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ORIGINAL ARTICLE

Molecular phylogeny and pathogenicity of *Fusarium oxysporum* f. sp. *elaeidis* isolates from oil palm plantations in Cameroon

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Abstract

In Cameroon, oil palm (*Elaeis guineensis* Jacq.) is of economic importance. However, it is affected by vascular wilt presumed to be caused by *Fusarium oxysporum* f. sp. *elaeidis* (FOE). Accurate species identification requires molecular-based comparisons. The aim of this work was to molecularly identify Fusarium species associated with diseased oil palms and to determine the pathogenicity of selected isolates. Fungal samples of diseased palms were collected from the canopies and the soil of five oil palm estates of the Cameroon Development Corporation and characterized by sequencing and comparing the translation elongation factor 1a gene. The results revealed the presence of FOE from approximately 80% of the isolates. Cameroonian isolate within FOE clade 1 exhibited the greatest variability grouping with isolates from Suriname, Brazil and Democratic Republic of Congo. Other isolates found in FOE clade 2 formed a unique group which was comprised solely of isolates originating from Cameroon. Twenty-two isolates were chosen for pathogenicity tests. After a short time, 14 isolates were found to be pathogenic to oil palm seedlings. This study revealed the pathogenicity of FOE isolates from Cameroon and demonstrated that FOE in Africa is more diverse than previously reported, including a lineage not previously observed outside of Cameroon. Comparisons between all isolates will ultimately aid to devise appropriate control mechanisms and better pathogen detection methods.

Keywords: oil palm, pathogenicity, phylogenetics, symptomatic, vascular wilt

Introduction

One of the highest oil yielding and important crops in the world is the oil palm, *Elaeis guineensis* Jacq. The fruit of oil palm is utilized to produce crude palm oil and palm kernel oil which is used for diverse purposes. These uses include the production of food, oleochemicals, health supplements, and biofuels as well as pharmaceutical uses. Farmers who cultivate oil palm face a number of challenges including diseases such as basal stem rot, bud rot and vascular wilt which reduce the yield of the oil and affect growth (Ntsomboh-Ntsefong *et al.* 2012, 2015). Vascular wilt seriously damages oil palm in Cameroon and other production areas of Africa (Corley and Tinker 2003; Flood 2006; Tengoua and Bakoumé 2008) and can cause as much as 70% mortality. Fusarium is a fungal genus that is abundant in soils, whole parts of a plant, plant debris and other

organic substrates (Summerell *et al.* 2003). Different control measures such as soil renewal, defense stimulation, potassium (K) fertilizer, cover crops, resistant or tolerant plant material selection and chemical applications have been used to manage vascular wilt disease and improve yields (Ntsomboh-Ntsefong *et al.* 2012).

Serious economic damage occurs in oil palm in Cameroon due to the prevalence of vascular wilt disease (Ntsomboh-Ntsefong *et al.* 2012). In mature oil palm plantations, great economic losses occur because palm trees die 3 to 4 months after symptoms appear; hence vascular wilt disease is lethal in its acute form (Flood 2006). In its chronic form, fruit production is prevented, and growth of the vegetative organs is retarded, weakening the tree and rendering it almost unproductive (Ntsomboh-Ntsefong *et al.* 2012). In Cameroon's Southwest Region, vascular wilt disease is common and it affects small farms and several oil palm estates in commercial plots of the Cameroon Development Corporation (CDC). On other oil palm estates in different regions in Cameroon, there have been no reports of high incidence of vascular wilt disease. The reason for this is because almost all estates in commercial oil palm plantations are located in the southwest and neighboring Littoral regions, which may account for the low disease incidence in other regions of the country. Vascular wilt disease of oil palm has not been investigated in almost 70% of Cameroon's suitable land area available for the cultivation of oil palm. This implies that the pathogen may already be present in the soil before oil palm cultivation takes place. This final observation is strengthened by the fact that the pathogen could have readily spread over the last 10 years throughout Cameroon because of the uncontrolled and intensive movement of oil palm seedlings (Ngando *et al.* 2013).

Morphological characters have been mainly used in Cameroon to study *Fusarium* species on agricultural crops (Ntsomboh-Ntsefong *et al.* 2015). That notwithstanding, limitations exist on the usage of morphological characters alone for *Fusarium* species identification. This is because similar or overlapping ranges of morphological characters exist for many species. Morphological characters, despite their limitations, still play a major role in placing isolates into smaller groups before other identification methods are used (Leslie and Summerell 2006; Hsuan *et al.* 2011). Comparisons of DNA sequence and differences of gene-coding loci support enhanced resolution of *Fusarium* species using morphological identification. The genetic relationship between closely related *Fusarium* species were assessed and differentiated by DNA sequences using phylogenetic analysis (Yli-Matilla *et al.* 2002) and within the *Fusarium oxysporum* species complex (O'Donnell *et al.* 2009). In particular, the DNA sequence encoding a portion of the translation elongation factor 1-α gene (TEF-1α) is widely used for identification of *Fusarium* species and formae speciales within the *F. oxysporum* species complex. GenBank and the more focused and curated FUSARIUM-ID database have sequences of the TEF-1α (Geiser *et al.* 2004). The internal transcribed spacer region (ITS) of the nuclear rRNA is also a useful nucleotide sequence for comparison purposes, as this region evolves the fastest along with the intergenic spacer (IGS) (O'Donnell *et al.* 2009), and often there is variability with a genus among species or among populations (White *et al.* 1990). However, confirmation of *F. oxysporum* formae speciales is still dependent on pathogenicity experiments as many non-pathogenic *F. oxysporum* are present in the environment.

To develop effective quarantine measures and disease control methods and to make resolutions to preserve crops of agricultural importance and other natural resources from pathogenic fungi, identification of the correct species and sub-species is essential (Rossman and Palm-Hernandez 2008). A previous study examining sequence types in the *F. oxysporum* species complex included *F. oxysporum* f. sp. *elaeidis*, but the isolates were from Suriname, Brazil and Zaire (now Democratic Republic of Congo) (O'Donnell *et al.* 2009). Interestingly, there were two sequence types – one associated with the isolates from South America and the second associated with the isolates from Africa (O'Donnell *et al.* 2009). Therefore, the present study aimed to identify, compare, and characterize *F. oxysporum* f. sp. *elaeidis* isolates from different oil palm plantation estates in Cameroon by DNA sequencing, phylogenetic relationships, and pathogenicity testing.

Materials and Methods

Field symptoms of *Fusarium oxysporum* f.sp. *elaeidis* (FOE), fungal isolation and morphology

This study took place on five oil palm estates of the Cameroon Development Corporation consisting of Bota, Musaka, Mondoni, Debundscha and Idenau all in the Southwest Region of Cameroon (Fig. 1). Bota, Debundscha and Idenau are coastal estates while Musaka and Mondoni are inland estates. Symptomatic plants were identified by opportunistic sampling (that is by identification of symptoms and disease lesions on the plant as they were encountered in the field). For comparison purposes, 200 *Fusarium* isolates were recovered from the canopy tissue (rachis) showing symptoms of fusarium wilt and from the soil associated with roots of symptomatic palms (*E. guineensis*). Sampling was done on all of the five oil palm plantation estates of the Cameroon Development Corporation (CDC) in the Southwest Region of Cameroon.

