percentage, total green yields, number of pods/plant, weight of ten pods, pod characters, weight of 100 green seeds and total protein[11].The highest numbers of shedding flowers and shedding percentage% were obtained the control. Foliar spraying of GA3 or IAA at 100 ppm exceeded the control by 31.6 and 4.10%, respectively in number of shedding flower/plant[12].Number of pods/plant, pods yield /plant, 100 seed weight and biological yield/plant, protein %and seed yield/hawere significantly enriched with foliar spraying of 50 mg/l IAA + 75 mg/l Kinetin[13].The objectives of this investigationwas aimed to investigate the effect of foliar spraying of different concentrations of Kinetin,Naphthalene Acetic Acid on growth, shedding percentage, seed yield and its quality.

II. MATERIALS AND METHODS

2.1 *Research time and location*:

Two field experiments were conducted in extensive field in Awish El-Hagar village Dakahlia district during 2014/2015 and 2015/2016 seasons to investigate the effect of foliar spraying of different levels of both Naphthalene Acetic Acid (NAA), i.e. 0, 20, 40 and 60 ppm and Kinetin (Kin), i.e. 0, 15, 30 and 45 ppm. The experimental design, layout used was strip plot design with four replicates. Each plot contained of five ridges, 3.5 meters'length and 60 cm in width. The size of each plot being 10.5m^2. The vertical plots were occupied with the following foliar spraying of Naphthalene Acetic Acid (NAA) rates, i.e. 0, 20, 40 and 60 ppm. The horizontal plots were occupied with foliar spraying of Kinetin (Kin) rates, i.e. 0, 15, 30 and 45 ppm. Growth hormone was sprayed twice at 35 and 50 days from sowing.The experimental units were fertilized with calcium super phosphate (15.5% P_2O_5) at a rate of 240kg/hawas added to the soil during tillage operation and before sowing. 115 kg K_2O/ha of potassium sulphate (48% K_2O) was added to field in two equal portions, before the first and second irrigation. Nitrogen in the form of ammonium sulphate (20%N) at the rate of 35 kg N/ha as starter dose and was added before irrigation. However, other agricultural practices were done as commonly followed in the district.Sowing date of faba bean (Cv. Nobaria) was on the 10thof November in both seasons. Faba bean seeds were soaked in water for 24 hours before planting to raise seed germination. Planting was performed on both sides of ridges at 25 cm between hills. Thinning was done in 21 days from sowing to leave healthy two plants/hill. Hand digging was done every 21 days to control weeds i.e. before, time of irrigations.

2.2. *Studied traits:*

All studied characteristics were applied to harvest time. From each plot, ten guarded plantswere booked from the outer ridges from each sub plot to estimate:1.Total chlorophyll (SPAD): Chlorophyll content in leaf samples was assessed by SPAD-502 (Minolta Co. Ltd., Osaka, Japan). 2. Plant height (cm): It was measured for each plant of the samples of the soil surface to the top of the plants. 3.Number of branches/plant. 4. Fresh weight/plant. 5. Dry weight/plant. 6. Shedding percentage: It was determined by using the following equation:

$$\text{Shedding \%} = \frac{\text{Shedding}}{\text{Shedding + number of mature pods}} \times 100$$

At harvest time marketable pods per plant was picked and let to dry up normally and data were recorded for the following traits:7. Number of pods/plant. 8. Seed yield (g)/plant. 9. 100-seed weight (g). 10. Seed yield (ton/ha): whole plants produced from the three inner ridges of each plot were harvested and left to dry in the air, then they were threshed and the seeds (which were at 13 % moisture) were weighted (kg), then converted to (Kg/ha). 11. Protein%: Total nitrogen was estimated by the improved Kjeldahl - method according to[14], modified by distilling the ammonia into saturated boric solution and titration in standard acid. Protein % was estimated by multiplying the total nitrogen values of faba bean flour by 6.25.

2.3. *Experimental analysis:*

All obtained data were statistically analyzed according to the technique of analysis of variance (ANOVA) for the split-plot design to each experiment (row spacing), then combined analysis was done between row spacing trails as published by [15]by using "MSTAT-C" computer software package. To test the differences between treatment means, the least significant difference (LSD) method was used at the 5 % level of probability as designated by[16].

III. RESULTS AND DISCUSSION

3.1. Naphthalene Acetic Acid concentration effects:

The results presented from Tables 1 and 2 clearly revealed that foliar spraying of Naphthalene Acetic Acid (NAA) and Kinetin (Kin) concentrations significantly affected total chlorophyll, plant height (cm), number of branches/plant, number of shedding flowers, shedding %,pods and seeds number/plant,number of seeds/pod,

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seed yield (g) /plant, 100-seed weight (g), seed yield (ton/ha) and protein %.The results showed that increasing NAA concentrations of 60 ppm significantly increased total chlorophyll, plant height (cm), number of branches/plant, number of shedding flowers, shedding %, pods and seeds number/plant, number of seeds/pod, seed yield (g) /plant, 100-seed weight (g), seed yield (ton/ha) and protein % in both seasons. From our results, it could be stated that incensing Naphthalene Acetic Acidup to 60 ppm increased total chlorophyll, plant height (cm),branchesnumber/plant, number of shedding flowers, podsnumber/plant, seedsnumber/pod, seedsnumber/plant, seed yield/plant, 100-seed weight (g), seed yield (ton/ha) and protein % by 11.47, 23.92, 92.88, 20.53, 11.87, 23.48, 14.16, 24.91, 26.15 and 13.23%, respectively as the average of twoseasons.But, reduced the shedding % by 11.91% as the average of two seasons.Foliar application of Naphthalene Acetic Acid increased the growth, viz, plant height, number of branches/plant, leaf area index, dry weight/plant and plant attributes. Plant growth regulators are known to modify the growth and development patterns of plant by exertinga profound effect on various physiological processes and hence regulating seed productivity[17].Application IAA caused a reduction in the flower abscission %,hence producinga highest number of pod set, seedweight/plant, number of seeds/pod and weight of 100 seeds[7]. The increase in seed yield/plant due to foliar spraying of Naphthalene Acetic Acid could be attributed to the more increases in vegetative growth characters, which might provide more vegetative area and increases pod set%, reduction in abscission %, increasing the seed number/pod, as mentioned by[18].These results are in agreement with those described by[6,8,10,11,12].

3.2. Kinetin (Kin) concentration effects:

The results presented from Tables 1 and 2 clearly suggested that increasing foliar spraying of Kinetin (Kin) concentrations significantly exaggerated total chlorophyll, plant height (cm), number of branches/plant, number of shedding flowers, shedding %pods and seeds number/plant,number of seeds/pod, seed yield/plant, 100-seed weight (g), seed yield (ton/ha) and protein %. Our results clearly showed that increasing foliar spraying of Kinetin (Kin) levels up to 45 ppm significantly augmented total chlorophyll, plant height (cm), number of branches/plant, number of shedding flowers, shedding %, pods and seeds number/plant,number of seeds/pod, seed yield/plant, 100-seed weight (g), seed yield (ton/ha) and protein % percentage.In addition, it could be observed that incensingfoliar spraying of Kinetin (Kin) concentrationsof 45 ppm increased total chlorophyll, plant height (cm), branchesnumber/plant, number of shedding flowers,podsnumber/plant, seedsnumber/pod, seedsnumber/plant, seed yield/plant, 100-seed weight (g), seed yield (ton/ha) and protein % by 12.16, 19.39, 61.64, 5.60, 5.56, 6.96, 5.64, 18.75, 13.38 and 4.39%, respectively as average of both seasons. But, reduced the shedding % by 14.73 % as the average of both seasons. Foliar spraying ofKinetin (Kin) andNaphthalene Acetic Acid(NAA) promote photosynthetic rates, photo assimilates production, fruit set and growth. It could be summarized that the aptitude of cytokines and NAA to mobilize assimilates to the area of request is accountable for enhanced fruit set and seed productivity[19].Foliar spraying with growth regulators, enhanced expression and forms into SOD indicate the possible participation in growth regulators in delaying the membrane deterioration during abscission leading to increased fruit set [20].Seed yields and its attributes i.e. number of pods/plant, pods yield /plant, 100 seed weight, biological yield/plant and protein yield/ha were significantly better with application with 50 mg/l IAA + 75 mg/l Kinetin[13].These results are in harmony with[10,11,12,13].

3.3. Interaction Effect:

From our results, it could have suggested that the interaction among foliar spraying of Naphthalene Acetic Acid (NAA) and Kinetin (Kin) concentrations, results in Tables 1 and 2 designated that plant height (cm), fresh and dry weight/plant and total chlorophyll, number of pods/plant, seed yield/plant significantly affected only in the first season. Whilst, the results in the same Tables showed non-significant effects due to the interaction among foliar spraying of Naphthalene Acetic Acid (NAA) and Kinetin (Kin) concentrations on the number of branches/plant, shedding % and 100 seed weight in the two seasons. Concerning to the interaction between Naphthalene Acetic Acid (NAA) and Kinetin(Kin) concentrations on seed yield/ha, results in Figs 1 and 2 clearly indicated that this interaction significantly affected on seed yield/ha. From our results, it could be suggested that increasing Naphthalene Acetic Acid (NAA) up to 60 ppm and Kinetin (Kin) up to 45 ppm increased seed yield/ha by 38.2% compared without growth regulators foliar application.The growth promoter ofNaphthalene Acetic Acid (NAA) enhances the mobilization of photo assimilates into filling seeds[6]. Seed yield and its attributes i.e.number of pods/plant, pods yield/plant, 100 seed weight, biological yield/plant and seed, proteinyields/ha were significantly improved with foliar spraying at a rate of 50 mg/l IAA + 75 mg/l Kinetin[13].

IV. CONCLUSION

Accordingly, from above results, it could be suggested that increasing Naphthalene Acetic Acid (NAA) up to 60 ppm and Kinetin (Kin) up to 45 ppm increased seed

yield/ha by 38.2% compared without growth regulators foliar application.

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Table.1: Mean of plant height (cm), No. of branches/plant, fresh and dry weight/plant and total chlorophyll as affected by Naphthalene Acetic Acid (NAA) and Kinetin (Kin) concentrations during 2014/2015 and 2015/2016 seasons.

Treatments	**Plant height (cm)**		**No. of branches/plant**		**Fresh weight/plant (g)**		**Dry weight/plant (g)**		**Total chlorophyll**	
	2014/2015	2015/2016	2014/2015	2015/2016	2014/2015	2015/2016	2014/2015	2015/2016	2014/2015	2015/2016
A. Nitrogen fertilizer rates:										
0 ppm NAA	97.1	100.8	4.24	4.28	671.05	672.48	167.70	168.85	40.82	41.86
20 ppm NAA	102.6	108.7	5.31	5.42	794.05	781.57	182.45	179.51	42.85	43.28
40 ppm NAA	106.7	112.1	5.35	5.55	820.18	831.58	192.79	197.25	44.11	46.13
60 ppm NAA	109.2	114.3	5.49	5.71	864.22	870.83	207.95	215.57	46.71	47.11
F-test	*	*	*	*	*	*	*	*	*	*
L.S.D. 5%	0.8	0.8	0.05	0.04	5.28	3.06	0.51	0.91	0.15	0.16
B. Phosphorus fertilize rates:										
0 ppm kin	97.2	101.5	4.49	4.56	701.88	712.40	183.57	184.15	42.71	42.91
15 ppm kin	100.6	106.8	5.06	5.22	799.81	793.16	186.61	188.8	43.33	44.29
30 ppm kin	107.0	112.1	5.23	5.48	809.68	810.41	188.59	190.81	43.89	45.14
45 ppm kin	110.7	115.5	5.59	5.65	839.44	840.51	192.12	197.41	44.56	46.09
F-test	*	*	*	*	*	*	*	*	*	*
L.S.D. 5%	0.5	0.5	0.05	0.03	3.89	1.84	0.38	0.48	0.08	0.19
Interaction AXB										
F-test	*	N.S.	NS.	N.S.	*	N.S.	*	N.S.	*	N.S.

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Table.2: Mean of shedding percentage, No. of pods/plant, 100-seed weight seed yield/plant and per hectare and protein percentage as affected by Naphthalene Acetic Acid (NAA) and Kinetin (Kin) concentrations during 2014/2015 and 2015/2016 seasons.

Treatments	Shedding %		No. of pods/plant		100-seed weight (g)		Seed yield/plant (g)		Seed Yield t/ha		Protein %	
	2014/2015	2015/2016	2014/2015	2015/2016	2014/2015	2015/2016	2014/2015	2015/2016	2014/2015	2015/2016	2014/2015	2015/2016
A. Nitrogen fertilizer rates:												
0 ppm NAA	85.31	85.55	18.65	19.53	94.32	94.88	81.81	82.79	4.276	4.301	26.15	26.75
20 ppm NAA	83.66	83.74	21.69	22.83	98.75	99.45	88.45	88.85	4.746	4.801	27.66	28.50
40 ppm NAA	79.59	80.77	23.01	24.03	103.67	105.75	96.78	98.11	5.122	5.100	28.62	29.45
60 ppm NAA	71.61	78.89	24.55	25.35	110.90	111.16	109.11	110.26	5.791	5.822	30.31	30.36
F-test	*	*	*	*	*	*	*	*	*	*	*	*
L.S.D. 5%	0.25	0.35	0.26	0.15	0.27	0.61	0.18	0.73	0.079	0.119	0.12	0.25
B. Phosphorus fertilize rates:												
0 ppm kin	85.19	84.51	21.50	22.01	99.76	100.06	86.16	87.40	4.542	4.578	27.72	27.96
15 ppm kin	84.44	83.96	21.83	22.84	100.66	101.48	89.07	91.17	4.769	4.799	27.80	28.65
30 ppm kin	80.36	83.42	22.14	23.03	102.05	103.15	94.18	94.69	4.993	4.999	28.30	29.13
45 ppm kin	70.79	73.91	22.45	23.76	105.05	106.68	106.73	106.76	5.596	5.626	28.92	29.31
F-test	*	*	*	*	*	*	*	*	*	*	*	*
L.S.D. 5%	0.24	0.23	0.15	0.08	0.37	0.19	0.36	0.46	0.196	0.177	0.10	0.15
Interaction AXB												
F-test	N.S.	N.S.	*	N.S.	N.S.	N.S.	*	N.S.	*.	*	*	N.S.

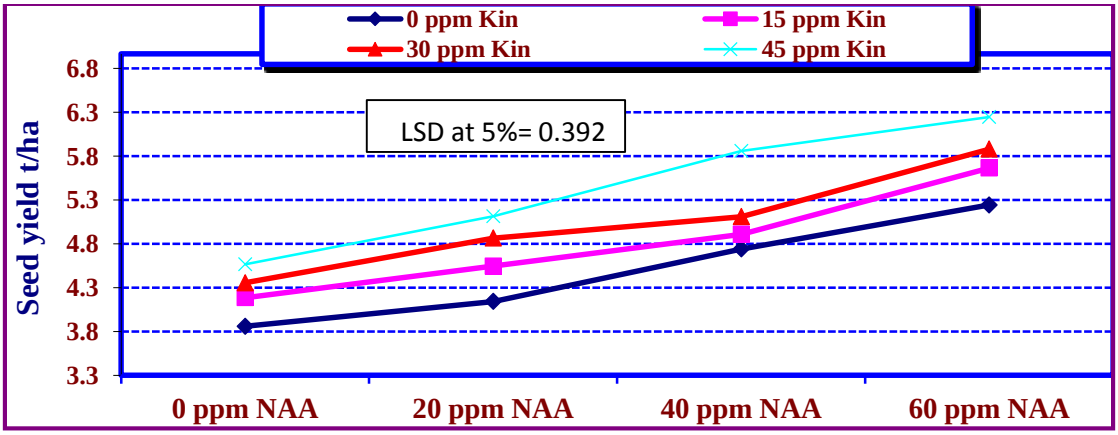

Fig.1: Seed yield t/ha as affected by the interaction between NAA and Kin concentrations during 2014/2015 season.

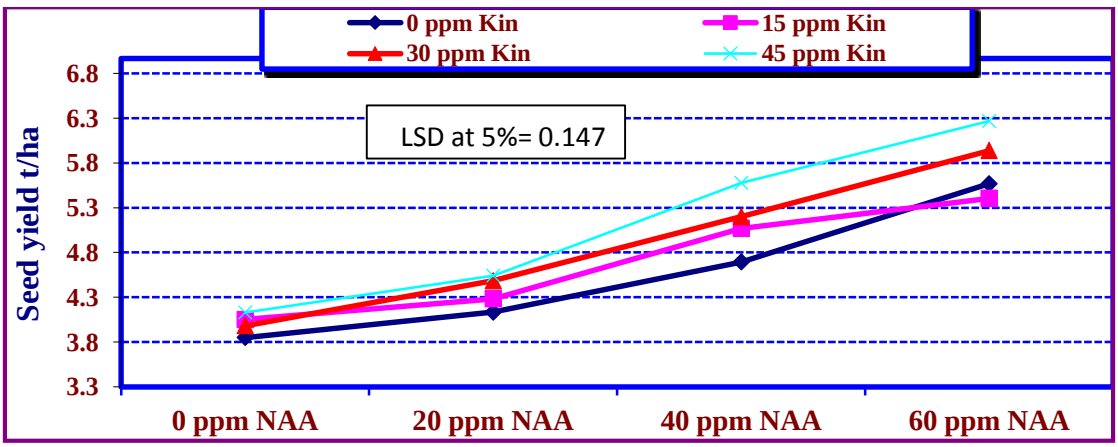

Fig.2: Seed yield t/ha as affected by the interaction between NAA and Kin concentrations during 2015/2016 season

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Effect of Irrigation Levels on Yield Performance of Black Cumin

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Abstract**—** *An experiment was conducted in the experimental field of Horticulture Department, Bangabandhu Sheikh MujiburRahman Agricultural University (BSMRAU), Salna, Gazipur during the period from 20 November, 2012 to 12 April, 2013 to determine the optimum level of irrigation for better yield and quality of black cumin. There were six different irrigation levels (I_1 - no irrigation, I_2 - three irrigation, I_3 - four irrigation, I_4 - six irrigation, I_5 - eight irrigation and I_6 - ten irrigation). Results revealed that the number of primary branches (6.33), secondary branches (11.84), tertiary branches (6.29), number of capsule per plant (18.64), capsule length (1.89 cm), diameter of capsule(1.05 cm), number of seed per capsule (107.8), fresh seed yield per plant (3.84g), dry seed yield per plant (3.26g), 1000 seed weight (2.40g) and seed yield (1.77 t/ha) were observed maximum in I_6(ten irrigation).*

Keywords**— Black cumin, capsule, irrigation, seed, yield.**

I. INTRODUCTION

Black cumin (*Nigella sativa* L.) is an annual aromatic plant native to Southwest Asia and the Mediterranean region. Presently, it is cultivated in various parts of the world, including Asia, the Middle East and North Africa. Seed of black cumin contain about 21% protein, 35% carbohydrates and 35-38% plant fats and oils [1]. It contains all essential amino acids and rich source of vitamins and minerals [2, 3]. Total cultivable area of Bangladesh is 14.86 million hectare but only 56 percent of cultivable area under irrigation coverage [4]. North-west part of the country mostly is affected by the droughts which generally has lower rainfall than the rest part of the country. Crop production will become impossible especially in drier northern and western regions of the country. The cost of irrigation is one the main obstacle for small farming. Optimizing irrigation management together with the cultivation of appropriate crops is desirable in these regions [5]. The total annual production of black cumin is 3675 tons from 3530 hectares of land with an average yield of 1.04 t/ha [6]. The yield is low compared to Iran(1.71- 2.1 t/ha)[7]. In farmers field of some places of Bangladesh, yield is 1.19 to 1.48 t/ha [8]. Black cumin is an annual plant, originally grown in arid and semi-arid regions[9]. One third of the world lands are classified as arid and semi-arid region and the remains are faced with water seasonal or local fluctuations[10]. Availability of water rather than land is the main constraint on agricultural production in arid and semi-arid environments [11]. Some studies shown that, the black cumin is able to tolerate moderatelevels of water stress [11, 12].Some researchers have focused on response of black cumin to different irrigation intervals [12, 13] and irrigation scheduling based on developmental stage [11]. Moreover, deficit irrigation is one way of maximizing water use efficiency (WUE) for higher yields per unit of irrigation water applied where crop is exposed to a certain level of water stress either during a particular period or throughout the entire growing season [14]. The cost of irrigation pumping and inadequate irrigation capacity as well as limited water sources is among the reasons that force many farmers in the region to reduce their irrigation applications. Irrigation scheduling based on developmental stage is the technique of applying water on a timely and accurate basis to the crop [15]. Irrigation cost in Bangladesh is 4 times higher than India, 6 times thanThailand and Vietnam. High irrigation cost in Bangladesh is one of the major obstacles for crop production. Considering the above facts the experiment was undertaken to determine optimum level of irrigation for obtaining better yield of black cumin.

II. MATERIALS AND METHODS

The experiment was conducted at the Horticultural Research Farm of Bangabandhu Sheikh MujiburRahman Agricultural University, Gazipur during the period from 20 November, 2012 to 12 April, 2013. The experimental site was located at the Centre of Madhupur Tract $24^0 09'$ N latitude and $90^0 26'$ E longitudes at 8.5 meter above the sea level and about 40 km north of Dhaka.

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2.1. Treatments of the experiment:

The experiment consisted of six treatments: I_1 = No irrigation I_2 = Three irrigationI_3 = Four irrigation I_4 = Six irrigation I_5 = Eight irrigation I_6 = Ten irrigation

2.2 Design and layout:

The field experiment was laid out in Randomized Complete Block Design (RCBD) with three replications. The whole experimental area was divided into three blocks which represented replication. The treatments were randomly allotted in each replication.

2.3**.** Plant material:

Exotic variety of black cumin was used as plant material.

2.4. Collection of data:

Data were collected from the inner rows of each plot to avoid the border effect. The following seed yield and yield contributing parameters were observed.

