http://dx.doi.org/10.22161/ijeab/4.2.43 *ISSN: 2456-1878*

Responses of date Palm Seedling to co-Inoculation with Phosphate Solubilizing Bacteria and Mycorrhizal Arbuscular Fungi

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Abstract**—** *With the broad aim of biologically improving P uptake by date palm, in this work we use the beneficial phosphorus solubilizing microorganisms like arbuscular mycorrhizal fungi (AMF) and phosphate solubilizing bacteria (PSB) characterized by a remarkable ability to solubilize phosphorus and tolerance to hard pedoclimatic conditions. For this study, a pot experiment was conducted under greenhouse condition to assess the effect of associations between mycorhizal fungus (Glomus mosseae) (M) with a range of ten bacterial strains (B).*

An essay of different treatments (control seedlings, seedlings inoculated by arbuscular mycorhizal fungi (AMF) and seedlings inoculated by a combination of PSB and AMF) showed the progressive evolution of microbial activity.

The results indicated that the rhizosphere interactions between Glomus mosseae and PSB strains (Pseudomonas striata, Bacillus subtilis) significantly improved growth parameters including root dry weight (23,66%), root length (24%), leaf dry weight (67,7%), leaf length (26,9%), promote P mineralization in soil P (142,3%) uptake by date palm seedling as compared to control, and percent root colonization in date palm seedling was also recorded by the combined inoculation of Glomus mosseae and PSB strains (Pseudomonas striata, Bacillus subtilis).

Keywords**— Bacteria, date palm seedling, fungi, growth, inoculation, interaction.**

I. INTRODUCTION

Phosphorus is an important key element in the nutrition of plants. Although phosphorus is present both in organic and inorganic forms in soils but its availability is limited to plants and mostly restricted due to complex formations with other nutrients (Sharma et al. 2013).

The soils in Djerid region are calcareous in nature and due to the high pH much of phosphorus is not available for plant uptake and growth promotion (Mtimet 2016; Zougari et al. 2016). However, to respond to the growing demands, in this region, to improve the absorption of phosphorus and compensate this poor availability of this element, it is necessary to manipulate phosphorus solubilizing microorganisms (arbuscular mycorhizal fungi and phosphorus solubilizing bacteria), which convert the insoluble forms of P to an accessible form by plants (ortho-phosphate) (Costa et al. 2015). Currently inoculation of these microorganisms have gained popularity (Parkash et al. 2011) to be used as biofertilizers instead of high input chemical fertilizers in crop production system (Kennedy et al. 2004).

A synergistic relation between arbuscular mycorhizal fungi (AMF) and phosphorus solubilizing bacteria (PSB) had been observed (Fazli et al. 2015). These observations also showed that a combined application of *Glomus fasciculatum* and *Azotobacter* increase the concentration of P, K and N uptake by the mulberry (Morus nigra) leaf of 10, 16 and 5.8 %, respectively.

Therefore, it seems more appropriate to consider this technique is more interesting. Indeed, it is becoming increasingly clear that influencing the microbial diversity of soil, it would be possible to improve fertility (Beauregard 2010).

Several research results on soil microorganisms were published during the last decade, the factors influencing the growth of these microorganisms and the changes in their community are likely to act together and their combined effects are difficult to predict (Oehl et al. 2003; Castillo et al. 2006). Nevertheless, the work of Schrey et al. (2007) is in keeping with the small number of previous studies that have addressed the practical use of co-inoculation with phosphate solubilizing bacteria (PSB) and mycorrhizal arbuscular fungi (AMF) in agriculture.

http://dx.doi.org/10.22161/ijeab/4.2.43 *ISSN: 2456-1878*

In Djerid region, no attempts have been made so far to investigate the effect of PSB and AMF inoculation on date palm crop. Therefore, the aim of the present study was to investigate the effect of AMF and PSB strains inoculation on growth, mycorrhization and P uptake of date palm seedling in the arid conditions.

II. MATERIALS AND METHODS

The material used in this work is the AM fungal spore and phosphorus solubilizing bacteria (PSB) of the host plant: *Phoenix dactilifera L.* (Date palm).