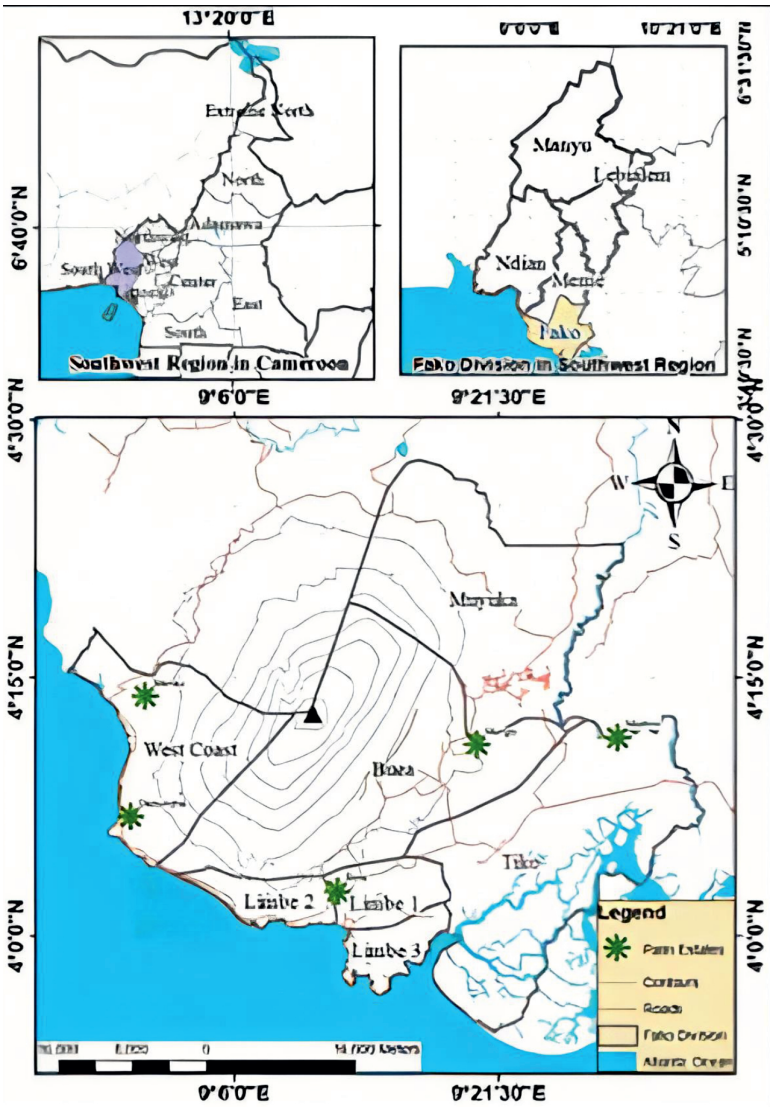

Fig. 1. Sample collection sites within the Cameroon Development Corporation, Cameroon. The Southwest Region of Cameroon. Fako division within the Southwest Region of Cameroon

Isolation and identification of FOE was performed using the following protocol: the rachis of the plants was thoroughly washed under running tap water to remove surface adherents. Subsequently, plant tissue of the affected fronds was selected and cut into segments (0.5 × 0.5 × 0.5 cm) with a pair of sterile scissors and air dried before surface sterilization. These samples were then immersed in 1% sodium hypochlorite (NaClO) for 3 minutes, then in 70% alcohol for 1 minute, and rinsed with three changes of sterile distilled water for 1 minute each. This procedure was aimed at eliminating saprophytes and non-pathogenic microorganisms which were found on the plant tissue surface. Six sections of the surface-sterilized pieces were separately plated onto (PDA; Difco Laboratories, Detroit, MI) and Van Wyk medium (VW) (Leslie and Summerell 2006). Soil samples were cultured by means of broadcasting and subsequently spreading on both media. These plates were then sealed with Parafilm® and incubated at 25°C for 7 days.

Wet mounts were made from the growth at the margins, stained using methylene-blue and observed under the microscope with a 40× objective lens. When a Fusarium identity was indicated, plates were single-spore sub-cultured to obtain pure cultures. New observations were conducted following 7 days of incubation on both media with the aid of keys proposed by Leslie and Summerell (2006). Single-spore isolates were stored at room temperature as mycelial agar plugs in cryogenic vials containing sterile distilled water. Additionally, isolates were frozen in 30% glycerol at –80°C. Variability in color, form, elevation, and colony margin of the single spore isolates was documented on potato dextrose agar (PDA). The frequency of isolation was calculated as: isolates obtained/isolates plated ×100.

DNA extraction, PCR and DNA sequencing

Each single-spore isolate was revived from storage by placing a single agar plug on 1/5 strength potato dextrose agar (1/5 PDA) (Difco Laboratories, Detroit, MI) and grown for 5 days at 28°C. Four 5 mm agar plugs from 1/5 PDA were transferred to a conical flask with 50 ml full strength potato dextrose broth (PDB) and incubated for 6 days at 28°C without shaking. Hyphae were collected by vacuum filtration, flash frozen with liquid nitrogen and lyophilized. DNA was extracted from lyophilized fungal hyphae using the DNeasy® Plant Mini Kit (QIAGEN® Group) according to the manufacturer's instructions and by the CTAB method following the protocol of Möller *et al.* (1992). The obtained DNA pellet, suspended in 30 µl of ddH_2O and the NanoDrop spectrophotometer (Thermo Scientific NanoDrop 2000) at absorbance A260/280, was used to determine the DNA purity and concentration. Ethidium bromide stained on a 1.5% agarose gel run at 7V/cm for 60 min was used to visualize the quality of the DNA. Working stock DNA was diluted to 40 ng · μl^{-1} and stored for further use at 4°C.

All 107 isolates genomic DAN was amplified by PCR using primers EF1 (EF1T): 5`-ATGGGTAAG GAGGACAAGAC-3` and EF2 (EF2T): 5`-GGAAG TACCAGTGATCATGTT-3` (Geiser *et al.* 2004). The translation elongation factor 1α gene (TEF-1α) phylogenetically informative portion was amplified by EF1 and EF2 primers. A Biometra TRIO Analytic Jena thermocycler was used to carry out PCR amplification, hot start Taq PCR premixed in a 25 µl reaction volume containing 40 ng template DNA, 0.18 µM of each primer, 2.5 µM of dNTPs and 15.1 µl Milli-Q water was added. The settings of the thermocycler were as follows: denaturation at 94°C for 1 minute; 34 cycles of denaturing at 94°C for 1 minute, annealing at 53°C for 1 minute, and extension at 72°C for 1 minute; and a final extension at 72°C for 7 minutes. To check the purity of the PCR product, 5 µl of the product was electrophoretically separated on a 1% agarose gel prepared in 0.5X TAE. The gel was run at 7 V · cm^{-1} for 45 min. Ethidium bromide was used in staining the DNA and photographed under UV exposure. Purification of PCR products was performed by ExoSAP-IT (Applied Biosystems) and on spin columns using the Wizard® PCR Preps DNA Purification System (Promega Corp., Madison, WI). Sanger sequencing of both DNA strands was conducted by Eurofins, USA with the same primers used for amplification.

