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Characterisation of several isolates of *Fusarium oxysporum* f. sp. elaeidis for the selection of fusarium-resistant oil palm varieties

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Abstract— *This work was aim to evaluate the pathogenicity and the level of aggressiveness of ten strains of Fusarium oxysporum f. sp. elaeidis (Foe) on palm oil seedlings susceptible to vascular wilt. For this purpose, the radial growth, sporulation and the morphological characteristics of ten strains of Foe from the farm palm of the Cameroon Development Corporation (South-west) were studied in vitro. In addition, 500 oil palm seedlings susceptible to vascular wilt and aged 5 months were distributed in a randomized complete block device, and inoculated with said strains. External symptoms of Fusarium wilt were observed on those seedlings and the correlation between these parameters was also investigated. The data collected in prenursery did not establish a significant difference in the aggressiveness of the isolates. However, regarding the pathogenicity, 7 strains have shown a high pathogenicity. As for the radial growth, strain F presented fastest growth was 8.12 cm in diameter, unlike the strain **I** whose growth was the lowest is 3.98 cm in diameter. The **C** strain is one that is abundantly sporulated126,5x10^{5}spores/ml; however, isolates **B** and **J** do not sporulate. No correlation was detected between the different parameters. But, considering the results obtained, the **F** and **I** strains could be the most aggressive and can potentially be used to test the timber yard of the specialized Centre for oil palm research of La DIBAMBA to select the most resistant to vascular wilt.*

Keywords— ***Oil Palm, Fusarium oxysporum, Pathogenicity, aggressiveness, strains.***

I. INTRODUCTION

In Cameroon, the exploitation of oil palm by local communities is a social and cultural heritage. Before the colonial period, the plant was the main source of vegetable fats and a major source of income for local people. Drinks, medicines, household and building materials, art objects, fuel, livestock and agricultural products and cosmetics can all be obtained from this crop (Temgoua and Bakoume, 2008).

Unfortunately, Cameroon's palm oil production is not keeping pace with population growth in order to satisfy the ever-increasing demand for this product. Similarly, on the international stage, Cameroon's position in the world palm oil supply is steadily declining. Ranked 11th in 2008, with 160,000 t of palm oil produced (Ntsomboh-Ntsefong, 2015), the country came 13th in 2012, with production estimated at almost 230.000 t (Diabate et 2010 ; Renard et Franqueville, 1989). This situation stems primarily from the many problems facing the country's olive oil sector, namely the low rate of oil extraction from artisanal units, the low

use of fertilisers due to their very high prices, the ageing of plantations, the economic crisis that has led to the government's withdrawal from the sector, the use of inadequate selected planting material and the resurgence of diseases and pests (Henni et al., 1994).

Fusarium is the most devastating of the oil palm diseases (Flood, 2006 ; Chehri et al. 2011). This disease is caused by a fungus of telluric origin, *Fusarium oxysporum*. f. sp. Elaeidis (Foe). It slows plant growth, reduces organ size and in severe cases leads to plant death, causing partial or total loss of production. Losses can be as high as 100 % (Diabate, et al., 2010 ; Assohoun et al. 2016). It is present in all CDC and SOCAPALM palm groves in the Littoral, South-West and Centre regions (Tengoua, 1994; Tengoua, 2003).

Given the telluric and vascular nature of this pathogen, no chemical control method is economically feasible (Renard et Franqueville., 1989 ; Gbongue et al., 2012). However, it very quickly became apparent that, as with other fusarioses, the selection of plant material tolerant to the disease could limit its development (Bachy and Fehling, 1957; Diabate et al. 2010 ; Gogbe-Dibi et al. 2022).

The method used, and indeed the most effective, is the preventive method involving the use of tolerant plant material (Lepoivre, 2003. However, as the durability of varietal resistance is a major issue for the use of palm varieties, we need to update our knowledge of the capacity of pathogen populations to adapt to partial or total resistance (Andanson, 2010).