The collection of a representative soil sample was taken from Jerid palm groves. AMF spores were isolated using the wet-sieving (125 and 45 µm) and decanting method described by Gerdemann and Nicolson (1963). Spores and spore clusters were transferred into Petri dishes and counted in three replications under binocular microscope with magnification of 40X and divided in groups in relation to morphological characteristics as shape, size, color, presence of structures like sporiferous saccule, subtending hypha. Although, the same morphotypes of mycorrhizal fungal spores (*Glomus mosseae)* were isolated in collaboration with Eastern cereal and Oilseed Research Centre (ECORC) Ottawa). Approximately 50 spores were isolated and counted for each pot and stored at 4°C for a maximum of two days before pots application.

The AMF colonization rate of date palm seedlings roots was determined using the magnified line-intersect method of McGonigle et al. (1990).

Phosphate solubilizing bacteria (PSB) were collected from the rhizosphere of date palm. Samples of soil were suspended in phosphate buffer saline and serial dilutions were spread on Pikovskaya's (PVK) agar containing $Ca_3H_2PO_4$ as the phosphate source. The bacterial isolates were characterized by conventional tests on API 20 E gallery, API 50CH gallery and on specific reactive media in collaboration with Environmental Health Laboratory (EHL) Tozeur.

The ability of the bacteria to solubilize insoluble phosphate was qualitatively determined from the clear light zone surrounding colonies (Figure 1) according to Edi-Premono et al. (1996) by the solubilization index = [ratio of the total diameter (colony + halo zone) to the colony diameter].

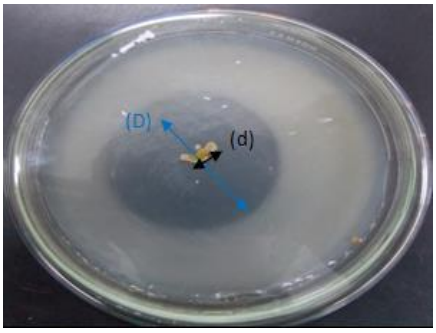

Fig.1: Halo zone around the colony on Pikovskaya (PVK) agar confirm phosphate solubilizing bacteria. (D: total diameter (colony + halo zone) and d: colony diameter).

For the inoculums preparation the selected PSB strains were inoculated separately in 250 ml flasks containing 100 ml of Pikovskaya's broth. The flasks were kept in a growth chamber at 28°C on an orbital shaker at 120 rpm. After 7 days 1 ml of the broth culture was spread on Pikovskaya (PVK) agar plates to determine the microbial concentration. Finally, the bacterial cultures were adjusted to a concentration of approximately 10^8 cfu / ml (Murray et al. 2003).

The soils of Djerid region were selected for cultivation of date palm seedlings grown in plastic pots under greenhouse conditions at the regional research center of oasis agriculture. Pots were filled with 3kg sterilized soils of Djerid region. Three replications were prepared for each treatment.

the considered soils studied at all depths are generally characterized by coarse textures, slightly alkaline to alkaline pH values, very low cation exchange capacity (CEC); the percentage of active limestone is moderately high (Table 1).

Table.1: Chemical and textural characteristics of the soils from Djerid region

Depth (cm)	pH	EC (mmhos/cm)	CEC (mmol (+)/kg)	CaCO3 (%)		Texture class
				Total	Active	
0-20	8.8 ±0.02	7.4 ±0.12	5.3	12.7 ± 1.01	7.3 ±0.09	SCL
20-40	8.7 ±0.05	5.3 ±0.01	4.5	12.5 ±1.17	8.1 ±0.38	SL
40-60	7.4 ±0.01	5.2 ±0.16	4.5	10.1 ±1.20	8.0 ±1.07	SL

SCL: Sandy Cay Loam, SL: Sandy Loam, EC: electrical conductivity, CaCO3: calcium carbonate

The date seeds were disinfected with 3.5% NaOCl and then rinsed with distilled water 3 times after 10 min. The disinfected date seeds were transferred to incubator at 28°C to accelerate pre-germination seed activities.

