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ORIGINAL ARTICLE

Development of pathogenicity assay and characterization of *Fusarium oxysporum* f. sp. *elaeidis* (*FOE*) based on *Secreted In Xylem* genes and *EF*-1α

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Abstract

The pathogenicity of four *Fusarium oxysporum* isolates collected from symptomatic oil palm (*Elaeis guineensis* Jacq.) from Ghana were tested for the first time to develop a new pathogenicity assay for *Fusarium oxysporum* f. sp. *elaeidis* (*FOE*) infection in oil palm seedlings. All four *FOE* isolates used for pathogenicity assay were pathogenic to oil palm seedlings within a relatively short time compared to other pathogenicity studies, for which infection/symptoms in oil palm seedlings was time consuming. *FOE* and *"presumed-FOE"* (i.e. *Fusarium* isolates *collected from symptomatic oil palm trees whose pathogenicity is not confirmed*) were characterised based on partial sequences of a housekeeping gene *EF*-1α and three *Secreted In Xylem* genes (*SIX8, SIX9* and *SIX11*). All the phylogenetic trees generated for *EF*-1α, *SIX8, SIX9* and *SIX11* showed some variation between *FOE*, and *"presumed-FOE"*, but could not cluster isolates based on geographical location. Phylogenetic trees for *EF*-1α and *SIX* (*SIX9* and *SIX11*) genes clustered both *FOE* and *"presumed-FOE"* from FUSARIUM-ID from GenBank, but *SIX8* could not.

Keywords Phylogenetics · *Presumed-FOE* · Symptomatic · Effector proteins · *Elaeis guineensis* Jacq.

Introduction

The pathogen *Fusarium oxysporum* f. sp. *elaeidis* (*FOE*) is a soil borne fungus and causes fusarium wilt disease in the world's highest oil producing crop, the oil palm (*Elaeis guineensis* Jacq.) (Corley 2009; Hansen et al. 2015). The pathogen is present in African countries including Ghana, Democratic Republic of Congo and Cameroon (Prendergast 1957; Aderungboye 1982; Corley and Tinker 2003) where oil palm is produced. In Ghana, management of the pathogen such as removal and burning of infected oil palm trees is the only method of control over years in various oil palm plantations.

Although the pathogenicity of *Fusarium oxysporum* isolates collected from oil palm showing symptoms of fusarium wilt disease have been successfully studied in oil palm seedlings, these pathogenicity tests were extremely time consuming and laborious (Flood et al. 1993; Mepsted et al. 1994a; Rusli et al. 2015). Molecular methods have a strong impact on characterising or fingerprinting isolates of plant pathogens and in the last two decades, use of molecular techniques have expanded due to their sensitivity and accuracy. Tools that have been used for identifying variation in a wide range of *F. oxysporum* f. sp. include Inter-Simple Sequence Repeat (ISSR) (Thangavelu et al. 2012; Baysal et al. 2013; Liu et al. 2014; Hannachi et al. 2015) or Simple Sequence Repeat (SSR) markers (Bogale et al. 2005; Datta et al. 2011; Datta and Lal 2013). In addition, efforts have been made to use other gene of interest such as housekeeping genes (Baayen et al. 2000; Bogale et al. 2005, 2009; Silva et al. 2014). More so, other studies have been conducted to associate pathogenicity with the presence or absence of effector proteins such as *Secreted In Xylem* (*SIX*) genes within other *F. oxysporum* ff. spp. (Sasaki et al. 2015; Taylor et al. 2016). These molecular tools have been applied to identify and study the relationship or genetic variation of different strains of *F. oxysporum* (Baayen et al.

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2000; Bogale et al. 2005; Silva et al. 2014; Sasaki et al. 2015; Taylor et al. 2016).

The primary objective of this research was to develop pathogenicity assay that would reduce the amount of time necessary for symptom development in oil palm (*Elaeis guineensis.* Jacq.) using four *FOE* isolates. Although many studies have tried to understand the association between pathogenicity either the presence or absence of *SIX* (*Secreted In Xylem*) genes in *F. oxysporum* ff. spp. (Sasaki et al. 2015; Taylor et al. 2016), in this present study, the authors considered only to determine the presence or absence of *SIX* (*1–14*) in the four *FOE* isolates used for the pathogenicity test and *"presumed-FOE"* (i.e. *Fusarium* isolates *collected from symptomatic oil palm trees whose pathogenicity is not confirmed*). Phylogenetic trees were also generated to characterise *Fusarium oxysporum* f. sp. *elaeidis* (*FOE*) and *"presumed-FOE"* isolates via sequencing genes based on three *SIX* (*8, 9* and *11*) genes and *EF-1α*.

Materials and methods

Field sampling

Sampling was done from mature oil palm randomly selected in plantations from two major regions in Ghana (Western and Eastern). In the Eastern Region, the Oil Palm Research Institute (OPRI), and in the Western Region, two different oil palm plantations, namely Norpalm and Benso Oil Palm Plantation (BOPP) and collected samples coded as shown in Table S1. Oil palms showing symptoms of fusarium wilt disease were found at all three sites. In addition to these samples (Table S1) collected from Ghana (West Africa), two other *Fusarium* isolates, DR CONGO-Z and SURINAME-S (originally from the Democratic Republic of Congo and Suriname respectively) were obtained from the CBS-KNAW culture collection, the Netherlands and two isolates DR CONGO-F3 and Ivory Coast-16F (Table S1) used by Rusli et al. (2015) from the University of Bath-UK. Originally, these two isolates of *FOE* (F3 and 16F) were from diseased oil palms from the Democratic Republic of Congo (DRC) and Ivory Coast respectively. Isolate 16F was also used by Institut de Recherches pour les Huiles et Oleagineux (IRHO) as an isolate for screening fusarium wilt disease in oil palm (Mepsted et al. 1994a, b).

Plant materials

Oil palm seedlings Tenera or 'commercial type' were obtained using the assisted pollination method (Teo 2015). Pregerminated seeds were transplanted to seed trays (9 mm) filled with a mixture of soil (Levington F2 seed Modular Compost) and perlite at the ratio of 8:1 and maintained in a controlled glasshouse environment (30 °C, 90% RH and 12 h photoperiod), with light [240 μ mol m^{-2} s^{-1} photo flux density (PFD)]. The RH in the glasshouse was reduced to 80% after 2 months when the oil palm seedlings were transplanted into Soparco black pots of diameter 13 cm filled a mixture of sand based soil (John Ines No. 3), perlite and vermiculite at a ratio of 8:1:1. Light levels were maintained between 800 and 500 μ mol m^{-2} s^{-1} with a day length of 14–17 h; humidity and temperature ranged from 60 to 80% and 20 to 35 °C, respectively. The seedlings were watered at the base at regular intervals when necessary. Conditions in the glasshouse were maintained throughout the experiments.

