Int.J.Curr.Microbiol.App.Sci (2022) *11*(12): 216-229

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 11 Number 12 (2022)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2022.1112.023

Pathogenic, Morphological and Molecular Diversity of *Fusarium oxysporum* f sp *elaeidis* Isolates, Causal Agent of Fusarium Wilt of Oil Palm in Côte d'Ivoire

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Keywords

Diversity, PCR-ITS-RFLP, *Fusarium oxysporum* f.sp. *elaeidis*, oil palm, Côte d'Ivoire

Article Info

Received:
10 November 2022
Accepted:
05 December 2022
Available Online:
10 December 2022

A B S T R A C T

The main means of controlling Fusarium wilt of oil palm is the use of tolerant hybrids. The objective of this study was to show the pathogenic, morphological and molecular differences of Fusarium oxysporum f sp elaeidis (Foe) isolates from Ivory Coast. A total of 13 *Fusarium oxysporum* (Fo) strains were isolated from fusarium-damaged oil palm organs from *Héania*, *La Mé*, *Anguédedou*, *Dabou* and *Grand-Bereby*(SOGB) localities. Fo isolates were subjected to pathogenicity tests, morphological, and morphometric characterization, molecular diversity (PCR-ITS-RFLP) and DNA sequencing. Results showed that six strains are pathogenic to oil palm. Isolates from Anguededou (A10), Dabou (D2-6) and La Mé (L60) were more aggressive, with incubation time ranging from five to six weeks, while those from Héania (E69), SOGB (So-19) and Mono 179 strains recorded from 8 to 20 weeks incubation time and showed less aggressive. Morphometric characterization showed that length of microconidia and macroconidia (two partitions) of Foe are shorter than Fo. For molecular level, electrophoretic profiles were different from one enzyme to another and identical for the same enzyme. The high aggressive strains obtained in this study can be use to screen hybrids varieties.

Introduction

Oil palm is a tropical perennial plant grown for its fruit (Demol *et al.*, 2002). Palm oil and palm kernel oil are extracted from its fruits for food and industrial use (Monde *et al.*, 2008; Jacquemard, 2012). Palm oil is also used as a biofuel. It was the most consumed vegetable oil in the world (FAO, 2010). Côte d'Ivoire is the second largest producer after Nigeria in Africa. However, it is the largest

exporter of palm oil in Africa. It ranks 5th in the world with 450,000 tons of raw oil palm per year as annual production (Anonymous, 2016). For area occupied by palm plantations, 140,000 ha belong to small producers (village plantations) and 230,200 ha belong to industrial plantations. Côte d'Ivoire is far behind Indonesia (3.7 million hectares) and Malaysia (3.5 million hectares) (Carrere, 2013). To increase oil palm production, the Côte d'Ivoire govenment decided to expand the area of land cultivated, fight against insects and disease pests of oil palm. Fusarium wilt of oil palm is afungal-based disease which causes the most damage in many African countries (Durand *et al.,* 2002; Rusli *et al.,* 2013). The pathogen of this tracheomycosis is a telluric fungus *Fusarium oxysporum* f. sp. *elaeidis* (Foe) (Ascomycetes) specific to oil palm (Diabaté *et al.,* 2013). The fungusis inserted into the plant through root wounds and its aggression enzymes. The pathogen crosses the epidermal barriers to migrate along the xylem intra and intercellularly to the pseudobulb, this action helps in the accumulation of gums into xylem vessels. These gums can completely obstruct light of vessels and completely block the flow of sap, often leading to the rapid decline of affected palms (Flood, 2006 ; Tengoua and Bagkoumé, 2008). In some cases, this has lead to the death of more than 70% of oil palms plantations (Cooper, 2011; Ntsomboh *et al.,* 2012). In Côte d'Ivoire, this desease is present in all oil palm production areas, except Iboké locality (Gogbé *et al.,* 2016). The control method used against this disease is mainly the implementation of resistant genetic hybrids, which has resulted in ther significantly reduction of infection. In addition, hybrids continue to be bred in order to increase oil palms production and improve fusarium desease tolerance. Obtaining such palms requires genetic crossing to obtain hybrids and also make selection through pathogenicity tests in green house and *ex-situ*. The assessement of pathgen tolerance of hybrid oil palm varieties must be done by using most aggressive strains of Foe. The pathogenic, morphological and genetic diversity of the Foe strains of Côte d'Ivoire has not yet been studied. However, studies of Mouyna *et al.,* (1996) showed that strains from Côte d'Ivoire were different from those of other African sub-regions. So, if the fungus presents a genetic diversity divergent from one country to another, it is very likely that it is the same for the different traditional oil palm growing areas in Côte d'Ivoire. It is therefore necessary to characterize the Ivorian pathogenic strains of Foe in order to use them in breeding programs for hybrids tolerant to Fusarium head blight in oil palm.

