Improving the detection and control of *Fusarium oxysporum* f. sp. *elaeidis*

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Abstract

Fusarium wilt of oil palm, caused by a soil-borne fungal pathogen *Fusarium oxysporum* f. sp. *elaeidis* (*Foe*), is a major problem to oil palm cultivation and palm oil yield in Africa, Asia, and parts of North America. There is currently a shortfall in palm oil to meet local and international demands. Thus, factors constraining maximum yield are critical to address. This research was done to find potential methods of controlling *Foe* in Ghana and if possible extended to other countries experiencing similar problems. Effective disease control will come from understanding the genetic variability of *Foe*, a combination of selection and release of resistant germplasm, and rapid detection of *Foe*. In-depth molecular fingerprinting via AFLP, SSR, ISSR as well as using the "housekeeping" genes (*TEF* and *ITS*) and effector proteins specifically *SIX* genes as molecular markers revealed genetic variations among different isolates of *Foe* from Africa (Ghana, Ivory Coast, DR Congo) and South America (Suriname) as shown in the various constructed phylogenetic trees in the study. This research has introduced improved measures to obtain resistant oil palm germplasm from the high local genetic diversity via pathogenicity studies. Pathogenicity assessment under green-house conditions through different inoculation techniques revealed four *Foe* isolates could cause infection in oil palm genotypes from Ghana and Malaysia. Effectors based on fourteen *SIX genes* were studied and primers with higher specificity designed for the LAMP technique for only *Foe* detection or diagnosis on site developed. Different LAMP primer sets were developed in this study to distinguish or discriminate only *Fusarium oxysporum* isolates from other pathogens. A *P-450* cytochrome gene LAMP primer was also successfully developed in this research. Survey of the disease was conducted in four different oil palm plantation fields in Ghana. Tissue samples were collected from symptomatic oil palm trees neighbouring asymptomatic oil palm trees in fields of

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Ghana as part of the survey. All tissue samples from both symptomatic and asymptomatic oil palm tested positive for *Foe* infection via PCR. General observations in the oil palm plantations in Ghana showed that infection of *Foe* occurred in clusters among oil palm trees. Application of different control mechanisms against *Foe* with biological agents (*Trichoderma* sp.), chemical (sodium silicate pentahydrate - $Na_2O_3Si.5H_2O$) or photochemical (Conventional UV or High Intensity Pulsed Polychromatic source) techniques all proved to be potential methods to suppress or totally inhibit *Foe.* Among the three *Trichoderma* sp. used in the study, *Trichoderma harzianum* (T3) was the most suppressive to the four *Foe* isolates, though the other two *Trichoderma* isolates, *T. cerinum* (T1) and *T. atroviride* (T2) showed potential as biological control agents (BCAs). The three different concentrations of $Na_2O_3Si.5H_2O$ test yielded varying results as increasing concentrations increased the percentage inhibition in all four *Foe* isolates used. The lethal dose for *Foe* spores with either a High Intensity Pulsed Polychromatic or Conventional UV sources was 60 KJ but oil palm pollen exposed to this dose did not germinate. Generally, the higher the dose of UV, the lower the percentage germination of the oil palm pollen in this study.

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List of Abbreviations

AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
Avr2	Avirulent 2
BCA	Biological Control Agent
BOPP	Benso Oil Palm Plantation
bp	Base pair
BW	Bunch weight
CPO	Crude palm oil
CWDE	Cell wall degrading enzymes
D	Dura
DNA	Deoxyribonucleic acid
DRC	Democratic Republic of Congo
f.sp.	formae speciales
FAO	Food and Agriculture Organization of the United Nations
FAPRI	Food and Agricultural Policy Research Institute
FDR	Fluorescein Diacetate Reaction
FFB	Fresh fruit bunches
Fg	*Fusarium graminearum*
Fo	*Fusarium oxysporum*
Foc	*Fusarium oxysporum* f. sp. *cepae*
Foc	*Fusarium oxysporum* f. sp. *cubense*
Foe	*Fusarium oxysporum* f. sp. *elaeidis*
Fol	*Fusarium oxysporum* f. sp. *lycopersici*
Fon	*Fusarium oxysporum* f. sp. *niveum*
Fov	*Fusarium oxysporum* f. sp. *vasinfectum*
Foxy	*Fusarium oxysporum*

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gao	Galactose oxidase
gDNA	Genomic deoxyribonucleic acid
GOPDC	Ghana Oil Palm Development Company
I2CD	I-2-mediated cell death
ISSR	Inter Simple sequence repeat
ITS	Internal transcribed spacer
Kbp	Kilo base pair
KJs^{-1}	Kilo Joules per second
LAMP	Loop Mediated Isothermal Amplification
LS	Lineage specific
M/F	Mesocarp to fruit ratio
MAT	Mating type
MPOB	Malaysia Palm Oil Board
mtDNA	mitochondrial deoxyribonucleic acid
NCBI	National Centre for Biotechnology Information
nm	Nano meter
NORP	Norpalm
O/B	Oil to bunch ratio
OPRI-CSIR	Oil Palm Research Institute-Council for Scientific and Industrial Research
P	Pisifera
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PKO	Palm kernel oil
pl	Pectate lysase
plg	Polygalacturonase