2.5. Number of branches per plant:

The primary, secondary and tertiary branches per plant were counted at the time of harvesting. Ten randomly selected plants from each plot were used for counting number of primary, secondary and tertiary branches.

2.6. Length of capsule:

A slide calipers was used to measure the capsule length in centimeter. Ten randomly selected capsules were taken from each plot to take average length of capsule.

2.7. Capsule diameter:

A slide calipers was used to measure the capsule diameter in centimeter. Ten randomly selected capsules were taken from each plot to measure average diameter of capsule.

2.8. Number of seeds per capsule:

Ten randomly selected capsules were taken from each plot and average seeds per capsule were counted.

2.9. Number of capsule per plant:

Ten randomly selected plants were taken from each plot and average capsules per plant were counted.

2.10. Fresh seed yield per plant:

Fresh seed yield per plant was calculated from the total fresh weight of seed often randomly selected plants dividing by ten.

2.11. Dry seed yield per plant:

Dry seed yield per plant was calculated from the total sundried weight of 10 randomly selected plants dividing by ten. Moisture content of seed was about 10%.

2.12. 1000 seed weight:

Thousand seeds of randomly selected 10 samples were weighted and its averages were taken with the help of an electric balance.

2.13. Seed yield:

The mature seeds of all plots were harvested, cleaned and dried. First plot yield was obtained in kg by the help of electric balance. Then plot yield was converted into t/ha.

2.14. Statistical analysis:

The recoded data on different parameters were statistically analyzed and partitioning the variance with the help of "MSTAT-C" software. The difference between treatment means was compared by Duncan's Multiple Range Test (DMRT).

III. RESULT AND DISCUSSION

3.1. Number of branches per plant:

Due to effect of irrigation, the number of primary branches found significantly different in black cumin (Fig. 1). The maximum number of primary branches (6.33) was recorded in I_6 (Ten irrigation) which was statistically similar to I_5 and I_4 while the minimum number of primary branches (4.92) was observed in I_1. The effect of irrigation on the number of secondary branches found significant (Fig. 1). The maximum number of secondary branches (11.82) was recorded in I_6 which was statistically similar to I_5, I_4 and I_3. The minimum number of secondary branches (5.87) was observed in I_1.The effect of irrigation on the number of tertiary branches also found significant (Fig.1). The plants irrigated ten times (I_6) produced the highest number of tertiary branches (6.26) and the lowest number of tertiary branches (0.16) was observed in I_1(No irrigation). The number of primary, secondary and tertiary branches increased with the increasing number of irrigation.These results may be due to the effect of short watering intervals, plants received sufficient moisture to enhance the rates of physiological processes and increasing the hydrostatic pressure on the cell wall, which is necessary for the enlargement of cell.Hence, enhancement of the assimilated food and increase the cell elongation and division consequently, the whole growth of plant as well as branching could be increased [16].

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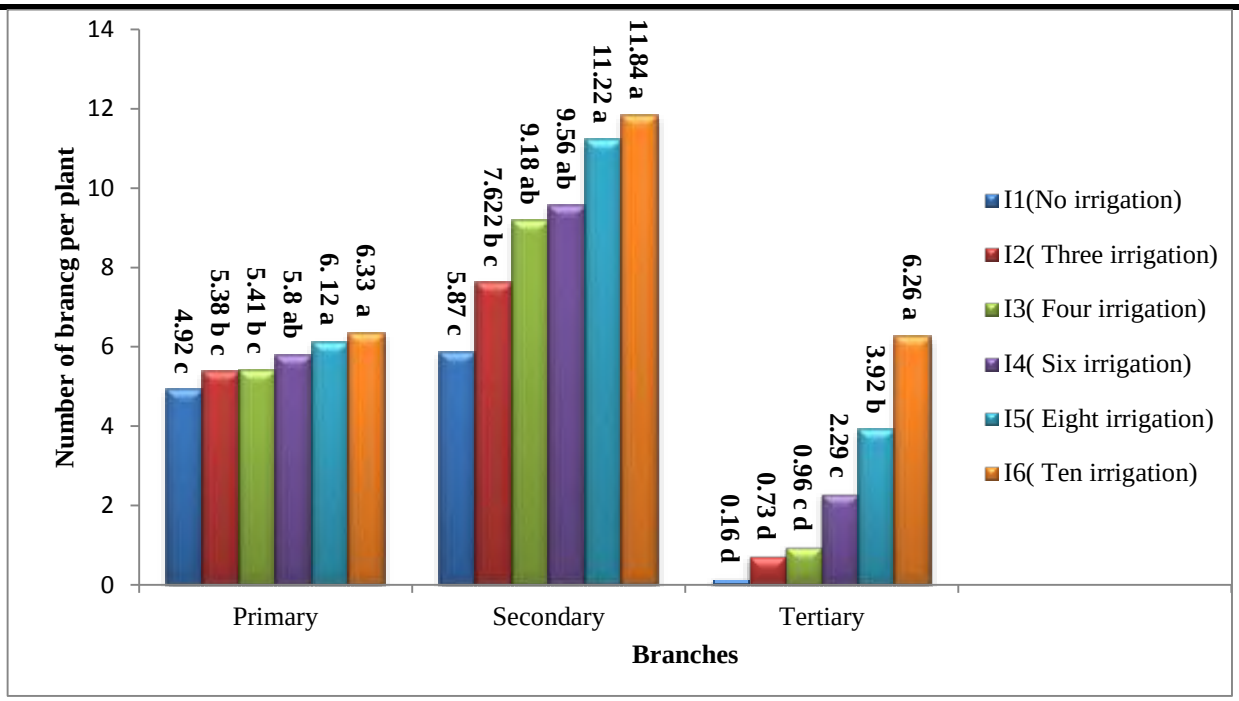

Fig.1: Effect of irrigation on the number of branches per plant.

3.2. Length & diameter of capsule:

The effect of irrigation on the length of capsule was found significant (Table 1). The maximum capsule length (1.89cm) was recorded in I_6 which was statistically similar to I_5 and I_4. The minimum capsule length (1.13cm) was observed in I_1 (No irrigation) which was statistically similar to all except I_6.

The effect of irrigation on the diameter of capsule was found significant (Table 1). The maximum capsule diameter (1.05cm) was recorded in I_6 which was statistically similar to I_5, I_4 and I_3. The minimum capsule diameter (0.78cm) was observed in I_1 which was similar to I_2, I_3 and I_4. The diameter of capsule increased with the increasing of irrigation number.

3.3. No. of capsule per plant & no. of seed per capsule:

The effect of irrigation on the number of capsule per plant was also found significant (Table 1). The highest number of capsule (18.64) was observed in I_6 (Ten irrigation) which was statistically different from others. The lowest number of capsule (10.42) was found in I_1 which was statistically at par with I_2 and I_3. Number of capsule increased with the increasing number of irrigation. The higher number of irrigation facilitated the plant to produce more branches resulting more capsules per plant.

The effect of irrigation on the number of seed also found significant (Table 1).The plants treated with I_6 produced maximum number of seed (107.8) closely followed by I_5 (97.48). The minimum number of seed (81.77) was observed in I_2 which wasstatistically identical to I_1 (87.07), I_3 (84.62) and I_4 (85.81). The number of seed increased with the increasing of irrigation. Due to less irrigation during the growing period caused a decrease of water use efficiency, biomass and number of seeds. And drought that occurred in the late growing season affected seed filling. Decrease in number of seeds per capsule and seeds per plant in water stress conditions can be the result of water shortage during the seed filling stage that shortens the flowering [17]. Irrigation had a significant effect on number of follicles per plant [11].

Table. 1: Effect of irrigation on capsule length, diameter, number of capsule per plant and seed per capsule in black cumin.

Irrigation frequency	Capsule length(cm)	Capsule diameter	No. of capsule per plant	No. of seed percapsule
I_1-No irrigation	1.09 b	0.78 b	10.42 d	87.07 c
I_2- Three irrigation	1.13 b	0.84 b	10.61 d	81.77 c
I_3- Four irrigation	1.21 b	0.91 ab	11.99 cd	84.62 c
I_4- Six irrigation	1.30 ab	0.92 ab	13.52 c	85.81 c
I_5- Eight irrigation	1.42 ab	1.04 a	16.11 b	97.48 b
I_6- Ten irrigation	1.89 a	1.05 a	18.64 a	107.8 a
Level of significance	*	*	*	*
CV%	7.70	9.88	9.16	6.08

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Means bearing same letter (s) in a column do not differ significantly at 5% level of probability by DMRT.

3.4. Fresh and dry seed yield per plant (g)

Fresh seed yield per plant is an important yield contributing character varied significantly due to different genotypes. The effect of irrigation on fresh seed yield per plant was found significant (Fig.2). The plants irrigated ten times (I_6) showed maximum fresh seed yield (3.84g) which was statistically identical to I_5 (3.53g). The minimum fresh seed yield (1.86g) was observed in I_1 which was statistically similar to I_2. The supply of sufficient water from the soil due to more irrigation might have helped in maintaining better substrate for photosynthetic activities in the leaves. It is well known fact that proper supply of moisture help in maintaining high photosynthetic rate and turgidity, which could increase the cell elongation and its multiplication at much faster rate resulting higher seed yield per plant.

The effect of irrigation on dry seed yield per plant was also found significant (Fig.2). The maximum dry seed yield (3.26g) was recorded in I_6 which was statistically similar to I_5 (3.00g). The minimum dry seed yield (1.45g) was observed in I_1 which was statistically identical to I_2.

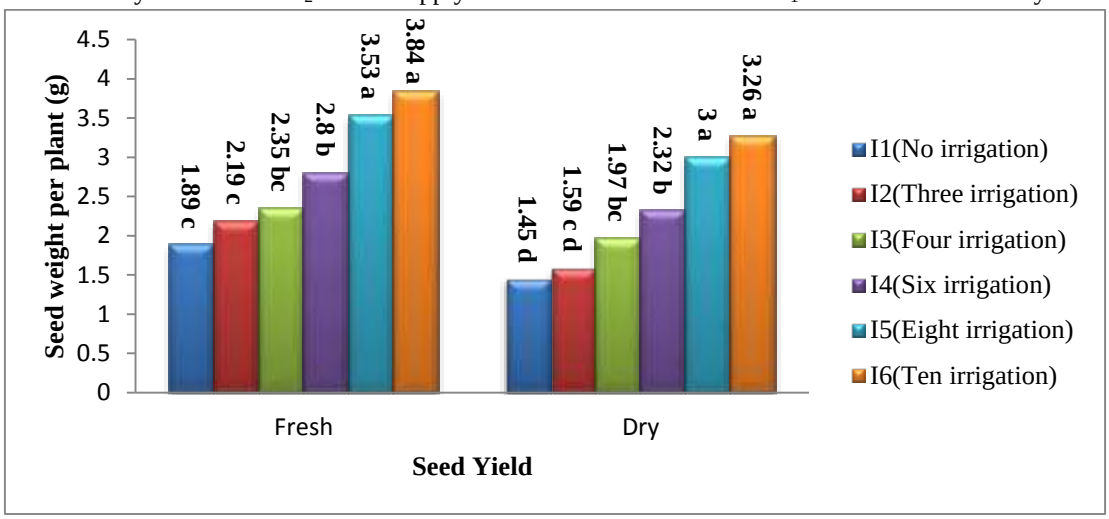

Fig. 2: Effect of irrigation on fresh & dry seed yield per plant.

3.5. 1000 seed weight (g)

Thousand seed weight is an important yield contributing character. The effect of irrigation on 1000 seed weight was found significant (Fig. 3). The maximum 1000 seed weight (2.40g) was recorded in I_6 (Ten irrigation) which was statistically similar to I_5 (2.31g) and the minimum 1000 seed weight (1.85g) was observed in I_1 (No irrigation). The results were similar to those of [11, 18] where significant difference in 1000 seed weight was reported in different irrigation treatments for black cumin.

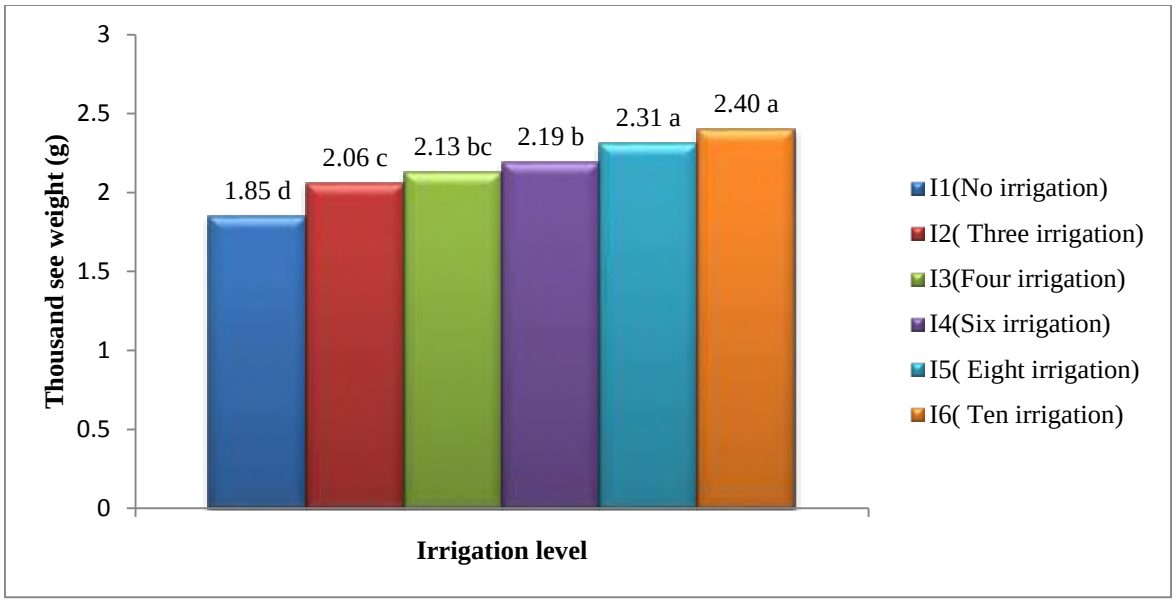

Fig.3: Effect of irrigation on 1000 seed weight.

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3.6. Seed Yield

Seed yield per hectare varied significantly due to influence of irrigation (Fig. 4). The highest seed yield (1.77 t/ha) was found in I_6 (Ten irrigation) which was statistically different from others. The minimum seed yield (1.31 t/ha) was observed in I_1 (No irrigation). Reduced yield as impact of stress mainly is due to shortening of plant growth stages [17]. Yield of black cumin per hectare increased with increase of irrigation which was similar to results obtained by [19, 20, 21, and 22]. All of them reported to have an increasing yield with increase in irrigation water supplied. The yield and growth characters were maximum at 0.8 IW/CPE (fiveirrigation) [23]. It was due to higher physiological activities favoring higher nutrient uptake and photosynthesis which might be responsible for formation of more photosynthesis under this treatment resulting more yields. Besides, improvement in physical, chemical and microbial environment of soil, it might have also increased the availability of nutrients and water. The supply of sufficient water from the soil might have helped in maintaining better substrate for photosynthetic activities in the leaves. It is well known fact that proper supply of moisture help in maintaining high photosynthetic rate and turgidity, which could increase the cell elongation and its multiplication at much faster rate. Drought stress made changes in photosynthetic pigments and components [24] damaged photosynthetic apparatus [25] and diminished activities of Calvin cycle enzymes, which are important causes of reduced crop yield.

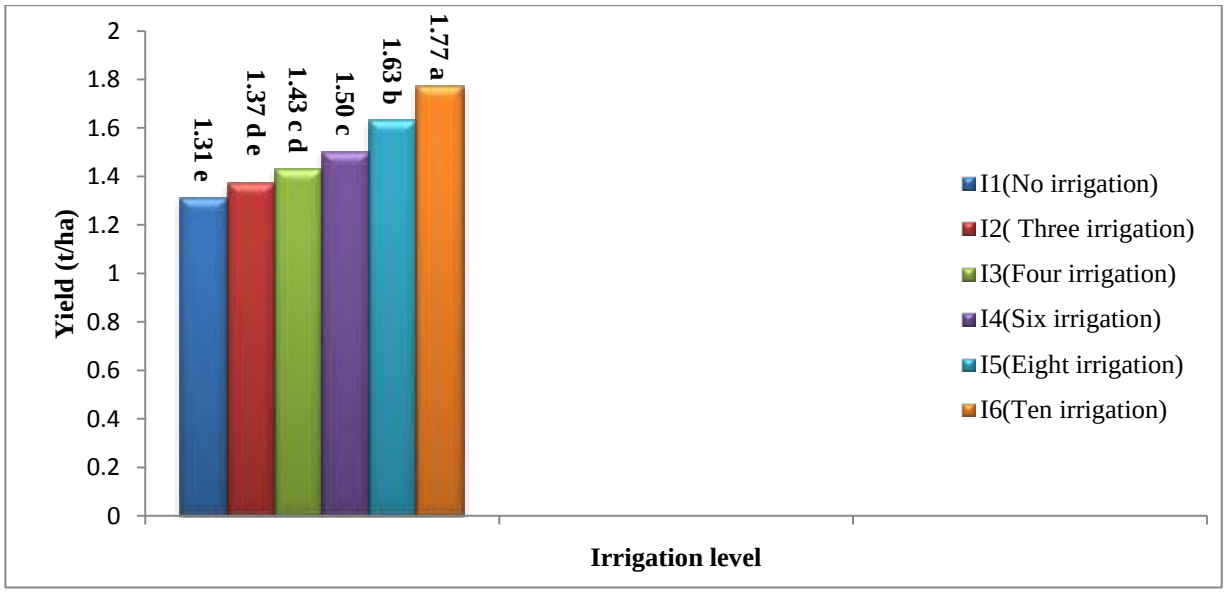

Fig. 4: Effect of irrigation level on seed yield.

The result revealed that almost all the parameters studied significantly influenced by different level of irrigation, the highest primary branches (6.33), secondary branches (11.84), tertiary branches (6.26) at 75 DAS were found in I_6 (Ten irrigation) and lowest was found in I_1 (No irrigation).

The maximum capsule length (1.89 cm), capsule diameter (1.05 cm), number of capsule per plant (18.64), number of seed per capsule (107.8), fresh seed yield per plant (3.84g) and dry seed yield per plant (3.26g) were found in I_6 (Ten irrigation).

1000 seed weight and seed yield were maximum (2.40g and 1.77 t/ha respectively) in I_6 (Ten irrigation).

IV. CONCLUSIONS AND RECOMMENDATIONS

The plants received higher number of irrigation I_6 (Ten irrigation) produced maximum number of primary branches, number of secondary branches, number of tertiary branches, capsule length, capsule diameter, number of capsule per plant, number of seed per capsule and seed yield per hectare. Similar type of experiment may be conducted in different Agro-Ecological Zones (AEZs) to confirm the results.

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Combined Toxicity and Bioconcentration of Fluoride and Arsenic in African Catfish *Clarias gariepinus* (Burchell, 1822)

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Abstract— *Laboratory experiments were performed to examine the combined toxic effects of two important aquatic contaminants viz., arsenic and fluoride on African catfish, Clarias gariepinus. Additionally, the bio concentration factors (BCFs) of the two contaminants in tissues and blood of catfish were also determined. The LC_{50} for sodium fluoride and arsenic trioxide were determined to be 619.3 mg L^{-1}, 30.3 mg L^{-1}, respectively. Erratic swimming movements with hyperactivity, loss of equilibrium, augmented air gulping and decreased food consumption were observed in the experimental groups. In co-exposure groups of arsenic and fluoride, the concentration of fluoride in fish tissues increased with increasing water fluoride concentration in the test aquaria with significant differences ($P<0.01$) between different groups. Also significant differences ($P<0.05$) in tissue concentrations of arsenic between groups were observed in response to different concentrations of water arsenic. However, the differences in blood fluoride and arsenic concentrations were not significantly dissimilar ($P>0.05$) among the exposure groups. Arsenic was observed to exceedingly bioaccumulate and biomagnify in the tissues. Perhaps due to the complex formation of arsenic and fluoride the bio concentration of arsenic in tissues was observed to decrease with increasing water fluoride concentration and vice-versa. The study concludes that fluoride may interfere with the bio-concentration of arsenic.*

Keywords— Arsenic, Bio-concentration factor, Combined toxicity, Fluoride, LC_{50}.

I. INTRODUCTION

Fluoride and arsenic are two stern drinking water contaminants recognized worldwide [1] with natural sources contributing to the bulk of their environmental load. Fluoride is found in freshwater at concentrations less than 1.0 mg L^{-1}; however, its natural concentrations may exceed even 50.0 mg L^{-1} [2]. While lower concentrations, viz., <1.0 mg L^{-1} according to Bureau of Indian standards and 1.5mg L^{-1} according to World Health Organization are beneficial, higher concentrations may lead to various health problems [1]. Fluoride causes fluorosis, a slow degenerative disease affecting teeth and bone tissues. It also induces neurological defects, infertility, mental retardation, depression of thyroid activity [3, 4, 5, 6, 7] and persistently bioaccumulates in aquatic animals continuously exposed to the contaminated medium [8, 9, 10].In India, 19 out of 35 states and union territories have ground water highly contaminated with fluoride [11].

A heavy metal, arsenic is more toxic than fluoride at the same dose and exposure duration [1]. Symptoms of toxicity during short term exposures in humans include vomiting, abdominal pain, encephalopathy, and watery bloody diarrhea. Long-term exposure may result in thickened pigmented skin, abdominal pain, diarrhea, heart disease, numbness, and cancer. Globally, arsenic toxicity is mostly prevalent in West Bengal (India), Nepal, and Bangladesh [12, 13] with contaminated drinking water being the most common source. A higher concentration of arsenic is lethal to many organisms in the aquatic environment [14, 15] inducing the synthesis of stress related proteins [16] and alterations in B and T cell functions [17] in the fish body. Like other heavy metals, it is non degradable and considered hazardous to aquatic ecosystem due to its environmental persistence and tendency for bioaccumulation [18, 19, 20]. Donohue and Abernathy [21] reported that total arsenic (µg g^{-1} dry weight), in marine fish, shellfish, and freshwater fish tissues ranged between 0.19 to 65, 0.2 to 125.9, and 0.007 to 1.46, respectively.