Materials and Methods

Vegetal material

Plant material consisted of healthy germinated nuts of susceptible oil palm cultivar C1001 from cross breeding ‘’D115D x L2T AF’’ of CNRA research center (La Mé). These seeds were sown in nursery bags and put in a greenhouse. Three months later the seedling plants were used for pathogenicity tests (Figure 1).

Fungal material

The fungal strains of *Fusarium oxysporum* used in this study were isolated from rachis, stipe and roots samples. The different samples have been collected in Éhania, La Mé, Anguédédou, Dabou, and Grand Béréby oil plantation. Mono 179 strain from CNRA plant pathology laboratory was used as control fungal strain during the pathogenicity test.

Isolation, identification and purification of *Fusarium oxysporum* isolates

Infected rachis, stipe and root samples collected were used for fungal isolation. Five samples from each organ types (roots, rachis and stipe) were disinfected by soaking in 70% alcohol for 1 min. Before desinfection, brown fibers of samples were removed with a knife and seeded into Petri dishes containing MM medium and incubated at 28°C in oven. Seven days later, fungal colonies developed were subcultured separately on fresh MM medium. They were discriminated and classified as Fusarium oxysporum or not on the basis of macroscopic and

microscopic characters by using identification keys of Tivoli (1988) and Barnett *et al.,* (2000). The purification was done according to the method of Henni *et al.,* (1994)

Pathogenicity test of 13*Fusarium oxysporum* isolates

This test was carried out to select *Fusarium oxysporum* pathogenic isolates of oil palm to estimate each aggressiveness level. Three-month-old seedlings in bags, were arranged in a completely randomized block, spaced 1 m, with three replicates. For each 13 Fusarium oxysporum and control isolates, 10 seedlings were inoculated per replicate corresponding to 30 seedlings per isolate.

Inoculation of young oil palms plants with *Fusarium oxysporum* inocula

The inocula of 13 *Fusarium oxysporum* isolates were prepared, following the method of Gbongué *et al.,* (2012). Scratch inoculation method of Asssohoun *et al.,* (2016) was used to test pathogenicity. It consisted of wounding roots by scratching with a piece of sterile wood.

Next, 20 ml ($8,10^6$ spores) of pathogen inoculum, were inoculated into the wounded roots and covered with sterile soil. On control plants, after wounding and rinsing, the roots received 20 ml of tap water before being covered with soil.

Determination of pathogenicity parameters of *Fusarium oxysporum* isolates

Estimation of symptoms

After inoculation, external symptoms caused by *Fusarium oxysporum* pathogenic isolates were recorded weekly over a five month period. Seedlings were removed, rinsed with tap water, and pseudobulbs were cut longitudinally to watch all internal symptoms characteristic of Fusarium disease. The data collected concern incubation latency period of each isolate, disease gravity index, disease severity index and rate of fusarium plants (FP) were determined.

Latency time of the different isolates

The latency period, expressed in weeks, is period between the inoculation of the plant with the pathogen and the appearance of the first disease symptoms.

Disease severity index (DSI)

The disease severity index induced by each pathogen isolate was calculated by leaf symptoms observed on each plant (Beye & Lafay, 1985). Observationdone at 4, 8, 12, 16 and 20 weeks later inoculation according to modified formula of Besri *et al.,* (1984):

$$DSI\ (\%) = \frac{NYL + NSL + NPL + NDL}{NTF} \times 100$$

DSI (%): Disease Severity Index; NYL: Number of Yellowed Leaves; NSL: Number of Stunted Leaves; NPL: Number of Punctured Leaves; NDL: Number of Desiccated Leaves; NTL: Total Number of Leaves on Plant.

Average disease severity

For each isolate and symptoms observed, plants were divided into six classes according to scoring scale (Couteaudier *et al.,* 1985). This scale was modified and adapted to our plant material (oil palm):0 : no symptoms; 1: yellowing or perforation of one leaf ; 2: yellowing or perforation on two leaves; 3: yellowing, perforationor stunting on more than two leaves; 4: yellowing, perforation, stunting, and drying of more than two leaves; 5: dead plant. The mean severity (SM) was determined according to Yao *et al.,* (2017) formula:

$$SM = \sum 05\ (NPn \times VnS) \times NTP.10^{-1}$$

SM: Mean severity of Fusarium head blight; NPn: Number of plants with a given severity score; VnS:

Value of that Severity score; NTP: Total number of plants observed.

Rate of *Fusarium wilt* Plants

Rate of Fusarium wilt Plants (RFP) is the number of plants that developeddisease symptoms per total number of plants for each Foe isolate. For each pathogen isolate, rate of fusarium wilt plants (RFP) was determined after 5 months of cultivation, according to the following formula:

RFP (%) = NPF/NTP × 100

RFP = Rate of Fusarium wilt Plants ; NFP = Number of Fusarium Plants ;TNP= Total Number of Plants

Study of the molecular diversity of Foe

DNA extraction of *Foe* isolates

DNA from oil palm *Fusarium oxysporum* pathogenic isolates was extracted using the modified method of Doyle and Doyle (1990). The concentration and quality of extracted DNA were analyzed on electrophoresis gel and Nanodrop and the isolated DNA was stored at -20°C.