List of Abbreviations

QPCR	Quantitative Polymerase chain reaction
RAPD	Random amplified polymorphism
RFLP	Restricted fragment length polymorphism
RNA	Ribonucleic acid RNA
RT-LAMP	Reverse Transcription Loop-Mediated Isothermal Amplification
RT-PCR	Real Time-Polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SCAR	Sequence Characterized amplified region
SIX	Secreted in xylem
SSA	Sub-Sahara Africa
SSR	Simple sequence repeat
T	Tenera
t ha^{-1}	Tonnes per hectar
TEF	Translation elongation factor
TEs	Transposable elements
TOPP	Twifo Oil Palm Plantation
UK-DEFRA	United Kingdom-Department for Environment Food and Rural Affairs
UPGMA	Unweighted pair group method using arithmetic mean
UV	Ultra voilet
uWcm^{-2}	Micro watts per centimetre squared
USDA	United States Department of Agriculture
VCGs	Vegetative compatibility groups
W/A	West Africa
xly	Xylanase

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UoN	University of Nottingham

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CHAPTER ONE: INTRODUCTION

1.0 *General introduction*

Oil palm (*Elaeis guineensis* Jacq.) is currently the highest oil yielding crop in the world and contributes close to 30 % of edible vegetable oil requirements across the globe (Corley, 2009; Hansen *et al.,* 2015). *Elaeis* is the genus, according to Jacquin (Williams and Hsu, 1970), and the botanical name of *Elaeis* was derived from the Greek word *elaion*, which means oil. In addition, its specific name *guineensis* indicates that it was believed by Jacquin to originate from the Guinea coast. The genus includes two species, *E. guineensis* and *E. oleifera* from West African and American origin respectively (Zeven, 1965). From its home in West Africa, the oil palm (*Elaeis guineensis* Jacq.) has been spread throughout the tropics and is now grown commercially in more than 16 countries. It is common in almost all the ten regions of Ghana, especially the Eastern region, and in Ghana, oil palm production is divided between large estates managed by publicly listed companies, smaller independent estates, independent small holders and government small holder settler schemes.

The oil palm has been projected to be the world's highest oil producing crop in 2020 (Kushairi *et al.,* 2010). There are three major different forms of oil palm, including the Dura (D), Pisifera (P) and Tenera (T) varieties. These are distinguished on the basis of their shell and mesocarp thickness (Muniran *et al.,* 2008). The

commercially produced oil palm crop is the Tenera (T) form which is a cross between the D×P and it is normally supplied to farmers as living plant material.

Oil palm starts producing oil-bearing fruit bunches as early as two and a half years old if good quality planting materials and appropriate agronomic practices are used. The lifespan of oil palm, as demonstrated by specimens planted in the Oil Palm Research Institute (OPRI), Kusi, Ghana, is at least 70 years. However, the crop is generally grown for 25-30 years then replaced with young palms.

The significant contribution of oil palm globally is as a result of committed research in marketing its oil and fat. In some parts of the world such as Malaysia, the success of the oil palm is associated with many factors including favourable climatic conditions, good infrastructure, management skills or technology for oil palm cultivation and a land ownership structure which favours an estate type of agriculture (Basri *et al.*, 2003). On the contrary in Ghana, the only successes for oil palm production are linked to favourable weather and private investors, with a lack of the remaining factors that make Malaysia production superior, even though the crop was introduced into Ghana before Malaysia. To become competitive and to ensure agricultural sustainability of the oil palm in Ghana, appropriate disciplines such as crop physiology, agronomy, plant genetics, entomology, plant tissue culture and plant biotechnology, must be strategically planned and implemented along with effective pathological studies.

Basri *et al*. (2003) reported that, aside from increasing the total yield of Fresh Fruit Bunches (FFB) in oil palm, several areas of oil palm are being improved, which include breeding and selection focusing on achieving high FFB oil and kernel content, and vegetative characters where reduced rates of trunk extension and long bunch stalks are desirable attributes to facilitate harvesting, while compact palms may allow higher planting densities. However, for all these to be successful the oil palm needs to be healthy, hence the need for studies on its diseases and their control.

Oil palm is crucial to Ghana providing income to major companies including GOPDC - Ghana Oil Palm Development Company, BOPP - Benso Oil Palm Plantation, TOPP - Twifo Oil Palm Plantation and NORPALM, and these vibrant companies are associated with local employment. Ofosu-Budu and Sarpong (2013) confirmed that palm oil is a key source of income through trade at border districts. They further reported that after cocoa, oil palm is the second most important cash crop in Ghana (Angelucci, 2013; Ofosu-Budu and Sarpong, 2013). However, significant amounts of palm oil are still imported. For example, in 2011, 74,431 t palm oil at a cost of US$ 83 million was imported into Ghana (FAO, 2014). This is due to reduction in yield of oil palm fruit bunches (FB) in Ghana from 6.5 t ha^{-1} in 1990 to 5.4 t ha^{-1} in 2012 (FAO, 2014). This reduction has been associated with poor climatic conditions (Rhebergen *et al.,* 2016) and issues in relation to disease, such as fusarium wilt. It has been reported in other countries that fusarium wilt (vascular wilt) disease is reducing the yield and killing oil palms across the world making targets set by stake holders in the oil palm industry unreachable (Lai *et al,* 2012).