Research is therefore constantly being carried out to improve the tolerance level of oil palms. The most recent study is the confrontation of pathogenic and non-pathogenic strains of *Fusarium oxysporum* f.sp. elaeidis in the acquisition of resistance against fusariosis of oil palm (Assohoum et al., 2016 ; Kablan et al. 2016) carried out in Côte d'Ivoire, it stated that susceptible oil palm plants can be protected against Fusarium head blight by antagonism between saprophytic F. oxysporum strains and the pathogen *F. oxysporum* f.sp.elaeidis (Foe). Other studies are also being undertaken to assess the reaction to Fusarium head blight of seedlings from a Fusarium tolerant cross and a Fusarium non-tolerant cross of oil palm previously protected from a non-pathogenic strain of *Fusarium oxysporum* (Armstrong and Armstrong, 1981).

Furthermore, in order to produce tolerant oil palm seed in Cameroon, fusariosis-tolerant pollen grains have to be purchased from foreign partners at excessively high cost, notwithstanding the large number of genitors (4,000) available in Cameroon. Most of these genitors cannot be used because they have not been tested for Fusarium head blight (Tengoua, 2003). Indeed, to this day, the production of fusarium-tolerant seedlings for oil palm in Cameroon depends on tolerant pollen bought abroad at exorbitant prices (around 50,000 CFA francs for a unit of 0.0625g of fusarium-tolerant pollen grains).

This work consisted of testing several strains of F. oxysporum f.sp.elaeidis Foe on a few palm trees in order to identify the most aggressive strains to be used for tolerance testing of the broodstock used at CEREPAH (Dibamba Oil Palm Research Centre) for seed production or to better guide the oil palm variety improvement and selection programmes.

II. METHOD

Plant material

Five-month-old fusarium-susceptible oil palm seedlings were used in this trial. They were supplied by IRAD's Centre Spécialisez on oil palm reserch (CEREPAH) in Dibamba.

Fungi material

The fungal material used consisted of ten strains of F. oxysporum isolated from palms affected by chronic fusariosis in the palm groves of the CDC located in the South-West region, Department of Fako, more specifically in the localities of Matango, Ekona and Powo. The ten strains tested were labelled with the following designators: A; B; C; D; E; F; G; H; I; J. (Table 1).

Table 1 : Informations on isolates used (Temgoua and Bakoume, 2008).

Isolate	Sampling area on the trunk	Locality
A	Approximately 1 m from the ground	IRAD Ekona
B	Approximately 1 m from the ground	IRAD Ekona
C	Middle of the trunk	CDC Matango
D	Middle of the trunk	CDC Powo
E	Middle of the trunk	CDC Matango
F	Middle of the trunk	CDC Matango
G	Approximately 1 m from the ground	CDC Matango

H	Approximately 1 m from the ground	CDC Matango
I	Middle of the trunk	CDC Matango
J	Approximately 1 m from the ground	CDC Matango

Preparation of mycelium medium (MM) and strain maintenance

This is the most appropriate medium for the growth of Fusarium strains isolated from oil palm (Ntsomboh-Ntsefong, 2015). The various components [dipotassium phosphate (1 g), magnesium sulphate (0.5 g), iron sulphate (0.100 g), asparagine (1.5 g), agar agar (25 g), glucose (20 g), yeast extract (1 g)] were dissolved in 1 litre of distilled water. After homogenisation and autoclaving, the medium was poured into Petri dishes in a laminar flow hood at a temperature of 50-55ºC. After the medium had cooled and solidified in the dishes, the isolates were subcultured.

Isolate maintenance consisted essentially of renewing the isolates in the new media after one month..