Amplification of the ITS region of ribosomal DNA

The universal primer pair ITS1 and ITS4 was used to perform PCR (White *et al.*, 1990). Each amplification reaction was performed in a total reaction volume of 25µl containing 5 ng of DNA.

PCR reactions were performed in a thermal cycler according to thefollowing program : 1 cycle of initial denaturation at 94°C for 5 min, followed by 35 cycles each consisting of denaturation at 94°C for 30 s, hybridization at 55°C for 30 s, elongation at 72°C during 30 s, and a final elongation at 72°C for 10 min. The amplification products were developed on agarose gel (1.5%) and visualized using a transilluminator.

Study of amplified region diversity by PCR-RFLP

PCR-ITS-RFLP is aamplicons digestion with restriction enzymes. Three restriction enzymes were used (MSPI, MSEI and AluI). Each digestion reaction was performed in 15 µl of reactionnal volume with 5 ng as DNA concentrationin PCR product.

Tubes containing the prepared reaction medium were put in water bath at 37°C during 30 minutes. Migration was performed on agarose gel (2.5 %) and visualized under UV light at 360 nm.

Sequencing and Phylogenetic Analysis

PCR products were sequenced in forward direction withITS1 universal primer. Sequencing has been done by INQABA BIOTHEC in South Africa. The amplified fragments were compared with ITS sequences of databases, using the Basic Local Alignment Search Tool (BLAST) (web ref: http:// www.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1990).

Phylogenetic study lead to phylogenetic tree construction usedfor fungi identification, by analyzing their homology with the sequences of databases. Phylogenetic analyses were realized with MEGA 6 software (Tamura *et al.*, 2007) and phylogenetic tree constructed by neighbor-joining method (Saitou and Nei, 1987).

Statistical analysis

Statistica 7.1 software was used for all variance analyses (ANOVA). Analysis of variance (ANOVA) with one or two classification criteria was done at 5% threshold to determine isolates pathogenicity, mycelial growth on culture media and morphometric measurements. When a significant difference was detected ($p<0.05$), ANOVA was completed by Fisher's Least Significant Difference (LSD) test for homogeneous groups constitution.

Results and Discussion

Fusarium oxysporum isolates pathogenicity

Disease symptoms presence

Oil palm seedlings, inoculated with *F. oxysporum* isolates at pre-nursery stage, developed external and internal Fusarium disease symptoms. Among 13 isolates tested, six isolates including five new strains (A10, L60, E69, D2-6 and So-19) and the reference strain Mono 179 caused disease on oil palm plant.

External symptoms of disease were manifested by leaf midrib perforation, yellowing, drying, and stunting of leaves or plant (Figure 2). Internal symptoms, observed five months after fungal inoculation, were manifested by browning of pseudobulb driving vessel. However, control seedlings (not inoculated), showed noFoe disease symptoms.

Pathogen latency

The results (Table 1) showed that no seedlings expressed *Fusarium*wilt during the first four weeks (1 month) after inoculation with different isolates. First external symptoms of disease were observed on plants inoculated wiht isolates A10 and D2-6 with 40% and 20%, respectively, five weeks after inoculation. Seedlings inoculated with isolate L60, 40% expressed the disease symtôms sixweeks after inoculation. According to isolate E69 and reference Mono 179, thefirst disease symptoms appeared eight weeks later and reach 40% of the fusaried seedlings. 20 weeks later, isolate So-19 were expressed first disease symptoms on 20 % of seedlings.

Fusarium wilt severity index

The severity index of induced Fusarium wilt varied significantly ($P< 0.001$) according to fungal isolates tested (Table 2). The ppds test classified isolates into four groups or aggressiveness levels. The highest aggressiveness level (92%) was caused by isolate L60; the second (55%) caused by isolate A10, the third (35.94%) by isolate D2-6, and the fourth level (12.5 ; 9.72 and 6.67%), by isolates E69, reference Mono 179 and So-19, respectively.

Disease severity average

The mean severities of Fusarium wilt varied significantly ($P< 0.001$) from 1,6 to 3.8 depending on the strain of *F. oxysporum* used for oil palm seedlings inoculation (Figure 2). On disease-susceptible oil palm seedlings, isolates L60 (3.8), A10 (3.4) and D2-6 (3.2) induced the highest severity score. Low severity average 2 ; 2 and 1.6 were scored by Mono 179, So-19 and E69 respectively.

Rate of fusarium-damaged plants

There were no significant difference rates in fungal isolates ($P= 0.35$). However, we noted that isolate L60 caused disease in all inoculated seedlings (100%) and isolate D2-6 expressed disease symptômsin 80% of inoculated seedlings. Isolates A10 and E69 caused disease in 60% of inoculated seedlings. Isolates So-19 and Mono 179 caused disease in 40% of inoculated seedlings.