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Koch et al., [22] demonstrated that total arsenic in freshwater fish ranged from 0.28 to 3.1 for whitefish (*Coregonus clupeaformis*), 0.98 to 1.24 for sucker (*Catostomus commersoni*), 0.46 to 0.85 for wall eye (*Stizostedion vitreum*), and 1.30 to 1.40 µg g^{-1} dry wt. for pike (*Esox lucius*).

Geological structures and expanding human activities contribute to the high concentrations of both fluoride and arsenic. Although, their concurrent chronic poisoning is a sprouting disease prevalent in India and many other countries, however, few reports exist suggestive of their chronic co exposure [23]. Li et al., [24] studied effects of arsenic-fluoride co-exposure on rat teeth and observed no effects on dental tissues. Distinct damage on the nervous system of the offspring with decreased learning and memory ability was reported by Zhang et al., [25]. Altered histology of cerebral hemisphere subsequent to combined arsenic-fluoride exposure was observed by Chinoy and Shah [26] with arsenic having more prominent effects as compared from fluoride. Marked genotoxic effects were apparent in case of combined exposure to arsenic and fluoride as compared to their individual exposures [27, 28]. Exposure of mice to higher doses of fluoride and arsenic revealed their antagonistic effects [29] while, low doses showed synergistic effects [30]. González-Horta et al. (2015) [31] studied urinary arsenic and fluoride in human residents of Chihuahua, Mexico exposed to concurrent arsenic and fluoride in drinking water. Positive correlations between As and F in drinking water and between urinary arsenic and fluoride were observed.

Fishes, the major source of protein in many countries [32] are often contaminated with high concentration of water borne pollutants and act as a major vector for contaminant transfer to humans. Their ability to detect sudden changes in the environment and to monitor short or long term changes in water quality, make them efficient biomarkers. Although, toxic effects of elevated levels of fluoride [33, 34, 35, 36] and arsenic [16, 17, 37, 38] individually on various aquatic species are well documented, however, no work has been done on their combined toxic effects. The present study includes the determination of the Median Lethal Concentrations, bio concentration and behavioral effects of fluoride and arsenic separately and in combination on the freshwater fish *Clarias gariepinus* (Burchell, 1822).

II. MATERIALS AND METHODS

2.1. Determination of Median lethal Concentration (LC_{50}) of Fluoride and Arsenic

2.1.1. Experimental design

Seventy African catfish (*Clarias gariepinus*) of either sex weighing between 100 and 250 g were procured live from local hatcheries in Raipur. The animals were housed in an air conditioned animal house at 24 ± 2°C under 12 hours of light and dark cycles and acclimatized for a period of seven days using de-chlorinated tap water. Feeding was done with Tokyo fish food.

2.1.2. LC_{50} estimation of Arsenic and Fluoride

Post acclimation, the fishes were divided into seven groups of ten fish each and acute toxicity bioassay conducted by exposing the fish to diverse concentrations (100, 200, 300, 600, 800, 1000, 1200ppm) of sodium fluoride in glass aquaria. Similar experiments were conducted with 7 different concentrations (10, 20, 25, 40, 45, 60 ppm) of arsenic trioxide. The control group was kept in an aquarium having tap water without addition of sodium fluoride and arsenic trioxide. The bioassay was conducted in a static system. Mortality was recorded at every 24, 48, 72 and 96 hour of exposure. LC_{50} was calculated by Probit analysis using SPSS 16.0 [39].

2.2. Combined arsenic and fluoride toxicity assay

On the basis of 96 hr LC_{50} values and the 95% confidence limits of sodium fluoride and arsenic trioxide obtained from the preliminary tests, various concentrations viz., Group I (600ppm F+10ppmAs), Group II (350ppm F+20ppm As), Group III (600ppm F+20ppm As) and Group IV (350ppm F+40ppm As) of sodium fluoride and arsenic trioxide were selected for combined toxicity testing. Blood and tissue (liver, kidney and muscle) samples were collected at the end of 96 hours along with water samples for quantitative analysis of fluoride and arsenic. Behavioral changes during the 96 hours duration were also recorded. Physicochemical properties of the test water during exposure were measured according to standard methods. Water quality during experiment varied as follows: Ambient temperature 24-26°C, Water temperature 21-22°C, pH 7.5-8.5, Conductivity 350-460 µs cm^{-1}, Ammonia nitrogen 0.005-0.01 mg L^{-1}, TDS 200-250 mg L^{-1}.

2.3. Arsenic analysis

Water was collected in polyethylene vials and kept in refrigerator at 4°C until further analysis. 100 ml aliquots of water samples were taken in 250 ml beaker, covered with watch glass and digested with concentrated HNO_3 on a hot plate [40].Blood was collected in EDTA coated tubes and stored in refrigerator at 4°C until further analysis. 0.5 ml blood was taken in 100 ml conical flask, covered with watch glass and digested by the addition of 5 ml conc.HNO_3: $HClO_4$ (6:1) on a hot plate [40]. Liver, kidney and muscles were isolated and dried in an oven at 100°C for

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24 hours. 0.5 g dried tissue samples were taken in 100 ml beaker. The sample was covered with watch glass and digested on a hot plate by the addition of HNO_3: $HCLO_4$ (3:1) [41].All the digested samples were filtered through Whatman filter paper No. 541 and diluted to 25 ml using distilled water. Sample analysis was done by Thermo Fisher Scientific Atomic Absorption Spectrophotometer (Model no.ICE 3000).

2.4. Fluoride analysis

Collection and storage of all samples was done as mentioned in arsenic analysis. Water fluoride was measured by direct determination method by adding TISAB buffer (1:1) .Tissue samples were digested with a mixture of 1:1 (HNO_3: $HCLO_4$) and neutralized with citrate buffer. Final sample solution was obtained by adding TISAB buffer (1:1). Fluoride content was measured by direct determination method [42] with required modifications. Blood fluoride was measured by Analyte addition method using the following equation (Thermo scientific Orion ion selective electrode manual):

$$\mathbf{C_U = C_S\,[(V_U / V_{S+}V_U)* 10^{\Delta E/S}]}$$

Where: $\mathbf{C_U}$ = concentration of unknown sample, $\mathbf{C_S}$ = concentration of standard sample, $\mathbf{V_U}$ = volume of unknown sample, $\mathbf{V_S}$ = volume of standard sample, $\Delta E = E_2 - E_1$= is the change in the electrode potential after addition, E_2= **mV** after addition of sample, E_1=**mV** before addition of sample S = slope of the electrode

In all samples, quantification of F^- ion was done with the help of Thermo Fisher Scientific Orion 9609 BNWP ion selective fluoride electrode.

2.5. Bioconcentration factor (BCF): Bio-concentration factors of arsenic and fluoride in the fish samples were obtained using following equation:

$$BCF = C_{org} / C_{water}$$

Where BCF- bio-concentration factor, C_{org} = concentration of chemical in aquatic organism, C_{water}= concentration of chemical in ambient environment, water in this case.

III. RESULTS

No fishes were observed dead in the control aquarium at the end of the experiments. Highest fluoride concentration caused mortality with increasing exposure time. 100% mortality was observed at 1200 ppm, 60 ppm and 40+350 ppm for fluoride, arsenic and arsenic+ fluoride, respectively.

3.1. Determination of LC_{50} value of sodium fluoride for *Clarias gariepinus*

The observed percentages of mortality of *Clarias gariepinus* for sodium fluoride are shown in Tables 1, 2, 3 &4. The observed LC values and 95% confidence limits for LC_{25} (333.445-590.361), LC_{45} (454.322-743.495), LC_{75} (649.953-1141.940), LC_{96} (938.671-2279.511) are shown in Table 4. In this study, 96 hour LC_{50} of sodium fluoride on *Clarias gariepinus* was estimated to be 619.3 mg L^{-1}.

3.2. Determination of LC_{50} value of arsenic trioxide for *Clarias gariepinus*

The observed percentages of mortality of *Clarias gariepinus* for arsenic trioxide are shown in Tables 5, 6, 7 & 8. The observed LC values and 95% confidence limits for LC_{25} (13.5-29.2), LC_{45} (20.8-35.9), LC_{75} (31.8-59.39.0), LC_{96} (44.3-148.4) are shown in Table 8. In the present study, the 96 hour LC_{50} value of arsenic trioxide on *Clarias gariepinus* was estimated to be 30.3 mg L^{-1}.

3.3. Concentration of fluoride and arsenic in fish sample

The levels of arsenic and fluoride obtained in fish tissues and blood are depicted in Figures 3 and 4, respectively. Bio-concentration factors of arsenic and fluoride in blood and tissues are depicted in Fig. 5. Fluoride concentration in fish tissues increased with increasing water fluoride concentration in the test aquaria and significant differences exist ($P<0.01$) between different groups. Similar to fluoride, arsenic concentration increased in tissues with increasing water fluoride and arsenic concentration and accumulation in liver was higher than blood. Significant differences ($P<0.05$) in tissue concentrations of arsenic was observed in response to different concentrations of water arsenic levels. However, the differences in blood fluoride and arsenic concentrations were not significantly different ($P>0.05$) among the exposure groups. Bioaccumulation of arsenic in tissues was observed decreased by increasing water fluoride concentration. Similarly, bioaccumulation of fluoride in tissue was observed to decrease with increasing water arsenic concentration.

IV. DISCUSSION

Highly variable 96 hour Median Lethal Concentration (mg L^{-1}) values have been reported for fluoride in diverse species of fishes including *Oncorhynchus mykiss* (107.5) and *Salmo trutta* (160.5) at a water temperature between 15-16ºC [43, 44]; *Labeo rohita* (935) [45] and *Oreochromis mossambicus* (54.0)[46] at a water temperature of 18–30ºC and *Puntius sophore* (126.12) [48] at 19-20 ºC . The 96 hour LC_{50} of sodium fluoride on *Clarias gariepinus* (619.3 mg L^{-1} at 21-22 ºC of water temperature) obtained in this study undoubtedly, proves the hardy nature of *Clarias gariepinus* which appears to be far more tolerant to fluoride

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as compared from *Oreochromis mossambicus, Oncorhynchus mykiss, Puntius sophore and Salmo trutta.*

On the basis of 96 hours LC_{50} values (30.33 mg L^{-1}) observed in this study for arsenic, *Clarias gariepinus* appears to be equally tolerant than *Oryzias latipes* (30) [47] and *Cyprinus carpio* (32) [49], less tolerant than *Ctenopharyngodon idella* (89) [50] and *Channa punctatus* (76) [16] and more tolerant than *Carassius carrassius auratus* (10), *Anabas testudineus* (18.21) [51], *Danio rerio* (8.91) [52] and *Clarias batrachus* (8.4) [17].Erratic swimming activity, increased opercular movement and mucous secretion, loss of equilibrium and body dispigmentation ,changes in feeding behavior, similar to reports by Bhavani and Karuppasamy [52], Narwaria and Saksena [48] were also observed.

Although numerous studies exist on evaluation of the individual effect of fluoride and arsenic on mammals and fishes, there are no studies related to understanding the potential combined effects of fluoride and arsenic on fish. Cao et al., [53] reported that the concentration of fluoride in the gills and other tissues in *C. carpio* increased with exposure time and exposure concentration and were in the order of gills > liver > brain > kidney > muscle > intestine. In this study, tissue fluoride content of *Clarias gariepinus* increased significantly ($P<0.01$) with increasing water fluoride and arsenic concentration and exposure time. Similar results were obtained by Aguirre-Sierra *et al.,* [54] after exposing white clawed crayfish (*A. pallipes*) to different concentration of fluoride. Our results also depict BCF values of blood arsenic (0.48 - 0.50), blood fluoride (0.024 - 0.384) and tissue fluoride (0.58 - 0.87), to be in the lower range ie. (<01). However, tissue arsenic was observed to be very high (0.63 - 3.11), indicating that arsenic exceedingly bioaccumulated and biomagnified in the tissues. Data show bioaccumulation of arsenic and fluoride to occur predominantly in liver, it being responsible for detoxification and elimination of toxic elements. Differences in blood fluoride and arsenic concentrations were not significantly different ($P>0.05$) among the exposure groups.

Bio concentration of arsenic in tissues was observed to decrease with increasing water fluoride concentration and vice-versa. We agree with [23] that the possible reason of this antagonistic behavior could be the presence of an empty *d* orbital of fairly low energy in arsenic which predominately binds with the halogen due to its electro negativity. In trivalent oxidation state it shows SP_3 hybridization with the formation of AsF_3, while in pentavalent oxidation state it shows SP_3d hybridization and forms AsF_5 which is a potent ion acceptor forming AsF_6^- ions or more complex species. Hence, fluoride possibly suppresses the ionization of sodium arsenite thereby reducing its retention.

V. ACKNOWLEDGEMENTS

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VI. DECLARATION OF INTEREST

The authors declare that there are no conflicts of interest to disclose.

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TABLES

Table.1. *Parameter Estimates for fluoride vs. Clarias gariepinus*

	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Probit[a]	Concentration	5.769	1.278	4.513	.000	3.264	8.275
	Intercept	-16.107	3.584	-4.494	.000	-19.691	-12.523

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Table.2: Log concentration, observed responses in fish Clarias gariepinus

Number	Concentration	Number of Subjects	Observed Responses	Expected Responses	Residual	Probability
1	2.000	10	0	.000	.000	.000
2	2.301	10	0	.023	-.023	.002
3	2.477	10	0	.347	-.347	.035
4	2.602	10	0	1.367	-1.367	.137
5	2.699	10	5	2.960	2.040	.296
6	2.778	10	6	4.684	1.316	.468
7	2.903	10	7	7.394	-.394	.739
8	3.000	10	8	8.851	-.851	.885
9	4.079	10	10	10.000	.000	1.000

Table.3: Confidence Limits for fish Clarias gariepinus

Probibility Probit point	95% Confidence Limits for Concentration			95% Confidence Limits for log(Concentration)[a]		
	Concentration	Lower Bound	Upper Bound	Concentration	Lower Bound	Upper Bound
LC1	244.701	112.764	343.699	2.389	2.052	2.536
LC25	473.113	333.445	590.361	2.675	2.523	2.771
LC45	588.976	454.322	743.495	2.770	2.657	2.871
LC50	619.269	484.202	789.417	2.792	2.685	2.897
LC75	810.576	649.953	1141.940	2.909	2.813	3.058
LC96	1245.487	938.671	2279.511	3.095	2.973	3.358
LC99	1567.196	1119.693	3366.831	3.195	3.049	3.527

a. Logarithm base = 10.

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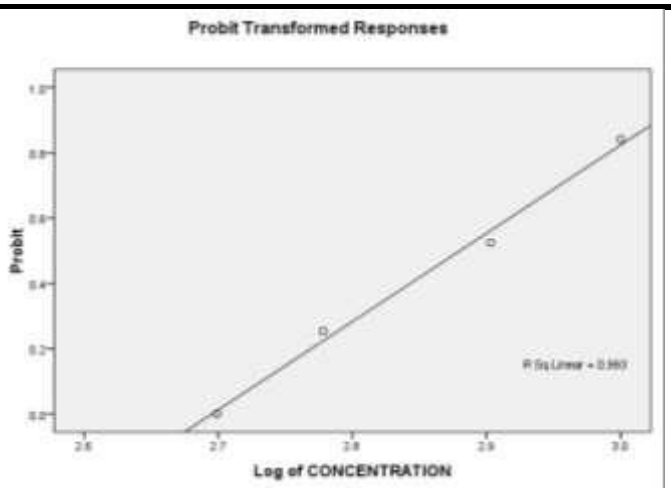

Fig. 1: The graph showing linear curve between Log concentrations of sodium fluoride against probit mortality of fish Clarias gariepinus

Table.4: Parameter Estimates for arsenic vs. Clarias gariepinus

	Parameter	Estimate	Std. Error	Z	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Probit[a]	Concentration	6.110	1.845	3.312	.001	2.495	9.726
	Intercept	-9.056	2.795	-3.240	.001	-11.851	-6.260

a. PROBIT model: PROBIT(p) = Intercept + BX (Covariates X are transformed using the base 10.000 logarithm.)

Table.5: Log concentration, observed responses in fish Clarias gariepinus

Number	Concentration	Number Of Subjects	Observed Responses	Expected Responses	Residual	Probability
1	1.301	6	1	.807	.193	.134
2	1.398	6	2	1.823	.177	.304
3	1.602	6	3	4.611	-1.611	.768
4	1.653	6	6	5.114	.886	.852
5	1.778	6	6	5.789	.211	.965

Table.6: Confidence Limits for fish Clarias gariepinus

Probibility Probit Point		95% Confidence Limits for Concentration			95% Confidence Limits for log(Concentration)[a]		
		Concentration	Lower Bound	Upper Bound	Concentration	Lower Bound	Upper Bound
	LC1	12.626	3.133	18.568	1.101	.496	1.269
	LC25	23.528	13.540	29.175	1.372	1.132	1.465
	LC45	28.934	20.762	35.956	1.461	1.317	1.556
	LC50	30.337	22.603	38.210	1.482	1.354	1.582
	LC75	39.116	31.793	59.390	1.592	1.502	1.774
	LC96	58.678	44.318	148.423	1.768	1.647	2.172
	LC99	72.893	51.518	248.919	1.863	1.712	2.396

Table.7: Behavioral changes in Clarias gariepinus after exposure to various concentration of fluoride and arsenic

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Parameter	**Control group**	**F^- exposed group**	**As exposed group**
Body position	Horizontal at the bottom of the aquarium	Both horizontal and vertical	At the bottom of the aquarium
Operculum movement	2 per 5 minutes	6-7 per 5 minutes	7-8 per 5 minutes
Sensitivity to food	Immediate	Slow	Initially unresponsive then sluggish
Swimming movements	Normal	Erratic	Erratic

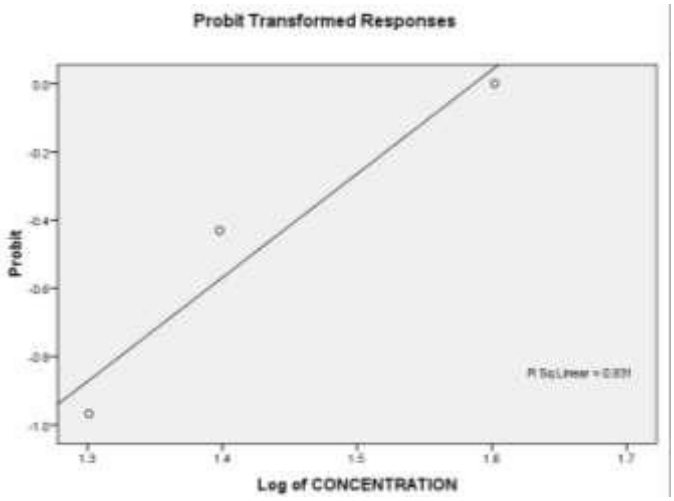

Fig. 2: *The graph showing linear curve between Log concentrations of arsenic trioxide against probit mortality of fish Clarias gariepinus*

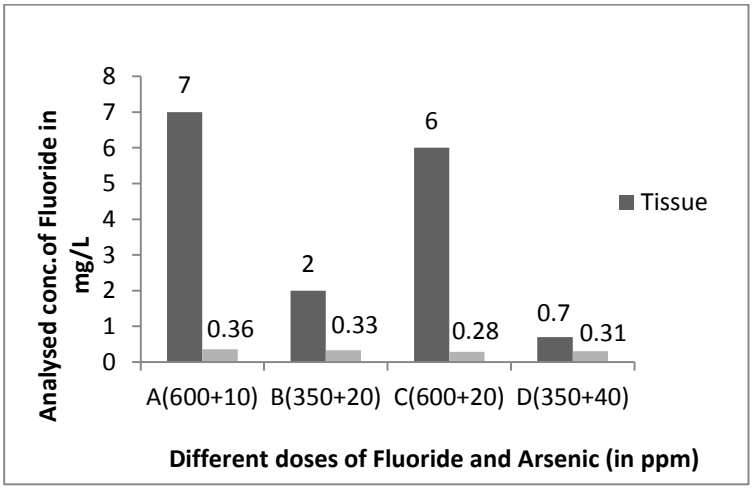

Fig. 3: Comparison in the concentrations of arsenic in blood and tissue of Clarias gariepinus.