Disease control can best be achieved through a combination of selection and release of resistant germplasm and understanding epidemiology and the variability of *Fusarium oxysporum* f. sp. *elaeidis.* According to Elliot *et al.* (2010), screening for resistance at the nursery stage has been introduced in many plantations and research stations over a period of 30 to 40 years, and successful breeding programme in West Africa (W/A), notably in the Ivory Coast, have resulted in more resistant oil palm materials becoming available. The success of eliminating the fusarium wilt disease in W/A, which includes Ghana, is vital as there is rapid expansion of oil palm cultivation across the globe due to the rise in demand for palm oil. Because of the reduction of favourable land availability in some parts of the world such as Southeast Asia, there are prospects for future expansion in other areas, especially in sub-Saharan Africa (SSA), and in Central and South America as reported by Laurance *et al*. (2014).

1.1 *Origin and distribution of oil palm*

It is generally accepted that the oil palm originated in the tropical rain forest region of West Africa. The main belt runs through the southern latitudes of Ghana, Cameroon, Côte d'Ivoire, Liberia, Nigeria, Sierra Leone, Togo, equatorial region of Angola and the Congo (Soh *et al.,* 2009). In Ghana, the most popular region for oil palm cultivation is in the south (Fig. 1.1).

Oil palm seeds were brought by the Dutch from West Africa to Indonesia and propagation started there in the 19th Century. This process finally resulted in four

seedlings planted in Bogor Indonesia in 1848. The oil palms were Dura and the progenies from these seedlings were planted as ornamentals in Deli and became known as Deli Dura. Most of the planting materials since 1961 have been Tenera (i.e. the progeny from the crossing of Deli Dura x Pisifera), which has become the sole commercial planting material first cultivated in Malaysia (Corley and Tinker, 2003) and has gradually been extended to other countries including Ghana.

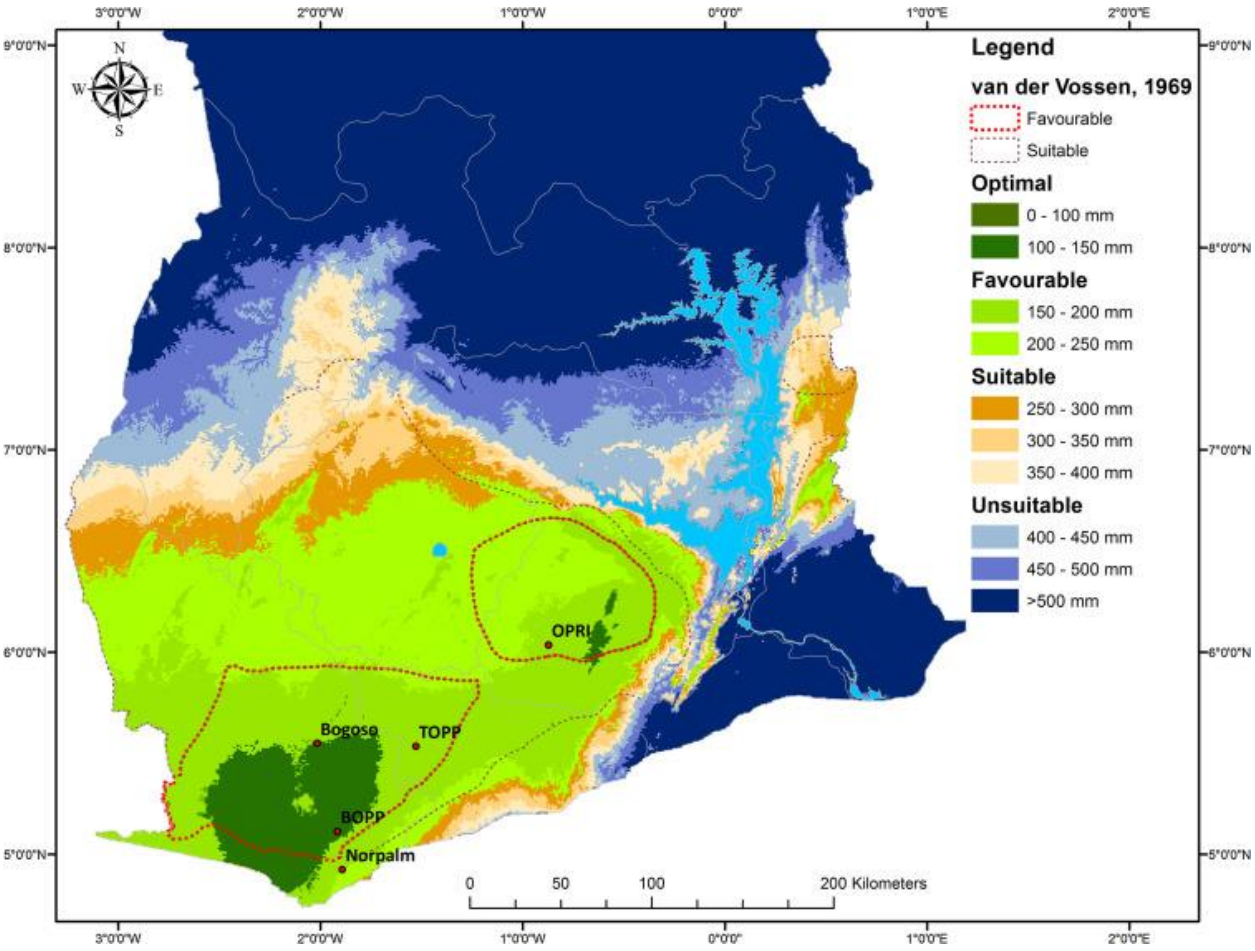


Figure. 1.1. Map of southern Ghana showing areas climatically suitable for oil palm production (≤400 mm year^{-1} water deficit) in steps of 50 mm water deficit, compared to suitable areas identified by van der Vossen (1969).