Morphological diversity of pathogenic strains (Foe) of oil palm

Cultural characteristics

Fourteen days after seeding on MM medium, colonies from *F. oxysporum* f. sp. *elaeidis* strains were grouped into four types according to color (Figure 3). The first type, represented by the D2-6 strain, produces short mucous mycelia with a translucent coloration. The second type, which includes A10 and Mono 179 strains, develops white-cotton mycelia with a light orange reverse. The third type is represented by the So-19 strain with a pink-salmon colored fluffy colony at the growth front with a white inoculation point and a purple background on the reverse side of the Petri dish. The fourth type (L60 and E69) is characterized by a white cottony mycelium with a white reverse.

Microscopic characteristics

Microscopically (Figure 3) strains showed a hyaline branched and septate mycelium. Two weeks after culture, strains produced monophialides bearing ovoid or elliptical, mostly unicellular microconidia

and fusoid macroconidia with three or at most four septa.

Morphometric characteristics

Microscopic observations revealed that Foe microconidia are statistically different ($P < 0.001$) as well as macroconidia (Table 3). The length of microconidia, ranging from 2.8 to 3.95 µm. was used to classify the strains into two groups. Microconidia of the first group (L60, A10 and E69) measure 3.95; 3.65 and 3.65 µm in length, respectively. The second group (D2-6, Mono 179 and So-19) measure 2.9, 2.8 and 3.05 µm respectively.

According to macroconidia length, strains were divided into five groups. The reference strain Mono 179 (13 µm) is placed in group I. Group II is represented by strains A10 and E69 with macroconidia lengths of 12.67 and 12.57 µm respectively. Group III consists of strain L60 with 12.13 µm length and group IV, strain D2-6 with 11.53 µm length. Group V is represented by the strain So-19 whose macroconidia are 11.16 µm long.

Molecular diversity of oil palm *Fusarium oxysporum* pathogenic strains (Foe)

PCR amplification of all Foe strains with ITS1/ITS4 primer pair generated single DNA fragment with 550 base pairs (Figure 4A). PCR products from strains were digested with following restriction enzymes: *MSEI*, *MSPI*, and *AluI*. All enzymes digested corresponding rDNA region to Foe strains. Each restriction enzymes *MSPI* and *MSEI* generated two bands for all Foe strains (Figure 4B and 4C). AboutAluI enzyme, digestion of PCR product generated three individual fragments (Figure 4D). The total size of the restriction fragments obtained with the *MSPI* enzyme is equivalent to total size of amplicons. On other hand, with *MSEI* enzyme, the total size of restriction fragments is less than size of fragment obtained during PCR process. With *AluI* enzyme, the total size of restriction fragments is greater than amplicons total size.

Table.1 Latency times of oil palm pathogen isolates.

Isolates	Time of appearance of the first symptoms (Weeks)	Rate of diseased seedlings (%)
A10	5	40
D2-6	5	20
L60	6	40
E69	8	40
Mono 179	8	40
So-19	20	20

Table.2 Severity index of Fusarium wilt caused by *Fusarium oxysporum* isolates pathogenic to oil palm

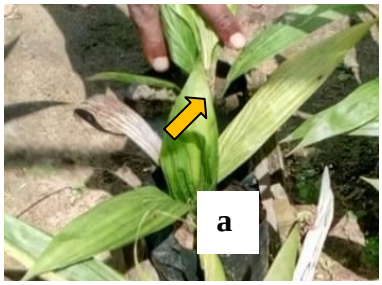
Isolats	Indice de gravité de la maladie (%)
L60	92,00 ± 8,00 a
A10	55,00 ± 21,13 ab
D2-6	35,94 ± 13,47 bc
E69	12,5 ± 6,84 c
Mono 179	9,72 ± 7,27 c
So-19	6,67 ± 6,66c
F	8,027
P	< 0,001

The values followed by the same letter in the same column are statistically identical according to Fisher's or Seuille's LSD test. 5 %. F = Fisher's F, P = Probability.

Fig.1 Different external and internal symptoms of Fusarium head blight of oil palm in the pre-nursery after inoculation with Foe.

Non-inoculated seedling

Leaf yellowing

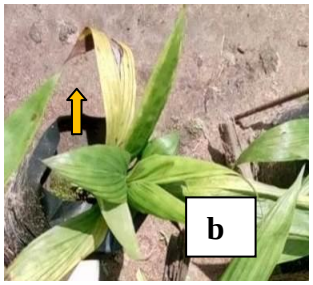


Stunting without yellowing

Yellowing and desiccation

Leaf puncture

Leaves yellowing

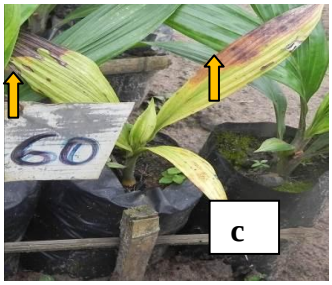


Yellowing and stunting

Yellowing and desiccation

Leaf puncture

Leaves yellowing



Yellowing and stunting

Browned vessels inside the infected pseudobulb on the right

Table.3 Average length of microconidia and two-walled macroconidia of *Fusarium oxysporum* f. sp. *elaeidis* strains five days after culture on Nash medium