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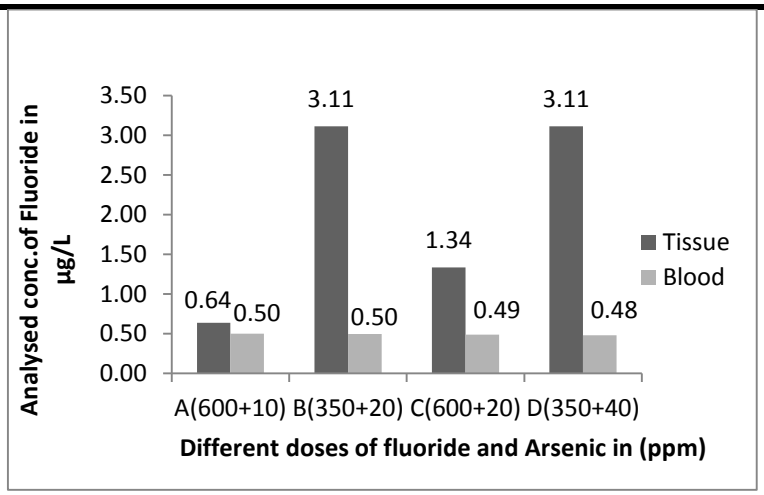

Fig. 4: Comparison in the concentrations of fluoride in blood and tissue of Clarias gariepinus

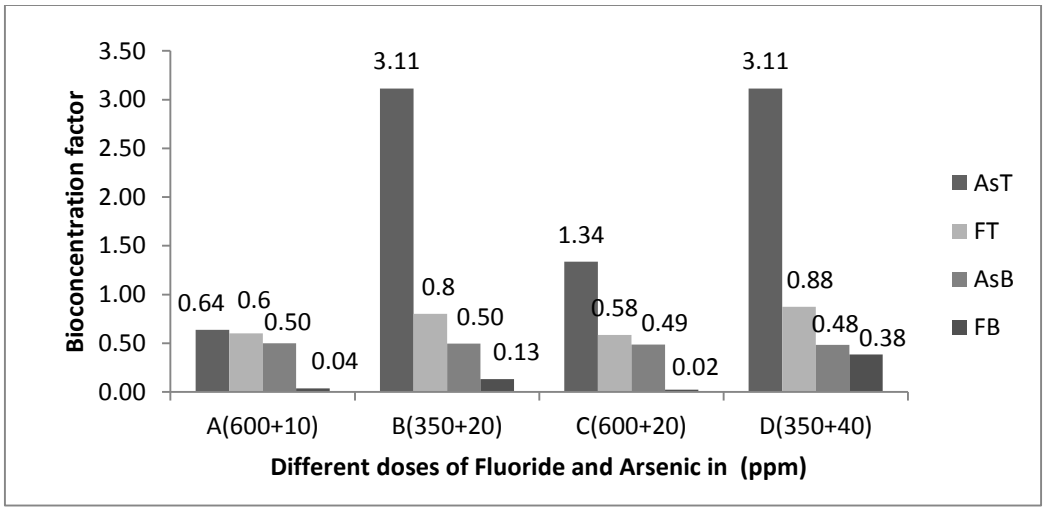

Fig.5: Comparison in the bio concentration factors of arsenic and fluoride in blood and tissues of Clarias gariepinus

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Physicochemical Properties of Two Fish Ponds in Akure, Implications for Artificial Fish Culture

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Abstract— *The physicochemical analyses of water samples collected from Ponds located at the Department of Fisheries and Aquaculture, Federal University of Technology, Akure, FUTA and Pond in Oda Road, Akure were conducted using standard laboratory techniques.The values of the parameters ranged from pH7.10±0.06^{b} - 8.39±0.01^{c}, conductivity 1.03±0.57^{c}- 7.72±1.16^{b}mS/cm, TDS 5.17±0.58^{c} - 3.85±0.58^{b}mg/l, turbidity 1.87±0.09^{c} - 0.60±0.06^{b} NTU, TSS 2.12±0.01^{c} - 1.17±0.01^{b}mg/l, BOD 12.60±0.06^{b} - 12.63±0.09^{b}mg/l, DO 62.77±0.03^{c}- 41.67±0.01^{b}mg/l, alkalinity7.64±0.01^{b}- 7.98±0.04^{b}mg/l, sulphate 1.10±0.01^{a} - 1.01±0.04^{a}mg/l, phosphate 2.13±0.09^{b} - 2.06±0.01^{b} mg/l, nitrate 4.13±0.01^{c} - 2.86±0.01^{b}mg/l.However, there should be periodic or constantwater qualitycontrol of fish ponds in order to ensure conducive environment for fish safety.The physico-chemical parameters investigated in this study, were within the range recommended for good fish production, indicating that the environmental conditions in these pond waters offer conducive conditions for fish survival and growth hence, increased productivity from fish ponds.*

Keywords— Fish pond, Physicochemicals parameters, water samples.

I. INTRODUCTION

Pond ecosystems are often teaming with rich vegetation and a diverse organismal life. Pond is a body of fresh water smaller than a lake. Ponds are naturally formed by a depression on the ground filling and retaining water. Streams or spring water is usually fed into these bodies. They can also be man-made ponds which can be created by damming a stream, and digging a holes (Burnett, 2008).The bottom of a pond is usually sediment of sand, decaying matter and micro-organisms. Pond water is usually stagnant. Ponds have a wide variety of microbial life. Nutrients are brought to the pond by streams that feed into, run off during rain, or by the human anthrogenic activities (Ehiagbonare and Ogunrinde, 2010). The water in soil, animal waste and decaying plant matter in the pond are broken down and used to fuel the pond ecosystem. Many animals that live in the surrounding area, migrating birds and nearby plants depends on these ponds for a rich source of nutrient and water (Ehiagbonare and Ogunrinde, 2010).

Water quality refers to the chemical, physical, biological, and radiological characteristics of water (Diersing, 2009). Water quality describes the condition of the water, including chemical, physical, and biological characteristics, usually with respect to its suitability for a particular purpose such as drinking, swimming or to ascertain the suitability of such ponds water for artificial fish culture (Ayanwale *et al*., 2012). Physical factors that are important in domestic fish farming can be measured by several factors, such as the concentration of dissolved oxygen, bacteria levels, the amount of salt (or salinity), or the amount of material suspended in the water (turbidity). In some bodies of water, the concentration of microscopic algae and quantities of pesticides, herbicides, heavy metals, and other contaminants may also be measured to determine water quality(Ayanwale *et al*., 2012). Water quality generallymeans the component of water which must be present for optimum growth of aquatic organisms. The determinant of good growth in water body includes dissolved oxygen, hardness, turbidity, alkalinity, nutrients, temperature, etc. Conversely, other parameters like biological oxygen demand, and chemical oxygen demand indicate pollution level of a given water body. In most water bodies, various chemical parameters occur in low concentrations (Diersing, 2009).

According to Burnett (2008), ponds have an average of 184.5 different types of microbes. There are many factors that determine the variety and number of organisms that live in a pond. These factors include oxygen, light, size, shelter and competition for food and nutrients. Quality of pond water could be detected through physico-chemical and microbial analysis so that people could be aware of its use for different purposes.The present study therefore investigate the physicochemical parameter of two selected pond water.

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II. METHODOLOGY

Study Area

This study was carried out in ponds in Ijigba layout, off Oda road and Federal University of Technology both in Akure, Ondo State, Nigeria. Ijigba layout lies between latitudes 7.2º North and longitude 5.2º East. Federal University of Technology, Akure (FUTA) lies between longitude 5.1º East and latitude 7.2º North. Operations such as integrated fish pond production having both concrete and earthen fish ponds stocked with tilapia fish and catfish are been performed in these ponds.

Plate.1: Oda Road Pond

Plate.2: FUTA Fish Pond

Collection of Fresh Water Samples

Water samples were collected aseptically from ponds water surface using sterile cap bottles. The water samples were transported to the laboratory, FUTA in an ice packed container for physico-chemical analyses.

Analysis of Water Quality in Pond Using Physico-Chemical Parameters

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Test for pH

This test was carried out to determine the hydrogen ion concentration in the pond water sample. The pH was determined using a pH meter (Suntex model sp-701), which was first calibrated with buffer 4 and 7 (using 1M NaOH and 1M HCL). The probe of the pH meter was rinsed with distilled water. Samples of 20 ml of pond water were measured in a labeled beakers, then the probe was inserted into the sample collected. The reading was taken when the pH meter displayed a stable value. The probe was rinsed with distilled water and cleaned with tissue paper after each insertion in the various samples (Ehiagbonare and Ogunrinde, 2010).

Test for Turbidity

This test was carried out to measure the degree to which water loses its transparency due to the presence of suspended solid particles. The turbidity was determined using a turbidometer (Nach model 2100N). The turbidometer was turned on to initialize. The cuvette of the meter was rinsed with distilled water, and 10 ml of distilled water was dispensed into the cuvette then placed in the meter to calibrate it and later poured off. After the meter had been calibrated, 10mL of pond water samples was dispensed in the cuvette and placed in the turbidometer (Nach model 2100N) at a wavelength of 810 nm. Readings were taken and recorded in units known as Formation Turbidity Unit (FTU) (Ademoroti, 1996).

Test for Conductivity and Total dissolved Solid (TDS)

This test was carried out to determine the concentration of the dissolved mineral salt and to determine the ionic effect in water sample. The conductivity was determined using conductivity meter (Labtech model 648). Pond water samples of 20 ml was measured and dispensed into the labeled beakers. The meter was switched on and its probe rinsed with de-ionized water, and the probe was inserted into the pond water samples, and the read button was pressed on the meter to take the readings. The unit is µs/cm (micro Siemens per cm) and the total dissolve solid (TDS) ions such as potassium, sodium, chloride, carbonate, sulfate, calcium, and magnesium that contribute to the dissolved solids in water were determined using the formular: TDS = 0.5 × Conductivity (Ehiagbonare and Ogunrinde, 2010).

Test for Total Suspended Solid (TSS) and Total Solid (TS)

This test was carried out to determine the concentration of suspended solid particles in pond water samples, it basically refers to matter suspended or dissolved in water. Beakers was rinsed and labeled, then pond water samples was homogenized with industrial blender which was later poured into the labeled beakers. Ten (10) ml of homogenized pond water sample in the beaker was dispensed into the cuvette and placed in the spectrophotometer (Hach 3900). Readings were taken and recorded; the unit of measurement is mg/l. Total solid test was conducted to determine the concentration of both suspended and dissolved solids in pond water samples. The formular used was: TS = TSS + TDS (Ehiagbonare and Ogunrinde, 2010).

Dissolved Oxygen (DO)

This was carried out to determine the amount of oxygen present in water. Two (2) ml of manganese sulphate and 2ml of alkali iodide azide reagent were added to the 20 ml of pond water measured and a brownish colour was obtained. The solution was then made to stand until it formed clear supernatants and concentrated sulphuric acid was added for preservative purpose and was shaken to distribute iodide evenly. Sodium thiosulfate was used in titrating to get a pale yellow and 1ml of 1% starch was added to get blue-black colour. At a point, the blue-black colour disappeared which is referred to or known as the end point. (Ehiagbonare and Ogunrinde, 2010).

Biological Oxygen Demand (BOD)

Water samples were aerated for five days at 20^{o}C in an incubator in a BOD bottle with a volume of 355 ml and 105 ml volume of the samples was used. Distilled water was used as water solution for dilution and as a blank which were aerated for five days using clean supply of compressed air, later the dissolved oxygen (DO) was then taken after five days of incubation and the BOD was determined using the mathematical formula below:

BOD (mg/l) = DO_o - DO_d , DO_o =dissolved oxygen of the first day, DO_d = dissolved oxygen after five days (Ehiagbonare and Ogunrinde, 2010).

Test for Sulphate, Nitrate, and Phosphate using Colorimetric Method

Sulphate was determined using colorimetric method where 25ml of each sample was measured in a Nessier tube; 25 ml of Barium chloride was added. The solution, which gave various colours, was allowed to stand for 15 minutes and its turbidity was measured at a wavelength of 420 nm in a spectrophotometer. Nitrate was determined by measuring 10 ml of the sample into a Nessier tube and the same amount of distilled water was measured into a Nessier tube, 0.5 mL of brucine and 20 mL of concentrated sulphric acid was added to each, thereafter the turbidity of colour produced by each tube was measured using a spectrophotometer (Hach 3900) at a wavelength of 470 nm (Ehiagbonare and Ogunrinde, 2010). Phosphate were measured using Nessler

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reaction and Ascorbic acid method respectively (Njoku *et al*., 2015)

III. RESULTS

Physicochemical Analysis of Pond waters

The results for the physicochemical analysis (pH, Conductivity, Turbidity, TSS, TDS, TS, BOD, DO, Nitrate, Sulphate, Phosphate) of the pond waters are shown in Table 1. The Oda Road pond has a lower conductivity value of 1.03 ms/cm,andhigher value of dissolved oxygen (DO) 62.77 ms/cmwhile FUTA pond had a lower turbidity value of 0.60 NTU.

Table.1: Physicochemical Parameters of Pond waters

Parameters	Oda Road Pond	FUTA Pond
Temperature	26.80±0.06^{b}	28.40±0.12^{b}
pH	7.10±0.06^{b}	8.39±0.01^{c}
Conductivity ms/cm	1.03±0.57^{c}	7.72±1.16^{b}
Turbidity (NTU)	1.87±0.09^{c}	0.60±0.06^{b}
Alkalinity (mg/l)	7.64±0.01^{b}	7.98±0.04^{b}
TSS (mg/l)	2.12±0.01^{c}	1.17±0.01^{b}
TDS (mg/l)	5.17±0.58^{c}	3.85±0.58^{b}
TS (mg/l)	5.19±0.58^{c}	3.88±1.16^{b}
BOD (mg/l)	12.60±0.06^{b}	12.63±0.09^{b}
DO (mg/l)	62.77±0.03^{c}	41.67±0.01^{b}
Nitrate (mg/l)	4.13±0.01^{c}	2.86±0.01^{b}
Sulphate (mg/l)	1.10±0.01^{a}	1.01±0.04^{a}
Phosphate (mg/l)	2.13±0.09^{b}	2.06±0.01^{b}

Data along the same row are not significantly the same

IV. DISCUSSION

The pH is a major factor that determines the growth of microorganisms. The pH measurement helps to determine if the water is a proper environment for fish, plants and algae. The pH obtained from both ponds in this study were within the range of pH 7.0 to 10.0 required for aquaculture as reported by Njoku *et al*. (2015). Ehiagbonare and Ogunrinde (2010),and Kamal *et al*. (2007).

The temperature obtained from this study ranged from 26^{o}C - 29^{o}C which is still within the limit that supports fish productivity. Ntegwu and Edema (2008) has reported optimum temperature 20^{o}C - 30^{o}C for increased fish productivity. This finding corroborates the report of Fafioye (2011) who observed a temperature of 27^{o}C - 28^{0}C in the preliminary studies and water characteristic of microbial population in Kojalo fish pond.

The results of conductivity from this study ranged from 1.03 ± 0.57 µs/cm to 7.72 ± 1.16 µs/cm. Higher conductivities (µs/cm) of water were observed in FUTA Pond which might be due to discharge of pollutants into the pond water.. The FAO acceptable limit for conductivity in aquaculture is between 20 and 1500 µs/cm (DWAF, 1996). This limit was not exceeded in the ponds studied. Thus, the parameter is suitable for fish production. However, the result is in contrast to the findings of Ehiagbonare and Ogunrinde (2010) who reported conductivity value of 0.012 µs/cm – 0.017 µs/cm for fish pond water in Okada and its environs.

It was observed that the turbidity values obtained from the FUTA pond and Oda Road pond were 0.60NTU and 1.87NTU respectively. Turbidity hinders the penetration of sunlight in the pond making it difficult for aquatic habitat such as algae that require light for photosynthesis to receive the positive effect of light (Ali *et al*., 2004). However, the results of turbidity obtained from this study, is in contrast to the findings of Ehiagbonare and Ogunrinde (2010) who reported higher turbidity value of 5NTU - 170NTU.

The BOD obtained from Oda Road pond and FUTA pond were 12.60mg/l and 12.63mg/l respectively. The BOD obtained from this study was higher than the findings of Ehiagbonare and Ogurinde (2010) who reported BOD of 3.38mg/l in Oloku and 2.4mg/l in concrete ponds in Igusa and 1.6mg/l in earthen pond at Afugle during the study of physiochemical analysis of pond water in Okada and its environs. The increase in BOD might be due to excreta of the fish, feed use or high organic matter (Kay *et al.*, 2008). However, these values are below the BOD standard of 30 mg/l recommended by the Federal Environmental Protection Agency, FEPA (FEPA, 1991). This implies that the pond water is devoid of pollution and the fishes are not affected negatively.

The dissolved oxygen (DO), obtained in this study ranged from 41.67mg/l to 62.77mg/l. The result obtained from this study is higher than the result obtained by Onome and Ebinimi, (2010) who reported DO of 4.34 mg/l and 6.33mg/l from the surrounding industries near fish farm, and Ehiagbonare and Ogunrinde (2010) who reported DO value of 9.3mg/l – 16.2mg/l. The high value of dissolved oxygen recorded in study could be attributed to elevated temperature, increased microbial and organic loads and resultant increase in metabolic activity (FAO, 2005). Similar findings was also reported by Okpokwasili and Ubah, (1991) on water quality and bacterial disease in fish pond. However, Saloom and Duncan, (2005) suggested that the minimum DO should be 5mg/l for tropical fish.

The alkalinity values obtained from Oda Road Pond and FUTA pond were 7.64mg/l to 7.98mg/l respectively.

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Ehiagbonare and Ogunrinde (2010) reported alkalinity values of between 35mg/l and 135mg/l for fish pond water. Njoku *et al*. (2015) on the other hand, reported alkalinity values of between 200mg/l and 290mg/l in concrete pond and between 18mg/l and 24mg/l in earthen pond. He suggested the optimum alkalinity for increased fish production is between 20mg/l and 300mg/l. The reason for the variations in the alkalinity values might be due to high limestone in the fish pond water.

Findings from the result of nitrate concentration obtained in this research work ranged from 2.86mg/l to 4.13mg/l and was lower than the maximum permissible limit of 10mg/l. The low concentration of nitrate might be due to absence of eutrophication. However, this findings corroborate the findings of Ehiagbonare and Ogunrinde (2010) who reported nitrate value of between 2.21mg/l and 4.91mg/l. Eze and Ogbaran (2010) observed that nitrate is a skin irritant and will cause the fish to display symptoms of irritability such as rubbing themselves, jumping and skimming across the surface of the pond. High nitrate value concentration causes algal bloom while nitrate deprivation leads to increased lipid content in algae and in turn affect the water ecosystem. Furthermore, Dourborow *et al*. (1997) stated that high concentration of nitrate prevents the blood cells from absorbing oxygen from water and this process turns their blood to a dull brown color and hence the popular name of nitrite poisoning "brown blood disease".

It was revealed from this findings, that the phosphate level in FUTA pond and Oda Road pond was below the 3mg/l standard. However, this findings is in agreement with the work of Ehiagbonare and Ogunrinde (2010) who reported phosphate values of between 1.40mg/l and 4.51mg/l. This low values of phosphate may be due to the type of fish feed introduced into the pond or surface run off, and could also be from the building materials used in the construction of the ponds. According to Durborow *et al*. (1997), high concentration of phosphate may cause algal bloom leading to killing of fishes in the pond. Also, Eze and Okpokwasili (2010) stated in their findings that this limit should be controlled to avoid eutrophication of the ponds. These fishes can also store phosphate in their organs and when they die, they release the previously absorbed Phosphate into the water which triggers the growth of new algae and create algal bloom or eutrophication.

The result of sulphate concentration in the ponds from this study varied from 1.01 to 1.10 (mg/l) with the Oda Road pond higher than the FUTA pond. The result obtained from this study was similar to the findings of Ehiagbonare and Ogunrinde (2010) who reported sulphate concentration of between 0.66 and 1.09m/l. However, Utang and Akpan (2012) had reported sulphate concentration of 42.46 and 57.36mg/l. Sulphate that leach from soil fertilized with animal manure which got into the water body may be responsible for the high value of sulphate (Ehiagbonare and Ogunrinde (2010).

V. CONCLUSION

Analysing water is an important part of environmental monitoring which is essential part of keeping the planet healthy and sustainable. When water quality is poor, it affects not only aquatic life but the surrounding ecosystem as well which were carried out in this study. Findings from this work revealed that regularly monitoring water parameter such as temperature, pH etc provide insight to the health of the aquatic ecosystems. The results may be used to pinpoint any changes or trends that appear in water bodies over a period of time.Conclusively, as we continue to build cities, clear land for farming such as aquaculture and make other man-made changes to the natural environment, water quality monitoring should be done to ensure good functioning of the aquatic ecosystem increased fish productivity.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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The Family of Carabidae (Coleoptera) in Artvin Hatila National Park of Turkey

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Abstract**—** *The faunistical studies on the family Carabidae (Coleoptera) species in Artvin Hatila National Park in Turkey. In this study, totally 32 species belonging to Pterostichinae, Brachininae, Carabinae, Platyninae, Lebiinae, Nebriinae and Harpalinae subfamilies of Carabidae were collected from Artvin province during 2011-2014. Among these, Amara lucida Duftschmid, Amara aulica Panzer, Brachinus elegans Chaudoir, Brachinus crepitans Linné, Carabus scabrosus Olivier, Anisodactylus binotatus Fabricius, Carabus coriaceus Linnaeus, Carabus mulsantianus Paykull, Carabus graecus Dejean, Harpalus affinis Schrank, Harpalus caspius Panzer, Ophonus cribricollis Dejean, Ophonus azureus Fabricius, Ophonus subquadratus Dejean, Pterostichus anthracinum Illiger are the first records from Artvin.*

Keywords— Carabidae, Artvin Hatila National Park.

I. INTRODUCTION

The family of Carabidae is one of the biggest family of the Coleoptera order in terms of the number of species as about 40.000 in the worldwide [1]. The species of the Carabidae family lives under the stones, logs and crusts [2]. 73,5% of these species lives carnivorously, 19,5% of them lives omnivorous and 8,1% of them lives fitofag [2]. The carnivore Carabides are usually eat insects, earthworms, snails, caterpillars and tube lights [3,4]; and fitofags eats plants [5,6]. Some carnivore species of carabids are used significant elements of the biological contention [7]. Carabidae where they live are one of the best bioindicators for the protection of ecosystems [8,9,10,11].

The Carabidae family is pullulated with the species and more than 170 species have been determined in the based on 1100 species in Turkey. Also 41% of them are endemic. This number does not reflect the actual number of the Carabidae species in Turkey [12,13,14].

There are several studies about the family of Carabidae in Turkey [12,15,16,17,18,19,20,21,22,23,24,25,26,27,28, 29,30,31,32,33]. But there has not been conducted any study about the Carabidae fauna of Artvin. It is aimed that this research define the fauna of Carabidiae family in Hatila Valley National Park and around by using of the pitfall trap method.