Phylogenetic analysis

Sequences obtained using EF1 and EF2 primers (Geiser *et al.* 2004) were queried against known sequences in GenBank using BLASTn and against the FUSARIUM ID database (Geiser *et al.* 2004), to determine their associated species identity. MEGA version 7 (Kumar *et al.* 2016) was used to carry out a phylogenetic analysis independently for the data sets and a phylogram was produced. MUSCLE using the default parameters was used to perform a multiple sequence alignment (Edgar 2004). The heuristic search option Tree-Bisection-Reconnection (TBR) branch swapping algorithm with 1000 random addition sequences with 1000 bootstrap replications was used to construct a phylogenetic tree using the neighbor-joining method. Evolutionary relationships between isolates were inferred by constructing unrooted TEF-1α based phylogenetic trees (Kinene *et al.* 2016). Also, a phylogenetic tree was constructed between the *F. oxysporum* f. sp. *elaeidis* from our isolates with other FOE from GenBank and culture collection centers. Another phylogram was constructed between *F. oxysporum* f. sp. *elaeidis* and other *F. oxysporum* f. spp. Isolates used in producing the phylograms are shown in Tables 1, 2 and 3. The sequences of Aduse-Fosu and Dickinson (2019) could not be used in the analysis to compare with our sequences because they erroneously compared sequences from two different regions of the TEF-1α gene, when comparing their isolates to the reference sequences in GenBank.

Pathogenicity tests

Grove topsoil from 0–10 cm was heat sterilized for the pre-nursery experiments. The sterilization process was as follows: a 45 cm tripod stand was placed in a "drum" and placed over a heat source. Water was poured in the "drum" just beneath the tripod. Biodegradable bags were then filled with grove topsoil from 0–10 cm and placed on the tripod, covered with a thick layer of jute bags to prevent the escape of heat during sterilization. The water was allowed to boil, then the top of the "drum" was tightly sealed with plastic and secured in place with rope. Constant heat was maintained for 2 hours and then the covering over the drum was taken off. The soil was allowed to cool and then removed from the drum.

After cooling, the sterilized soil was aliquoted to 30 × 12 cm polythene bags and used for the nursery experiment. Treated oil palm (*E. guineensis* Jacq.) seeds (*Fusarium* susceptible palm 'Tenera' seeds) were bought from IRAD La Dibamba and planted in polythene bags in the sterilized soil. 'Tenera' is a hybrid of Dura and Pisifera which are parental varieties. Dura and Pisifera are raised in nurseries for hybridization breeding programs whereas Tenera is the commercial cultivar for plantations (Hartley 1988). The palms were watered every 24 h with sterile distilled water while observations were being made. Seedlings were grown to the 3 to 4-leaf stage. Oil palm seedlings in the nursery are shown in Figure 2.

Table 1. Isolation frequency of *Fusarium oxysporum* f. sp. *elaeidis* (FOE) from oil palm plantations in CDC

Estates	Source of material	Number plated		Isolates obtained		% Isolation frequency	
		PDA	VW	PDA	VW	PDA	VW
Debundscha	rachis	15	15	3	5	20	33.3
	soil	15	15	8	9	53.3	60
		30	30	11	14	36.6	46.6
Idenau	rachis	10	10	4	6	40	60
	soil	10	10	4	4	40	40
		20	20	8	10	40	50
Mondoni	rachis	25	25	12	17	48	68
	soil	25	25	21	22	84	88
		50	50	33	39	66	78
Mungo	rachis	25	25	20	21	80	84
	soil	25	25	20	23	80	92
		50	50	40	44	80	88
Bota	rachis	25	25	4	7	16	24
	soil	25	25	11	11	44	44
		50	50	15	18	30	36
TOTAL		200	200	107	125	53.5	62.5

PDA – potato dextrose agar; VW – Van Wyk medium

The single spore isolates prepared during molecular studies, previously stored in sterile distilled water, were sub-cultured on PDA media and incubated at 26°C for 5 days. After 5 days of growth, eight 5 mm diameter plugs were aseptically cut from the colonies and transferred to 100 ml of full strength PDB and stationary incubated at 26°C. After 2 days of incubation, the bottles were shaken to disperse the hyphae and the cultures were allowed to grow for 14 days. After 14 days of growth, to loosen asexual spores from the mycelia, approximately 7 ml of sterile distilled water (SDW) was added to each plate and a sterile, bent glass rod was used to gently rub the agar surface. The suspension was poured into a sterile bottle and approximately 10 ml of SDW was used to rinse the plate. To remove mycelia, layers of sterile cheesecloth were used to filter the suspension and it was then diluted with SDW to obtain 10^6 spores · ml^{-1}. The number of spores was quantified with a hemocytometer and varied for each isolate, so they were harmonized to 25 ml of 10^6 spores · ml^{-1} per PDA plate. This represented the standard amount of inoculum applied to each plant during pathogenicity testing.

Using a sterile syringe, apparently healthy 3-month-old oil palm seedlings in polythene bags (Fig. 2) ('Tenera' variety) were injected with a 2.5×10^7 spore·ml^{-1} FOE suspension at the roots. Subsequently, the remaining FOE suspension of 2.5×10^7 spores · ml^{-1} was poured around the injected area on the roots and kept overnight. Assessment of the presence or absence of symptoms of inoculated seedlings was done on a weekly basis until the first symptoms were observed. Qualitative assessment was done on the roots of symptomatic plants based on the fact that discoloration of roots is one of the main symptoms of FOE infection in oil palm (Rusli *et al.* 2015). The roots of oil palm seedlings used as negative controls for pathogenicity were inoculated with the same volume of water with no spores. A completely randomized design was used, and the replication for each treatment was done three times.

Results

Field symptoms of *Fusarium oxysporum* f. sp. *elaeidis* (FOE), fungal isolation and morphology

Field symptoms were based on the stage of the vascular wilt disease. Infected palms showed a range of symptoms. Some of the basic symptoms observed in the field were dry, older lower fronds that were desiccated towards the bud that separates from the lower trunk. Death in a one-sided manner of affected fronds was noticed on the spines and lower leaflets out to the tip of the frond as shown in Figure 3A. On the other side of the rachis, dieback extended from the tip to the base of the frond. Concurrently, some leaves were found to be dead beginning at the tip of the frond on

Table 2. Identity of *Fusarium oxysporum* f. sp. *elaeidis* isolates based on BLASTn and FUSARIUM ID accession numbers deposited in GenBank used in this study