Pathogenicity test: Preparation of *FOE* inoculum and inoculation procedures

Spores were collected from BOPP-B5, OPRI-5, NORPALM-N5 and 16F *Fusarium oxysporum* isolates that were cultured on PDA and incubated at 29 °C for 7 days. Spore suspension was prepared, filtered through Miracloth (CALBIOCHEM) to remove mycelial fragments and the filtrate centrifuged at 670 x *g* for 10 min with the Centaur 2 (MSE). The sedimented spores were gently resuspended in 50 ml Falcon tubes with sterile distilled water and adjusted to 3×10^6 spores ml^{-1}. Three (3) month-old oil palm seedlings (Fig. 2a) (Tenera or commercial type) were removed from the pot, roots were washed with distilled water followed by wounding the roots with sterile syringe. After wounding the roots, inoculation with 3×10^6 spore ml^{-1} *FOE* suspension was carried out in two different treatments as described below.

1. The washed roots of oil palm seedlings soaked in *FOE* suspension, $3X10^6$ spores ml^{-1} (Fig.2b) overnight with slight wounding around the roots.
2. The washed roots of oil palm seedlings soaked in *FOE* suspension, $3X10^6$ spores ml^{-1} (Fig. 2b) overnight without wounding.

The treated oil palm seedlings were assessed (i.e. presence or absence of symptoms) on weekly bases until first symptom observation, followed by testing with primer pairs (Table S2) via PCR. The roots of the symptomatic plants were qualitatively assessed since one of the major symptoms for *FOE* infection in oil palm is discolouration of roots (Rusli et al. 2015). All oil palm seedlings used as negative controls for pathogenicity test had the roots either wounded or not-wounded, soaked in sterilised distilled water overnight. *FOE* isolate 16F pathogenicity was confirmed (Flood et al. 1993; Mepsted et al. 1994a, b; Rusli et al. 2015) in oil palm, hence this was selected as the reference (positive control) *FOE* isolate. Experimental design used was Completely Randomised Design (CRD) and experiment repeated (conducted) three times. Each treatment was replicated three times. Statistical analysis of variance (ANOVA) was done with GenStat 16th Edition.

Root samples with length (0.5–1.0 cm) were collected from both asymptomatic and symptomatic oil palm seedlings and plated on Potato dextrose agar (PDA) purposely for *FOE* (BOPP-B5, OPRI-5, NORPALM-N5 and 16F) identification and confirmation via PCR. This was done for every repeated pathogenicity test or experiment. The *"presumed-FOE"* isolates collected from symptomatic oil palm from various sampling sites, were also tested for the presence or absence of *SIX* (*1–14*), although not used for inoculation/pathogenicity trials. *"Presumed-FOE"* isolates were not used for pathogenicity test due to the limited numbers of oil palm seedlings available.

DNA extraction, PCR and DNA sequencing for *FOE* or "*presumed-FOE*" identification

Mycelium (50–100 mg) of *FOE* and *"presumed-FOE"* was scraped from the PDA plates using a sterile surgical blade. Tissue disruption was carried out using glass beads and homogenizer (FastPrep®) at a speed of 6.5 m/s for 45 s in the presence of liquid nitrogen. DNA extractions of the cultures were then carried out using DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. Polymerase chain reaction (PCR) of various regions of the template DNA was performed using primer pairs of interest (Table. S2). PCR was carried out in 30 µl volumes consisting of 15 µl of master mix (MangoTaq™ DNA Polymerase), 1 µl (of 10 pmol / ul) each, 12 µl sterile distilled water and 1 µl of template DNA of the isolates of interest. The reaction was performed in a BIO-RAD S1000 Thermal Cycler with the amplification conditions of 95 °C for 2 min for initial denaturation, followed by 35 cycles of denaturation at 95 °C for 2 min, annealing at temperatures suitable for amplification for each primer pair of interest and extension/elongation at 72 °C for 1 min 30 s. The final extension was set at 72 °C for 5 min. PCR products were cleaned using the QIAquick PCR Cleanup kit (Qiagen) following manufacturer's instruction and DNA concentration was estimated by nano-drop and using 1 kb ladder (Promega). Sequencing reactions were performed by Fisher Scientific or MyGATC. BLAST searches were performed using the GenBank sequence database to confirm the identity of the fungal isolates sequences based on the translation elongation factor-*1α* (*EF-1α*) and *Secreted In Xylem* (*SIX-1, 8, 9, 10, 11 and 12*) genes. The output from BLAST algorithms was used to query any unknown sequences against the database of all the fungal gene regions. Sequences for *EF-1α, SIX8, SIX9* and *SIX11* were subsequently submitted to National Centre for Biotechnology Information (NCBI) GenBank for accession number (Table S1) for the eleven isolates of *Fusarium oxysporum* from symptomatic oil palm.

Phylogenetic analysis

The *EF-1α, SIX8, SIX9* and *SIX11* partial sequences were manually edited and aligned with the MEGA6 software (Tamura et al. 2013). Phylogenetic analyses were conducted with MEGA6 on the combined selected data set for eleven *Fusarium oxysporum* isolates collected from symptomatic oil palm with seven reference genes from GenBank (Table S3) to support the *EF-1α*