1.2 *The oil palm plant*

The oil palm is a perennial tree crop that grows to a height of between 5 -20 m. In middle age, the leaves extend nearly parallel to the soil for about 3 - 7 m. Each is composed of some 250 linear leaflets, which grow out of the sides of the stalk, but in an irregular manner. The disorderly appearance of the leaves is one of the species characteristic features. The oil palm's productive life can last as long as 50 years; however, after 20 or 25 years its stalk reaches a height that makes it difficult to harvest and it is therefore necessary to replace it in commercial plantations. The stalk of the oleaginous plants is erect and has the form of an inverted cone. When young it is rough, because of the stalk bases that cover it. At maturity, even though the stem of the plant is smooth, it looks segmented because of the scars left by its approximately 40 leaves when they dry and fall off. The age of the oil palm can be determined from how smooth or rough the stem is (Okwuagwu *et al.,* 2011). The smoother the stem of the oil palm tree, the older it is and vice versa.

The oil palm is a monoecious plant. It has male and female inflorescences that develop in cycles and as such self-pollination is reduced (Turner and Gillbank, 1974). From the female inflorescence appear thousands of spherical, oval or elongated branches that make up compact bunches weighing between 10 and 40 kg or more when pollination takes place. The oil palm fruit, when developing, is initially dark purple in colour and almost black, after which it turns into a bright reddish orange colour when it matures. Inside the oil palm fruit is a seed, the

kernel, which is protected by the cover or stone (shell), and the endocarp is surrounded by a fleshy pulp. Oil can be obtained from both the kernel and the pulp. The kernel oil is processed from the kernel while the red palm oil is produced from the pulp.

1.3 *Classification on fruit form bases*

There are three fruit-forms of the oil palm. The first, homozygote dominant (TT) known as Dura (D) has a thick shell while the other homozygote recessive (tt) Pisifera (P), is shell-less. The product of DXP is Tenera (T) (Fig. 1.2), the commercial type to produce palm oil. Tenera has a thin shell, surrounded by a ring of fibres in the mesocarp. This results in more oil-bearing mesocarp in the Tenera than in the thick shelled Dura fruit (Seng, 1987). In the Dura fruit-form, the percentage of mesocarp to fruit (M/F) is variable, usually within the range of 35 - 50 %. The shell is comparatively thick, ranging between 2 – 8 mm, and has no ring of black fibre around it. The kernel is usually large. Extractable oil content of Dura mesocarp in proportion to bunch weight (BW) is quite low at 17 - 19 %. Most Dura materials from Asia have been derived from the four palms grown in Bogor, Indonesia. Comparatively, the Indonesian form is superior to most Dura materials found in Africa, though oil palm prospection has revealed some very promising additions from other countries such as Ghana. Dura is normally used as the female parent in oil palm breeding. Whereas the Dura and Tenera palms have shells, the Pisifera fruit-form is characterised by the absence of a shell, vestiges of which are

represented by the ring of fibres around the kernel. The absence of shell in Pisifera gives it a high ratio of mesocarp to total fruit (M/F) size. In addition, the mesocarp oil content is also high. Pisifera is usually described as sterile female since the majority of its bunches abort at an early stage of development. Fully-fertile Pisifera are known but most have proven to be poor parents, although it has been suggested that certain Pisifera individuals could be used on a commercial scale. Generally, Tenera is the standard variety planted for commercial purposes and supplied to farmers in Ghana; Tenera has the combined characteristics of the parent material (D x P) or is simply called a hybrid. The shell thickness ranges from about 0.5 – 4 mm, with a characteristic ring of black fibres. The ratio of mesocarp to fruit (M/F) is comparatively high, usually in the range of 60 - 95 %. Commercial Tenera palms generally produce more fruit bunches (FB) than Dura, although mean bunch weight (BW) is lower. In all, the Tenera is a much better variety for industrial and economic purposes. Turner and Gillbanks (2003) reported that the extractable ratio of oil to bunch (O/B) is normally in the range of 22 - 25 %, but individual progenies are known to produce much higher amount, with figures of up to 30 %.