Preparation of inocula

The inoculum for each strain was prepared on liquid medium (Armstrong medium) following the methodology described by Tengoua and Bakoume (2008), which consisted of taking a fragment of a five-day culture on solid medium and introducing it into a 100 ml flask containing 75 ml of Armstrong medium (Table 2). After shaking every ten minutes for four days, 2 ml were taken and placed in one-litre Roux dishes containing 100 ml of liquid medium. After shaking at the same rate for 10 days, the inoculum from each Roux dish was mixed with its counterpart from the flask, ground in a blender for 30 seconds and diluted in 4 litres of tap water for seedling inoculation.

Table 2 : composition of Armstrong culture medium

Compounds	Quantity (g)
Glucose ou sucrose	20,000
Magnesium sulphate	0,400
Chloride of potash	1,600
Potassium dihydrogen phosphate	1,100
Calcium nitrate	5,900
Iron chloride	Take 1ml of a stock solution 0,2g/L 1 cristal
Manganese sulphate	
Zinc sulphate	
Distilled water	1,000 (l)

Morphological characterisation of the various isolates

The different isolates were compared on the basis of colour, mycelial appearance, colony margin and colony surface.

a) Evaluation of radial growth of strains in Petri dishes

Each strain was transplanted into Petri dishes containing MM culture medium and repeated 3 times. The daily radial growth of the mycelium was recorded for each strain using a graduated ruler on 2 perpendicular axes joining at the centre of the Petri dish. Daily radial growth (V) was calculated using the following formula:

$V= (d_1+d_2)- d_0/2$ **(**Ntsomboh-Ntsefong, 2015).

d_1= diamèter 1 ; d_2= diamèter 2; d_0= 5 mm (diameter of mycelial disc).

b) Assessment of sporulation of the various isolates

It was carried out using a Malassez cell (Hematimeter), by microscopic observations of the isolate preparations. The aim was to count the asexual reproductive organs (microconidia and macroconidia) produced by each isolate. The chlamydospores were simply observed. For this quantification, each isolate was grown for four days in 100ml of liquid medium (Armstrong medium) from a four-day-old culture on solid medium. At the end of the 6 days, the cultures were filtered and appropriate dilutions were made to facilitate counting. For each strain, counting was repeated 2 times (Tengoua and Bakoume, 2008).

Pathogenicity test

a) Construction of the shade house

The shed was covered with oil palm leaves to limit sunlight penetration to 50% and reduce the kinetic energy of raindrops. In addition, a one-metre-high wire mesh fence surrounds the prenursery and prevents rodents from entering.

b) Experimental plan design

A randomised complete block design with five replicates (blocks) was used, with the ten strains as treatments (Fig. 1 a).

Each elementary plot contained 10 seedlings arranged in two rows of five (Fig. 1 b), and the distance between two elementary plots in the same block was 50 cm as long as two neighbouring blocks were 75 cm apart**.**

Block 1	Block 2	Block 3	Block 4	Block 5
P_1F	P_2E	P_3G	P_4J	P_5C
P_1H	P_2A	P_3B	P_4F	P_5B
P_1C	P_2B	P_3J	P_4D	P_5F
P_1A	P_2G	P_3E	P_4H	P_5D
P_1I	P_2D	P_3C	P_4G	P_5J
P_1G	P_2H	P_3A	P_4I	P_5E
P_1B	P_2I	P_3H	P_4C	P_5A
P_1D	P_2F	P_3I	P_4E	P_5G
P_1E	P_2J	P_3F	P_4A	P_5I
P_1J	P_2C	P_3D	P_4B	P_5H

Fig. 1 : a) plants in nursery ; b) differents plots

a) Plants maintenance

In order to prevent any interference from one or more undesirable factors, watering, fertilisation, phytosanitary treatments and weeding were carried out throughout the experiment.

The seedlings were watered every two days if it was not raining, at a rate of 10 litres of tap water per 500 seedlings, i.e. around 20 ml per seedling.