II. MATERIAL AND METHODS

Artvin Hatila Valley National Park was announced as a national park in 1994 and became one of the 40 national parks in our country. This national park area has 16998-hectare area and the GPS coordinates of 41° 11′ 11″ N, 41° 44′ 09″ E′. The altitude of the highest point is 3224 m (Kurt Mountain), and the lowest point is 160 m (Çoruh River). This national park area has relict and endemic plant cover, unusual geological and geomorphological structure, unique scenic beauty, rich fauna and recreational potential. Semi-arid, subhumid and humid climates are seen in the area. According to the grid system that was applied by Davis that this area in A8 square and Colchis part of Euro-Siberian flora area of Holarctic flora region concerning the phytogeography and flora areas [34]. The field of the study has characteristics of a transition region between Black Sea climate and Continental climate [35].

The samples were collected from the Artvin Hatila National Park in between 2011-2014 years constitute the primary material of the study. Other materials are as follow; pitfall traps for collecting the species, pliers, stereomicroscope, numbered insect stings, glass materials and various chemicals for preparation. The samples of the species belong to Carabidae family were collected from the weeds (meadow grass, ferns, etc.) unopened areas for agriculture and forest lands.

The most of the species of Carabidae lives under the soil and several studies show that the pitfall traps were used to catch them in several studies. 80 pitfall traps were placed in 20 different areas by embedding till on the ground level. The traps has plastic containers were places and specially prepared malt solution and land snails belong to the families of Cochlostomatidae, Pomatiidae, Truncatellidae, Aciculidae. These traps were controlled every 15 daysand insects were collected from their in and the malt solution and snails were changed as well. The malt solution was prepared by boiling one litter beer and 250g sugar together, then refrigerated. The preparation was applied to the Carabidae species in the laboratory conditions. The

diagnosis of these collected samples was actualized by using the determination keys of Lindroth [36], Geigenmüller & Trautner [37], Forsythe [38] and Hurka [1].

The similar samples were recognized in the forest entomology laboratary in Artvin Çoruh University After that some species were determineted Protection Department Entomology Laboratory and Entomology Museum of Plant Protection of Agricultural Faculty of Erzurum Atatürk University. The studies about Carabidae species were useful for publishing Lindroth [36], Lodos [12], Gueorguiev & Gueorguiev [37], Kryzanovsky *et al.*, [39], Hurka [1], Casale and Taglianti [40], Neculiseanu and Matalin [41], Casale *et al.*, [13] and Kesdek & Yıldırım [42] the distribution of species in Turkey and the world.

III. RESULTS AND DISCUSSION

As a result of the studies, 32 Carabidae species belonging to Pterostichinae (9), Brachininae (2), Carabinae (14), Platyninae (1), Lebiinae (1), Nebriinae (1), Harpalinae (4) 15 subspecies and 7 subfamilies were recorded in Hatila National Park in Artvin.

Pterostichinae

***Amara* Bonelli, 1810**

***Amara similata* (Gyllenhal, 1810)**

Material examined: Artvin – Hatila National Park: 27.VI.2014, 2 ♂♂, ♀ ; 17.VII.2014, 3 ♀♀, 2 ♂♂

Distribution in Turkey: Artvin, İzmir, Kars [28,40].

Distribution in the world: Algeria, Anatolia, Asia, Caucasus Crimea, Czech Republic, Europe, Moldova, North Africa, Northwest Himalaya, Russia, Siberia, Slovakia, South of Armenia, Turkestan, Turkey, Ukraine [1,37,39,40,41,43].

***Amara (Paracelia) saxicola* (Zimmermann, 1831)**

Material examined: Artvin – Hatila National Park: 29.VI.2014, 3 ♂♂, ♀; 17.VII.2014, 2 ♀♀.

Distribution in Turkey: Artvin, Erzurum, Kars [40,44].

Distribution in the world: Anatolia, Armenia, Azerbaijan, Georgia, Caucasus and Mediterranean Countries, Crimean Mountains, Kazakhstan, Moldova, Southeast of Central Asia, Turkey, Ukraine [39,40,41].

***Amara aenea* (De Geer, 1774)**

Material examined**:** Artvin – Hatila National Park: 18.VI.2014, 4 ♂♂, 2 ♀♀; 11.VII.2014, ♂, 3 ♀♀.

Distribution in Turkey: Ankara, Antalya, Ardahan, Artvin, Bitlis, Bolu, Erzincan, Eskişehir, Erzurum, Giresun, Gümüşhane, Iğdır, İzmir, Kars, Kastamonu, Konya, Manisa, Muğla, Nevşehir, Ordu, Rize, Sinop, Tokat [23,29,40,43,45,46].

Distribution in the world: Armenia, Bosnia and Herzegovina, Bulgaria, Canary Islands, Corsica, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Georgia, Greece, Jordan, Netherlands, Iraq, Iran, Ireland, Israel, Italy, Latvia, Liechtenstein, Lithuania, Lebanon, Luxembourg, Macedonia, Malta, Moldova, Nearikovo North Africa, Norway, Portugal, Romania, Russia, Sardinia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Syria, Turkey, Ukraine, United Kingdom, Yugoslavia [46].

***Amara lucida* (Duftschmid, 1812)**

Material examined: Artvin – Hatila National Park: 20.VI.2012, 5 ♂♂, ♀; 14.VII.2013, 2 ♀♀, 2 ♂♂.

Distribution in Turkey: Anatolia, Erzurum, Kahramanmaraş [20,40,44].

Distribution in the world: Armenia, Austria, Azerbaijan, Belarus, Bosnia and Herzegovina, Bulgaria, Caucasus, Czech Republic, Denmark, Estonia, Finland, France, Georgia, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Macedonia, Moldova, Montenegro, Moravia, Netherlands, North Africa, Norway, Poland, Portugal, Romania, Russia, Serbia, Slovakia, Slovenia, Transcaucasia, Ukraine [1,6,37,39].

***Amara ovata* (Fabricius, 1792)**

Material examined: Artvin – Hatila National Park: 20.VI.2012, 5 ♂♂; 14.VII.2013, 3 ♀♀, 2 ♂♂

Distribution in Turkey: Artvin, Erzurum, Kars [20,44].

Distribution in the world: Algeria, Austria, Azerbaijan, Belgium, Belarus, Bosnia and Herzegovina, Bulgaria, Caucasia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Germany, Japan, Italy, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Luxembourg, Hungary, Macedonia, Moldova, Norway, Poland, Portugal, Romania, Russia, Serbia, Slovenia, Slovakia, Sweden, Switzerland, Syria, Tajikistan, Transcaucasia, Transbaikalia, Turkey, Turkmenistan, Ukraine, Urals, Uzbekistan [1,6,37,39].

***Amara (Curtonotus) aulica* (Panzer, 1796)**

Material examined: Artvin – Hatila National Park: 28.VI.2013, 2 ♂♂; 10.VII.2014, ♀; 24.VII.2014, ♀.

Distribution in Turkey: Kahramanmaraş [20,40].

Distribution in the world: Albania, Armenia, Austria, Azerbaijan, Bosnia and Herzegovina, Canary Islands, Czech Republic, Denmark, Estonia, Faroe Islands, Finland, France, Georgia, Germany, Greece, Hungary, Ireland, Italy, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Macedonia, Mongolia, Moldova, Netherlands, North America, Montenegro, Norway, Poland, Romania, Russia, Serbia, Siberia, Slovakia, Tajikistan, Turkey, Turkmenistan, Ukraine, Uzbekistan [1,6,47].

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***Pterostichus* Bonelli, 1810**

***Pterostichus (Melanius) anthracinum* (Illiger, 1798)**

Material examined: Artvin – Hatila National Park: 20.VI.2012, 3 ♂♂, ♀ ; 14.VII.2013, 2 ♀♀, 2 ♂♂.

Distribution in Turkey: Anatolia (no locality) [40].

Distribution in the world: Europe, Bulgaria, Czech Republic, Iran, Kyrgyzstan, Moldova, Central Asia, Russia, Slovakia [1,37,39,40,41].

***Pterostichus (Melanius) nigrita* (Paykull, 1790)**

Material examined: Artvin – Hatila National Park: 20.VI.2012, 2 ♂♂; 14.VII.2013, 3 ♀♀, ♂

Distribution in Turkey: Ardahan, Artvin, Eskişehir, Erzurum, Kars [21,40,42].

Distribution in the world: Bulgaria, Caucasus, Czech Republic, England, Central Asia and Europe, Moldova, Russia, Siberia Slovakia, Turkey, Turkistan [1,37,39,40,41].

***Zabrus* Clairville, 1806**

***Zabrus spinipes* (Fabricius, 1798)**

Material examined: Hatila National Park: 30.VI.2014 ♂; 22.VII.2014, 2 ♀♀.

Distribution in Turkey: Ankara, Artvin, Trabzon [15,48].

Distribution in the world: Austria, Bulgaria, Czech Republic, Sweden, Hungary, Poland, Slovakia, Turkey, Greece, Yugoslavia [6].

Brachininae

***Brachinus* Weber, 1801**

***Brachinus elegans* (Chaudoir, 1842)**

Material examined: Artvin – Hatila National Park: 20.VI.2012, 3 ♀♀; ♂, 17.VII.2013, 2 ♀♀.

Distribution in Turkey: Tokat [48].

Distribution in the world: Armenia, Azerbaijan, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, France, Georgia, Iraq, Iran, Italy, Kazakhstan, Moldova, Morocco, Romania, Russia, Slovakia, Slovenia, Spain, Ukraine, Yugoslavia [6].

***Brachinus crepitans* (Linné, 1758)**

Material examined: Artvin – Hatila National Park: 20.VI.2012, 2 ♂♂; 14.VII.2013, 3 ♀♀, ♂.

Distribution in Turkey: Amasya, Giresun, Kahramanmaraş [20,40,48].

Distribution in the world: Azerbaijan, Belarus, Bosnia and Herzegovina, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Georgia, Germany, Greece, Hungary, Luxembourg, Latvia, Moldova, Montenegro, Norway, Romania, Russia, Slovakia, Slovenia, Syria, Tajikistan, Turkmenistan, Ukraine, Yugoslavia [6].

Carabinae

***Calosoma* Weber, 1801**

***Calosoma inquisitor* (Linnaeus, 1758)**

Material examined: Artvin – Hatila National Park: 20 IV.2012, 2♂♂; 11.VII.2014, 2 ♀♀, ♂.

Distribution in Turkey: Artvin, Erzincan, Tunceli, Antalya [49].

Distribution in the world: Armenia, Azerbaijan, Belarus, Belgium, Germany, Greece, France, Hungary, Japan, Italy, Iran, Luxembourg, Morocco, Netherlands, North Africa, Norway, Poland, Russia, Slovakia, Slovenia, Sweden, Switzerland, Ukraine, United Kingdom, Turkey, [37].

***Calosoma sycophanta* (Linné, 1758)**

Material examined: Artvin – Hatila National Park: 20.VI.2012, 2 ♂♂; 14.VII.2013, 2 ♀♀, 2 ♂♂.

Distribution in Turkey: Ankara, Artvin, Adıyaman, Kahramanmaraş [15,20,40].

Distribution in the world: Albania, Armenia, Austria, Azerbaijan, Belgium, Bosnia and Herzegovina, Bulgaria, Czech Republic, China, Cyprus, Denmark, Estonia, Germany, Greece, France, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Macedonia, Moldova, Montenegro Morocco, South Moravia, Netherlands, Croatia, North Africa, North America, Poland, Portugal, Romania, Russia, Serbia, Siberia, Slovakia, Slovenia, Syria, Spain, Tunisia, Turkey, Turkmenistan, Ukraine, Uzbekistan [1,6,37,39,47].

Carabus Linnaeus, 1758

***Carabus convexus* (Fabricius, 1775)**

Material examined: Artvin – Hatila National Park: 20.VI.2012, 3 ♂♂, 2 ♀♀ ; 14.VII.2013, 2 ♀♀, ♂

Distribution in Turkey: Black sea region of Turkey, Marmara Region, Rize [13,48].

Distribution in the world: Andorra, Austria, Belarus, Belgium, Bosnia and Herzegovina, Bulgaria, Denmark, Finland, France, Germany, Greece, Hungary, Italy, Kazakhstan, Luxembourg, Macedonia, Moldova, Norway, Netherlands, Poland, Romania, Russia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey, Ukraine, Yugoslavia [6].

***Carabus scabrosus* (Olivier, 1795)**

Material examined: Artvin – Hatila National Park: 28.VI.2014, ♂, ♀; 07.VII.2014, 2 ♀♀.

Distribution in Turkey: Ordu [48].

Distribution in the world: Armenia, Azerbaijan, Bulgaria, Greece, Georgia, Iran, Turkey, Ukraine [6].

***Carabus (Procrustes) coriaceus* (Linnaeus, 1758)**

Material examined: Artvin – Hatila National Park: 20.VI.2012, 2 ♂♂, 2 ♀♀; 14.VII.2013, 3 ♀♀, ♂.

Distribution in Turkey: Izmir, Canakkale, Marmara Region, Muğla Western Mediterranean [13,29,46,49].
Distribution in the world: Armenia, Austria, Azerbaijan, Belarus, Bosnia and Herzegovina, Czech Republic, Denmark, Estonia, France, Georgia, Germany, Greece, Hungary, Iran, Iraq, Ireland, Israel, Italy, Jordan, Kazakhstan, Kyrgyzstan, Latvia, Lebanon, Lithuania, Luxembourg, Macedonia, Moldova, Netherlands, Norway, Poland, Romania, Russia, Sardinia, Sicily, Slovakia, Slovenia, Syria, Tajikistan, Turkey, Ukraine, Uzbekistan, Yugoslavia [13].

***Carabus mulsantianus* (Paykull, 1790)**
Material examined: Artvin – Hatila National Park: 20.VI.2012, 2 ♂♂; 14.VII.2013, 2 ♀♀, 2 ♂♂.
Distribution in Turkey: Kahramanmaras [15,50].
Distribution in the world: Turkey [6].

***Carabus (Pachystus) graecus* (Dejean, 1826)**
Material examined: Artvin – Hatila National Park: 20.VI.2012, 3 ♂♂; 14.VII.2013, 3 ♀♀, 2 ♂♂.
Distribution in Turkey: Aegean and Marmara Regions, Ankara, Canakkale, Western Mediterranean [15-50].
Distribution in the world: Armenia, Azerbaijan, Bulgaria, Caucasus Republics, Egypt, Greece, Georgia, Jordan, Iraq, Iran, Israel, Lebanon, Peninsula, Russia, Scandinavia, Syria, Turkey [13,51].

***Harpalus* Latreille, 1802**
***Harpalus affinis* (Schrank, 1781)**
Material examined: Artvin – Hatila National Park: 20.VI.2012, 4 ♂; 14.VII.2013, 4 ♀.
Distribution in Turkey: Rize [48].
Distribution in the world: Albania, Andorra, Azerbaijan, Belarus, Bosnia and Herzegovina, Czech Republic, Denmark, Egypt, Estonia, Finland, France, Germany, Greece, Hungary, Iran, Ireland, Israel, Italy, Kazakhstan, Kyrgyzstan, Lichtenstein, Lithuania, Macedonia, Moldova, Mongolia, Netherlands, Norway, Poland, Portugal, Romania, Russia, Slovakia, Slovenia, Turkey, Ukraine, Yugoslavia, Sweden, Switzerland [6].

***Harpalus smaragdinus* (Duftschmid, 1812)**
Material examined: Artvin – Hatila National Park: 20.VI.2012, 4 ♂♂; 14.VII.2013, 2 ♀♀, 3 ♂♂.
Distribution in Turkey: Antalya, Ardahan, Artvin, Bitlis, Bolu, Çanakkale, Çankırı, Erzurum, Giresun, Gümüşhane, İzmir, Kahramanmaraş, Kars, Malatya, Manisa, Sivas [20,29,40,42,50].
Distribution in the world: Albania, Austria, Azerbaijan, Belarus, Bosnia and Herzegovina, Bulgaria, Caucasus, Central Asia, Crimea, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Georgia, Germany, Greece, Hungary, Italy, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Luxembourg, Macedonia, Moldova, Montenegro, Norway, Poland, Romania, Russia, Serbia, Siberia, Slovakia, Slovenia, Sweden, Switzerland, Tajikistan, Turkey, Turkmenistan, Ukraine, United Kingdom, United States, Urals, Uzbekistan [1,6,37,39,47].

***Harpalus caspius* (Panzer, 1797)**
Material examined: Artvin – Hatila National Park: 20.VI.2012, 3 ♂♂, ♀; 14.VII.2013, 3 ♀♀, ♂.
Distribution in Turkey: Anatolia (no locality) [39].
Distribution in the World: Armenia, Austria, Azerbaijan, Bulgaria, Caucasus, Crimea, Croatia, Czech Republic, Georgia, Germany, Hungary, Iran, Kazakhstan, Moldova, Montenegro, Slovakia, Slovenia, Turkey, Ukraine [6,37,39].

***Harpalus distinguendus* (Duftschmid, 1812)**
Material examined: Artvin – Hatila National Park: 30.VI.2013, 4 ♂♂.
Distribution in Turkey: Ankara, Antalya, Artvin, Aydın, Bergama, Bayburt, Burdur, Çanakkale, Erzincan, Erzurum, Giresun, Gümüşhane, Isparta, İçel, İstanbul, İzmir, Kahramanmaraş, Konya, Tokat, Trabzon [29,40,42,50].
Distribution in the world: Afghanistan, Azerbaijan, Caucasus, Central Asia, Czech Republic, Europe, Iran, Kazakhstan, Korea, Middle Mediterranean Countries, Mongolia, North East Africa, North East and West China, Northern Russia, Siberia, Slovakia [1,40,51].

***Poecilus* Bonelli, 1810**
***Poecilus cupreus* (Linnaeus, 1758)**
Material examined: Hatila National Park: 30.VI.2014, 4 ♂♂; 22.VII.2014, 2 ♀♀, 2 ♂♂.
Distribution in Turkey: Ankara İzmir-Kemalpaşa, Antalya, Ardahan, Artvin, Aydın, Balıkesir, Bolu, Çanakkale, Çankırı, Erzurum, Kars, Kastamonu, Konya, Ordu, Sinop [29,40,50].
Distribution in the world: Albania, Armenia, Austria, Azerbaijan, Belarus, Belgium, Bosnia and Herzegovina, British Isles, Bulgaria, Caucasian Republics, Corsica, Crete, Croatia, Czech Republic, Denmark, Eastern Palearctic Region, Estonia, Finland, France, Georgia, Germany, Greece, Hungary, Iraq, Iran, Ireland, Israel, Italy, Jordan Latvia, Lebanon, Liechtenstein, Lithuania, Luxembourg, Macedonia, Moldova, Netherlands, Northern Ireland, Norway, Poland, Romania, Russia, Sardinia, Sicily, Slovakia, Slovenia, Spain, Sweden, Switzerland, Syria, the Arabian Peninsula, Turkey, Ukraine, Yugoslavia [51].

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***Pseudoophonus* Motschulsky, 1844**

***Pseudoophonus rufipes* (De Geer, 1774)**

Material examined: Hatila National Park: 20.VI.2012, 2 ♂♂; 14.VII.2013, 4 ♀♀, 2 ♂♂.

Distribution in Turkey: Adana, Adıyaman, Ankara, Antalya, Ardahan, Artvin, Bartın, Bingöl, Bursa, Çankırı, Diyarbakır, Erzincan, Erzurum, Eskişehir, Gaziantep, Giresun, Gümüşhane, Iğdır, Isparta, İçel, İzmir, Kahramanmaraş, Kayseri, Malatya, Karaman, Kars, Konya, Kütahya, Malatya, Muğla, Ordu, Osmaniye, Sivas, Tokat, Trabzon, Yalova [15,20,23,40,42,48,52,53].

Distribution in the world: Afghanistan, Albania, Albania, Austria, Azerbaijan, Algeria, Armenia, Belarus, Belgium, Bosnia and Herzegovina, Bulgaria, China, Crimea, Croatia, Czech Republic, Denmark, Egypt, Estonia, Finland, France, Georgia, Greece, Hungary, Ireland, Italy, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Luxembourg, Macedonia, Malta, Moldova, Morocco, Netherlands, North America, Norway, Poland, Portugal, Romania , Russia, Serbia, Siberia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Tajikistan, Tunisia, Turkey, Turkmenistan, Ukraine, United Kingdom, Urals, Uzbekistan [1,6,37,39,47,52].

***Pseudoophonus calceatus* (Duftschmid, 1812)**

Material examined: Hatila National Park: 30.VI.2014, 2 ♂♂; 22.VII.2014, 3 ♀♀, ♂.

Distribution in Turkey: Artvin, Trabzon [48].

Distribution in the world: Armenia, Austria, Azerbaijan, Belgium, Bosnia and Herzegovina, Bulgaria, China, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Greece, Georgia, Germany, Japan, Hungary, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Luxembourg, Macedonia, Moldova, Mongolia, Norway, North Korea, Poland, Portugal, Romania, Russia, Slovakia, Slovenia, Tajikistan, Turkmenistan, Ukraine, Uzbekistan, Yugoslavia [6].

Platyninae

***Dolichus* Bonelli, 1810**

***Dolichus halensis* (Schaller, 1783)**

Material examined: Hatila National Park: 30.VI.2014, 3 ♂♂, ♀; 22.VII.2014, 4 ♀♀.

Distribution in Turkey: Ankara, Artvin [15,49].

Distribution in the world: Armenia, Caucasus, Central Asia, Central and South Russia, Kazakhstan, Siberia, Turkey, Ukraine [39,40].

Lebiinae

***Cymindis* Latreille, 1806**

***Cymindis axillaris* (Fabricius, 1794)**

Material examined: Hatila National Park: 20.VI.2012, 2 ♂♂; 14.VII.2013, ♀.

Distribution in Turkey: Artvin, Çorum, Kahramanmaraş [20,48].

Distribution in the world: Austria, Azerbaijan, Bosnia and Herzegovina, Cyprus, Czech Republic, Denmark, Egypt, France, Germany, Greece, Hungary, Ireland, Israel, Italy, Latvia, Lebanon, Libya, Lithuania, Luxembourg, Moldova, Norway, Poland, Portugal, Romania, Russia, Slovakia, Slovenia, Syria, Turkey, Turkmenistan, Ukraine, Ukraine, United Arab Emirates, United Kingdom, United States, Yugoslavia [6].