Strains	Microconidia (µm)	Macroconidia (µm)
L60	3,95 ± 0,29a	12,13 ± 0,39bc
A10	3,65 ± 0,23a	12,67 ± 0,27ab
E69	3,65 ± 0,20a	12,57 ± 0,27ab
D2-6	2,9 ± 0,17b	11,53 ± 0,17cd
Mono 179	2,8 ± 0,15b	13 ± 0,45a
So-19	3,05 ± 0,13b	11,16 ± 0,14d
F	5,273	5,42
P	< 0,001	< 0,001

Numbers followed by the same letters in the same column are statistically identical according to the LSD test at the 5% threshold ; F = Fisher's F, P = Probability ; L60, An, D2-6, E69, Mono and So-19 are the strains of F. oxysporum pathogenic to oil palm.

Table.4 Comparison of ITS1 sequences of oil palm pathogenic *Fusarium oxysporum* strains with ITS1 sequences of GenBank fungal strains

Pathogenic Strains	Closest organism with their accession number after comparison by Blast	Homology Proportion (%)
E69	*Fusarium oxysporum* MH790278.1	99
A10	*Fusarium oxysporum* GU371875.1	99
L60	*Fusarium oxysporum* KX421425.1	100
So-19	*Fusarium oxysporum* KU170716.1	100
Mono 179 reference	*Fusarium oxysporum KU324799.1*	100

Fig.2 *Fusarium wilt* severity average caused by *Fusarium oxysporum* isolates pathogenic on oil palm 5 months after inoculation. Histograms with the same letter are statistically identical (5% LSD test) and bars correspond to standard errors

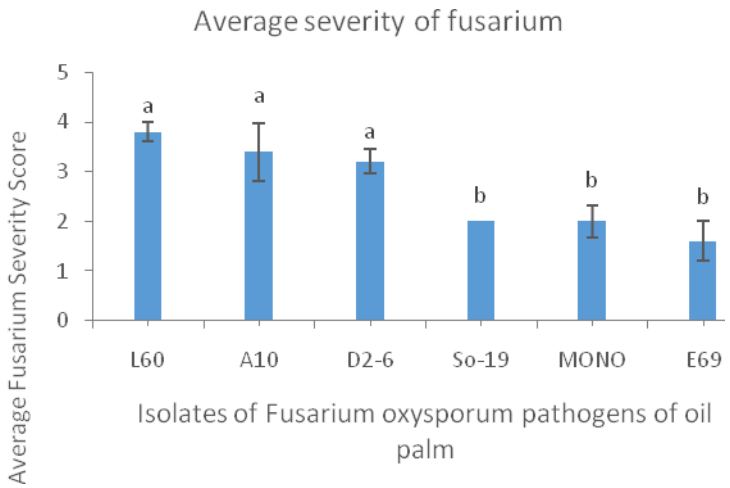


Fig.3 Cultural characteristics of *Fusarium oxysporum* f sp. *elaeidis* strains after 14 days of culture on MM medium. a: microconidia without septum; b: septate microconidia; c: macroconidia; d: mycelium; e: monophyalid (Grossis x 400)