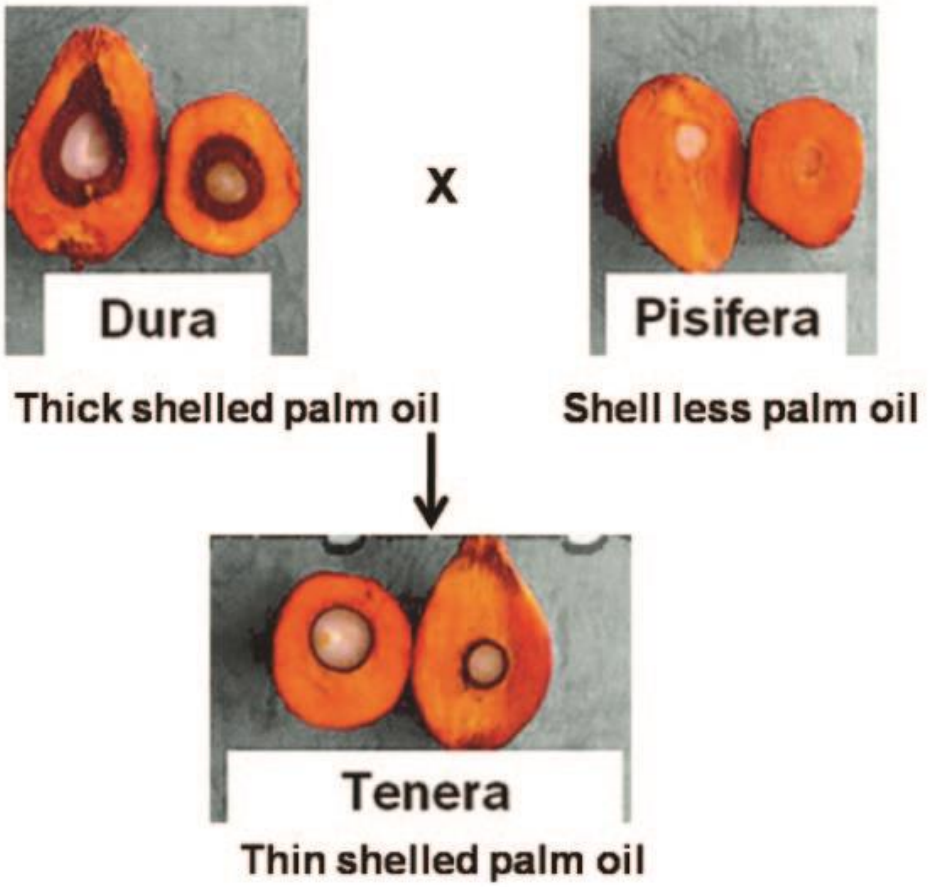


Figure. 1.2 Fruits forms of oil palm (Source: Sharifah Shahrul Rabiah Syed Alwee, FGV R&D)

1.4 *Importance of oil palm*

Pauron and Ballong (2012) reported that approximately 2.5 million hectares of land have been recently given to oil palm major investors in about twenty countries of the Western, Central and Southern parts of Africa ranging from Gambia to Cameroon, Angola, Mozambique up to Madagascar. The period for which these lands are to be used by the major foreign groups for the establishment of oil palm plantations has not been made clear. This extensive planting is due to the economic importance attributed to the oil palm crop. Recently, according to Ngando *et al.* (2013), the oil palm has overtaken soya bean since the mid-2000s to become the most important oil crop in the world. This report is supported by Soh *et al.* (2010) who reiterated that the oil palm is the world's most important oil crop, producing 24.9 % of total vegetable oils and fats, surpassing soybean at 23.9 %. This increment of palm oil production has been mainly supported by intensive planting of the oil palm during the two last decades in Malaysia and Indonesia which are by far the two main producers of palm oil in the world. Further to this, currently there have been tremendous FB yields ranging between 18.5 – 19.0 t ha^{-1} in Southeast Asia and Latin America (FAO, 2014). In addition, it has also provided employment to millions across the world in areas where it is cultivated such as Ghana. In the West African region governments are paving the way for local and foreign investors (Ofosu-Budu and Sarpong, 2013).

The oil palm produces two types of oil from its fruits, mesocarp oil and kernel oil, known in international trade as crude palm oil (CPO) and palm kernel oil (PKO), respectively. Soh *et al.* (2010) reported that oils are produced from some 13 million hectares of plantations in the humid tropical countries of Asia, Africa and Latin America: Indonesia (5.3 million ha), Malaysia (4.2 million ha), Papua New Guinea, Colombia, Ivory Coast, Nigeria and Thailand with the first two countries having the bulk of the plantings. Total world production of palm oil (CPO) in 2015 stood at approximately 58.84 million tonnes (USDA, 2016).

Palm oil is the largest internationally traded vegetable oil 80 % is used in food such as in cooking oil, margarine, vegetable ghee and the remaining 20 % used as oleochemicals replacing mineral oil to feed the detergents, cosmetics, pharmaceuticals or nutraceuticals, plastics and lubricants industries (Soh *et al.,* 2010). Furthermore, in terms of 'green' or renewable energy substitution, there has been a tremendous demand for palm oil as a source of biofuel (biodiesel). Yunus *et al.* (2012) reported that responding to consumer health and environmental concerns, secondary and by-products from the palm oil industry have spawned new industries. Vitamins A, E and other antioxidant health supplements from the oil, animal feed and organic fertilizers from the kernel, and sludge cakes and wastes from oil extraction mills have served as value added products. The Food and Agricultural Policy Research Institute (FAPRI) has also reported on the projection of palm oil production around the world until year 2025 and confirmed the increasing demand (Fig. 1.3).