The experiment includes the following treatments:

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1- Control treatment (T): untreated soil

2- M: Spores of the AMF (*Glomus mosseae*) (50 spores per pot)

3- Bi + M; (Bi: (B1, B2, …, B10)) ; M: Spores of the AMF (*Glomus mosseae*)

All date palm seedlings were harvested after 135 days and the growth parameters, such as_root length, root dry weight, leaf length and leaf dry weight were recorded. The roots were washed out with tap water to remove the soil particles. After drying for 48 hours at 60°C, the samples were weighted, grinded and further analyzed for phosphorus concentration and accumulation by date palm seedlings by the method as described by Pauwels et al. (1992).

To determine root percentage colonization, the roots were cleared in 10% KOH, rinsed once with water, soaked in 2% HCl and stained with Trypan blue solution. After rinsing three times with water, roots were stored in lactoglycerol. Percent colonization of various AMF structures and the overall association was determined under the microscope by the magnified intersections method (McGonigle et al. 1990)

Soil samples were collected, from each treatment, 135 days after inoculation. These soil samples were air dried ground and sieved through a 2 mm sieve. Soil samples were analyzed for assimilable P by the following procedures described by Pauwels et al. (1992).

The statistical treatment of results is achieved through the STATISTICA Version 5 software, (Beaux et al. 1991). Analysis of variance of two factors by Fisher's F test is performed to verify the equality of the means of hypothesis risk threshold of 5%. It is supplemented by multiple comparisons of means by the Newman Keuls test when the equality of averages hypothesis is rejected, according to Dagnelie (1986).

III. RESULTS

3.1. Phosphorus solubilizing bacteria associated with date palm

We isolated and purified a total of 10 bacterial strains from the rhizosphere of date palm seedling by repeated streak culture on PVK medium. Initially, all isolates were tested for their phosphate solubilizing activity using PVK medium.

The most efficient and competitive phosphorus solubilizing bacteria are selected: B1: *Pseudomonas horyzihabitans*, B2: *Pseudomonas aeruginosa*, B3: *Serratia liquefacien*, B4: *Pseudomonas striata*, B5: *Bacillus subtilis*, B6: *Citrobacter freundii*, B7: *Moraxella spp*, B8: *Serratia odorifera*, B9: *Non-fermenter spp* and B10: *Bacillus cereus)*

3.2. The determination of the bacterial effectiveness

The phosphate solubilization index of the date palm seedling isolates varied from 2.4 to 4.3 (Figure 2).

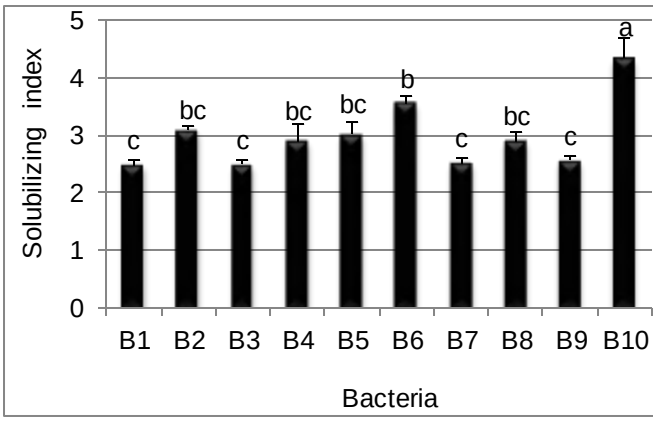

Fig.2: The phosphate solubilization index of the date palm seedling isolates (Bars with different letters represent statistical significant differences according to Newman Keuls test (p≤0.05).

Bacillus cereus (B10) exhibited the highest phosphate solubilization index (4.3).

Several early studies revealed that many phosphorus solubilizing bacteria are normally present in the soil. They can remove and release phosphorus from soil.

3.3. Phenotypic analysis

The influence of combinations (*Glomus mosseae* (M) and PSB) on growth and phosphorus nutrition was studied nine months after inoculation

3.3.1. Root length

Test results showed that the inoculation of *Glomus mosseae* (M) and PSB strains influenced the root length of date palm seedlings as compared to the treatments having single or no inoculation. The maximum (31 cm) root length with 24% increase over control (T) and single inoculation (M) was observed. The treatments (B4+M; B5+M and B10+M) have somewhat higher values than the other treatments, but statistical analysis shows some stability of the root length for all treatments (Table 2).

Table.2: Root length, root dry weight, leaf length and leaf dry weight as influenced by the inoculation of AMF (Glomus mosseae (M)) and PSB.