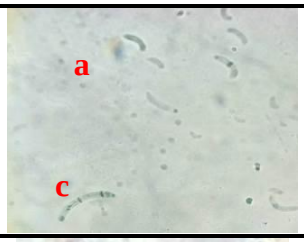
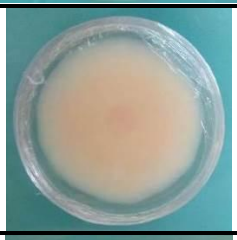
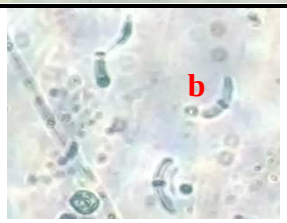
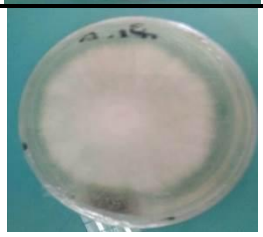
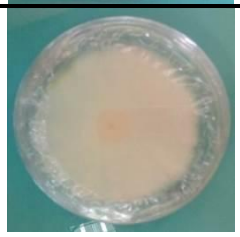
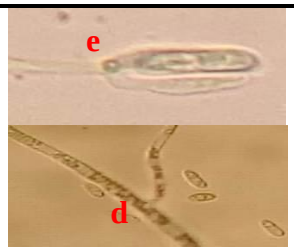
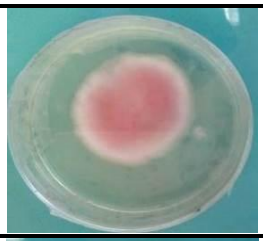
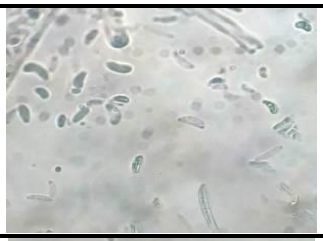
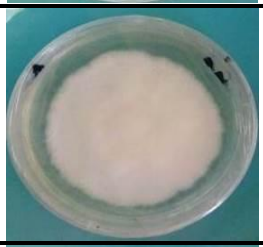
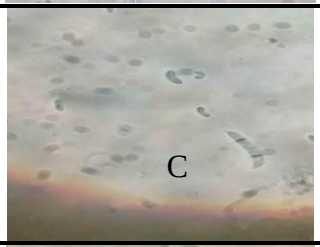
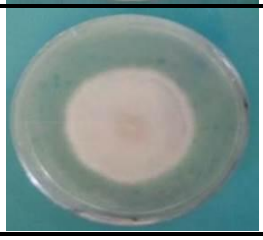
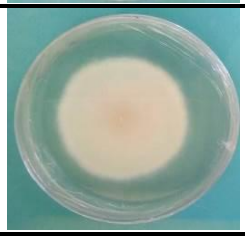
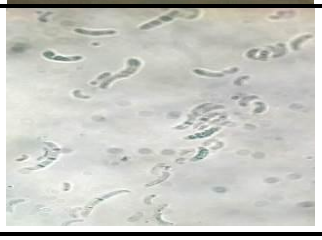
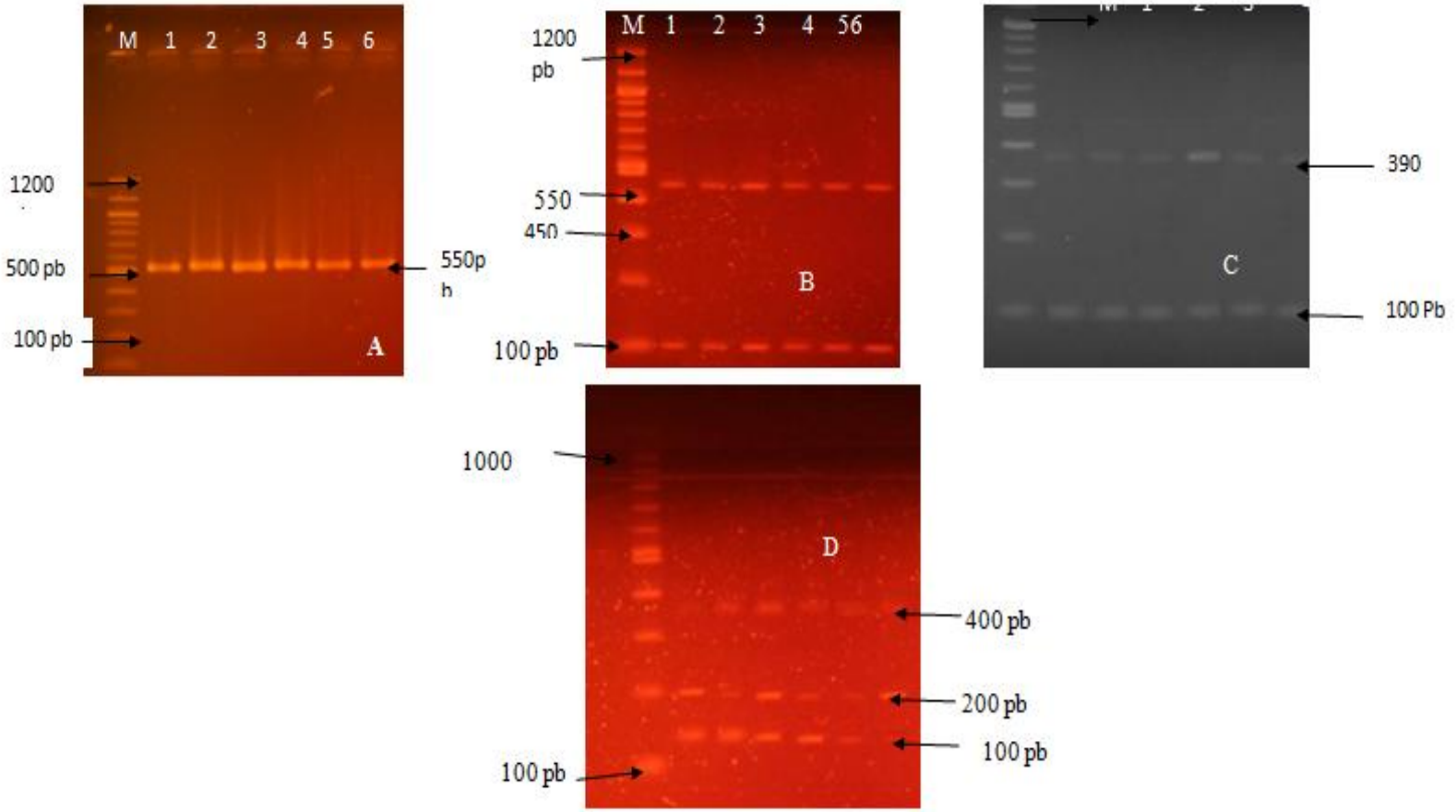
Foe Isolates	Upside of the Petri dish	Backside of the Petri dish	Microscopic observation
L60			
A10			
Mono 179			
So-19			
L60			
E69			