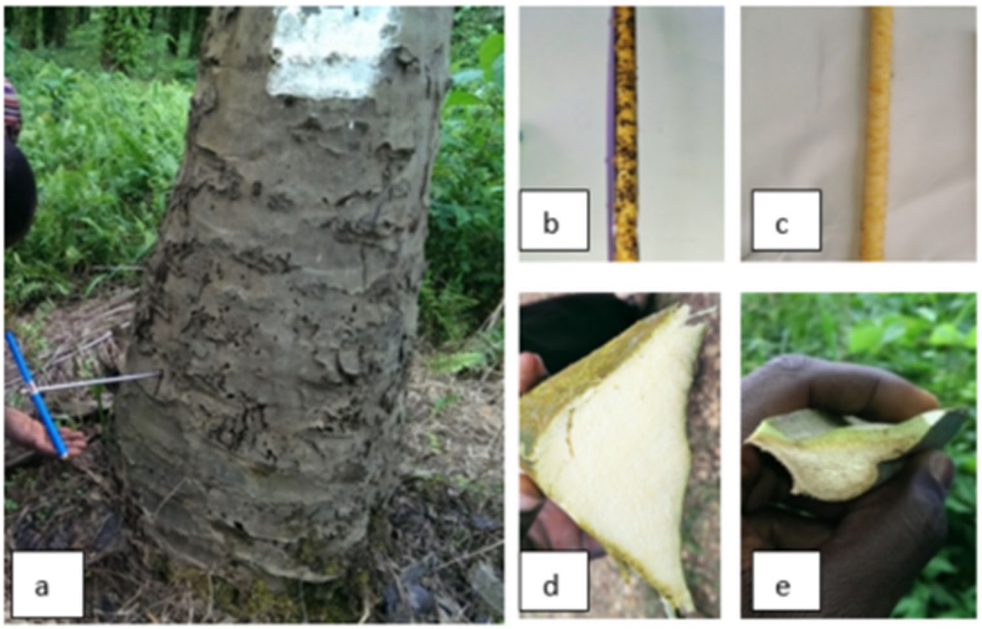

Fig. 1 Pictures showing **(a)** Drilling process with incremental stem borer in oil palm trunk **(b)** Drilled core (discoloured/brown/spotted) sample from trunk of diseased oil palm **(c)** Drilled core (clean) sample from trunk of a healthy oil palm **(d)** Cross-sectional view of a healthy oil palm lower branch or frond **(e)** Cross-sectional view of diseased oil palm blocked (brownish part) vascular section lower branch or frond

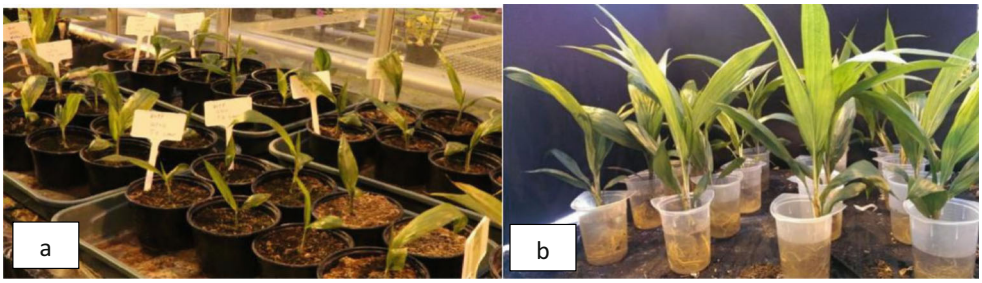

Fig. 2 Picture of oil palm seedlings **(a)** already potted with soil at the base to be inoculated and **(b)** removed from pots with roots washed and soaked in spore suspension in beakers

phylogenetic tree in addition to three known *EF-1α FOE* sequences host specific to other palms and not oil palm (Table S3). The phylogenetic trees for *SIX8, SIX9* and *SIX11* were eleven partial sequences of *Fusarium oxysporum* isolates from symptomatic oil palm with various reference genes from GenBank (Table S3). Six reference genes were used to support *SIX8* phylogenetic tree, whereas seven reference genes from GenBank supported both *SIX9* and *SIX11* phylogenetic trees (Table S3). Unweighted parsimony analyses were performed. Clade stability was assessed by 1000 parsimony bootstrap replications and the grouping statistical method was Unweighted Paired Group Method with Arithmetic Average (UPGMA). Bootstrapping values were shown in the branch nodes of the phylogenetic trees. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was assessed to determine the clade stability.

Results

Sampling

In the study, infected oil palm were identified in the Eastern Region - Oil Palm Research Institute (OPRI), and in the Western Region, two different oil palm plantations, namely Norpalm and Benso Oil Palm Plantation (BOPP). Generally, sampled tissues (Fig. 1a) from the stem or trunk of symptomatic oil palm showed discolouration (Fig. 1b). A cross-section view (Fig. 1e) of symptomatic oil palm leaf frond or branch showed brownish colour (Fig. 1e). Discoloured tissues were