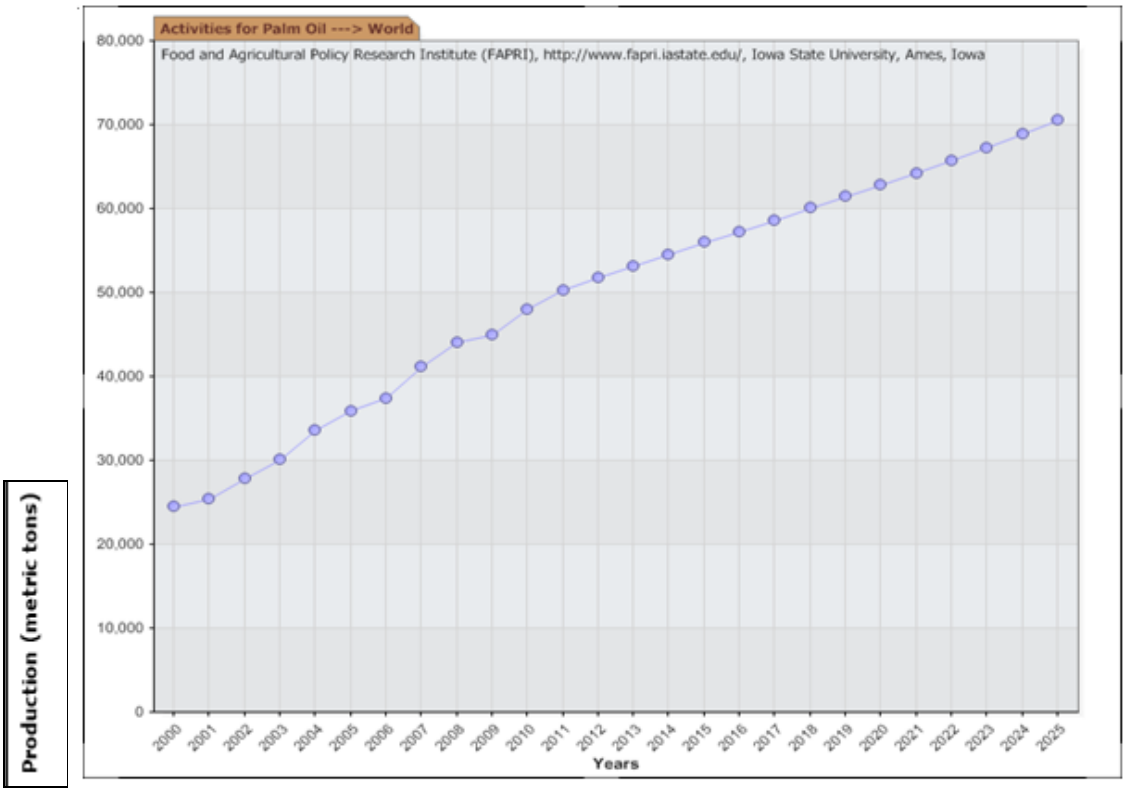


Figure. 1.3 World Production of Palm Oil Projected to 2025 (Source: FAPRI World Agriculture Outlook 2000-2025)

1.5 *Diseases of oil palm*

Diseases of oil palm in Africa include fusarium wilt or vascular wilt (which are widespread across oil palm growing regions), bacterial bud rot (in the Democratic Republic of Congo), and dry basal rot. Fusarium wilt is a serious problem in some African countries including Ghana but the disease is not significant in East Asia where rigorous quarantine measures have been imposed by the Malaysian and Indonesian authorities to prevent introduction of the pathogen. *Fusarium oxysporum* is a very large complex of pathogens with wide divergence in host

species that are attacked. Vascular wilt is the most destructive disease in oil palm and can cause severe losses. Other diseases of oil palm include; basal stem rot, and bud rot (Turner, 1981; Torres *et al.,* 2010).

1.5.1 *Fusarium wilt disease*

Plant diseases affect the physiological make-up of the host plant they attack and mostly end up causing yield reduction. Fusarium wilt is currently the major threat to oil palm cultivation yield and palm oil production in Africa and this disease threatens the other remaining continents. The causal agent is *Fusarium oxysporum* f. sp. *elaeidis* (*Foe*). *F. oxysporum* is divided into *formae speciales* (f. sp.) based on pathogenicity to one or more plant hosts. Over 150 f. sp. have been described, but many isolates are saprobes (Baayen *et al.*, 2000; Lievens *et al.*, 2008). There are four *F. oxysporum* f. sp. that affect palms worldwide namely, *canariensis, albedinsis, palmarum* and *elaides* in *Phoenix canariensis*, *Phoenix dactylifer*, *Washingtonia robusta* and *Elaeis guineensis,* respectively.

1.5.2 *The Fusarium*

The genus Fusarium is a filamentous fungi distributed widely on plants and in the soil. Pitt *et al.* (1994) reported that it is found in the normal mycoflora of commodities such as rice, bean, soybean, and other crops. *Fusarium* is a cosmopolitan soil inhabiting fungus which is known to be phylogenetically diverse.

Fusarium is a genus of the hyphomycetes, formerly classified in the Deuteromycetes or Fungi Imperfecti. Sixteen sections have been recognised within the genus namely *Eupionnotes, Macroconia, Spirarioides, Submicrocera, Pseudomicrocera, Arachnites, Sporotrichiella, Roseum, Arthrosporiella, Gibbosum, Discolor, Lateritium, Liseola, Elegans, Martiella* and *Ventricosum*. Though many different articles have emerged on the classification of *Fusarium* species, the most widely accepted is that of Nelson *et al.* (1981). Based on examination of fruiting structures (sporodochia) on plant material, an estimated 1000 *Fusarium* species had been described by the year 1900. Wollenweber and Reinking (1935) reduced the number of species to 65, 55 varieties and 22 forms, in 16 sections, and since that time all taxonomic classifications have been based on this system (Burgess *et al.,* 1994). Burgess *et al.* (1994) further suggested de-limitation and stressed the importance of analysing differences in a large number of cultures from a broad range of geographic sources and substrates.