Fertilisers were applied at a rate of 10 g of Urea, 05 g of potassium chloride and 05 g of kieserite dissolved in 05 litres of water per 500 seedlings, starting one month after inoculation, i.e. as soon as the first symptoms of mineral deficiency were observed. It continued on a monthly basis. Immediately after spraying, the seedlings were watered abundantly to prevent leaf burn.

Insecticide treatments were applied to prevent insect attacks. Parastar 40 EC, a systemic and contact insecticide (20g/l imidachloprid + 20g/l lambdacyhalothrin) was used against Temnoschoita quadripustulata. Anti-slug (metaldehyde) was used against slugs and snails. Weeding was carried out by hand.

Inoculatioon

This operation involved removing the soil from the neck of each seedling to free the roots and wounding them with a pointed stick. The scarified roots were then rinsed with tap water before receiving 20 ml of diluted inoculum from each strain. After inoculation, the roots were covered with soil (Fig. 2).

Fig. 2 **:** inoculation of plants A= Root injury; B= Root washing; C= Administration of the inoculum.

Assessment of external and internal symptoms of fusariosis

Observation of symptoms in the prenursery began two months after inoculation of the seedlings and continued every fortnight until the symptoms stabilised. External symptoms characterised by yellowing and/or stunting of the leaves were noted, and the number of leaves of each type (green, yellow, stunted and dry) was counted.

At the end of the experiment, the seedlings were dissected in order to observe the internal symptoms of fusariosis (browning of the vessels).

Seedlings showing external symptoms were compared with those showing external symptoms in order to confirm or refute the origin of the external symptoms. Vessel atrophy is often considered to be a primary symptom that can cause secondary damage to the aerial part of the plant.

III. DATA ANALYSIS

The data obtained were analysed using SAS software to determine the effect of the treatments. Correlations between growth rate, spore count and external symptoms of Fusarium head blight were studied using SPSS 20.0 software.

IV. RESULTS

Morphological characteristics of strains

The development of isolates on MM medium varies from one isolate to another in terms of the appearance of the mycelium, its pigmentation and that of the culture medium (Fig. 3 a). However, these isolates can be grouped together in clusters with more or less similar cultural characteristics.

With the exception of isolate C, all the other isolates have cottony white aerial mycelium

Isolates A, B, D, E, F and I have abundant, well-developed aerial mycelium, which is whitish in colour at the start of growth. This pigmentation slowly turns yellow in isolates B and F, while D takes on a pink colouration. Strains G, H and J show a thin, short, pale white mycelium.

The view from below shows a purplish coloration evolving from the centre to the periphery of the dish as the cultures age (Fig. 3b). For strains A, E, F, I and J, this pigmentation conferred on the medium by the strain is more pronounced. Strain J in particular shows progressive darkening, but rapidly blackens the entire bottom of the dish when it is full. Strain C stands out the most for its strong brown pigmentation seen from both the front and the bottom.

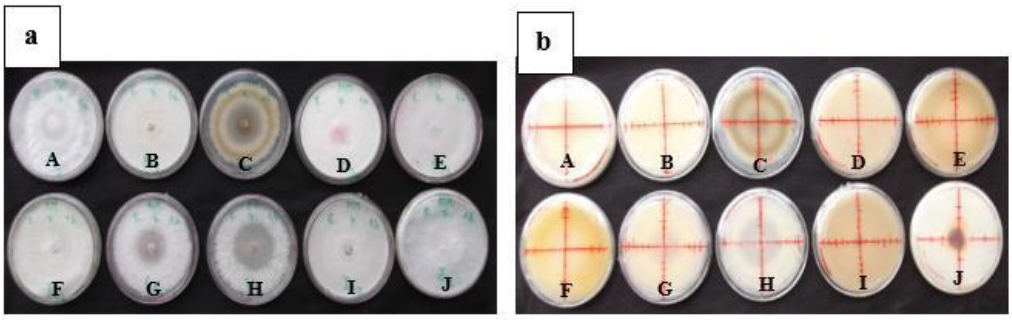

Fig. 3 : Morphological appearance of 10 Foe isolates grown on MM (a)= top view; (b) = bottom view.