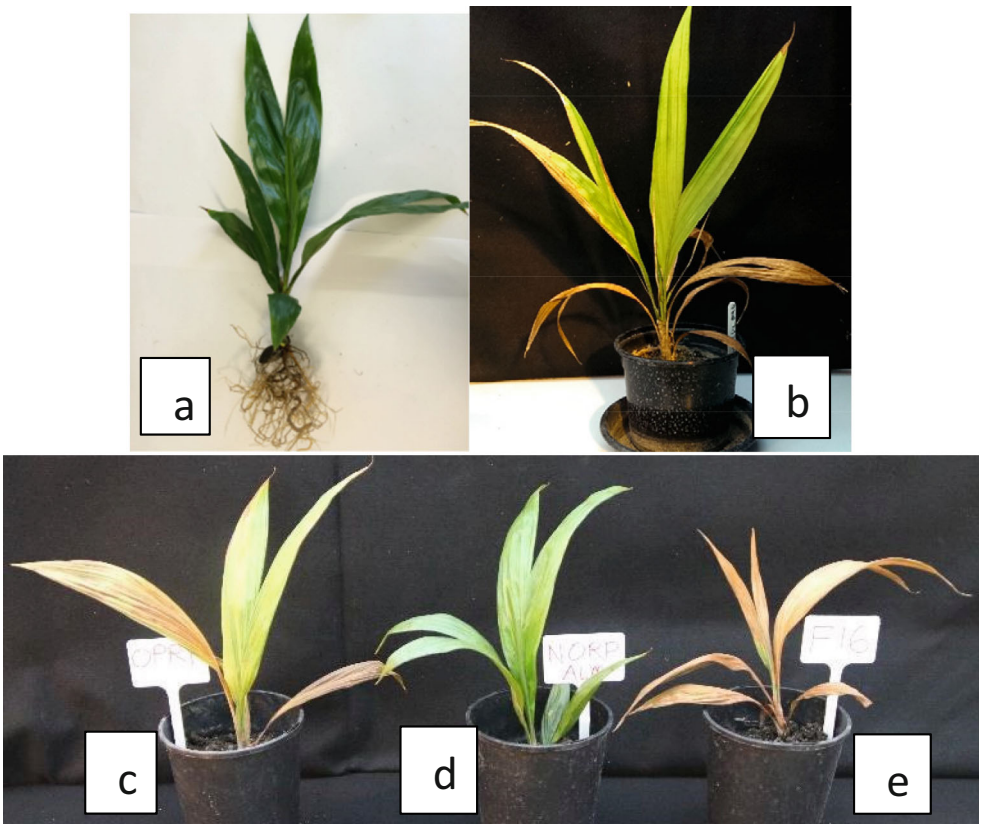

Fig. 3 Oil palm seedling showing **(a)** no symptoms for control and seedlings showing symptoms of fusarium wilt disease after inoculating with *FOE* isolates **(b)** BOPP-B5 **(c)** OPRI-5 **(d)** NORPALM-N5 **(e)** IVORY COAST-16F

Fig. 4 Oil palm roots **(a)** healthy (control treatment) or *FOE* inoculated **(b)** mildly discoloured **(c)** severely discoloured

absent in both the sampled tissues from stem or trunk and the cross-section view of the leaf frond or branch was also without discouloration in asymptomatic oil palm (Fig. 1c, d).

Pathogenicity test

In the pathogenicity assay for this study, 16F *FOE* isolate was used as the reference isolate besides *Fusarium oxysporum* isolates collected from Ghana (OPRI-5, BOPP-B5 and NORP-N5). Oil palm seedlings that had either minor wounds or no wounds on the roots soaked in 3×10^6 *FOE* spores overnight (Fig. 2) before potting also showed symptoms at 4 and 7 months respectively (Fig. 3). However, ANOVA showed there was no significant difference ($P > 0.05$) among the *FOE* isolates in the time of first symptoms. The discolouration of the roots as a result of infection by *FOE* are shown in Fig. 4 b, c. Root discolouration progressed with time in infected plants but did not vary among *FOE* isolates. Data was not analysed for root discolouration. Roots were tested for the presence of *FOE* by plating on PDA supplemented with antibiotics (Fig. 5 a, b) and DNA followed by PCR (Table 1).

Identification of *SIX* genes and *EF-1α* in four FOE isolates and *Fusarium oxysporum* isolates from symptomatic oil palm

Secreted In Xylem (*SIX8, SIX9* and *SIX11*) genes were identified within the pathogenic *FOE* and *"presumed-FOE"* from Ghana, Ivory Coast, DR Congo and Suriname (Table 2). The *SIX* gene primers were from published research (Lievens et al. 2009; Taylor et al. 2016). *Secreted In Xylem* genes (*SIX2, SIX3, SIX4, SIX5, SIX6, SIX7, SIX10, SIX13* and *SIX14*) were not identified within any of the *FOE* and *"presumed-FOE"* isolates but *SIX1* was present in only *FOE* (DR Congo-F3) isolate (Table 2) whereas *SIX12* was present in *FOE* OPRI-5 and OPRI-11. *EF-1α* was identified in all the *FOE* and "*presumed-FOE"* isolates based on the similarity with known *FOE* isolates.

Molecular characterisation of four *FOE* isolates and *Fusarium oxysporum* isolates from symptomatic oil palm based on *SIX8, SIX9, SIX11* and *EF-1α*

Characterising the *Fusarium oxysporum* isolates collected from oil palm, the phylogenetic trees showed *SIX* gene sequences considerably varied across the *FOE* or

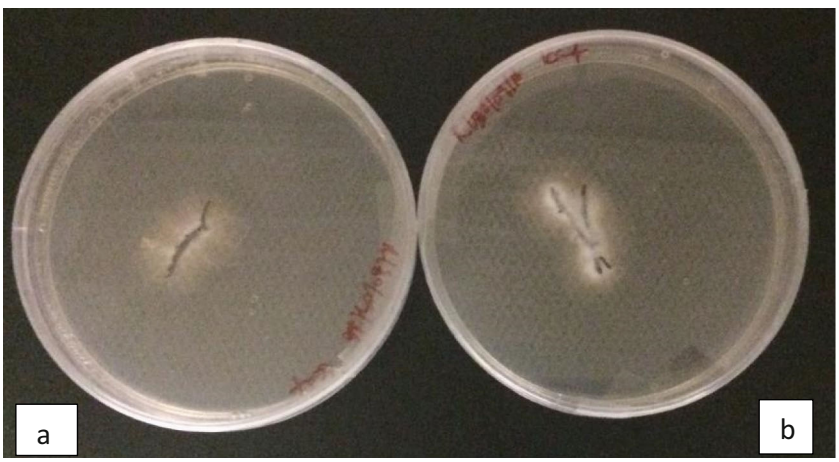

Fig. 5 Growth of *FOE* **(a)** OPRI-5 and **(b)** BOPP-B5 isolated from oil palm roots

Table 1 Result of *FOE* infected oil palm seedlings via root wounding or no-wounding inoculation methods

Oil palm type	*FOE* isolates inoculated				Inoculation mechanism
	OPRI-5	BOPP-B5	NORPALM-N5	16F	
Tenera	+	+	+	+	W
	+	+	+	+	W
	+	+	+	+	W
	+	+	+	+	NW
	+	+	+	+	NW
	+	+	+	+	NW

Each *FOE* isolate was replicated three times per treatment. There was no significance difference ($P > 0.05$) via both inoculation mechanisms for time of infection among the four *FOE* isolates according to Fisher's protected least significant difference test

NW = Non – wounded roots

W = Wounded roots

+ = Infected seedlings

*Selected oil palm seedlings inoculated via soaking wounded roots and non-wounded roots in $3X10^6$ in spore suspension

"presumed-FOE". The phylogenetic trees showed that *FOE* and *"presumed-FOE"* isolates could not be clustered based on geographical location using *SIX8, SIX9* and *SIX11* (Figs. 6, 7 and 8). The *SIX9* and *SIX11* genes could cluster both *FOE* and *"presumed-FOE"* as one group separated from the known FUSARIUM-ID sequences from the GenBank (Figs. 7 and 8), but *SIX8* could not (Fig. 6). Characterisation of *Fusarium oxysporum* isolates from oil palm using housekeeping gene partial sequences of *EF-1α,* grouped all oil palm *FOE* and *"presumed-FOE"* isolates from the known FUSARIUM-ID sequences in the GenBank (Fig. 9).