Number	Isolate number*	GenBank accession number	Tissue type	Geographic location (estate)
1	TRBO6	MH684539	canopy	Bota
2	TRBO8	MH684540	canopy	Bota
3	TRBO80	MH684557	canopy	Bota
4	TRBO98	MH684558	canopy	Bota
5	TRBO47	MH684579	canopy	Bota
6	TRDE29	MH684547	canopy	Debundscha
7	TRDE41	MH684551	canopy	Debundscha
8	TRDE92	MH684582	canopy	Debundscha
9	TRDE21	MH684544	canopy	Idenau
10	TRID42	MH684552	canopy	Idenau
11	TRID100	MH684559	canopy	Idenau
12	TRID58	MH684576	canopy	Idenau
13	TRMO54	MH684554	canopy	Mondoni
14	TRMO120	MH684561	canopy	Mondoni
15	TRMO62	MH684568	canopy	Mondoni
16	TRMO48	MH684574	canopy	Mondoni
17	TRMO64	MH684580	canopy	Mondoni
18	TRMO68	MH684581	canopy	Mondoni
19	TRMU2	MH684537	canopy	Musaka
20	TRMU43	MH684553	canopy	Musaka
21	TRMU14	MH684563	canopy	Musaka
22	TRMU26	MH684572	canopy	Musaka
23	TRMU101	MH684577	canopy	Musaka
24	TRMU103	MH684578	canopy	Musaka
25	TRBO9	MH684541	soil	Bota
26	TRBO11	MH684542	soil	Bota
27	TRBO12	MH684543	soil	Bota
28	TRBO16	MH684544	soil	Bota
29	TRDE37	MH684549	soil	Debundscha
30	TRDE39	MH684550	soil	Debundscha
31	TRDE66	MH684556	soil	Debundscha
32	TRDE40	MH684567	soil	Debundscha
33	TRDE71	MH684569	soil	Debundscha
34	TRDE50	MH684575	soil	Debundscha
35	TRID31	MH684562	soil	Idenau
36	TRMO4	MH684538	soil	Mondoni
37	TRMO25	MH684546	soil	Mondoni
38	TRMO57	MH684555	soil	Mondoni
39	TRMO51	MH684560	soil	Mondoni
40	TRMO20	MH684565	soil	Mondoni
41	TRMO28	MH684566	soil	Mondoni
42	TRMU27	MH684536	soil	Musaka
43	TRMU32	MH684548	soil	Musaka
44	TRMU15	MH684564	soil	Musaka
45	TRMU97	MH684570	soil	Musaka
46	TRMU99	MH684571	soil	Musaka
47	TRMU38	MH684573	soil	Musaka
48	TRMU74	MH684583	soil	Musaka

*TR – Tonjock Rosemary, BO – Bota, DE – Debunscha, ID – Idenau, MO – Mondoni, MU – Mungo

Table 3. List of *Fusarium oxysporum* f. sp. *elaeidis* (FOE) and other *Fusarium oxsporum* f. sp. isolates used for phylogenetic analyses in the study

Location	FOE isolates	NCBI accession numbers	Gene of interest
Suriname	*Fusarium oxysporum* f. sp. *elaeidis*	NRRL22543	EF-1α
Zaire (now the Democratic Republic of Congo)	*Fusarium oxysporum* f. sp. *elaeidis*	NRRL36358	EF-1α
Zaire (now the Democratic Republic of Congo)	*Fusarium oxysporum* f. sp. *elaeidis*	NRRL36359	EF-1α
Zaire (now the Democratic Republic of Congo)	*Fusarium oxysporum* f. sp. *elaeidis*	NRRL36386	EF-1α
Brazil	*Fusarium oxysporum* f. sp. *elaeidis*	NRRL38313	EF-1α
Brazil	*Fusarium oxysporum* f. sp. *elaeidis*	NRRL38314	EF-1α
Florida, USA	*Fusarium oxysporum* f. sp. *palmarum*	KX530818	EF-1α
Texas, USA	*Fusarium oxysporum* f. sp. *palmarum*	KC897693	EF-1α
Florida, USA	*Fusarium oxysporum* f. sp. *palmarum*	PLM246B	EF-1α
Florida, USA	*Fusarium oxysporum* f. sp. *palmarum*	PLM195C	EF-1α
Florida, USA	*Fusarium oxysporum* f. sp. *palmarum*	PLM249A	EF-1α
Florida, USA	*Fusarium oxysporum* f. sp. *palmarum*	PLM181C	EF-1α
Florida, USA	*Fusarium oxysporum* f. sp. *palmarum*	PLM153B	EF-1α
Florida, USA	*Fusarium oxysporum* f. sp. *palmarum*	PLM140B	EF-1α
Florida, USA	*Fusarium oxysporum* f. sp. *palmarum*	PM119C	EF-1α
Florida, USA	*Fusarium oxysporum* f. sp. *canariensis*	PLM754A	EF-1α
Florida, USA	*Fusarium oxysporum* f. sp. *canariensis*	PLM724A	EF-1α
California, USA	*Fusarium oxysporum* f. sp. *canariensis*	PLM706A	EF-1α
Florida, USA	*Fusarium oxysporum* f. sp. *canariensis*	PLM696A	EF-1α
South Carolina, USA	*Fusarium oxysporum* f. sp. *canariensis*	PLM511A	EF-1α
Texas, USA	*Fusarium oxysporum* f. sp. *canariensis*	PLM385B	EF-1α
China	*Fusarium oxysporum* f. sp. *conglutinans*	LC165018	EF-1α
South Africa	*Fusarium oxysporum* f. sp. *cepae*	GU165966	EF-1α
France	*Fusarium oxysporum* f. sp. *dianthi*	AF363406	EF-1α
California, USA	*Fusarium oxysporum* f. sp. *niveum*	MN057702	EF-1α
New Mexico, USA	*Fusarium oxysporum* f. sp. *vasinfectum*	MK953690	EF-1α
China	*Fusarium oxysporum* f. sp. *momordicae*	MK642292	EF-1α
USA	*Fusarium oxysporum* f. sp. *raphani*	FJ985273	EF-1α
East and Central Africa	*Fusarium oxysporum* f.sp. *cubense*	KX365407	EF-1α
Spain	*Fusarium oxysporum* f. sp. *pisi*	KF913727	EF-1α
Peoria, IL, USA	*Fusarium oxysporum* f. sp. *niveum*	FJ985346	EF-1α
Australia	*Fusarium oxysporum* f. sp. *cubense*	KX434905	EF-1α
Peoria, IL, USA	*Fusarium oxysporum* f. sp. *gladioli*	FJ985302	EF-1α
Peoria, IL, USA	*Fusarium oxysporum* f. sp. *glycines*	AF008496	EF-1α
Finland	*Fusarium oxysporum* f. sp. *cepae*	KT239478	EF-1α

NRRL, ARS Culture Collection, NCAUR-ARS-USDA, Peoria, IL

both sides of the rachis. At variable distances from the base of the frond, there was an extension of brown stripes which developed from the lower surface of the rachis frond (Fig. 3B). Fig. 3C shows a healthy oil palm plant. When the rachis was observed in cross and longitudinal sections, vascular discoloration and brown tissue with discrete pockets were seen. Necrotic streaking was observed in some spines and pinnae. Figures 3D, E and F show vascular discoloration in the cross section of diseased palms. Fusarium wilt and several rachis blight diseases can cause death of the frond in the same manner.

During fungal isolation, 200 samples were plated from rachis and soil, and 107 and 125 isolates were recovered from PDA and VW media, respectively, with an isolation frequency of 53.5 and 62.5%, respectively.