Radial growth of isolates

Analysis of the results shows that there are highly significant differences in the radial growth of the isolates studied (p = 0.0001). The fastest-growing strain filled the Petri dish after 4 days, i.e. 8.12 cm, whereas the slowest-growing strain filled the dish on day 10. This means that the best time to compare the diameters of the isolates is day 4.

Statistical analysis of the mean diameters observed on day 4 reveals 5 statistically homogeneous groups (Table 2). The behaviour of the isolates varied from low growth (group e) to high growth (group a). Isolate F showed rapid and significantly different growth (8.12 cm) compared with the other isolates. It was followed by isolates B (6.56 cm), A (5.94 cm) and J (5.35 cm). In contrast, isolate I had the lowest growth rate (3.98 cm) and was also significantly different from the other isolates (H, E, G and D), which had intermediate values. Isolate C was statistically significantly different from isolates J, A and B. Isolates H, E, G and D were also statistically different from isolates A and B (Table 3).

Table 3 : Mean diameter of Foe isolates, 4 days after plating on MM medium

Isolates	mean diameter (cm)
F	8,12 a
B	6,56 b
A	5,94 bc
J	5,35 dc
D	4,77 de
G	4,58 de
E	4,50 de
H	4,26 de
C	4,02 e
I	3,98 e

Means followed by the same letter are not significantly different according to the Duncan test at the 5% threshold.

Sporulation of different isolates

Spores counted from four-day-old isolates on liquid medium showed that, with the exception of strains B and J,

all other strains sporulated. Furthermore, the concentration of spores varied significantly from one strain to another. Strains C and H were the most abundant sporulators, with average sporulation of $126.5x10^5$ spores/ml and $112.25x10^5$ spores/ml respectively. They were followed by strains G, I and F, which respectively had $45.1x10^5$, $39.75x10^5$ and $31.75x10^5$ spores/ml, while strains A, D and E sporulated weakly, with an average sporulation of less than $22x10^5$ spores/ml (Fig. 4).

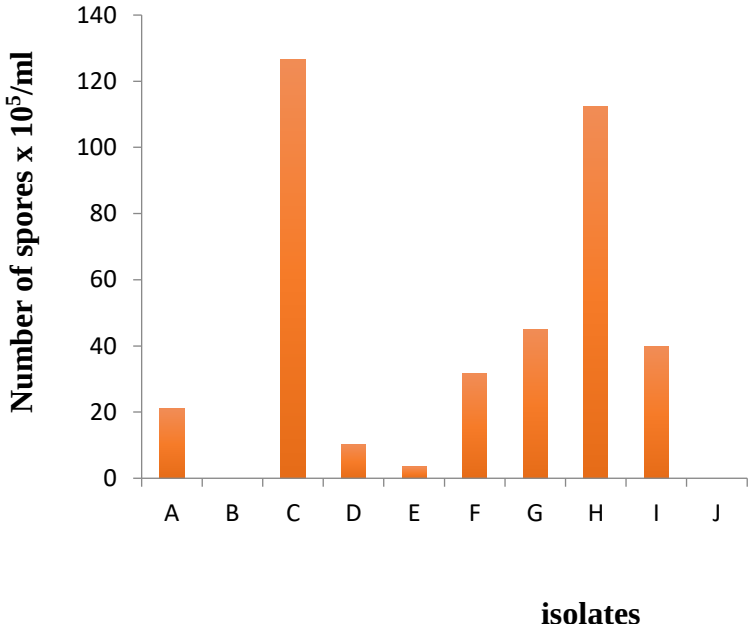

Fig. 4 : Rate of sporulation of differents strains

Types of spores observed

The main organs observed are microconidia, macroconidia and also chlamydospores, which are abundant in highly sporulating isolates (Fig. 5). Microconidia are unicellular, ovoid in shape and smaller than the other organs. Macroconidia are multicellular, septate, sickle-shaped and larger than microconidia. Chlamydospores have the same characteristics as macroconidia, the only difference being that they are larger and thicker..