	Root length	Root dry weight	Leaf length	Leaf dry weight
B1+M	26,6 a	1,136 ab	29,1 ab	1,763 cd
B2+M	26,6 a	1,312 a	28,2 b	2,121 bc

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B3+M	28,2 a	1,174 ab	31,3 ab	2,009 c
B4+M	30,9 a	1,372 a	32,8 a	2,520 a
B5+M	31,2 a	1,388 a	32,9 a	2,677 a
B6+M	29,0 a	1,339 a	31,3 ab	2,335 b
B7+M	29,4 a	1,177 ab	30,4 ab	2,405 ab
B8+M	29,6 a	1,209 ab	31,1 a	2,386 ab
B9+M	27,6 a	1,341 a	25,0 c	1,961 c
B10+M	31,2 a	1,434 a	31,5 a	1,994 c
M	25,1 a	1,356 a	30,1 ab	1,779 cd
T (Control)	25,4 a	1,031 b	26,1 bc	1,550 d

(Values followed by different letters represent statistical significant differences according to Newman Keuls test ($p \leq 0.05$)).

3.3.2. Root dry weight

The dual inoculation (AMF and PSB) increased root dry weight of date palm seedlings significantly ($p \leq 0.05$) over control (T). The maximum (1.42 g) root dry weight with 37.9% increase over control was observed.

The influence of combination (PSB (B_i and *Glomus mosseae* (M)) on root dry weight was studied and the results showed that treatments (B10+M) gives the highest value, but there are no significant differences for the majority of treatments (Table 2).

3.3.3. Leaf length

Based on comparisons of treatments it's fair to say that treatments ((B4+M; B5+M; B8+M and B10+M) give the highest values (Table 2), but the minimum leaf length of date palm seedling (25 cm) was noted in the combined inoculation (B9+M). The experiment showed that there are bacteria that are auxiliaries of mycorhization, so-called "mycorrhiza helper bacteria". While others have inhibitory activity on the development of mycorrhizae.

3.3.4. Leaf dry weight

The highest total dry weight was obtained when date palm seedlings were inoculated with AM fungi (*Glomus mosseae)* incorporated with bacteria B4 and B5 as compared to the control and other treatments. Plant inoculated with *Glomus mosseae* (M) plus bacteria B5 was found to be two times greater of leaf dry weight than that of the control (T)

The results showed that co-inoculation of fungus and phosphorus solubilizing bacteria (PSB), in comparison with single inoculation (M) and the control (T), of any of them caused a significant increase in leaf dry weight (Table 2).

3.4. Mycorrhization of date palm seedling

The majority of treatments generate a sizeable rate of mycorrhizal hyphae that exceeds 50% (Figure 3).

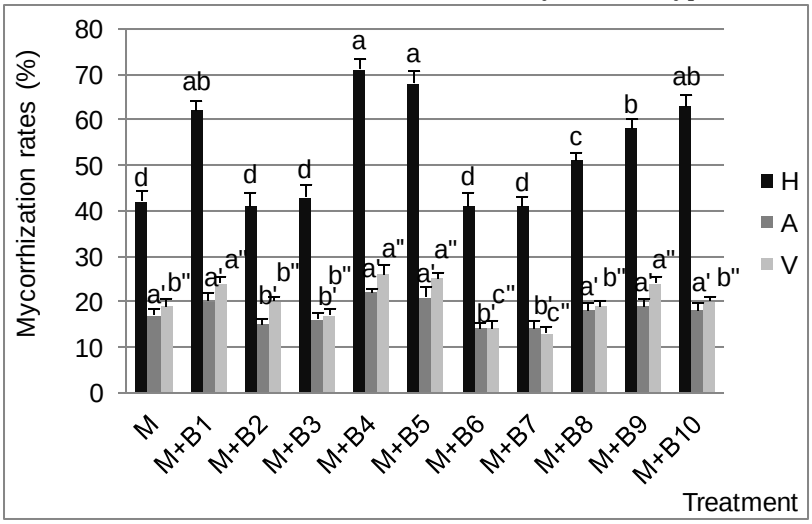

Fig.3: Effects of treatments on mycorrhization rate; hyphal ( ), arbuscular, ( ) and vesicular ( ) in the roots of date palm seedling. (Bars with different letters represent statistical signicant differences mycorrhization rate hyphal (a,b,c); mycorrhization rate arbuscular (a',b',c') and mycorrhizationrate vesicular (a",b",c") according to Newman K euls test (P<0.05)).