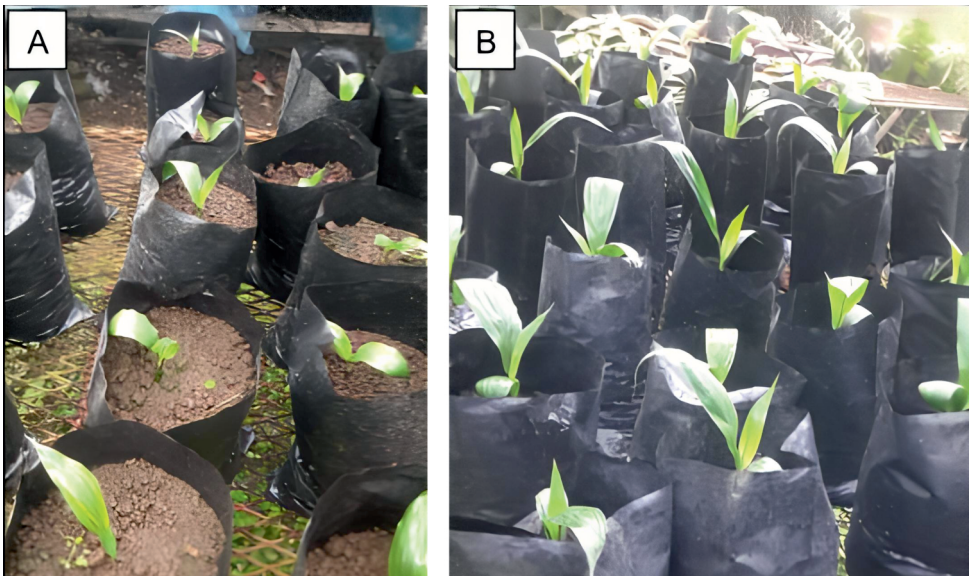

Fig. 2. *Fusarium* susceptible palm seedlings in the nursery at the A – 2 leaf stage, B – 3 leaf stage

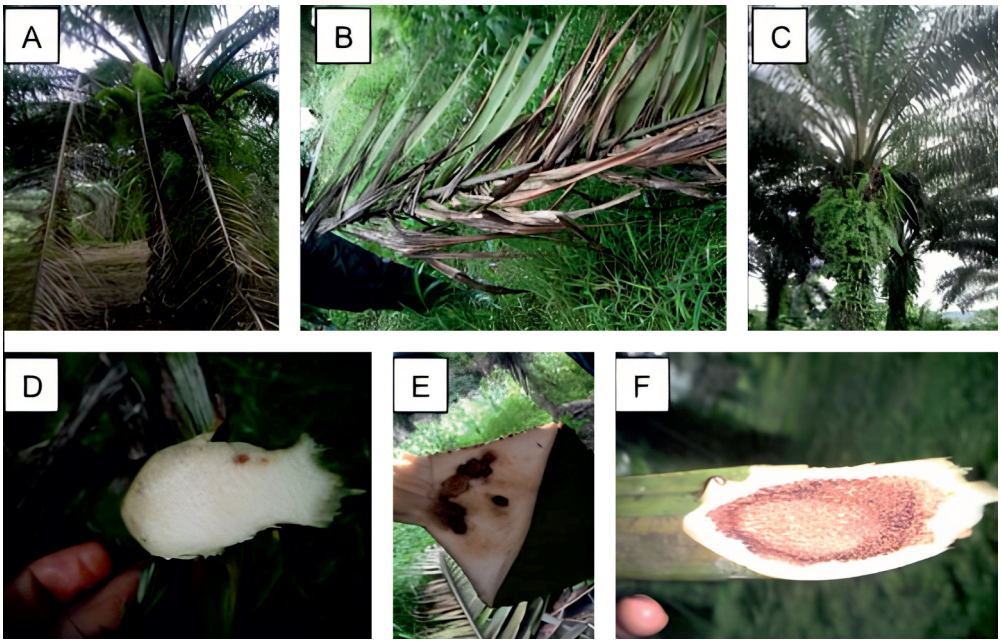

Fig. 3. Field symptoms of *Fusarium oxysporum* f. sp. *elaeidis* (FOE): A – an external symptom is seen as dry leaves, B – leaves become weak, form a skirt-like shape on the plant, C – healthy oil palm plant, D – symptoms begin as a brown spot in xylem, E – xylem tissue of frond gets more infected as disease progresses, F – Progression of FOE in the xylem

The isolation frequency on Mungo Estate was 80 and 88% on PDA and VW, respectively, yielding the highest number of isolates. Bota Estate had an isolation frequency of 30 and 36% on PDA and VW, respectively, with the least number of isolates. Debundscha, Bota and Mondoni Estates had soil samples which yielded a higher number of isolates than in Mungo and Idenau. Thirty to 66% was the range in the isolation frequency on Bota and Mondoni Estates when PDA was used. The number of isolates from soil and rachis from Idenau and Mungo were equal, with isolation frequencies of 40 and 80%, respectively. The isolation frequency for Idenau Estate was 60% and had more isolates from the rachis. The general pattern of isolation frequency of Fusarium from oil palm is shown in Table 1.

Morphologically, on the basis of the mycelium growth pattern, single-spored isolates could be categorized into several morpho groups exhibiting fluffy

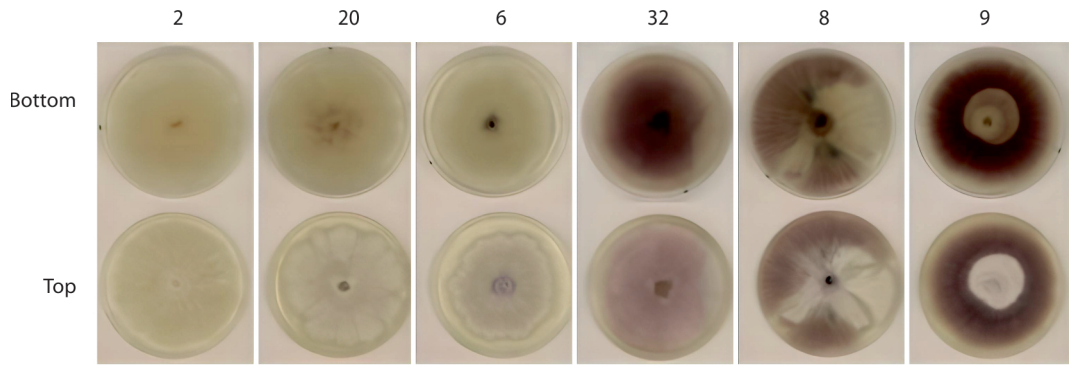

Fig. 4. Representative colony color variation of single spore *Fusarium oxysporum* f. sp. *elaeidis* (FOE) isolates cultured on PDA. The numbers 2, 20, 6, 32, 8 and 9 represent different isolates showing color variation

growth or adherent smooth growth with most isolates exhibiting fluffy growth. Undulating margins, irregular form and flat elevations were most commonly observed. Color variability of FOE isolates ranged from white, creamy white, light pink, pink to salmon pink and light purple to violet. The four main morpho-groups by color were cream white (group 1, represented by isolate TRMU2), white (group 2, represented by isolate TRMO2), light purple (group 3, represented by isolate TRBO6), and dark purple (group 4, represented by isolates TRBO8, TRBO9, and TRMU32). These different morpho-groups are shown in Figure 4.

DNA extraction, PCR and DNA sequencing

Using genomic DNA for all 107 single-spored isolates on PDA, a portion of the TEF-1α gene was amplified using EF1 and EF2 primers. Resulting amplicons ranged between 627–630 bp corresponding to the expected DNA target region. The amplicons were sequenced, and the sequences queried against GenBank using BLASTn and FUSARIUM ID databases to confirm the identities of the different isolates. Of the 107 single-spored isolates examined, 48 were identified by sequence similarity as *F. oxysporum* f. sp. *elaeidis*. The diagnostic TTα sequences were deposited in GenBank with associated accession numbers (Table 2). The list of other selected *F. oxysporum* f. sp. *elaeidis* (FOE) and *F. oxysporum* f. sp. isolates used for phylogenetic analysis in this study is shown in Table 3.