Table 2 Identified *Secreted In Xylem* (*SIX) 1–14* in *FOE* and Presumed-*FOE*

Fusarium species	Host	Isolate code	*Secreted In Xylem (SIX) genes*												
			1	2	3	5	6	7	8	9	10	11	12	13	14
F. oxysporum* f. sp. *elaeidis	Oil palm	IVORY COAST-16F	–	–	–	–	–	–	+	+	–	+	–	–	–
F. oxysporum* f. sp. *elaeidis	Oil palm	GHANA NORPALM-N5	–	–	–	–	–	–	+	+	–	+	–	–	–
F. oxysporum* f. sp. *elaeidis	Oil palm	GHANA BOPP-B5	–	–	–	–	–	–	+	+	–	+	–	–	–
F. oxysporum* f. sp. *elaeidis	Oil palm	GHANA OPRI-5	–	–	–	–	–	–	+	+	–	+	+	–	–
F. oxysporum f. sp. *elaeidis*	Oil palm	DR CONGO-F3	+	–	–	–	–	–	+	+	–	+	–	–	–
F. oxysporum f. sp. *elaeidis*	Oil palm	DR CONGO-Z	–	–	–	–	–	–	+	+	–	+	–	–	–
F. oxysporum f. sp. *elaeidis*	Oil palm	SURINAME-S	–	–	–	–	–	–	+	+	–	+	–	–	–
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA NORPALM-N2	–	–	–	–	–	–	+	+	–	+	–	–	–
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA NORPALM-N3	–	–	–	–	–	–	+	+	–	+	–	–	–
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA BOPP-B4	–	–	–	–	–	–	+	+	–	+	–	–	–
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA OPRI-11	–	–	–	–	–	–	+	+	–	+	+	–	–
F. oxysporum f. sp. elaeidis	Oil palm	GHANA OPRI-18	–	–	–	–	–	–	+	+	–	+	–	–	–
F. oxysporum f. sp. *lycopersici*	Tomato	Foxy.Forla-C	+	–	–	–	–	+	+	–	+	–	+	–	–
F. oxysporum f. sp. *lycopersici*	Tomato	Foxy.Forlb-D	+	–	–	–	–	+	+	–	+	–	+	–	–

Present = (+)

Not present = (−)

None in bold prints = *Presumed FOE* or non-*FOE*

Fol = *Fusarium oxysporum* f. sp. *lycopersici* (Foxy.Forla-C or Foxy.Forlb-D) used as positive control since *SIX* was first reported in *Fol* (Lievens et al. 2009)

*Bold prints = Pathogenic *FOE*

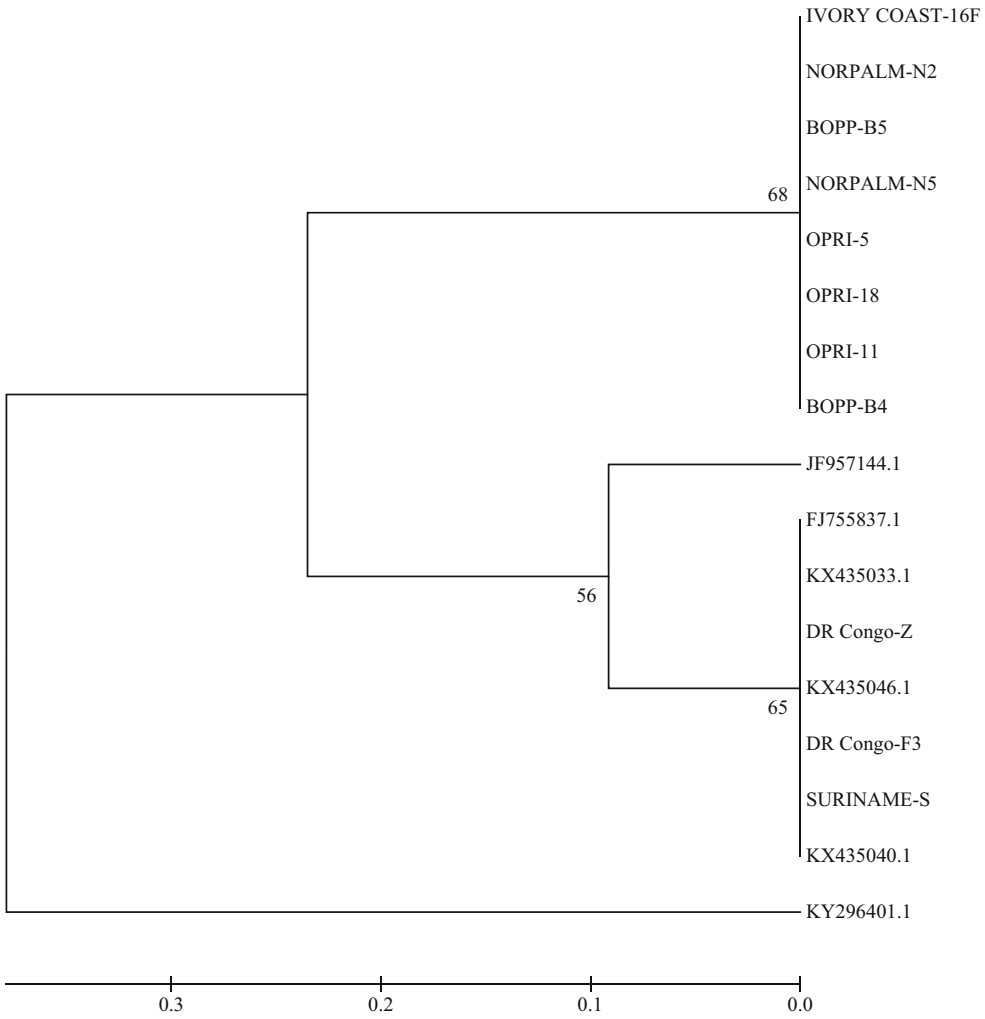

Fig. 6 Phylogenetic tree generated using statistical model Unweighted Pair-Group Method with Arithmetic Average (UPGMA) cluster composite date set from *Fusarium oxysporum* isolates from symptomatic oil palm and other host from GenBank based on *SIX8*. Bootstrapping was done for 1000 with bootstrap values shown in the nodes