It is evident that the treatment (M+B7) is characterized by a limiting effect on the appearance and the development of fungal structures (hyphae, arbuscule, and vesicle). The minimum values of hyphal growth rate, arbuscular growth rate and vesicular growth rate were 41%, 14% and 13% respectively.

http://dx.doi.org/10.22161/ijeab/4.2.43 *ISSN: 2456-1878*

The present study demonstrated the benefits of combinations between M+Bi (*Glomus mosseae* (M)) and phosphorus solubilizing bacteria strains (Bi)) for growth of fungal structures in comparison with single inoculation (M). The bacterial community can change the result of mycorrhizal establishme.

In addition, it was found that *Glomus mosseae* spores development was positively (R^2= 0.69) correlated with PSB population (Figure 4)

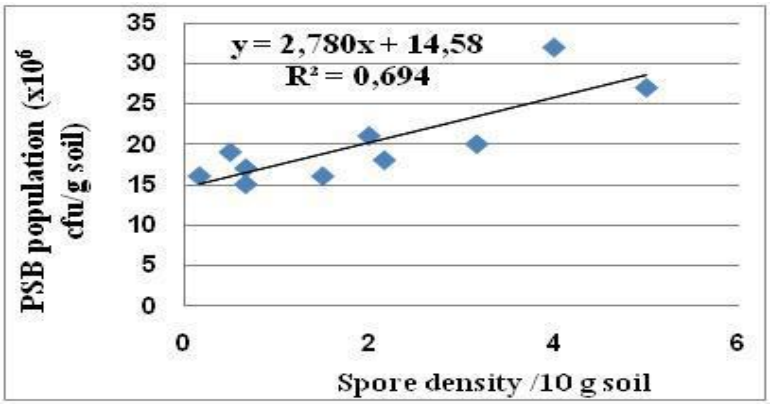

Fig.4: Correlation between spores density of the mycorrhizal fungus (Glomus mosseae) and PSB population in date palm seedlings

3.5. Phosphorus content

Based on comparisons of treatments it's fair to say that the highest P uptake was recorded in the treatment of AMF inoculated with *Pseudomonas striata* (B4) *and Bacillus subtilis* (B5).The minimum P uptake was found in "B6+M" treatment.

Based on Figure 5, some of the treatments have low phosphorus levels in comparison with treatment (M), which implies the ineffectiveness of these associations for biological uptake of this mineral element.

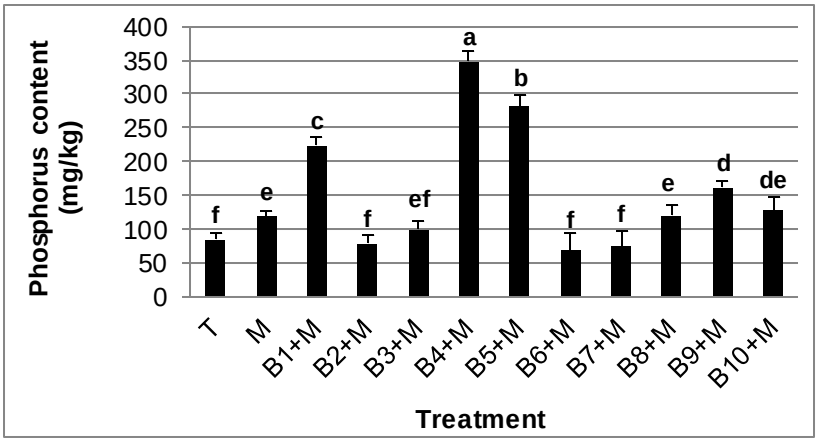

Fig.5: Effect of different treatments on phosphorus content of date palm seedlings. (Bars with different letters represent statistical significant differences according to Newman Keuls test (p≤0.05).

Interference and correlation of all these results showed several types of biological relationships between different microorganisms in their habitat (symbiosis, mutualism, antagonism).