Fig.4 Electrophoresis profile (A) :of rRNA amplicons from the six oil palm pathogenic *Fusariumoxysporum* f. sp. elaeidis isolates(B) :of the products of the digestion of amplicons with the MSPI restriction enzyme (C) : of the products of the digestion of amplicons with the MSE restriction enzyme (D) :of the products of the digestion of amplicons with the Alu restriction enzyme:



M : molecular weight marker in 100 PB scale (Invitrogen); wells 1-6 are the amplicons ; 2.5% agarose gel; bp: base pair

Ribosomal DNA ITS region amplification

rRNA ITS region encoding gène PCR amplification was achieved for all isolates (06). Primers used for all isolates amplification found a fragment with approximately 550 bp as size (Figure 4A). In order to identify isolated fungi, sequencing of the isolates, a representative sample from each collected area, was carried out. Sequences obtained were compared with ITS sequences from databases using the BLAST (Basic Local Alignment Search Tool) software (Altschul *et al.*, 1990), (Web ref : http:// www.ncbi.nlm.nih.gov/Blast.cgi). The accession numbers of the library sequences that were very close to our amplified ITS sequences were recorded in Table 4. These results allowed us to build a phylogenetic tree of isolated fungi population.

The phylogenetic analysis showed that six fungi identified belong to genus *Fusarium* with the species *oxysporum* (Figure 5). The pathogenicity test carried out with the 13 isolates of *Fusarium oxysporum* (Fo) showed six of them caused symptoms similar to those induced by pathogenic strains. The six isolates that led to infections in oil palm seedlings could be identified as strains of *Fusarium oxysporum* f. sp. *elaeidis* (Foe). Indeed, within the Fo species there are several specialised forms recognised on the basis on selective pathogenicity. Each specialised form of Fo has a susceptible specific host (Flood, 2006; Rusli *et al.*, 2013). Thus, in oil palm, the pathogen able to cause Fusarium wilt symptoms is *Fusarium oxysporum* f. sp. *elaeidis* (Foe). Isolates which didn't cause disease symptoms could be saprophytic strains in host tissues reported by Lechappé *et al.*, (1988) about beans. In fusarium-damaged plants, analysis of internal symptoms confirmed external symptoms. Various symptoms shown progressive degradation internal tissues, which are exteriorized on leaves.

Indeed, as the fungus develops (mycelium and conidia), physical defence reaction of plant is thylls and gums formation in the xylem vessels to block fungus ascent into the stipe, obstructing vessels and completely blocking sap flow (Beckman, 1987). The direct consequence of the reduction of hydromineral supply to leaves is the inhibition of chlorophyll synthesis, which causes the leaves tobecome yellow and dry more or less quickly (Flood, 2006 ; Tengoua and Bagkoumé, 2008).

The development of both external and internal symptoms, is a pathogenic symptom which is specific to *F. oxysporum* isolates (Renard and Ravisé, 1986; Beckman, 1987). The pathogenicity of particular strains was assessed basedon four pathogenici criteria : incubation period, average disease severity index, average disease severity and fusarium wilt plant rate. The incubation period, corresponds to elapsed time between infection and the first appearance of symptoms (Lepoivre, 2003). And, its depends onaction spontaneity or deadline with which pathogen acts. In this present study, this duration wasbetween five and 20 weeks. This result demonstrates pathogenic diversity within Foe strains. Indeed, the induced disease can be acute when incubation period is short (Semal, 1993). Thus, strains A10, D2-6, L60, E69 and reference Mono 179 could cause Fusarium wilt acutely, whereas strain So-19 would lead to chronic Fusarium wilt in oil palm. Studies by Asssohoun *et al.*, (2016) estimated this latency period to four weeks. However, results obtained in this study with reference strain Mono 179 are in agreement with those of Kablan *et al.*, (2016) who showed that the incubation period of reference strain Mono179, reach eight weeks after inoculation.The severity index of Fusarium wilt on inoculated plants depends to Foe strains. The highest disease indices were obtained with strains L60 (92%), A10 (55%) and D2-6 (35.94%), while strains E69, mono 179 and So-19 were less aggressive with indices below 15%. These results could be explained by ability of those six strains to invade the plant cells. Indeed, aggressive pathogens have many ways to attack their hosts. Their hydrolytic enzymes, such as pectate lyase and laccase, degrade the plant cell wall (Yao *et al.*, 2009). If the plant's response is not fast enough, the pathogen colonises the cell, destroys cell components and inhibits its metabolism. No warning can be given either to neighbouring cells or to those