Discussion

After testing a new pathogenicity assay in oil palm seedlings which took considerable shorter time for symptoms to show in plants, this study successfully confirmed the pathogenicity of four *Fusarium oxysporum* f. sp. *elaeidis* (*FOE*) isolates based on qualitative assessment and PCR. This current pathogenicity assay used in the current study differed from previous assays used by Rusli et al. (2015). Rusli et al. (2015) applied *FOE* spore suspension with a sterile syringe onto the soil surface around the base of each palm at age 3 months. Rusli et al. (2015) further reported that the inoculum was then watered with sterile distilled water for 2 weeks. However, symptoms in the current study were rapid compared to Rusli et al. (2015). Some of the isolates such as NORPALM-5 and BOPP-B5 showed mild infections compared to OPRI-5 and 16F. Previous studies have confirmed 16F to be more aggressive than other *FOE* isolates (Mepsted et al. 1994a, b; Rusli et al. 2015). Effector proteins (*Secreted In Xylem-SIX* gene) were for the first time studied in *FOE* and "*presumed-FOE"* isolates collected from two major regions in Ghana (Western and Eastern) in addition to other *FOE* isolates from Democratic Republic of Congo, Ivory Coast and Suriname. Characterisation of *FOE* and "*presumed-FOE"* isolates based on *SIX8, SIX9, SIX11* and *EF-1α*, was demonstrated via phylogenetic studies which showed non-clustering of *FOE* and "*presumed-FOE"* isolates on the bases of geographical location.

The oil palm-infecting *FOE* has not been classified into races (Gordon and Martyn 1997) unlike banana *F. oxysporum* f. sp. *cubense* isolates (Li et al. 2015). Some research on the pathogenicity of *FOE* isolates has been done (Flood et al. 1989; Flood 2006; Cooper 2011; Rusli et al. 2015) but was laborious and time consuming (Mouyna et al. 1996). The pathogenicity test in this present study showed that all four *FOE* were pathogenic to oil palm and this is congruent with reports (Flood et al. 1993; Rusli et al. 2015). The pathogenicity of 16F *Fusarium oxysporum* isolate collected

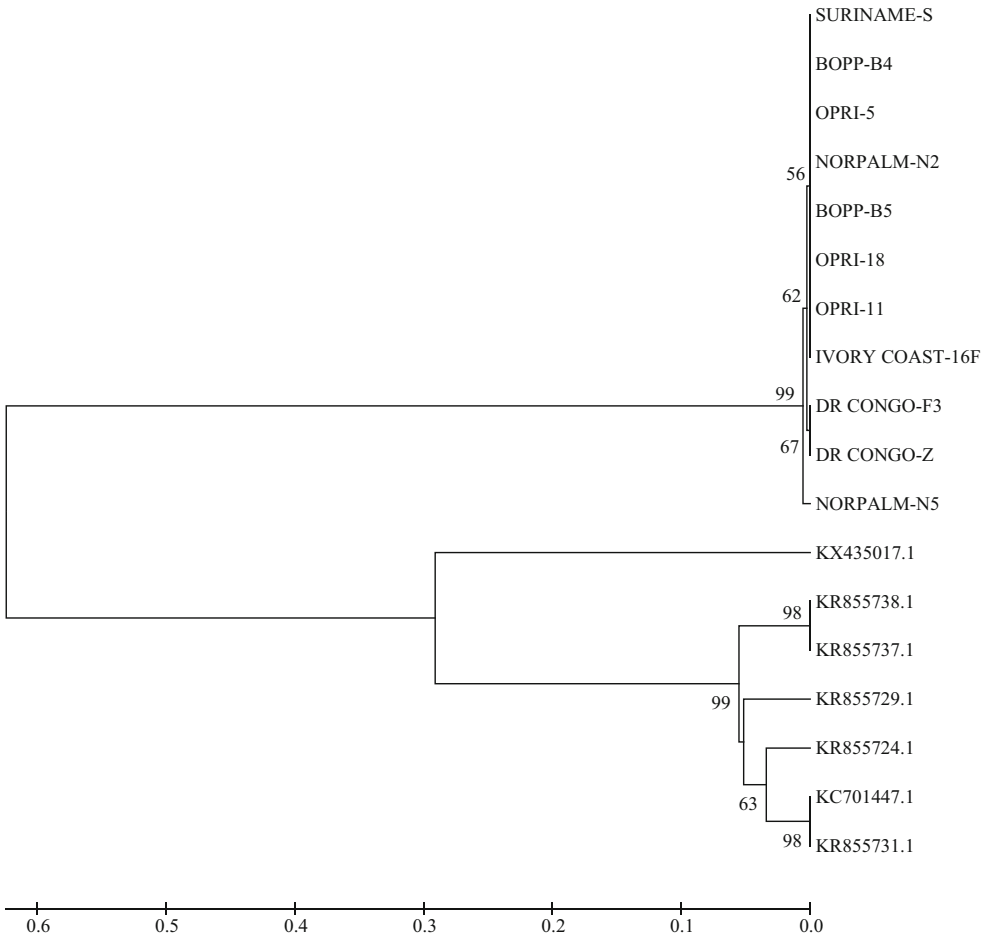

Fig. 7 Phylogenetic tree generated using statistical model Unweighted Pair-Group Method with Arithmetic Average (UPGMA) cluster composite date set from *Fusarium oxysporum* isolates from symptomatic oil palm and other host from GenBank based on *SIX9*. Bootstrapping was done for 1000 with bootstrap values shown in the nodes

from oil palm has been confirmed in a previous study (Rusli et al. 2015) but not the three *Fusarium oxysporum* isolates (BOPP-B5, NORP-5 and OPRI-5) collected from Ghana. Symptoms for *FOE* infection in oil palm seedlings showed at different times depending on the method of inoculation, indicating the complex nature of the *FOE* pathogen. The earliest time symptoms showed in inoculated oil palm seedlings via wounding of the roots and soaking in spore suspension, was 4 months under glasshouse conditions. However, Rusli et al. (2015) reported of severe symptoms at 25 weeks in Tenera (commercial type) oil palm. This shows that the protocol used for this current study, reduced the amount of time necessary for symptom development in oil palm seedlings compared to research conducted by Rusli et al. (2015), where higher concentration of 6 X 10^6 spores ml^{-1} was poured directly onto the soil surface around the base of the 3-month-old oil palm kept under glasshouse conditions. Other studies, Corley and Tinker (2003) pathogenicity test was in an open environment and the conditions optimized by providing shade to maintain the best temperature for pathogen invasion but the specific method of inoculation was not stated though plants showed late symptom development compared to the present study. Generally, there was yellowing of the leaves of the infected oil palm seedlings which is one prominent symptom of fusarium wilt disease (Flood et al. 1993; Flood 2006; Rusli et al. 2015).