Nebriinae

***Nebria* Latreille, 1802**

***Nebria brevicollis* (Fabricius, 1792)**

Material examined: Hatila Milli Parkı: 20.VI.2012, 2 ♂♂; 14.VII.2013, 3♀♀, ♂

Distribution in Turkey: Antalya, Artvin, Aydın, Denizli, Bolu, İzmir, İstanbul, Kastamonu, Ordu [29,40].

Distribution in the world: Albania, Andorra, Armenia, Azerbaijan, Balearic Islands, Belarus, Belgium, Bosnia and Herzegovina, British Isles, Bulgaria, Corsica, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Faroe Islands, Finland, France, Georgia, Germany, Greece, Jordan Netherlands Iceland, Iran, Iraq, Ireland, Israel, Italy, Latvia, Lithuania, Luxembourg, Macedonia, Moldova, Netherlands Norway, Russia, Romania, Russia, Russia, Sardinia, Sicily, Slovakia, Slovenia, Syria, the Arabian Peninsula, Turkey, Ukraine, Yugoslavia [51].

Harpalinae

***Anissodactylus* Dejean, 1829**

***Anisodactylus binotatus* (Fabricius, 1787)**

Material examined: Hatila National Park: 20.VI.2012, 2 ♂♂, ♀; 14.VII.2013, 3♀♀, ♂.

Distribution in Turkey: Adana, Anatolia (no locality), Kahramanmaraş [20,40].

Distribution in the world: Afghanistan, Albania, Algeria, Altai, Andorra, Armenia, Asor Islands, Austria, Azerbaijan, Bahrain, Belarus, Belgium, Bosnia and Herzegovina, Britain, Bulgaria, Caucasus, Crimea, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Faroe Islands, Germany, Greece, Iceland, Ireland, Italy, Kazakhstan, Kyrgyzstan, Latvia, Lithuania, Luxembourg, Macedonia, Malta, Moldova, Montenegro, Morocco, Netherlands, North Africa, Norway, Poland, Portugal, Romania, Russia, Serbia, Siberia, Slovakia, Slovenia, Syria, Spain, Sweden, Switzerland, Tajikistan, Turkey, Turkmenistan, Ukraine, Uzbekistan [1,6,37,47].

***Ophonus* Dejean, 1821**

***Ophonus (Hesperophonus) cribricollis* (Dejean, 1829)**

Material examined: Hatila National Park: 20.VI.2012, 2 ♂♂; 14.VII.2013, 3 ♀♀, ♂.

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Distribution in Turkey: Anatolia (no locality), Ankara [15,40].
Distribution in the world: Albania, Armenia, Austria, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, France, Georgia, Greece, Iraq, Iran, Spain, Israel, Italy, Macedonia, Moldova, Montenegro, Russia, Serbia, Slovakia, Switzerland, Turkey, Ukraine [1,6,37,39,47].

***Ophonus (Hesperophonus) azureus* (Fabricius, 1775)**
Material examined: Hatila National Park: 20.VI.2012, 2 ♂♂; 14.VII.2013, 4 ♀♀, 2 ♂♂.
Distribution in Turkey: Anatolia, Ankara, Bayburt, Erzurum, Sinop, Trabzon [15,40,42].
Distribution in the world: Andorra, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, Denmark, France, Georgia, Germany, Greece, Hungary, Iraq, Iran, Italy, Kazakhstan, Kyrgyzstan, Latvia, Luxembourg, Macedonia, Moldova, Netherlands, Poland, Romania, Russia, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Tajikistan, Turkmenistan, Ukraine, Uzbekistan [1,6,37,39,47].

***Ophonus (Hesperophonus) subquadratus* (Dejean, 1829)**
Material examined: Hatila National Park: 20.VI.2012, 3 ♂♂, ♀; 14.VII.2013, 2 ♀♀, 2 ♂♂.
Distribution in Turkey: Anatolia (no locality), Antalya, Erzurum, İzmir [40,42,43].
Distribution in the world: Africa, Algeria, Bosnia and Herzegovina, Bulgaria, Caucasia, Croatia, Cyprus, France, Georgia, Greece, Iran, Israel, Italy, Libya, Malta, Moldova, Russia, Spain, Syria, Turkey, Turkmenistan, Ukraine [6,37,39,47].

Turkey has a rich biological diversity in respect of geographical location. The scientific researches are being conducted for revealing the natural riches in Turkey. 32 species from Carabidae family were found and analyzed to determine the fauna of Carabidae Hatila National Park in Artvin (*A.similata, A.saxicola, A.aenea, A.lucida, A.ovata, A.aulica, A.binotatus, B.elegans, B.crepitans, C.inquisitor, C.sycophanta, C.convexus, C.scabrosus, C.coriaceus, C.mulsantianus, C.graecus, H.affinis, H.smaragdinus, H.caspius, H.distinguendus, P.cupreus, P.rufipes, P.calceatus, Z.spinipes, D.halensis, C.axillaris, N.brevicollis, O.cribricollis, O.azureus, O.subquadratus, P.anthracinum, P.nigrita*).
Calosoma sycophanta of Calosoma species from the subfamily of Carabinae is one of the most important species that was found in the area and its known that these species are predator life and used actively for biological contention. The species of the *Zabrus spinipes* and *Pseudoophonus rufipes* are from the fitofag species that cause the harmful effects in cultivated plants such as corn, wheat, and strawberry fields [52].
15 species were determined and accepted as the first record for the local fauna of Artvin at the end of the study (*A.lucida, A.aulica, B.elegans, B.crepitans, C.scabrosus, A.binotatus, C.coriaceus, C.mulsantianus, C.graecus, H.affinis, H.caspius, O.cribricollis, O.azureus, O.subquadratus, P. anthracinum*). Again in this study, totally ten species were accepted as the first record for the fauna of Eastern Black Sea (*A.lucida, A.aulica, B.elegans, A.binotatus, C.coriaceus, C.mulsantianus, C.graecus, H.caspius, O.cribricollis, P.anthracinum*).
When the evaluation is conducted based on the species it is clearly seen that the individuals of *Harpalus smaragdinus* are more comman than the others. The species of *Harpalus distinguendus, Poecilus cupreus, Pseudoophonus calceatus, Zabrus spinipes, Dolichus halensis* and *Ophonus cribricollis* have the lowest population in contrast with the other kinds.
When the insects in traps are evaluated, totally 864 insects were caught. 153 of them belong to the species of Amara and Brachinus, 378 of them belong to the species of Calosoma and Carabus, 172 of them belong to the species of Harpalus, Poecilus, Pseudoophonus and Zabrus, and finally 161 of them belong to Dolichus, Cymindis, Nebria, Ophonus and Pterostichus species. It is found that when we evaluate the feed amount in the traps we saw that live snails catch more than the traps have malt solution. Forestlands and cultivated areas are defined intense population of the species of Carabidae family [8,53]. Hatila National Park was exposed to the attacks bark beetles in previous years. There are many trees that broken and ruined in this park area. These trees enhance the insect diversity in the area. It is possible to increase the number of species if this study is applied provincial in Hatila National Park. In conclusion, it is based on this research that 32 species of Carabidae family in Hatila National Park is a sign for the richness of species in the region.

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The Relationship between Surface Soil Moisture with Real Evaporation and Potential Evaporation in Iraq

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***Abstract*— *The aim of this research is to determine the relationship between surface Soil Moisture (SSM) of both Real Evaporation (E) and surface Potential Evaporation (SPE) for thirty years during the period of (1985-2014) for the eight stations (Sulaymaniya, Mosul, Tikrit, Baghdad, Rutba, Kut, Nukhayib, Basrah) in Iraq, from (NOAA) and taking advantage of some statistics such as the Simple Linear Regression (SLR) and the Spearman Rho test. Calculated the monthly average for Soil Moisture, Real Evaporation and Potential Evaporation, and found to increase the values of SPE in hot months and decreased in cold months while opposite to SM There was a strong inverse relationship between them, where the correlation coefficient was in Sulaymaniya -0.91, in Mosul -0.89, in the Rutba -0.92, in Tikrit -0.89, in Baghdad -0.89, in Nukhayib -0.89, in Kut -0.87, and in Basrah -0.83, and there is a high correlation in stations (Basrah, Kut, Nukhayib, and Rutba), while there is an average correlation in the stations (Baghdad and Tikrit), and there is low correlation in the stations (Sulaymaniya, Mosul), we also note an inverse correlation between RE and PE, where there is a low correlation in Sulaymaniya and medium correlation in the Mosul and Rutba stations, and there is a high correlation in the stations (Tikrit, Baghdad, Nukhayib, Kut, and Basrah).*

Keywords*— *Soil moisture, Potential evaporation, Real evaporation, Spearman rho test, Iraq.

I. INTRODUCTION

The evaporation of the main processes in the water cycle is a link between energy and water budget balance as the water cycle include the release of energy and water vapor transmission and for leading to condensation for precipitation on the surface of the globe. Water flow on the surface of the earth is through surface runoff, groundwater, eyes and others. Then the water cycle ends to the starting point, which is the water surfaces such as lakes, rivers, oceans, seas and the surface of the soil. Unfortunately, this process is one of the most incomprehensible processes in the water cycle. Water is from the liquid to the gaseous state. There are three main reasons for evaporation from the surface of the soil. First, sufficient energy must be available to convert the water from the liquid phase to the gas phase. Second, there is a vapor pressure gradient in the atmosphere sufficient to transform the water from Liquid to vapor. Third, there is sufficient water level in the surface of the soil, which affects the moisture content [1]. Potential Evaporation (PE) can be defined as the amount of evaporation that can be obtained if sufficient water source is available. Real Evaporation (E) is a sum of both Potential Evaporation (PE) and Actual Evaporation (AE) in the soil. Several factors affect the potential evaporation (solar radiation as the most important atmospheric influences, temperature, relative humidity, and wind speed), other factors indirectly affecting (water level, atmospheric stability, soil temperature, latent heat and sensible heat emissions).

The task in the biological processes as it contributes to the process of formation of clouds and then precipitation, which is one of the most important determinants of the cycle of water in nature and therefore measure the amount of potential evaporation to assess the water requirements as well as the need to calculate when planning irrigation projects president widely in many environmental studies, including meteorology, hydrology, agriculture, climate change and affect the surface of the soil, especially at a depth of one to two meters, a key interaction between the earth and the atmosphere is one of the key variables that control the exchange of and the thermal energy between the surface of the Earth and the atmosphere through evaporation and plant transpiration [2]. This variable has multiple links with other anaerobic variables, which makes it very effective predictively although it constitutes a very small layer compared to the global total water but is very important in many of the basic processes of many hydrologists, chemists and biologists are important variables used in many applications (numerical weather predictions, global climate change monitoring, flow forecasting and evaporation modeling) [3]. Spatial and temporal differences of soil water content [4].

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Agriculture is the sector most economically affected by extreme weather events such as drought. Many other economic sectors of society rely on agro-ecosystems, which are a specific form of human-adapted ecosystems for food production. Can lead to many negative economic and social impacts such as loss of income in agriculture and food industries and high costs for water and production technologies such as irrigation systems [5] [6].

II. METHODOLOGY

A. Methods of Analysis

There were several statistical tests available. Spearman rho was selected. The regression analysis was also selected, particularly the simple linear regression and the use of the P-value for the relationship [7]:

1. Simple Linear Regression (SLR)

Is the study of the relationship between two variables only accessible to a linear relationship (i.e. a straight line equation) between these two variables, a parametric test as it is assumed that the data are distributed normally distributed and to find out the value of the regression slope of the regression is calculated by the following linear equation:

$$\bar{Y} = a + b\bar{X} \qquad (1)$$

$$b = \frac{\sum_{i=1}^{n}(X_i - \bar{X}) - (Y_i - \bar{Y})}{\sum_{i=1}^{n}(X_i - \bar{X})^2} \qquad (2)$$

Where b: slope of the regression and found a mile straight line equation (1), a: a constant gradient and demonstrate the value of the lump of axis $\bar{Y}$ for Straight equation (1).

2. Probability-Value

It is purely a statistical term, which is a number or the number of measurements used to evaluate the statistical value of a show that was a contrast factor it is an influential factor or not really? If the P-Value is less than 0.05, the contrast factor it is an influential factor in the variable that we are trying to study the change may consider factor affecting even the value of P-Value equal to 0.1, but that exceeds 0.1, this factor should be removed from the form it is ineffective.

3. Spearman Rho Test

It is a test of a set of observed data (x_i = 1,2,……,n) is based on the null hypothesis that is, all xi values are independent and have the same distribution and to calculate the Spearman Rho coefficient statistical ranks (r_s) must convert the original model to the ranks mediated arranged in descending order in terms of amount and then the value of the account through di ($d_i = k_i - i$) where the (i = 1,2,…..,n) and r_s is given by the following [8]:

$$r_s = 1 - \frac{6\sum_{i=1}^{n} d_i^2}{n(n^2\text{-}1)} \qquad (3)$$

If the value of n large can choose the value of r_s to their importance by calculating the value of t_s which is given by equation:

$$t_s = r_s \sqrt{\frac{n-2}{1-{r_s}^2}} \qquad (4)$$

If the value of t_s false calculated within the trusted boundary for the selection of a dual party from this we conclude that there is no trend in the data series and through the "Table 1". Can determine the value of the degree of correlation and interpretation of test transactions.

Table.1: The degree of correlation and interpretation of test transactions [8].

Value	Correlation	Interpretation of relation
Less 0.2	Few	No relation
0.2-0.4	Low	Small relation
0.4-0.7	Medium	Acceptable relation
0.7-0.9	High	Special relation
0.9-1	Very high	Strong relation

B. The Data and Study Stations

Was used the monthly average surface soil moisture, surface potential evaporation and real evaporation data for eight different stations in Iraq (Mosul, Sulaymaniya, Tikrit, Baghdad, Rutba, Kut Nukhayib, and Basrah) were used for thirty years (1985-2014) from The National Oceanic and Atmospheric Administration (NOAA) [9], (see "Fig. 1" and "Table 2").

Table.2: The latitude, longitude and altitude of the study stations in Iraq [10].

Stations	Latitude (°N)	Longitude (°E)	Altitude (meter)
Mosul	36.19	43.09	223
Sulaymaniya	35.33	45.27	853
Tikrit	34.56	43.70	103
Baghdad	33.14	44.14	34
Rutba	33.02	40.17	615
Kut	32.48	45.73	19
Nukhayib	32.02	42.15	305
Basrah	30.34	47.47	2

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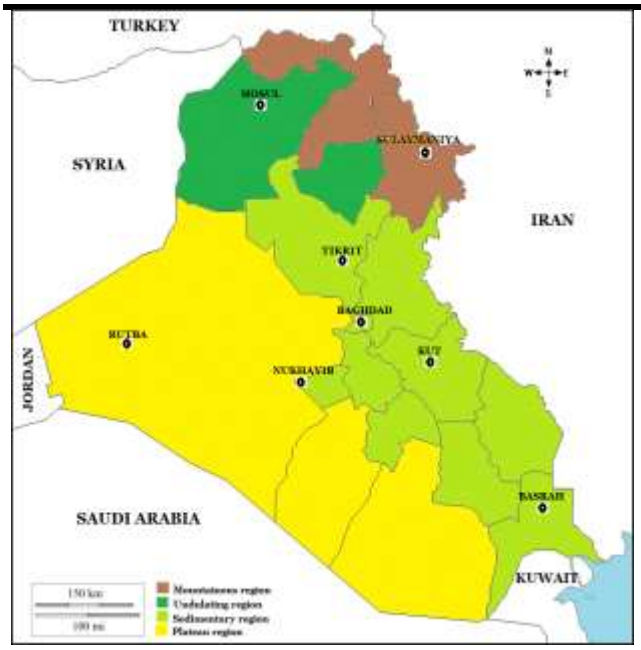

Fig.1: Iraq map, explaining the study stations [10].

III. RESULTS AND DISCUSSION

1. The Relationship between SPE and SSM

The "Fig. 2", shows the relationship between the surface PE with the surface SM, where there is strong inverse relationship where we note there is a high correlation in the eight stations where the highest value of the correlation coefficient was in Sulaimaniya and Rutba stations and the lowest value of the correlation coefficient in the Basra station and the reason for this relationship reverse that connects SPE and SSM, potential evaporative occurs only in the presence of moisture and when evaporation reaches the latent limit less moisture in the atmosphere where the high temperature and solar radiation directly affects the SPE and SSM, note that the southern stations characterized by high temperatures and this leads to the high in potential evaporation values and low values in SSM values, the opposite is happening in the northern stations (see "Table 3").

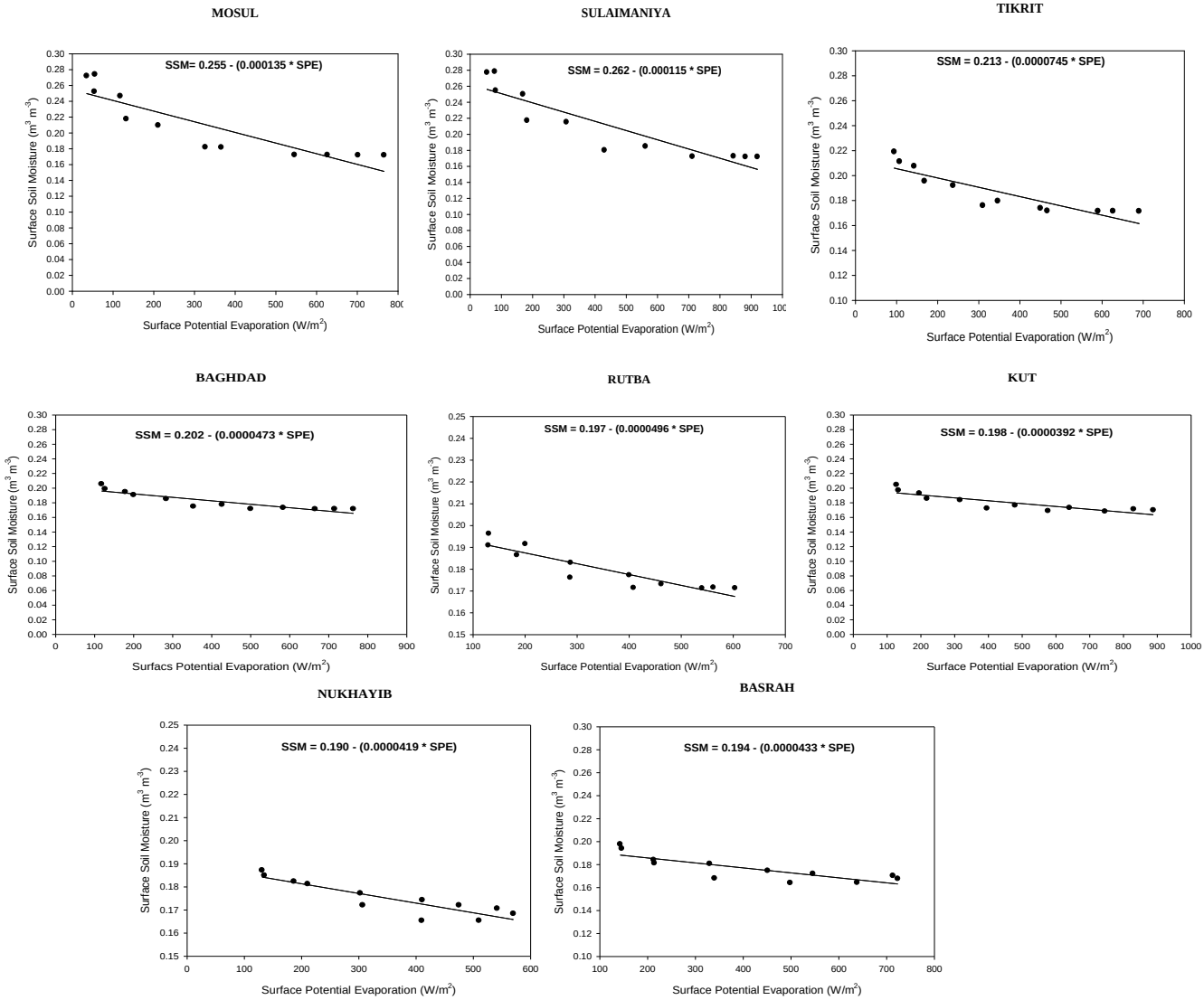

Fig.2: The relationship between the surface potential evaporation (SPE) and surface soil moisture (SSM) of eight different stations in Iraq for thirty years (1985-2014)

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Table.3: Spearman rho test results and Simple Linear Regression (SLR) to find the strength of the relationship between the SPE and SSM.

Station	Simple Linear Regression		Spearman Rho Test	
	P-value	Interpretation	r_s	Correlation
Mosul	0.0001	Linear relation	-0.89	High-inverse correlation
Sulaymaniya	0.0001	Linear relation	-0.91	Very high-inverse correlation
Tikrit	0.0001	Linear relation	-0.89	High-inverse correlation
Baghdad	0.0001	Linear relation	-0.89	High-inverse correlation
Rutba	0.0001	Linear relation	-0.92	Very high-inverse correlation
Kut	0.0002	Linear relation	-0.87	High-inverse correlation
Nukhayib	0.0001	Linear relation	-0.89	High-inverse correlation
Basrah	0.0009	Linear relation	-0.83	High-inverse correlation

2. The Relationship between E and SSM

The "Fig. 3", shows that there is a strong positive relationship between E and SSM, where there is a high correlation in the stations (Basrah, Kut, Nukhayib, and Rutba), while there is an medium correlation in stations (Baghdad and Tikrit), There is a low correlation in the stations (Sulaymaniya and Mosul), and also note through the P-Value, there is a nonlinear relationship in stations (Sulaymaniya and Mosul), while there is a linear relationship in the stations (Rutba, Tikrit, Baghdad, Nukhayib, Kut, and Basrah) for reasons the following is because evaporation occurs on the surface of the earth when the water moves into an atmosphere shaped like water vapor from the various confiscations as well as the evaporation plays a significant role in the occurrence of moisture and consequent upon the occurrence of condensation or fog or include rain dew, as well as the evaporation occurs only the existence and availability of sources of moisture (see "Table 4").