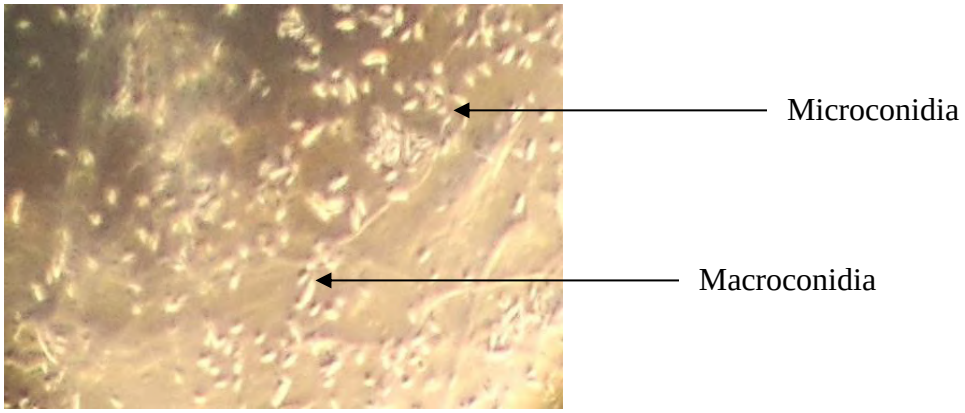

*Fig. 5 **:** Conidia of strain C under the light microscope (400X)*

External symptoms and pathogenicity of isolates

Seedlings inoculated with the strains tested showed secondary symptoms characterised by yellowing (Fig. 6 C), drying and stunting (Fig. 6 B) of the leaves (Fig. 6 A). After 12 weeks of incubation, the data collected showed expression by isolates I, F, E, C, H and G. The other isolates showed no perceptible external manifestations in the plautules in which they were inoculated. Yellowing

appeared to be the earliest symptom to appear. Strains D and G had a cumulative average of 0.32 chlorotic leaves for all the replicates, i.e. around 15 leaves per treatment and per block.

Fig. 6 : seedlings inoculated with isolates

A= without symptoms; **B**= stunted leaf; **C**= yellowed leaves.

After observation of the external symptoms, the data collected showed that there was no significant difference between treatments for the factors number of dry leaves and number of stunted leaves. However, for the number of yellow leaves factor, there was a significant difference (P>0.05) between treatments E and I compared with J (table 4).

Table 4 : external symptoms of plants (number of yellow leaves)

Isolates	Mean
J	1.660 b
H	1.380 ab
G	1.360 ab
C	1.300 ab
D	1.180 ab
B	1.160 ab
A	1.120 ab
F	1.080 ab
E	1.020 a
I	0.940 a

V. DISCUSSION

The pathogenicity of ten isolates was assessed in order to develop tolerance tests for Fusarium wilt for seed production. The morphological characteristics (colony appearance, radial growth, sporulation) and aggressiveness of the isolates on susceptible oil palm seedlings were observed..

The morphological parameters showed a variation in the pigmentation of the isolates, which increased with ageing. The colours observed were white, yellow, brown and pink respectively. Similarly, the appearance of the mycelium varied from thick to thin or short. This behaviour is consistent with that obtained by Tengoua (2003) and Dossa, (1993) who states that in *Fusarium oxysporum*, the morphology of the thallus is subject to strong variations under the influence of environmental factors (temperature, light). As a result, the morphological appearance of the mycelia is not an important criterion for identifying them. In fact, with the exception of isolate C, all the other isolates have a cottony white aerial mycelium (Tengoua and Bakoume, 2008).