that are far away. This situation weakens the entire plant, which can be rapidly invaded by the pathogen and thus show symptoms of the disease. The results obtained in this study showed a correlation between aggressiveness of Foe strains and disease severity. The rate of fusarium wilt plants confirms the aggressiveness of the inoculated strains. Fusarium isolates with infection rates below 50% were likely confronted with development of plant defence mechanisms. Proof of Foe isolates severity on oil palm seedlings is death. The time (5 months) allowed for our experiment did not allow us to observe mortalities that manifested themselves as complete desiccation of diseased seedlings. The variability of incubation period of pathogen, disease severity index and average disease severity revealed a pathogenic variability within Foe strains. Morphologically, the fungi show a diversity incoloration and a thallus of variable appearance. Thus, the characteristics of the species are a source of great morphological diversity (Dossa, 1993; Henni *et al.,* 1994) in Foe. Microscopically, in all strains, this result shows that Foe strains have the same microscopic characteristics as fungi of Fusarium genus, which makes it impossible to differentiate them from non-pathogenic *Fusarium oxysporum* strains. The length of microconidia and macroconidia of Foe are shorter than those obtained by Tivoli (1988). Indeed, the work of these researchers revealed that in *F. oxysporum*, microconidia lengths generally vary from 4.5 to 5.5 µm and macroconidia from 13 to 20 µm. The morphometric variability observed confirms morphological diversity within Foe strains.

All 6 strains (L60, A10, E69 ; D2-6, Mono 179 and So-19) grow on all culture media (MM, Nash and PDA) used in this study. However, their behaviour varied depending on culture medium. This difference would be linked to composition (nutritional constituents) of culture media. Similar observations were made by Ntsomboh-Ntsefong *et al.,* (2015) in Foe. The result of PCR corresponds to expected amplicon size with ITS1/ITS4 in Fusarium oxysporum (Fo) as reported by Leong *et al.,* (2009) and Chehri *et al.,* (2011). PCR product digestion with *MSPI*, *MSEI* and *AluI* revealed no genetic diversity among Fo strains regardless of locality. Similar results were obtained by Lee *et al.,* (2000) who showed special forms of Fo were 100% genetically identical. After analysis of restriction fragments of PCR product with the enzymes, only total size of the restriction fragments obtained with *MSPI* was equivalent to the approximate fragment size of PCR. This observation is similar to Leong *et al.,* (2009) and Chehri *et al.,* (2011) who used this enzyme to study intra-specific diversity in Fo special forms. However, digestion of PCR product with *MSEI* enzyme, provided restriction fragments of smaller total size than fragment size of PCR product. This fragment loss could be attributed to difficulties in visualising fragments smaller than 100 bp (Cooke and Duncan, 1997). Other reasons include fragments co-migration with same size on gel (Cooke and Duncan, 1997) or their loss on gel during electrophoresis (Gottlieb *et al.*, 2000). With the AluI enzyme, the total size of the restriction fragments was larger than the PCR fragment size. This could be due to the presence of typical mixed rDNA (Hibbett, 1992) or polymorphisms in the recognition sites (Cooke and Duncan, 1997). Strains sequencing confirmed that pathogen able to infect oil palm is Fo. However, not all strains of Fo are pathogenic to oil palm. According to Renard *et al.,* (1972), F. oxysporum species has a special form, *F. oxysporum* f. sp. *elaeidis* (Foe), which is responsible ofoil palm fusarium disease. In this genetic diversity study using ITS-PCR-RFLP, Foe strains were genetically identical regardless of locality.

Six isolates [Éhania (E69), La Mé (L60), Anguédédou (A10), Dabou (D2-6), Grand Béréby (So-19) and reference Mono 179] were found to be pathogenic to oil palm. These six pathogenic isolates are thought to be strains of *Fusarium oxysporum* f. *sp. elaeidis*. They were classified into two main groups according to their pathogenicity. Group I isolates contain the very aggressive A10, D2-6 and L60 strains with a short incubation time of five to six weeks. Group II isolates (Mono 179, E69 and So-19) showed a longer incubation time (eight to 20 weeks), indicating pathogenic variability among Foe

isolates. These strains show morphological diversity marked by variability in mycelium and its staining. Microscopically, Foe microconidia and macroconidia (with two partitions) are shorter than those of *Fusarium oxysporum*. Molecularly, PCR with the ITS1/ITS4 primer pair produced a single band of 550 base pairs. Digestion of the PCR products with the enzymes MSEI, MSPI and AluI did not reveal any genetic diversity between the strains. Similarly, sequencing of the strains showed that they are all strains of *Fusarium oxysporum*.

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How to cite this article:

Gogbe-Dibi Balé Françoise, Kouadjo Zézé Claude Ghislaine, Coulibaly Klotioloma, Diabate Sékou, Kouadio Ouattara Demby Laetitia Muriel, N'cho Achi Laurent, Dogbo Dénézon Odette. 2022. Pathogenic, Morphological and Molecular Diversity of *Fusarium oxysporum* f sp elaeidis Isolates, Causal Agent of Fusarium Wilt of Oil Palm in Côte d'Ivoire. *Int.J.Curr.Microbiol.App.Sci.* 11(12): 216-229.
doi: https://doi.org/10.20546/ijcmas.2022.1112.023