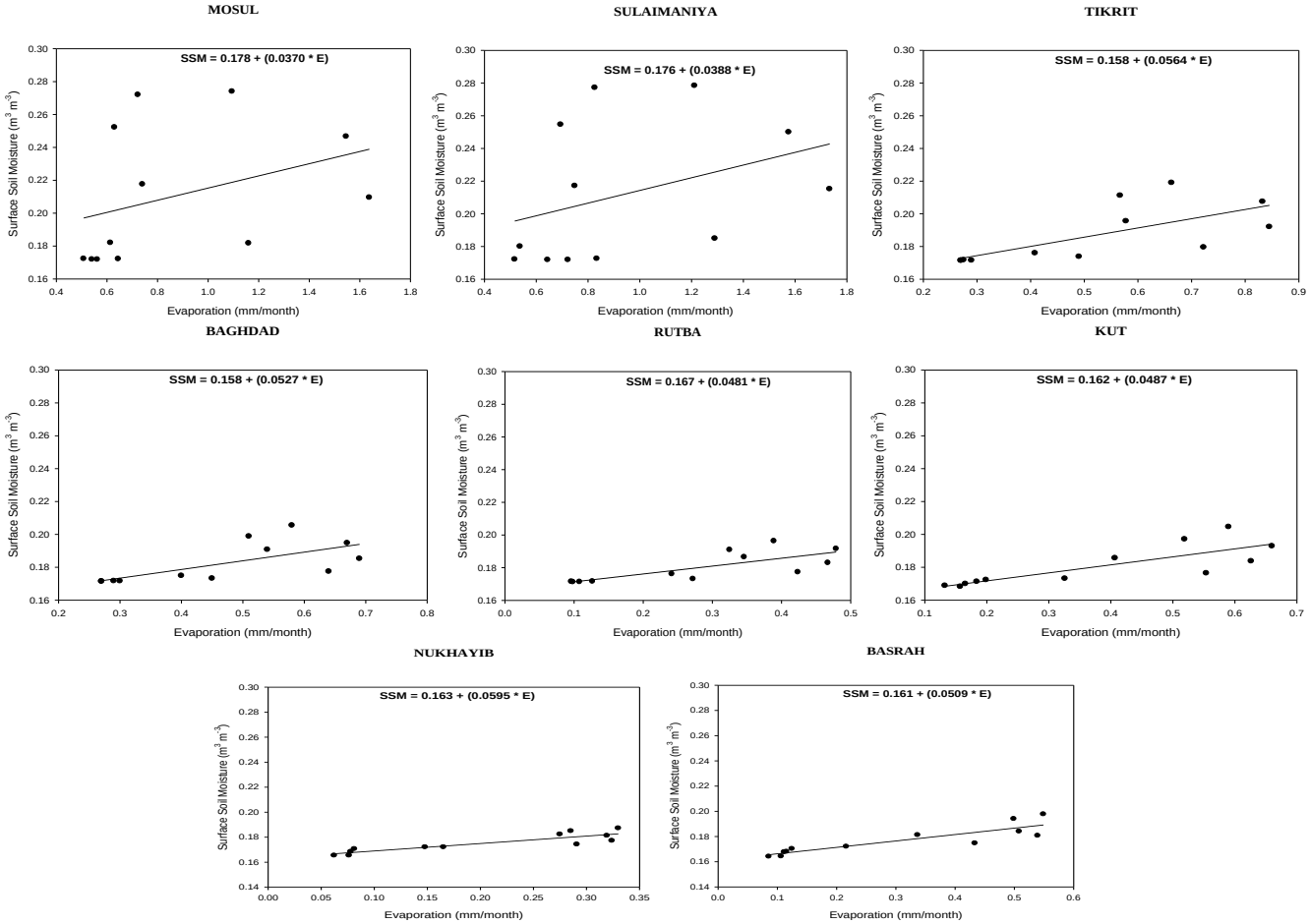

Fig.3: The relationship between the real evaporation (E) and SSM of eight different stations in Iraq for thirty years (1985-2014)

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Table.4: Spearman rho test results and Simple Linear Regression (SLR) to find the strength of the relationship between the E and SSM.

Stations	Simple Linear Regression		Spearman Rho Test	
	P-value	Interpretation	r_s	Correlation
Mosul	0.2528	Non-Linear relation	0.36	Low-positive correlation
Sulaymaniya	0.2364	Non-Linear relation	0.37	Low-positive correlation
Tikrit	0.0121	Linear relation	0.69	Medium-positive correlation
Baghdad	0.0139	Linear relation	0.69	Medium-positive correlation
Rutba	0.0039	Linear relation	0.76	High-positive correlation
Kut	0.0013	Linear relation	0.81	High-positive correlation
Nukhayib	0.0001	Linear relation	0.89	High-positive correlation
Basrah	0.0001	Linear relation	0.88	High-positive correlation

3. The Relationship between E and SPE

The "Fig. 4", shows the relationship between surface real evaporation (E) and potential evaporation (SPE) where there is a strong inverse relationship between them, where we note there is a low correlation in the Sulaimaniya station and the medium correlation in the (Mosul and Rutba) stations and high correlation in the stations (Tikrit, Baghdad, Nukhayib, Kut and Basrah) (see "Table 5").

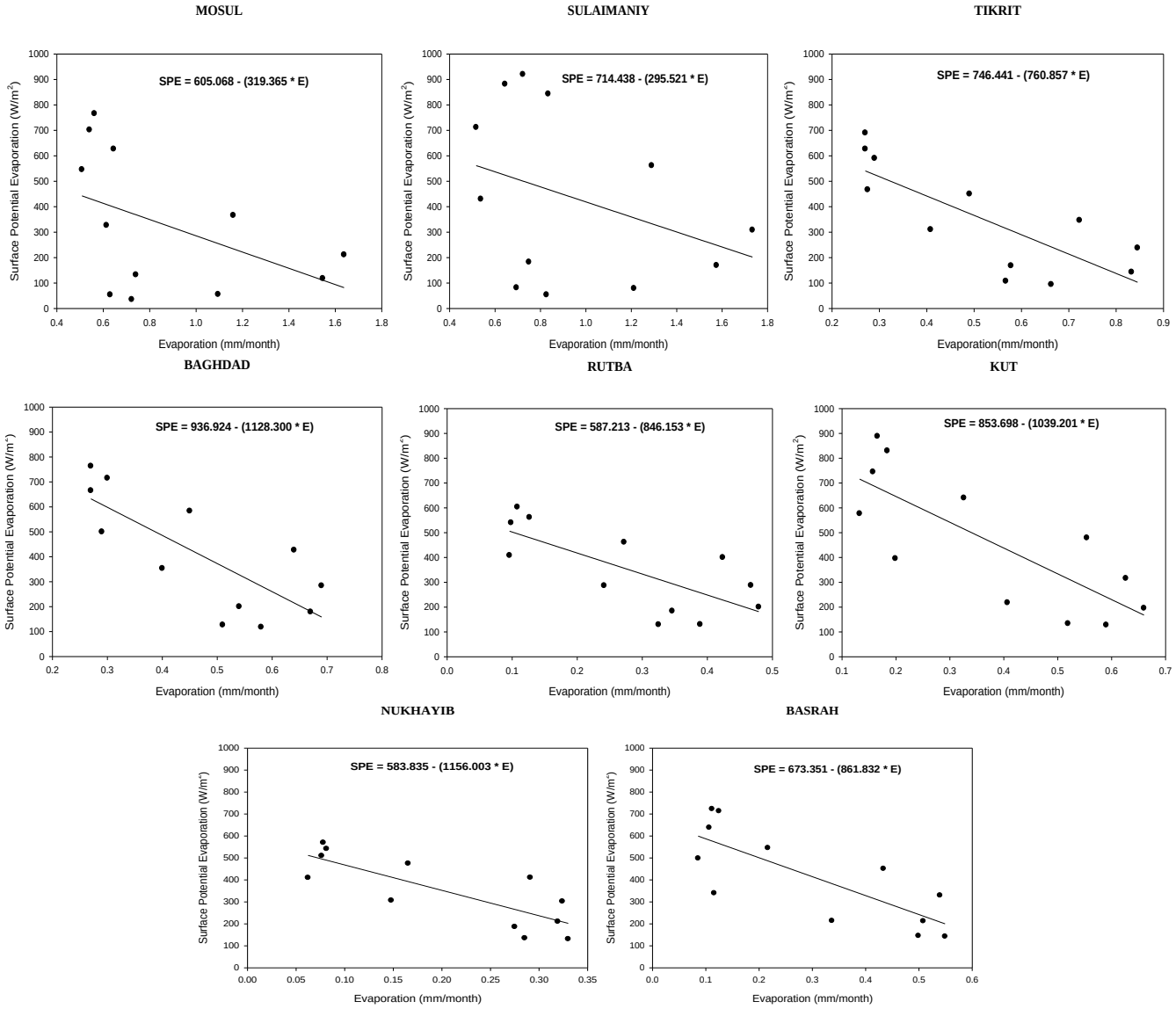

Fig.4: The relationship between the E and SPE of eight different stations in Iraq for thirty years (1985-2014)

Table 5: Spearman rho test results and Simple Linear Regression (SLR) to find the strength of the relationship between the E and SPE.

Stations	Simple Linear Regression		Spearman Rho Test	
	P-value	Interpretation	r_s	Correlation
Mosul	0.1239	Non-Linear relation	-0.47	Medium-inverse correlation
Sulaymaniya	0.2527	Non-Linear relation	-0.36	Low-inverse correlation
Tikrit	0.0024	Linear relation	-0.79	High-inverse correlation
Baghdad	0.0033	Linear relation	-0.77	High-inverse correlation
Rutba	0.0074	Linear relation	-0.73	High-inverse correlation
Kut	0.0026	Linear relation	-0.78	High-inverse correlation
Nukhayib	0.0016	Linear relation	-0.80	High-inverse correlation
Basrah	0.0026	Linear relation	-0.78	High-inverse correlation

IV. CONCLUSIONS

The results showed a strong inverse relationship between SPE and surface SSM. SPE was found to increase in the southern stations and increase in hot months, in contrast to SSM. It also showed an inverse relationship between E and SPE. Results there is a strong direct relationship between E and SSM where E occurs only with the presence of water (Soil Moisture). The results show that SPE is an evaporative energy in the atmosphere E of surface soil moisture because real evaporation is a process of transformation from the liquid phase to the gas phase. E occurs only with soil moisture. E is the sum of SPE and Actual Evaporation (AE) in the soil.

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Microclimatic Modeling and Analysis of a Fog-Cooled Naturally Ventilated Greenhouse

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Abstract*—In the present paper, a thermal model has been presented for predicting the thermal environment inside a fog cooled naturally ventilated greenhouse. Experiments were conducted on a polyethylene covered greenhouse having 250 m^2 ground area located at Coochbehar (latitude: 26.2^o N, longitude: 89^{o}E), West Bengal, India. The greenhouse was cooled by intermittent fogging with three distinct fogging cycles during the experiments. The greenhouse air temperature profiles as predicted by theoretical model were validated for different fogging cycle configurations. The model prediction and experimental results build up a good match (co-efficient of correlation was in range of 0.85 to 0.97). It was observed that fogging cycle configuration (spray time and spray interval combination) influences greatly the cooling performance of the fogging system. Further analysis revealed that greenhouse temperature could be maintained 2-5^{o}C below the ambient temperature by employing suitable fogging cycle, maintaining the relative humidity within acceptable level.*

Keywords*—*Cooling, Fogging cycle, Greenhouse, Natural ventilation, Spray time.

I. INTRODUCTION

Greenhouse is meant to provide optimum growing conditions of the plants inside it all over the seasons. In cold countries, the primary objective of the greenhouse is to increase the air temperature by the principle of "greenhouse effect" for sustainable growth of plants. However, a country in subtropical or tropical areas, temperature reduction is the main objective rather than the "greenhouse effect", which has been provided by "shading effect" (checking solar radiation) during the periods of high radiation, or providing a suitable air exchange, or incorporating evaporative type cooling. Nowadays, in hot climatic regions, evaporative cooling with some form of ventilation (natural or fan-induced) is used extensively to provide a suitable microclimate for plant growth during the hot summer season. In most of the cases, fan pad evaporative cooling is a common practice of greenhouse cooling. But fan pad cooling system creates temperature and humidity gradients along the length of the greenhouse; also the total equipment cost for the system is high. In order to maintain uniform temperature and humidity all through the greenhouse, fog cooling can be employed. It is based on fine water dispersion into the air stream to increase the heat exchange between water and air. Air circulation is very much important for fog cooled greenhouse and can be achieved by fan induced ventilation or natural ventilation. To reduce electric power consumption, the fog cooling system is often incorporated with natural ventilation, achieved by multiple ventilators which allow air to enter and leave the greenhouse.

This paper presents a thermal model of a fog cooled greenhouse located in the Indian subcontinent. The prime focus of the study was to investigate the fogging effect on a greenhouse micro-climate in a plastic greenhouse during summer under natural ventilation. To serve this purpose, a greenhouse equipped with fog system was selected, a thermal model has been established to characterize the fogging system, experiments were conducted and finally the model was validated with experimental data.

Many researches carried out studies on greenhouse cooling by employing fogging system. Arbel et al. (1999) developed a mathematical model to characterize the fog cooling system. They conducted an experiment in a four-span greenhouse which was equally divided into two parts. Each part of the greenhouse was equipped with fog system and with fan-pad evaporative cooling system. They did a comparative study by operating each system in the two parts alternately. It was observed that fog cooling system performed better than fan-pad evaporative cooling system. Arbel et al. (2003) presented a cooling arrangement for a greenhouse combined with high pressure fogging and fan-induced ventilation system. They reported that greenhouse air temperature and relative humidity can be kept at 28 ^{0}C and 80% respectively during mid-summer with such type of cooling arrangement. Ahmed et al. (2006) established a dynamic model for a naturally ventilated fog cooled greenhouse. The developed model was capable of predicting the greenhouse air temperature, plant temperature, cover temperature, floor surface temperature, relative humidity, transpiration and evaporation rate. The model results have been compared with an experimental greenhouse installed in Tokyo. Abdel-Ghany et al. (2006) suggested a new expression of cooling efficiency for a fog-cooled greenhouse system. They investigated the cooling efficiencies for different fogging cycles. Öztürk (2003)

carried out an experiment in a multi-span plastic greenhouse to determine the efficiency of the fogging system. The average represented fogging system efficiency was 50.5%. Ishigami et al. (2014) experimented on two separate fog- cooled greenhouses, each having 26.4 m^2 floor area. They observed that twin fluid nozzle system had higher evaporation rate and lower degree of wetting of plant foliage compared to single fluid nozzle system. It was observed that twin fluid nozzle system produced the same cooling effect as single fluid nozzle system. Li and Willits (2008) compared the performance of a low pressure (4.05 bar) fogging system with high pressure (40.5 bar) system. They observed that high-pressure systems provide better cooling than low-pressure systems, though high-pressure systems required much higher initial investment and operational costs. The cooling and evaporation efficiencies of the two systems were also compared.

controlled by low pressure fog cooling system; horizontal thermal shading screens were placed at gutter level and by adjusting the openings of side and roof vents. The side vents were set on both north and south walls; each side having of 14.4 m^2 area (0.9m× 16 m) and roof vent area was 16 m^2. The side vents were covered with insect proof net. The greenhouse side vent opening can be regulated by roll up curtain as per ventilation requirement.

Fogging System: The main elements of fogging system in the greenhouse are a pump unit and Fogging lines. Pump unit consists of pump, a water reservoir, a water softener, a fine filter, and a pressure adjusting regulator, valve, and the fogging lines consist of main pipe line, distributor line, LDPE (low density poly-ethylene) pipe

Fig.1: Experimental greenhouse

II. MATERIALS AND METHODS

Study Sites: The greenhouse located at Coochbehar (Latitude: 26.2^o N, Longitude: 89.0^{o}E) was selected for the experiment and data collection. The greenhouse is situated 700 Km away from Kolkata in India.

Experimental Greenhouse: The greenhouse was constructed to form single span arched-roof using single layer polyethylene as cover (200 micron thick). The greenhouse was East-West oriented and made by galvanized tubular steel structure. The side view of the experimental greenhouse is shown in Fig. 1. The greenhouse was 20 m in length and 12.5 m in width i.e. 250 square meter in ground area. The ridge of the greenhouse was 5.5 m high from the ground. The greenhouse floor was covered by young plants with a leaf area index of 0.25. The greenhouse has been provided with gravity fed drip irrigation system for the water requirement of the cultivated plants. The greenhouse microclimate was

lines with fog nozzles connected to it. Four fogging lines are equipped along with the length of the greenhouse at 2.5 m spacing and connected with a distributor line via main pipe line. There are total 32 four-way fog nozzles and each nozzle line consists of 8 nozzles which are located at 2 m spacing from one another. Fog nozzles are situated at 2.2 m above the ground surface and spray water to the greenhouse by an electrically operated pump at a pressure of 3 bar and at 0.175gm/m^2s fog rate.

Experimental Measurements: Experiments were conducted on the naturally ventilated greenhouse with both roof vent and side vents open and with intermittent spraying of water fog. Experiments were done considering three different fogging conditions (spraying time to interval time were 1-.5-3.5 min, 1-2 min, and 1-3 min respectively). The measurements were conducted at noon (12:10 pm to 1:00 pm) on clear hot sunny days of summer (20 and 21 June, 2015). Following parameters were recorded at 30 s intervals: (i) outside temperature and

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relative humidity using digital psychrometers (HTC HD304), (ii) inside temperature by aspirated temperature sensor, (iii) outside wind speed using an anemometer (HTC AVM06), (iv) outside solar radiation flux by pyranometer (WACO 206).

$$Q_{latent} = \lambda \beta m_w \quad (5)$$

Where λ is the latent heat of vaporization, β is the fraction of supplied water that would be evaporated into air. The fraction β is considered 0.6 [4].

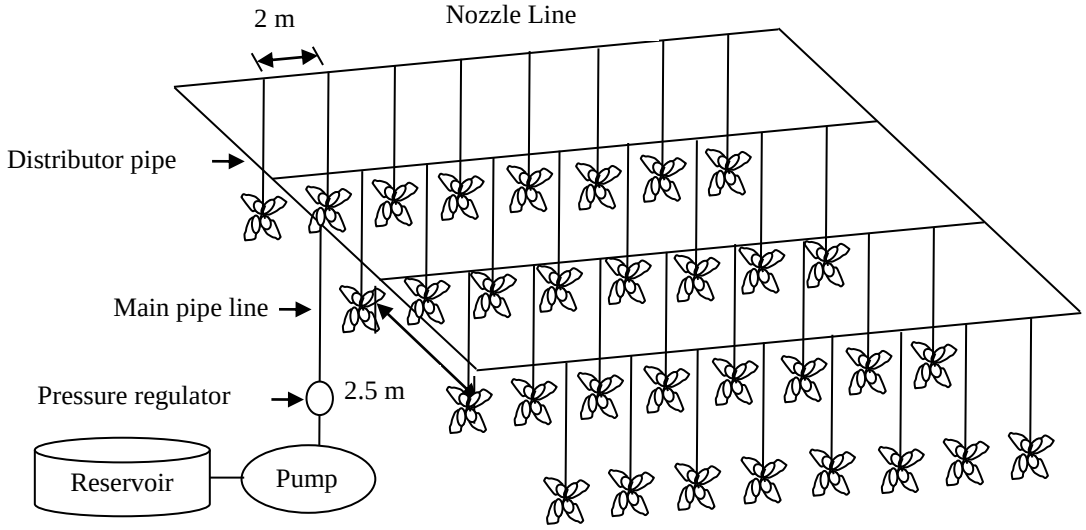

Fig.2: General layout of the fogging system

III. THERMAL MODELING

Instantaneous temperature of the greenhouse air is formulated by a simplified energy balance equation as follows

$$m_g C_p \frac{dT_i}{dt} = Q_{in} - Q_{cover} - Q_{vent} - Q_{latent} - Q_{crop} \quad (1)$$

Where m_g is the mass of the greenhouse air, C_p is the specific heat of greenhouse air, T_i is the temperature of the greenhouse air

Q_{in} is the net input solar energy to the greenhouse, and is given by

$$Q_{in} = \tau I_t (1 - SF) A_c \quad (2)$$

Where I_t is the solar radiation, τ is the proportion of the solar radiation entering into the greenhouse, SF shading factor, A_c is the greenhouse covering area.

Q_{cov} is related to the convective heat losses through the cover. Which is given by

$$Q_{cover} = UA_c (T_i - T_a) \quad (3)$$

Where U is the overall heat transfer coefficient and T_a is the temperature of the ambient air.

Q_{vent} represents heat exchange due to air infiltration through the greenhouse ventilators is given as

$$Q_{vent} = \rho_a m_v C_p (T_i - T_a) \quad (4)$$

ρ_a is the density of air and m_v is the volume flow rate of the ventilated air.

Q_{latent} refers to the latent heat transfer due to fog evaporation. Which is given by

During the interval period, when pump is off β is taken as zero. m_w is the mass of supplied water by fog nozzles.

The latent heat of vaporization of water λ (J/Kg) is given by [8]

$$\lambda = 10^3 \times (3.4702 \times 10^3 - 5.7352 \times T + 1.1687 \times 10^{-2} T^2 - 1.3478 \times 10^{-5} T^3) \quad (6)$$

Where T is the tempertaure in K.

Q_{crop} is related to is energy exchange due to crop transpiration, and given by

$$Q_{crop} = \lambda E_t \quad (7)$$

Where E_t transpiration rate of crop.