Arbuscular mycorrhizal (AM) associations have been shown to reduce damage caused by soil-borne plant pathogens.

IV. DISCUSSION

The dual inoculation with AMF and PSB is an important approach to decrease the use of chemical fertilizers and improve P supply for sustainable crop production. In our study, we inoculate ten strains of PSB with AMF and investigated its effect on different plant growth parameters. The synergistic effect of AMF and some PSB influenced P solubility and stimulated date palm roots to absorb nutrients from soil and thus enhanced the overall plant growth as compared to the treatments having single inoculation (M) and the control (T). The microbial activities stimulated nutrients uptake and plant growth may be due to hormones such as auxin or gibberellic acid production as stated by Minaxi *et al*. 2013; Kang *et al*. 2012.

http://dx.doi.org/10.22161/ijeab/4.2.43 *ISSN: 2456-1878*

In this study, we observed a significant growth benefit of the synergistic association of date palm seedlings with AMF and some PSB. Our results showed that combined inoculation of plants with AMF and PSB resulted in increased AMF colonization, which is an important indicator of plant nutrition. The synergistic interaction of some PSB and AMF significantly increasing plant biomass, plant growth and amelioration of phosphorus uptake compared with untreated plants (Osorio and Habte 2001 and Kohler et al. 2007). While, we found that some PSB strains reduced AMF colonization in date palm seedling, consistent with observations made by Alqarawi et al. (2014) in Ephedra aphylla and Hashem et al. (2015) in Vigna unguiculata.

The experiment showed that there are bacteria that are auxiliaries of mycorhization, so-called "mycorrhiza helper bacteria". While others have inhibitory activity on the development of mycorrhizae. However, plants inoculated with (M+B3, M+B6 and M+B7) showed significantly lower mycorrhizal colonization than plants inoculated with (M+B1, M+B4, M+B5 and M+B10). This result matches those found by Garbaye and Bowen (1989) who demonstrated that the rhizosphere microflora could have a positive or negative impact on the mycorrhizal symbiosis, depending on the bacterial isolates.

Several early studies revealed that many phosphorus solubilizing bacteria are normally present in the soil (Buchan 1983; Sidat et al. 1999). They are efficient in storing polyphosphate and can remove and release phosphorus from soil (Peix et al. 2003).

The bacterial community can change the result of mycorrhizal establishment (Marschner et al. 2001). Arbuscular mycorrhizal (AM) associations have been shown to reduce damage caused by soil-borne plant pathogens. These results confirm those from other authors (Ben Kaled et al. 2003; Chabot et al. 1993 and Baquall and Das 2006).

V. CONCLUSION

To improve phosphorus uptake by date palm, this work was realized to study the biological alternatives (phosphorus Solubilizing Bacteri (PSB) and mycorrhizal fungus (*Glomus mosseae*)) in order to solve phosphate's blocking assimilation characterizing soils in southern Tunisia.

This work has also highlighted the presence of bacterial strains that have phenotypical and genetical diversity. These bacteria seem to be adapted to the extreme conditions of the oasis.

Phenotypic analysis showed that treatments (B4+M; B5+M and B10+M) have somewhat higher values than the other treatments. These results suggest a synergistic relationship between some phosphorus solubilizing bacteria and arbuscular mycorrhizal fungi that can enhance date palm productivity.

According to the experimental results, it seems that co-inoculation (B4+M and B5+M) of date palm seedlings, can have a very strong effect on the uptake of phosphorus from soil. The other treatments showed the ineffectiveness of these associations for biological uptake of this mineral element.

The growth parameters, root colonization and phosphorus uptake responses of date palm seedlings to co-inoculation of AMF and PSB showed that they depend on a combination of fungal and bacterial strains. According to the results of this study, co-inoculation (B4+M and B5+M) significantly increased all parameters studied.

Generally, interference and correlation of all these results showed several types of biological relationships between different microorganisms in their habitat (symbiosis, mutualism and antagonism).

ACKNOWLEDGEMENT

The authors acknowledge the support of Dr. yoland Dalpé, Research Scientist for Agriculture and Agri-Food, (Eastern cereal and Oilseed Research Centre (ECORC) Ottawa), for collaboration to identify spores of AMF.

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