There was an initial thought that *SIX* genes were unique for *F. oxysporum* f. sp. *lycopersici* (FOL). (Fraser-Smith et al. 2014) but in other *F. oxysporum* f. spp. (Rep et al. 2004; Lievens et al. 2009; Laurence et al. 2015; Rocha et al. 2016; Taylor et al. 2016). The identified *SIX* genes common to all four *FOE* used for pathogenicity test were *SIX8*, *SIX9* and *SIX11*. However, *FOE* OPRI-5 isolate was detected to have *SIX12* which was however, absent in the other three *FOE* isolates used for pathogenicity test. Taylor et al. (2016) reported similar results for *F. oxysporum* f. sp. *cepae* (*Foc*) in onion, as some *SIX* genes were either present or absent in both pathogenic and non-pathogenic isolates. This is the first time fourteen *SIX* (*1–14*) genes have been studied in four *FOE* confirmed to be pathogenic to oil palm and *"presumed-FOE"* isolates from Ghana, Ivory Coast, DR Congo and Suriname.

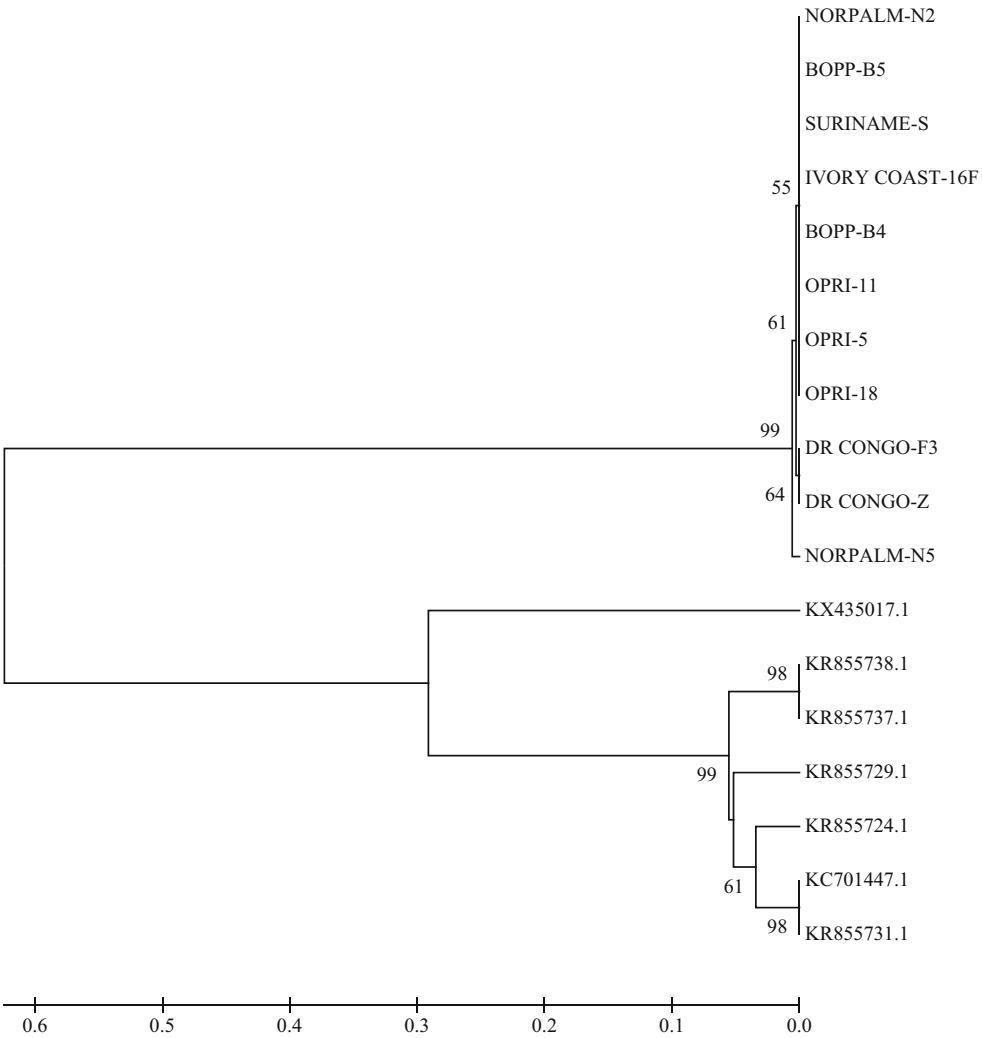

Fig. 8 Phylogenetic tree generated using statistical model Unweighted Pair-Group Method with Arithmetic Average (UPGMA) cluster composite date set from *Fusarium oxysporum* isolates from symptomatic oil palm and other host from GenBank based on *SIX11*. Bootstrapping was done for 1000 with bootstrap values shown in the nodes

However, conclusions cannot be made for this present study as to whether pathogenicity could be associated with the presence or absence of *SIX* genes in *FOE* as this would require further study. The phylogenetic tree generated from *SIX* genes (*SIX9* and *SIX11*) sequences grouped all *FOE* and *"presumed-FOE"* isolates into same cluster with known *Fusarium oxysporum* f. sp.-ID sequences from GenBank. On the contrary, *SIX8* did not cluster *FOE* and *"presumed-FOE"* from known *Fusarium oxysporum* f. sp. sequences-ID, as some isolates (DR Congo-Z, DR Congo-F3 and Suriname-S) clustered widely from the other *FOE* and "*presumed-FOE"* in the phylogenetic tree. The Genetic variation in *FOE* and *"presumed-FOE"* isolates based on the individual phylogenetic trees for *SIX8, SIX9* and *SIX11*is likely to be because *SIX* genes reside in the class II transposable elements (TEs) - enriched chromosomal sub-regions as reported (McDonald and Linde 2002; Ma et al. 2010; Schmidt et al. 2013). This characteristics of the *SIX* genes as reported (Ma et al. 2010; Schmidt et al. 2013) could have resulted in the failure of the clustering of the *FOE* isolates based on their geographical location where the isolates were sampled. A similar report showed the inability of *SIX* genes to differentiate Australian *F. oxysporum* f. sp. *canariensis* (*Foc*) isolates from international *Foc* isolates (Laurence et al. 2015). Laurence et al. (2015) further stated low sequence diversity among the *Foc* isolates based on the *SIX* genes and suggested horizontal gene transfer mechanisms as the cause. The diversity within *formae speciales* of *Fusarium* has been revealed by multiple studies that reported amino acid and nucleic acid sequences of *SIX* genes as highly conserved (Rep et al. 2004; Houterman et al. 2008; van der Doe et al. 2008; Thatcher et al. 2012) which is likely to have contributed to the genetic variation among *FOE, "presumed-FOE"* and known *Fusarium oxysporum* f. sp. sequences-ID from GenBank based on the *SIX8, SIX9* and *SIX11.*