Crop transpiration rate of the plants is given by [9]

$$E_t = A_f LAI \kappa (e_{ps} - e_a) \quad (8)$$

Where A_f is the area of floor and LAI is the leaf area index. e_{ps} is the saturated vapour pressure corresponding to plant temperature and e_a is the water vapour pressure corresponding to the greenhouse temperature of air. κ is the stomatal boundary layer conductance.

To find the instantaneous temperature of the greenhouse in a particular fogging cycle equation 1 has to be solved. The numerical solution of the differential equation of the greenhouse model required a set of initial conditions which are shown in table1.

In a naturally ventilated greenhouse, ventilation rate is due to mass flow rate due to the thermal buoyancy and wind velocity represented by Ganguly and Ghosh (2009). For fog cooled greenhouse, ventilation rate primarily depends on wind effect, buoyancy effect is being insignificant. A

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linear relationship of the type $y = Ax + B$ was assumed for the vent rate calibration and a co-relation was obtained by the fitting a regression line with an observed data points.

Table.1: Input parameters used for the model

Parameter	Values
Transmissivity of cover (τ)	0.75
Overall heat transfer coefficient (U)	4.5 $Wm^{-2o}C$
Covering area of the greenhouse(A_c)	312 m^2
Area of greenhouse floor (A_f)	250 m^2
Plant Leaf Area Index (LAI)	0.25
Mass flow rate of spraying water (m_w)	0.175 gm/m^2s
Fraction of fog water to be evaporate (β)	0.6

IV. RESULTS AND DISCUSSION

To solve the model equations, a program code which is written in C has been solved. Calculations were made using the measured solar radiation intensity and climatic parameters surrounding the greenhouse for clear sunny days of summer (20 and 21 June 2015).The program code is simulated in the two parts. In first part (spraying time), it simulates the greenhouse temperature profile with time, starting from initial temperature of the greenhouse till the attainment of the final temperature by spraying fog water under natural ventilation. In second part (interval period i.e. $\beta = 0$), it simulates the greenhouse temperature profile with time, starting from the temperature just after spraying off till the period of the commencement of next fogging cycle under natural ventilation. The ventilation rate of air in a greenhouse microclimate is difficult to predict as it depends on outer environmental conditions. Therefore, its value has been considered as input parameter to simulate the programme.

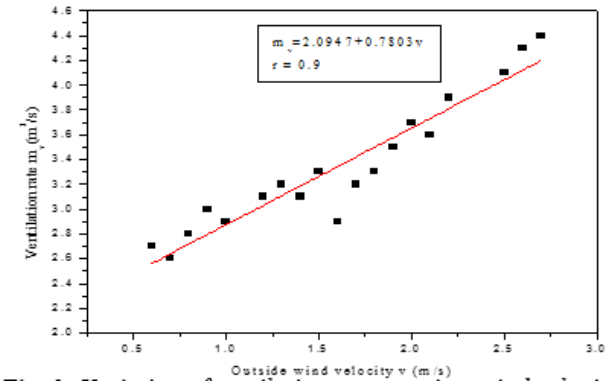

Fig. 3: Variation of ventilation rates against wind velocity

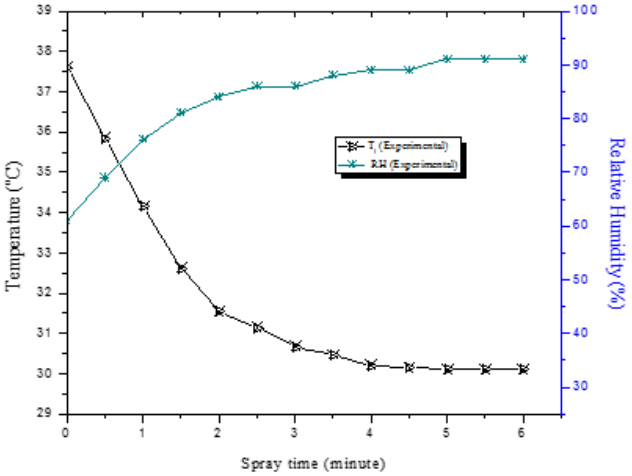

Fig. 4: Temperature and RH profiles with continuous fogging

Figure 3 shows the ventilation rate of air plotted against outside wind velocity from the experimental data. It is seen that ventilation rate was strongly correlated to the outside wind velocity. Since their correlation was good in agreement (coefficient of correlation r = 0.9), a regression equation (m_v=2.0947+0.7803v) was obtained.

Figure 4 represents the effect of continuous fogging on the greenhouse air temperature under natural ventilation (when side vents and roof vent were 100% open). It is clearly seen that temperature of the greenhouse air decreases sharply with fogging up to a certain time and thereafter temperature variation is very minimal or nearly constant. It is observed that major reduction of temperature occurs around 2 minute spraying of fog water. However spray (fogging) duration cannot be extended beyond certain time owing to RH limitation required for an operational greenhouse. It was observed that spraying time more than 1.5 minute results in exceeding the RH 80%. With 1 min spraying time RH can be kept within 75-80%. Thus to maintain the desired level of RH inside the greenhouse 1-1.5 min spraying time is advisable.

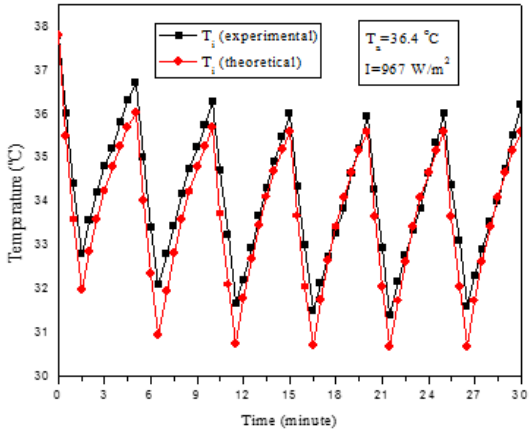

Fig. 5: Greenhouse temperature profiles with a fogging cycle of spray time- spray interval of 1.5- 3.5 min

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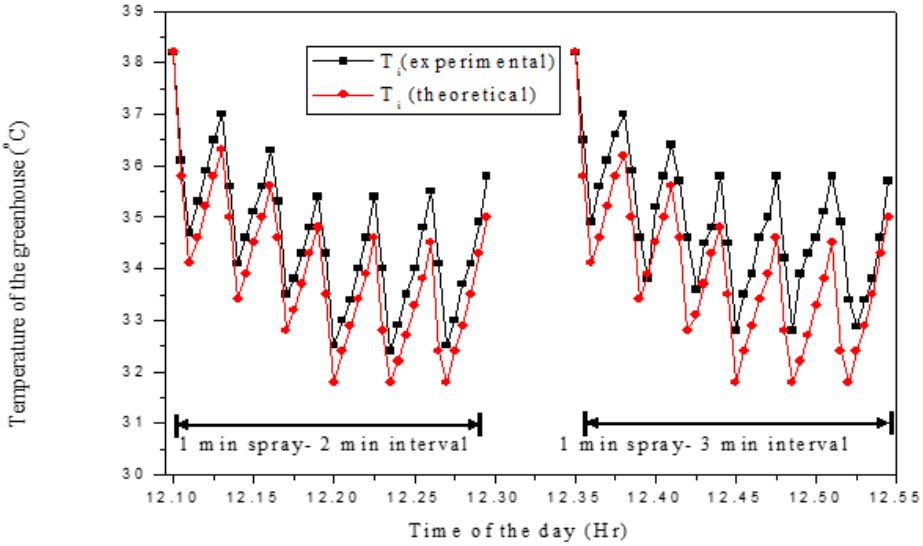

Fig. 6: Temperature profiles for two different fogging cycles in a summer day (21 June 2015)

Figure 5 shows the effect of repeated fogging cycles on greenhouse air temperature, considering a fogging cycle consisting of 1.5 min spray time and 3.5 min interval. Both model predicted temperature profile and actual greenhouse temperature profile are shown in the figure when the side vents and roof vent are fully opened. The experimental data were taken on 21 June 2015. During the experiment, the average global solar radiation intensity was 967W/m^2, average outside wind velocity was 1.3 m/s and the average ambient air temperature was 36.4^{o}C and 75% shading in place. From the figure it is seen that the temperature falls rapidly during fogging time of the cycle and increases during interval periods. The temperature reduction was in the range of 4 to 6 ^{o}C during fogging periods and rise was 4 to 5 ^{o}C during interval. It is observed that model predicted temperature profile closely matches the experimental temperature profile, the average coefficient of correlation being calculated to be 0.92.

Figure 6 shows greenhouse temperature variations in respect of time for two distinct fogging cycles on a hot summer day of June. The model predicted temperatures are obtained by the prevailing microclimatic data (solar radiation intensity, ambient temperature, wind velocity etc.) as input parameters. The model predicted temperature profiles are approaching nearer to the experimentally obtained temperature profiles. It is seen that measured and predicted temperatures disagreed for some fogging and interval periods. It is due to evaporation rate is assumed constant with time as well as free wind velocity, ambient temperature and solar radiation are considered constant during a fogging cycle in the present model.

Figure 7 and Figure 8 show the influence of fogging cycle configurations on greenhouse average temperature under natural ventilation. The study is done considering of a set of ambient condition, taken by the observed data on a hot and dry day of summer. Global solar radiation intensity, outside wind velocity, ambient RH and greenhouse initial temperature are assumed as 967 W//m^2, 1.3 m/s, 60% and 37.8 ^{o}C respectively. It is seen that average temperature depends on the fogging interval period; if the interval period increases, the average temperature increases too. It is due to heat gain by the incoming solar radiation into the greenhouse at interval period. The rate of decrease of temperature is higher for first 3-4 sequential cycles and thereafter temperature variation nearly constant.

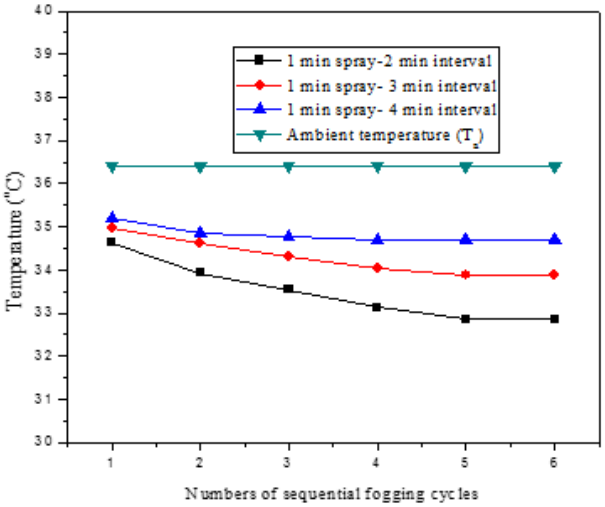

Fig. 7: Influence of spray intervals on greenhouse average temperature for fixed spray time of 1 min

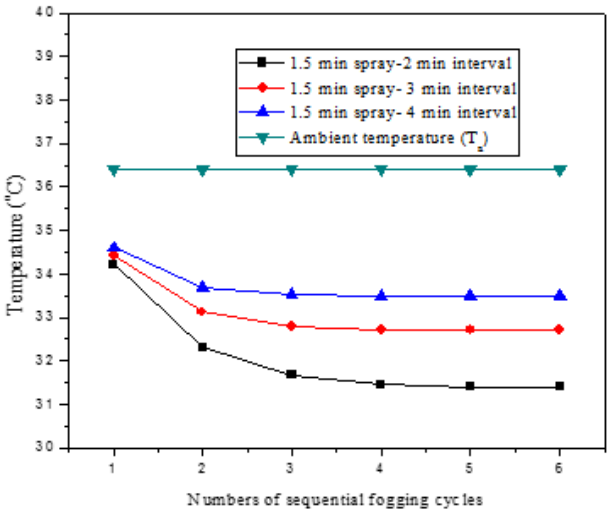

Fig.8: Influence of spray intervals on greenhouse average temperature for fixed spray time of 1.5 min

V. CONCLUSION

The thermal model developed in the present paper is capable to predict the greenhouse air temperature under different fogging configurations. To validate the thermal model, experimental data have been collected from a 250 m^2polyethylene covered greenhouse. The theoretical prediction of greenhouse air temperatures show a healthy match with measured experimental data. The value of coefficient of correlation is in the range of 0.85 to 0.97. It is observed that spray time and interval periods are significant for changing greenhouse air temperature. Performance study suggests that fogging cycle of 1.5 min spray time and 2 min spray interval is best choice, which can be reduced the greenhouse temperature up to 5 ^oC when free wind velocity is adequate and ambient condition is hot and dry. Thus it can be concluded that present naturally ventilated fog-cooled greenhouse is able for maintaining suitable environment inside the greenhouse.

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Pathogenicity of *Helminthosporium rostrata* on rice varieties widely grown in Morocco

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***Abstract*—** *The plants of rice varieties (Arco, Thaibonnet and Elio) were inoculated with three isolates of Helminthosporium rostrata (HR1 HR2 and HR3), isolated for the first time in Morocco from the rice seed of Taibonnet variety at the end of the growing season.*

The results obtained showed that all the isolates are able to induce the disease on rice plants and sporulate on the foliar lesions. HR1, HR2 and HR3 was respectively the most pathogenic on Elio (I.C = 113), Arco (I.C = 212.5), and Taibonnet (130.48).

The symptoms induced by the isolates are similar to those induced by Helminthosporium sativum on rice.

***Keywords*— Rice, Helminthosporium rostrata, symptoms, pathogenicity.**

I. INTRODUCTION

The helminthosporium disease is caused by fungi of the genus *Helminthosporium*. These fungi spend unfavorable season, as mycelium on infected plants and plant residues (Zambettakis, 1967; Lucas et *al.*, 1985). The inoculum is also carried by the seeds (Wells and Winstead 1965, Wilson *et al*., 1993). The host range of these fungi is not limited only to the plants, particularly cereals (Paul, 1926; Serghat *et al.,* 2005), but also extends to animals and humans (Pritchard *et al*., 1977).

The helminthosporium disease of rice is widespread in Moroccan rice fields. It is caused by a large number of species: *Helminthosporium oryzae* (Bouslim *et al*., 1997), *H. spiciferum* (Ennaffah *et al*., 1997), *H. australiensis, H. sativum* (Ouazzani Touhami *et al*., 2000), *H. cynodontis* (Zehhar *et al*., 2008) et *H. bicolor* (Kadri *et al*., 2013). Sometimes, rice foliar lesions can accommodate one or two *Helminthosporium* species, in addition to *Pyricularia grisea,* all these *Helminthosporium* may be encountered on the lesions of a single foot (Ouazzani Touhami *et al.*, 2000). This involves the estimation of losses due to these pathogens and the share due to each of them (Hannin, 2003). The main thing is that each species produces sporulating lesions on rice leaves (Bahous *et al.*, 2003).

In recent years, it was noted the presence of *Helminthosporium rostrata* on the rice grains harvested at the end of the vegetative cycle. This fungus has never been reported among rice mycoflora.

In this study, the symptoms of *Helminthosporium rostrata* are described and its pathogenicity was studied on three rice varieties.

Plant materiel

The grains of three varieties of rice plants (Arco, Thaibonnet and Elio) are disinfected by soaking in the hypochlorite of sodium in 0,6 % during ten minutes, then rinsed strictly in the sterile distilled water. After 24h of drying, grains are put in Petri dishes containing some sterile cotton soaked with distilled water. After 75h of incubation in the darkness and in 28°C, the obtained seedlings are planted in jars containing the soil of the forest Mamora. Then, they are watered with the tap water until the stage required for the inoculation (plant with 3 or 4 leaves).

Fungal material

The isolates of *Helminthosporium rostrata* (HR1, HR2 and HR3) studied are obtained from the rice grains of Taibonnet variety at the end of the growing season. These three isolates were previously transplanted from single conidia.

Inoculum preparation

H. rostrata isolates were pricked out on a rice flour medium (14 g of rice flour, 15 g agar-agar, 4g of yeast extract and 1000 ml of distilled water). The cultures were incubated for ten days at a temperature of 28 ° C in the dark. The surface charged with spores is scraped aseptically using a metal spatula. Conidial suspension obtained was then adjusted with sterile distilled water containing 0.05% of Tween 20 and 0.5% of gelatin, in order to have a final concentration of 10^6 spores / ml.

Inoculation and Result's evaluation

The plants were inoculated at the stage of 4 to 5 leaves by spraying the conidial suspension above the rice leaves using a charging pulverized. The inoculated plants are placed for 48 hours under a black plastic bag to maintain a high relative humidity. Control plants are sprayed with sterile distilled water containing 0.05% of Tween 20 and 0.5% of gelatin. The inoculated and control plants are

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then placed in the greenhouse for development of symptoms. Seven days after inoculation, the disease severity (S) is estimated using the scale of Notteghem *et al*. (1980), the disease incidence (I) represents the number of infected leaves. The infection coefficient (I.C) is calculated by multiplying I x S.

The disease severity index is determined by the percentage of the diseased leaf area estimated by the rating scale Notteghem *et al*. (1980).

Note	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
diseased leaf area	**0**	**0.05**	**0.5**	**1.5**	**3.5**	**7.5**	**17.5**	**37.5**	**62.5**	**87.5**

The statistical treatment of data focused on the variance analysis and p.p.d.s test at the threshold of 5%.

Sporulation on the host

Sporulation on host is estimated by the method of Hill and Nelson (1983) by counting the average number of spores produced per unit area of the host leaves carrying lesions (number of spores / ml).

Ten days after inoculation, leaves showing lesions are taken from inoculated plants of rice, cut into pieces of 1 cm and then placed in Petri plates containing two discs of filter paper soaked with sterile distilled water (1 leaf per plate). The plates are placed at 30 cm in continues light to 28ºC.

After 48 h, the fragments of each leaf were placed in a test tube containing 1ml of sterile distilled water and stirred by vortexing for 2 minutes in order to detach the conidia from the mycelium.

The spore's number in the suspension was determined using a Malassez blade (10 counts per sample). The observation is made at magnification factor x 100.

II. RESULTS

The leaves of rice three varieties, inoculated with *H. rostrata*, have tapered brown spots, and in the center, a dark brown aureole corresponding to the fungus penetration area on the leaves. Lesion size is variable and may be up to 5 mm length on certain varieties (Arco) (Figure 1).

The results of table 1 indicate that *H. rostrata* isolates showed pathogenicity, as estimated by the infection coefficient, varying depending on the tested rice varieties ; HR1, HR2 and HR3 was respectively the most pathogenic on Elio (I.C = 113), Arco (I.C = 212.5), and Taibonnet (130,48).

Fig.1: Helminthosporium rostrata : Conidia (A) and symptoms on rice leaves (B)

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Table.1: disease Incidence, severity and infection coefficient of rice varieties inoculated with H. rostrata isolates.

Isolates		Varieties								
		Arco			Taibonnet			Elio		
		I	S	I.C	I	S	I.C	I	S	I.C
Helminthosporium rostrata	HR1	28	6.8	191.33^{b}	37	5.74	117.67^{b}	16	5.16	113^{a}
	HR2	19	6.1	212.5^{a}	25	4.8	120^{b}	23	5.67	84.66^{b}
	HR3	17	6.68	82.61^{c}	20	4.23	130.48^{a}	15	5.37	80.6^{b}

Two results on the same column differ significantly at 5% level (p.p.d.s test) if they are not assigned by any letter in common, insignificant in the contrary case.

The results given in Table 2 show that all fungal species tested are able to sporulate on the leaves of rice varieties. Sporulation of HR2 isolate is very important on the three varieties. It is in the order of 17.33 10^5 conidia/cm^2 on Arco, 15.33 10^5/cm^2 on Taibonnet and 13.33 10^5 conidia/cm^2 on Elio. The isolate HR1 sporulates well on Arco variety (1710^5 conidia/cm^2), however sporulation is about 11.66 10^5 conidia /cm^2 on Taibonnet and decreases in the variety Elio (910^5 conidia/cm^2).

The isolate HR3 sporulates less on Arco varieties (2.33 10^5 conidia/cm^2) and Elio (4.3310^5 conidia/cm^2).

Table.2: Sporulation of Helminthosporium rostrata isolates on rice varieties (10^5 conidia/cm^2)

	Isolates sporulation (10^5 conidia/cm^2)		
Varieties	**HR1**	**HR2**	**HR3**
Arco	17.00^{a}	17.33^{a}	2.33^{ab}
Taibonnet	11.66^{b}	15.33^{a}	10.00^{b}
Elio	9,00^{b}	13.33^{a}	4.33^{c}

Two results on the same column differ significantly at 5% level (p.p.d.s test) if they are not assigned by any letter in common, insignificant in the contrary case.

III. DISCUSSION & CONCLUSION

The results obtained showed that *H. rostrata*, represented by the three isolates tested, is capable of altering the rice plants foliage. This pathogen encountered for the first time on the rice grains of Taibonnet variety, can induce the disease on the leaves of other varieties of rice (Arco and Elio).

Symptoms developed on the rice plants leaves varies from variety to another. They are similar morphologically to those induced by *Helminthosporium sativum* on Rice (Ouazzani *et al*., 2000), but different from those caused by *H. oryzae* (Bouslim *et al*., 1997), *H. spiciferum* (Ennaffah *et al*. 1997), *H. australiensis* (Ouazzani *et al*., 2000) and *H. bicolor* (Leopold, 2005).

All developed leaf lesions are sporulating, that is to say, that *H. rostrata* is capable, once inoculated to the rice plants leaves to produce secondary inoculum that can infect other healthy rice leaves and therefore to participate in the progression of the disease.

All these observations allow to incriminate *H. rostrata*, isolated from seeds, as a new rice leaf pathogen.

The introduction of some sensitive varieties, case of Arco variety, will favor the multiplication of this pathogen which probably finds in the Moroccan rice all favorable conditions for its development.

Furthermore, on other host plants, *H. rostrata* attack all aerial parts of corn plant, sorghum, and some plants such as pearled millet, which is an annual herb of the hot season (Mathur *et al*., 1973).

But according to some authors (Kadir and Ahmed, 2004), *H. rostrata* is unable to attack the bean. These authors suggested introducing in rotation this plant species to reduce the inoculum of this pathogen.

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