Phylogenetic analysis

Utilizing the TEF-1α sequences determined from FOE isolates, three unrooted consensus trees were generated by neighbor-joining analyses. The trees were deposited in TreeBASE (www.treebase.org). An analysis including unique sequences of all publicly available diagnostic FOE TEF-1α sequences produced two well-established FOE clades (Fig. 5). FOE clade 1 is made up of four subclades, with most of the Cameroonian FOE isolates in the three first subclades grouping with FOE isolates NRRL22543 from Suriname and isolates 38313 and 38314 from Brazil while subclade four in FOE clade 1 grouped with FOE isolates (36358, 36359, 36386) from Zaire (now the Democratic Republic of Congo), Africa. The second FOE clade 2 is represented by a new sequence variant of FOE isolates with three novel single nucleotide polymorphisms.

A second analysis utilizing TEF-1α sequences from other formae speciales produced a phylogram with three main clades. The first is the supported FOE clade (79% bootstrap value) with numerous subclades including different *F. oxsporum* ff. speciales. There was low bootstrap support (22 and 37%, respectively) in the subclades between FOE isolates and other *F. oxysporum* ff. spp. isolates. The second main clade had a 54% bootstrap value and was made up mainly of *F. oxysporum* f. sp. *palmarum* with other *F. oysporum* ff. spp. suchas *F. oxysporum* f. sp. *cepae* and *F. oxysporum* f. sp. *gladioli* while the third main clade was the *F. oxysporum* f. sp. *canariensis* clade and the grouping was supported by the bootstrap value of 99% (Fig. 6).

Pathogenicity tests

The pathogenicity tests revealed that eight isolates of FOE were aggressive agents of vascular wilt in oil palm. These isolates originated from five different oil palm plantations within the Cameroon Development Corporation. Symptoms were observed between 14 days and 28 days after inoculation. These included necrosis and brown discoloration of the roots and leaves as well as chlorosis of the canopy. Symptoms observed with six additional isolates included dry leaf tips, leaf mosaic stripes, and crinkled leaves. Representative symptoms are shown in Figure 7. Inoculations with the remaining seven isolates remained asymptomatic.

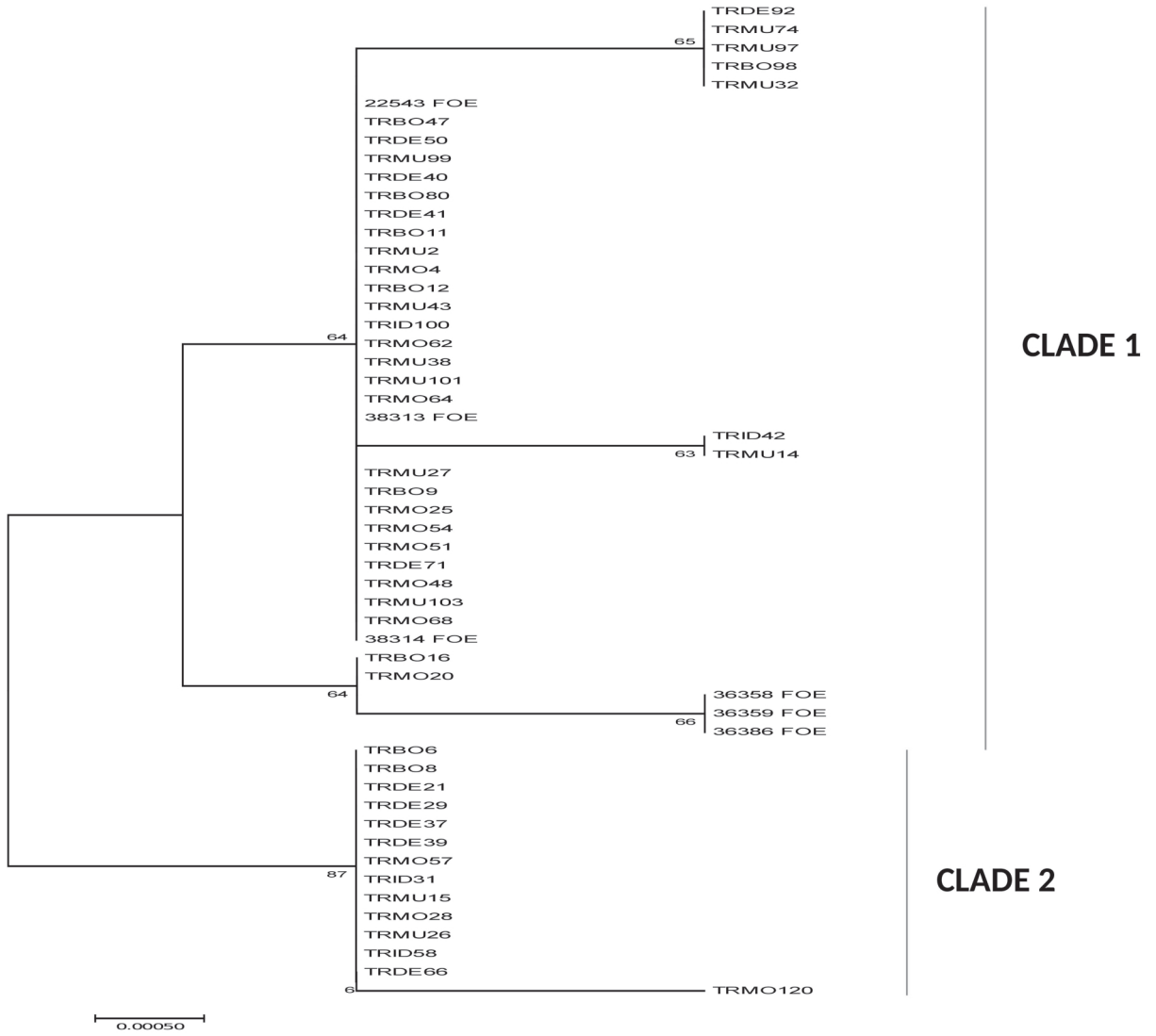

Fig. 5. Phylogenetic tree generated from TEF-1α sequences based on neighbor-joining analysis. FOE isolates from the current study were used plus all available *Fusarium oxysporum* f. sp. *elaeidis* (FOE) isolates. Isolate number and origin are indicated next to the sample numbers

Due to infection by FOE, there was discoloration of the roots as shown in Figure 8. Time was a major factor for the progression of root discoloration of infected plants even though there was no variation among isolates of FOE. FOE was consistently isolated from symptomatic plant tissue but not from asymptomatic plants or the non-inoculated control (Table 4); thus, Koch's postulates was fulfilled.