The ribosomal DNA (rDNA) genes are useful for identification of plant pathogens (Abd-Elsalam et al.

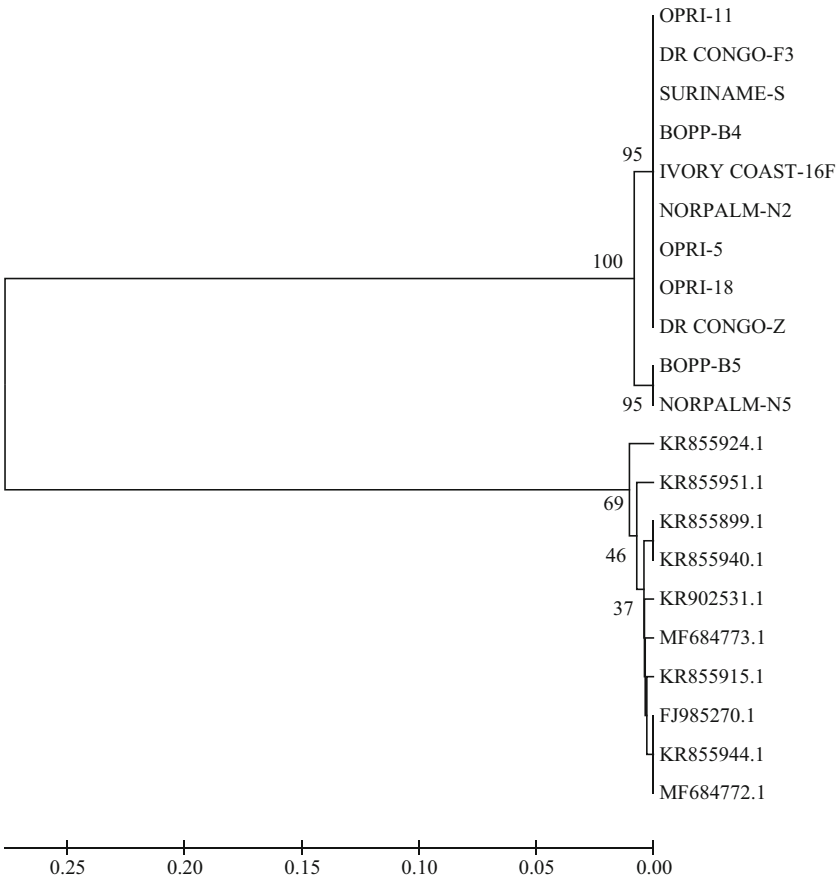

Fig. 9 Phylogenetic tree generated using statistical model Unweighted Pair-Group Method with Arithmetic Average (UPGMA) cluster composite date set from *Fusarium oxysporum* isolates from symptomatic oil palm and other host from GenBank based on *EF-1α*. Bootstrapping was done for 1000 with bootstrap values shown in the nodes

2003). Frequently used loci for studying the genetic variation within *Fusarium* species complexes is the *EF*-1α (Geiser et al. 2004; Laurence et al. 2015; Pinaria et al. 2015). The phylogenetic tree for *EF*-1α grouped all *FOE* and *"presumed-FOE"* isolates into a cluster separated from other *Fusarium oxysporum* f. sp.-ID sequences from GenBank. Similar results were reported for *EF*-1α phylogeny in *F. oxysporum* f. sp. *canariensis* (Laurence et al. 2015) and *F. oxysporum* f. sp. *vanillae* (Pinaria et al. 2015). The *EF*-1α showed genetic variation among the *FOE* and *"presumed-FOE"* as isolates from Ghana were mixed with Suriname, Ivory Coast and DR Congo within the same cluster in the phylogenetic tree. This observation is congruent with recent *EF*-1α phylogeny in *F. oxysporum* f. sp. *vanillae* sampled from Mexico (Flores-de la Rosa et al. 2018). Diversity among *FOE* and *"presumed-FOE"* based on *EF*-1α region is likely to be as a result of exchange of genetic material between the *FOE* and *"presumed-FOE"* isolates that lead to the switch of clades as reported for *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *radices-lycopersici* (Lievens et al. 2009).

In this study, pathogenicity assay developed has drastically reduced the time for symptom development and future researchers can be confident in this improved pathogenicity protocol developed. Different *SIX* genes have also been identified in both *FOE* and *"presumed-FOE"* isolates collected from Ghana, Suriname-S, Ivory-Coast and DR Congo. These isolates obtained from geographic origins where oil palm production is a significant part of their economy could tap into this knowledge and research further to improve on the management and control of fusarium wilt disease in oil palm. Future study should be conducted to verify if the three *SIX* genes identified within the four *FOE* isolates could be associated with pathogenicity.

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Compliance with ethical standards

Conflict of interest Author Dr. Kwasi Adusei-Fosu currently a Forest Pathologist with Scion (New Zealand Forest Research Institute), received research grant from Commonwealth Scholarship Commission-UK and was supervised by Co-author Prof. Matthew Dickinson, Senior Plant Pathologist, Diagnostician Researcher, and Lecturer at the University of Nottingham – UK.

Also, there is no conflict of interest in existence: Author Dr. Kwasi Adusei-Fosu declares that he has no conflict of interest. Author Prof. Matthew Dickinson also declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants performed by any of the authors. Further, this article does not contain any studies with animals performed by any of the authors.

This article does not contain any studies with human participants or animals performed by authors.

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