The radial growth rate on day 4 after culturing enabled the different isolates to be classified into 5 statistically homogeneous groups. Isolate F has showed the highest radial growth (8.12cm) while isolate I showed the lowest (3.98cm). This variability in the behaviour of the isolates

Correlations between sporulation, radial growth and external symptoms

The negative correlation coefficient (-0.002) shows a weak negative and significant correlation (p = 0.996) between the number of spores and the number of stunted leaves. The same result applies to the number of dry and yellow leaves. Similarly, there was a weak negative and significant correlation between the radial growth of isolates and the number of yellow and dry leaves. On the other hand, there was a weak positive correlation between the number of stunted leaves and the radial growth of the strains (Table 5). The only external parameter that showed a positive correlation, albeit a very weak one, was leaf stunting, which was correlated with the radial growth of the isolates..

Table 5 : Pearson correlation between spore number, radial growth of isolates and external symptoms of seedlings

		NS	D	YL	SL	SL
NS	r	1	-0.445	-0.249	-0.285	-0.002
	P		0.198	0.487	0.424	0.996
D	r	-0.445	1	-0.133	-0.189	0.109
	P	0.198		0.713	0.601	0.764

NS= Number of Spores; **D**= Diameter; **YL**= Yellow Leaves ;**FR**= Stunted leaves; **r**= coefficient of corrélation ; **P**= Probability.

can be explained both by the influence of genotypic and environmental factors such as light, temperature and humidity (Ntsomboh-Ntsefong et al 2015). The great variability observed in the radial growth rate of the different isolates shows that this parameter would be of interest for characterising these isolates. Radial growth can therefore be used for primary classification of the different isolates. (Renard and Revise, 1986)

Sporulation of isolates showed a significant difference between some treatments. However, all isolates sporulated with the exception of isolates B and J. This may be explained by the early spore count carried out on isolates that were only 4 days old. In fact, Kablan et al 2016 have shown that, the start of sporulation of the strains may not be at the same age for all strains. Furthermore, this absence may mean that the strains in question have lost their sporulation vigour as a result of permanent replanting during storage (Lepoivre, 2003). Isolates A, D and E showed a low sporulation rate, unlike isolates C and H, which showed abundant sporulation. This low sporulation rate is a limitation for the study of the pathogenicity of these isolates, as the spores are used as the infectious organ in the evaluation of pathogenicity.

Isolate J was the most aggressive, causing yellowing in a large number of seedlings. In contrast, isolates E and I were the least aggressive. It thus appears that all the strains tested are pathogenic to oil palm, but to varying degrees. The low pathogenicity may be due to successive subculturing of isolates in culture media. (Gbongue et al., 2012). Similarly, the use of 5-month-old seedlings may be sufficient to explain the delay in the edifying pronunciation of external symptoms, and hence the absence of any significant difference between treatments (Asssohoun et al., 2016).

The isolates with the greatest pathogenicity are not necessarily those that grow fastest in vitro, nor those that produce the most spores. This may explain the weak correlations obtained between the level of aggressiveness and morphological parameters (Flood, 2006). Nevertheless, a positive correlation was found between the number of stunted leaves and the radial growth of isolates. This means that strains with good radial growth can induce leaf stunting at an early stage.

At this stage of the trial, it is therefore difficult, if not impossible, to give an opinion on the aggressiveness of the strains. Notwithstanding this setback, an overview was given of the pathogenicity of the strains based on the isolates that showed external symptoms in advance. These are strains C, E, F, G, H, I and J, which, according to the observations made, are highly pathogenic. Once the external symptoms have stabilised, the strains can be classified according to their aggressiveness.

VI. CONCLUSION

The aim of this study was to classify ten isolates of *Fusarium oxysporum* f. sp. Elaeidis from the palm groves of the Cameroon Development Corporation (South-West Cameroon) according to their pathogenicity.

Isolate J was the most aggressive. In contrast, isolates E and I were the least aggressive.

The pathogenicity test can therefore be used to determine which varieties are resistant to fusarium wilt of oil palm.

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