Fusarium species comprise pathogens and saprophytes, and occur on most vegetative and reproductive parts of plants (Arif *et al.,* 2012); there are non-pathogenic strains as well. They are found on nearly all plant species in most parts of the world. Though *Fusarium oxysporum* (*Fo*) has a broad host range, individual strains usually infect only a single or a few plant species. These individual fungal strains usually show a high level of host specificity. Armstrong and Armstrong (1981) reported that this has resulted in classification of more than 120 *formae speciales*. However, according to Lievens *et al.* (2008), some *Fusarium oxysporum* (*Fo*) *formae speciales* such as *radicis-cucumerinum* and *radicis-lycopersici* have

broader host ranges. Thus, apart from infecting cucumber and tomato respectively, these can cause root and stem rot on multiple hosts from different plant families. Further sub-divisions based on cultivar specificity of isolates from a particular *forma specialis* are classified into physiological races. More so, *Fusarium oxysporum* (*Fo*) strains have been grouped into Vegetative Compatibility Groups (VCGs) (Puhalla, 1985). VCGs are defined as the genetic relationships of strains based on their ability to form heterokaryons. Based on VCGs, there are monophyletic, paraphyletic and polyphyletic groups. Monophyletic groups descend from a single common ancestor and are sometimes known as "clades". Paraphyletic groups include all but one descendent of a single common ancestor while polyphyletic groups have multiple origins and do not share a common ancestor. Katan *et al.* (1989; 1991) reported that different *formae speciales* and races may contain multiple VCGs, which This is important for identification below the species level.

1.5.2.1 *Fusarium oxysporum f. sp. elaeidis* (*Foe*)

The pathogen *Fusarium oxysporum* f. sp. *elaeidis* (*Foe*) is a soil borne fungus which causes fusarium wilt disease in oil palm. The perennial nature of the oil palm has made the management of the disease challenging (Ho *et al.*, 1985; Ho and Varghese, 1986; de Franqueville and Diabaté, 2004; Elliot *et al.,* 2010). The first description of the disease was provided by Wardlaw (1946) in the Democratic Republic of Congo (DRC), who isolated the pathogen from diseased palms. Fraselle (1951) later artificially reproduced the disease symptoms on seedlings and

confirmed the pathogenicity of the isolates. Following isolation in the Democratic Republic of Congo, further reports were made specifically in Western and Central Africa (Aderungboye, 1982; Corley and Tinker, 2003). In Cameroon, it was observed in the Ndian Estate of Pamol Plantations that the disease was spreading rapidly from the neighbouring Cowan Estate in Nigeria as far back as 1940 (Prendergast, 1957), indicating its ability to spread and survive from one country to another. In Ghana, Tagoe (2002) reported fusarium wilt symptoms in oil palm for some selected fields of the Oil Palm Research Institute (OPRI) of the Council for Scientific and Industrial Research (CSIR) - Ghana.

1.5.2.1.1 *Ecology and biology of Fusarium oxysporum*

The pathogen has been shown to penetrate roots through the loosely packed cells at the base of pneumathodes (Locke and Colhoun, 1973) as well as via wounds on the plant (Kovachich, 1948). Prendergast (1957) as well as Renard and de Franqueville (1989), suggested that in the field, spread occurred through root contact with dead infected palm tissues. This proposal is supported by the occurrence of infected palms in pairs or groups, and the greater infection of palms with missing neighbours than those without (Dumortier *et al*., 1992; Rusli *et al.,* 2012). Also, healthy palms in dispersed areas with fusarium wilt infected palms on the same field have a lower probability of being infected as compared to healthy palms that are in densely populated areas with infected palms. Mepsted (1992) and Mouyna (1996) further reported that, even in an area with a high incidence of wilt,

the vast majority of soil isolates of *F. oxysporum* are non-pathogenic. However, according to Flood *et al*. (1990) and Mepsted (1992), in a limited survey, pathogenic isolates of *F. oxysporum* were collected from pollen surfaces of male inflorescences as well as from both the outside and inside of oil palm seeds. It was concluded that epidemiological consequences of the aerial spread of the pathogen and the production of significant numbers of spores on the large male inflorescence are unknown; while contaminated seed and pollen may spread the pathogen to previously disease-free areas. Environmental factors have also been reported to support the development of *Foe* (Waterston, 1953; Prendergast, 1957; Aderungboye, 1982). After invading the host, *Foe* survives as thick walled chlamydospores in infected oil palms. It then systemically spreads to the entire plant via the vascular system (Turner, 1981). According to Lugtenberg *et al*. (2001), the spores of *F. oxysporum* sp. normally survive in the presence of several suitable nutrients such as organic acids with citric, malic, lactic, and succinic acid, which were present in tomato xylem sap including glucose, xylose, and fructose as the main monosaccharides; as well as amino acids but at a lower concentration than these organic acids.