Discussion

Fusarium is one of the most morphologically homogenous fungal genera, which makes identification difficult. Although *Fusarium* species have been defined by morphology of asexual and sexual reproductive structures, considerable overlapping variation occurs in these features and this variation is subject to environmental influence (Nelson 1991). Classification of species within this genus is made more difficult as many cryptic species lacking morphological character differences exist (Llorens *et al.* 2006). Morphologically indistinguishable pathogenic as well as non-pathogenic lineages are found within *F. oxysporum*. Thus, molecular approaches are essential tools for species identification within the Fusarium complex. The TEF-1α gene has found great utility in differentiating Fusarium because it is a single locus gene and contains adequate polymorphism to distinguish *Fusarium* species and formae speciales of *F. oxysporum* (Geiser *et al.* 2004). In this study, the cultural composition and variability of FOE from different oil palm estates were assessed using a culture-dependent approach. Morphological characteristics were used to identify FOE in the collected samples. The morphological method though

Table 4. Isolates used for pathogenicity, symptoms observed after 2 weeks of inoculation and relative virulence of the oil palm seedlings

Isolate number	Symptoms after 2 weeks of inoculation	Pathogenicity test		
		severe	moderate	asymptomatic
TRMU2	leaves drying rapidly at the tips	+		
TRBO12	asymptomatic			+
TRMU99	faint chlorotic symptoms on leaves		+	
TRBO98	leaf mosaic and chlorotic symptoms		+	
TRDE39	dried spots on the leaves and necrosis	+		
TRDE41	asymptomatic			+
TRMO25	mosaic leaves drying rapidly at the tip of leaves	+		
TRID100	asymptomatic			+
TRMO57	faint chlorosis of leaves		+	
TRBO6	leaves drying rapidly at the tips	+		
TRDE66	mosaic chlorotic symptoms on leaves and dried leaves	+		
TRBO16	leaf drying at the tips with chlorosis and necrosis	+		
TRBO8	leaf drying at the tips with chlorosis and necrosis	+		
TRMU32	asymptomatic			+
TRBO11	drying spots on leaves with necrosis	+		
TRID42	leaf base looks crinkled with chlorosis		+	
TRMU43	browning of leaves at the leaf base		+	
TRMO120	asymptomatic			+
TRMO51	asymptomatic			+
TRMO4	faint yellowing symptoms on leaves		+	
TRDE29	asymptomatic			+

TR – Tonjock Rosemary, BO – Bota, DE – Debunscha, ID – Idenau, MO – Mondoni, MU – Mungo
Virulent – mean lesion size is 2.5–1.80 cm^2, Moderately Virulent – mean lesion size is 0.8–0.5 cm^2, Non-Virulent – 0.0–0.0 cm^2

not sufficient, remains the most inexpensive and common method used in fungi identification in developing countries as is obvious in some publications (Kumar and Hyde 2004; Suryanarayanan *et al.* 2011). Cultural studies of *F. oxysporum* f. sp. *elaeidis* on potato dextrose agar (PDA) revealed great heterogeneity in radial growth but supported sporulation of all isolates examined. These results are similar to those reported for other *F. oxysporum* isolates by various authors. This is in agreement with the reported extensive colony heterogeneity observed by Kulkarni (2006). Also, Ochoa *et al.* (2000) reported *F. oxysporum* isolates from babaco tissues exhibiting cottony mycelium and a typical pale-pink to purplish discoloration of the species when it sporulated. However, *F. oxysporum* mycelia when grown on PDA medium produces dark purple or dark magenta pigments but never whitish yellow or clear (Leslie and Summerell 2006). Furthermore, yellow, violet or gray pigmentation was observed by Dubey *et al.* (2010). The variability in cultural characteristics was also observed in this study and demonstrated its limitation and non-suitability for utilization in *Fusarium* identification alone.

Fusarium oxysporum consists of both nonpathogenic and pathogenic isolates with the latter classified into formae speciales based on host pathogenicity (Gordon and Martyn 1997). Each forma specialis is confined to a limited host range normally comprised of only one or two host plant species. Based on the locations from which isolates were obtained, about 80% of isolates from the same estate tended to group together irrespective of whether they were isolated from the soil or the canopy. No geographical separation of isolates was apparent nor was there a distinction between isolates originating from soil versus canopy. It is also interesting that isolates from opposite sides of Mount Cameroon (Idenau versus Mungo) cladded together. The fact that the diversity of isolates from the soil was very similar to that of the canopy and the lack of geographical separation between genotypes suggests that FOE has been present for a long period of time and is distributed naturally or anthropogenically on the estates. Other rachis blight diseases do not lead to the death of the plant. In addition, in cross sections of the rachis, hard zones of necrotic tissue are seen when other fungi diseases infect plants. It is worth noting

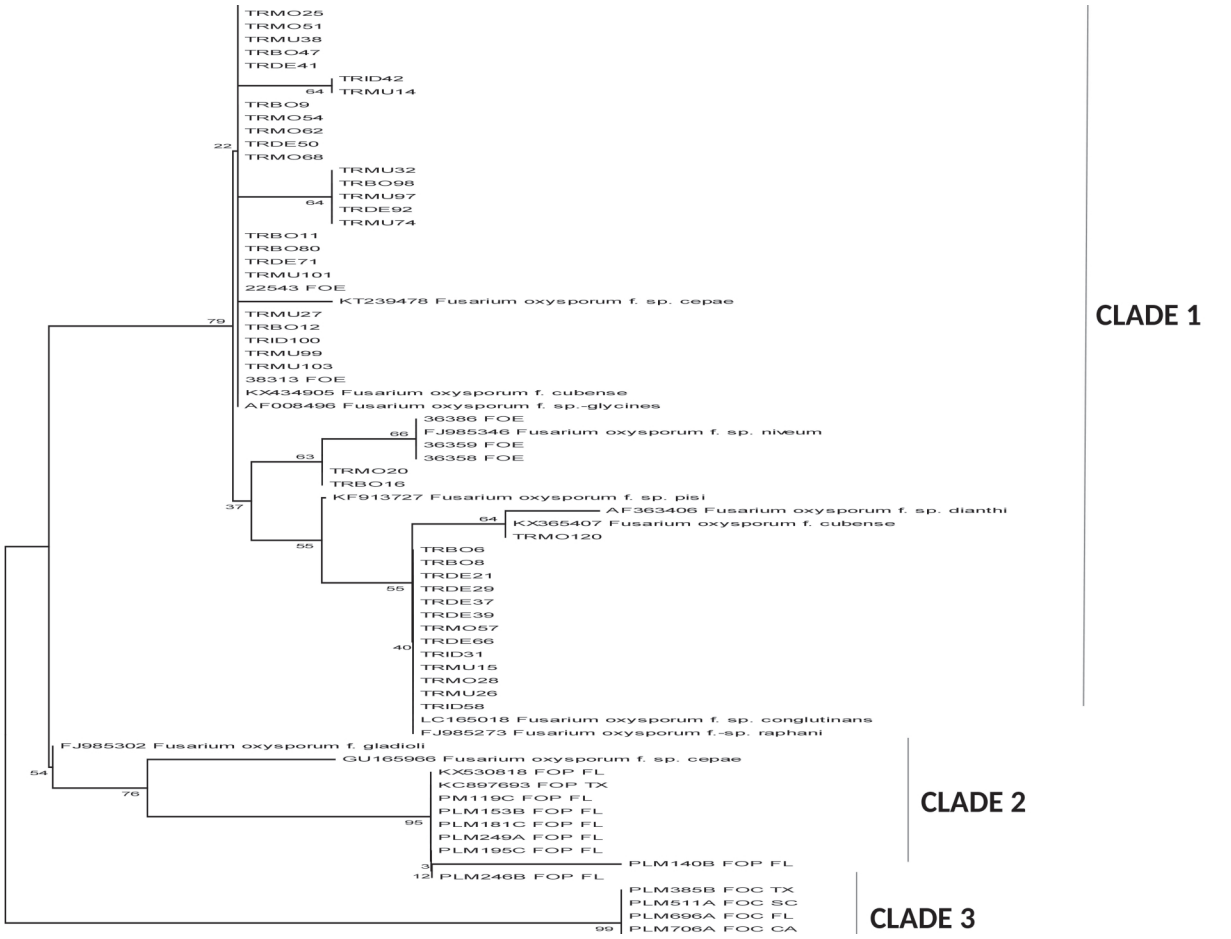

Fig. 6. Phylogenetic tree generated from TEF-1α based on neighbor-joining analysis of *Fusarium oxysporum* f. sp. *elaeidis* (FOE) and other *Fusarium oxysporum* f. sp. isolates. Isolate number and formae speciales are indicated next to the sample numbers

that on the same plant, the presence of two different syndromes can hide the symptoms of *Fusarium* wilt. For example, the existence of palm weevils in an area with *Fusarium* wilt can cause the insects to invade the trees which are already wilt stressed and this leads to unclear symptoms of *Fusarium* wilt and quicker palm death.

This study successfully confirmed the pathogenicity of 14 FOE isolates based on the observation of disease development and completion of Koch's postulates including confirmation of FOE recovery by sequencing the TEF-1α region. Fifty percent of the virulent isolates were from the Bota oil palm plantation. However, there was no clear pattern for isolates which produced moderate symptoms or no symptoms on the basis of the location from which the samples were isolated. The pathogenicity assay used in this study is similar to that of Rusli *et al.* (2015) in which they applied spore suspensions of FOE with a sterile syringe around the base of each palm on the soil surface at 3 months and kept moist for 2 weeks. However, more rapid symptom development occurred in the present study than in that of Rusli *et al.* (2015). Some isolates in this study showed mild infections while others showed aggressive infections. This might have been due to the variability of the different FOE isolates. The pathogenicity experiment in this study was conducted in an open greenhouse environment. However, shade was provided to optimize the conditions to maintain the best temperature for infection. This is similar to the study of Corley and Tinker (2003) but differs with Adusei-Fosu and Dickinson (2019) who found from their pathogenicity experiment that when palms were soaked in spore suspension and the roots wounded, the earliest time for symptoms to appear in oil palm seedlings inoculated under glasshouse conditions was 4 months. However, in our pathogenicity experiment it took 2 weeks for symptoms to show under greenhouse conditions. Our results differed to those of Rusli *et al.* (2015) who used Tenera (commercial type) oil palm seedlings and reported severe symptoms at 25 weeks after inoculation. Our more rapic symptom expression might have been due to the usage of a higher spore concentration of 2.5×10^7 ml^{-1} at inoculation than other studies such as Rusli *et al.* (2015) who used 6×10^6 ml^{-1} as well as different inoculation methods.

Fig. 7. Symptoms on some infected palms in the nursery

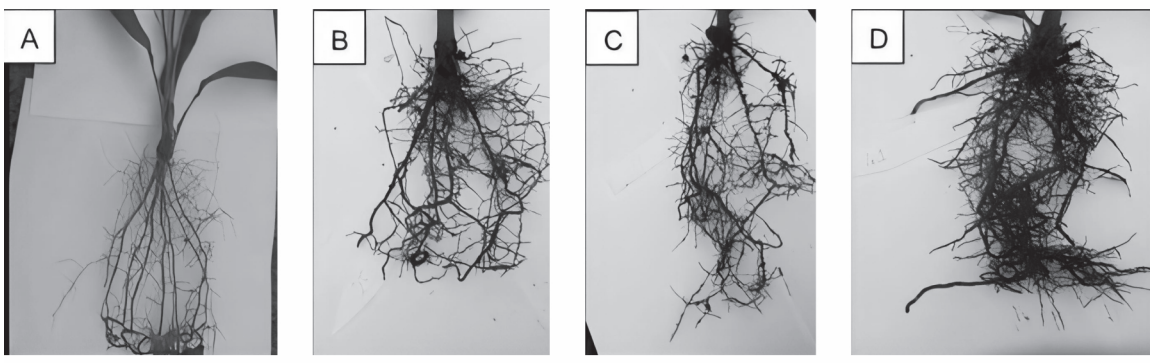

Fig. 8. Root samples of: A – control and B, C, D – infected palms

There was no phylogenetic separation of aggressive isolates from asymptomatic isolates. This is because the disease in its acute form is fatal and the palm dies 3 to 4 months after its appearance (Flood 2006), while in the chronic form, it keeps the palm alive, weakens and renders it almost unproductive (Ntsomboh-Ntsefong *et al.* 2012). Also, perhaps these different symptom types are environmentally controlled rather than based on the genetics of the pathogen. More robust disease assays for distinguishing acute versus chronic infections need to be developed and more detailed genetic characterizations of the FOE isolates are required to draw any significant conclusions.

FOE has been reported to occur in oil palm plantations in Cameroon based on field assessment and cultural studies (Ntsomboh-Ntsefong *et al.* 2012). However, this is the first study from Cameroon which utilizes molecular methods for *Fusarium* species identification and determination of their phylogenetic relationships. Forty-eight sequences of FOE were deposited in GenBank from these Cameroonian isolates. This may serve as a foundation for studies of FOE isolates obtained from other parts of Africa and South America to begin an assessment of the global diversity of the oil palm wilt pathogen. Evidence from the phylogenetic studies suggests that local FOE populations in Cameroon are monophyletic and although FOE DNA sequence resources from other oil palm cultivation regions with the disease are sparse (South America and the Democratic Republic of Congo), our phylogenetic analysis of the available data suggests that the global population is also monophyletic. This perhaps indicates that the pathogen has moved in conjunction with oil palm production rather than evolving from endemic *F. oxysporum* populations. In support of this, Flood *et al.* (1992) using vegetative compatibility groupings and Mouyna *et al.* (1996) using RFLP analysis reported a high degree of uniformity amongst isolates of FOE from Africa and South America. The results of Mouyna *et al.* (1996) indicated that isolates from soil associated with diseased palms were quite distinct from isolates obtained from diseased trees. This may be due to the fact that they assessed total FOE and other *F. oxysporum* diversity in their study while in this study only FOE diversity was assessed.

When the genetic variation within the *Fusarium* species complex is studied, the TEF-1α gene is frequently used (Geiser *et al.* 2004). The phylogenetic analysis of Adusei-Fosu and Dickinson (2019) characterized FOE isolates based on SIX 8, SIX 9, SIX 11 and TEF-1α. Our TEF-1α phylogenetic analysis differed significantly from theirs as they erroneously compared sequences from two different regions of the TEF-1α gene when comparing their isolates to the reference sequences in GenBank. This explains the phylogenetic separation of all FOE and "Presumed FOE" TEF-1α sequences generated in that study from all other TEF-1α sequences of FOE obtained from GenBank. From the sequences that were comparable, their results do show a lack of FOE clustering on the basis of geographical location similar to what we demonstrated.

In summary, cultural, morphological, pathogenicity and molecular studies based on single sequence comparison found that vascular wilt of oil palm in Cameroon was caused by FOE which is made up of at least three closely related lineages. Multilocus sequence comparison is recommended to confirm the results. That notwithstanding, the use of housekeeping genes, the TEF- α, are widely used for the identification of *Fusarium* species and formae speciales within the *F. oxysporum* species complex. This study revealed the pathogenicity of FOE isolates from Cameroon and demonstrated that FOE in Africa is more diverse than previously reported including a lineage not previously observed outside of Cameroon. Comparisons among all isolates will ultimately aid to devise appropriate control mechanisms and better pathogen detection methods.

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