In infected host plant, the disease is characterised by discolouration and blockage of xylem vessels with tyloses and gums (Wardlaw, 1950; Prendergast, 1957; Cooper, 2011). The presence of the tyloses and gums in the host xylem vessels is a plant response to a 'problem', in this case the presence of the *Foe* in the oil palm. On the other hand, *Foe* releases cell wall degrading enzymes (CWDE) to break down the vascular system of the host. These concurrent activities in the host and

the pathogen, cause a blockage of the xylem, occluding the flow of water in the host, which leads to wilt (Jonkers *et al*., 2009) and subsequent death of the host in extreme cases. This process normally results in yield losses (Mepsted *et al.,* 1995). According to Mepsted (1992), Prendergast (1957) and Wardlaw (1950), in highly diseased palms most roots show no signs of infection.

Prendergast (1957) reported that the pathogen can attack oil palm at all stages from seedling to mature palm. At the nursery stage, infected oil palms show progressive shortening of younger leaves and desiccation and death of older leaves (Prendergast, 1957). These symptoms are thought to result from a combination of water stress and changes to activities of the plant hormone levels such as gibberellin in the oil palm, as reported by Mepsted *et al.* (1995). However, clear symptoms are normally seen in palms of four years and above (Flood, 2006). On immature palms, leaves in the middle of the crown become yellow or brown; this symptom first spreads to lower neighbouring leaves but eventually the palm will totally desiccate and die (de Franqueville and Diabate, 1995). Further reports suggested that in mature palms the disease can exist in two forms, chronic (Fig. 1.4a) and acute which leads to subsequent death of the plant (Fig. 1.4b). In the chronic form, the older leaves become dried; the rachis breaks near or at some distance from the base and hangs down around the trunk. The disease continues to progress gradually, with younger leaves becoming successively affected whilst the erect young leaves in the crown are much reduced in size and may become chlorotic; the apex of the trunk may also reduce in diameter. Surprisingly, oil palms can exist in the chronic condition for several years, but fail to produce higher (FFB)

fresh fruit bunches (Wardlaw, 1950; Waterston, 1953; Guldentops, 1962; Renard and Quillec, 1983; de Franqueville and Diabaté, 1995) which eventually reduces the yield of the crop. Alternatively, an oil palm may display the acute form of the disease. Cooper (2011) reported that the acute form of the disease progresses rapidly and palms die within 2 or 3 months during which leaves dry out and die rapidly while retaining their original erect positions on the plant, until broken off by the wind. Various intermediate stages between the acute and chronic forms may occur. According to De Franqueville and Renard (1990), there is a third category of disease symptom that exists, where temporary wilt occurs, and the oil palm develops leaf symptoms but later recovers.

Losses ranging from death of 1 - 2 % of oil palms per annum were recorded by Cooper (2011) to chronic and acute wilt resulting in 30 % and 54 % reduction respectively.



Figure. 1.4 (a) Chronic symptom in oil palm tree and **(b)** Dead oil palm tree at the Oil Palm Research Institute, Ghana, West Africa.

1.5.2.2 *Pathogen host range and virulence*

The host range of *Foe* includes domesticated crops and wild hosts. *Foe* is pathogenic to the artificially inoculated South American oil palm *Elaeis oleifera* (Renard *et al*., 1980). Paul (1995) showed that under laboratory conditions, *F. oxysporum* isolates pathogenic to oil palm caused vascular wilt of date palm,

while date palm isolates (*F. oxysporum* f. sp. *albedinis*) were pathogenic to oil palm.

Most disease causing organisms are confirmed as virulent when they disrupt the physiology make-up of the host. As well as the cell wall degrading enzymes (CWDE) that *Foe* secretes when in contact with the host, protein effectors are secreted during the interaction between the pathogen and the host (Rusli *et al.,* 2014). To promote host colonization, many plant pathogens secrete effector proteins that either suppress or counteract host defences (Houterman *et al.,* 2009). When these effectors are recognized by the host defence system, they trigger resistance rather than promoting virulence. As such, effectors are therefore key molecules in determining disease susceptibility or resistance. *Fusarium oxysporum* has expressed several forms of virulence to its respective hosts upon the secretion of different effector proteins (Houterman *et al.,* 2007; Houterman *et al.,* 2008; Houterman *et al.,* 2009; Gawehns *et al.,* 2014). CWDEs are secreted by *F. oxysporum* upon interaction with a host; however, the activities of CWDEs such as pectate lysase gene *pl1*, xylanase genes *xly3*, *xly4* and *xly5*, polygalacturonase genes *pg1*, *pg5* and *pgx4* can be inactivated (Di Pietro *et al*., 1998; Huertas-Gonzalez *et al*., 1999; Di Pietro *et al*., 2003). More so, some avirulent gene (*Avr*) such as *Avr2* secreted by the vascular wilt fungus *F. oxysporum* f. sp. *lycopersici* (*Fol*), is required for full virulence in a susceptible host and also triggers resistance in tomato plants carrying the resistance gene *I-2* (Houterman *et al.,* 2009). Their study reported that point mutations in *Avr2*, causing single amino acid changes, were associated with *I-2*-breaking *Fol* strains. These point mutations prevent

recognition by I-2, both in tomato and when both genes are co-expressed in leaves of *Nicotiana benthamiana*. *Fol* strains carrying the *Avr2* variants are equally virulent, showing that virulence and avirulence functions can be uncoupled. Although *Avr2* is secreted into the xylem sap when *Fol* colonizes tomato, the *Avr2* protein can be recognized intracellularly by I-2. Further research on the *SIX6* (Secreted In Xylem) gene could suppress I-2-mediated cell death (I2CD) upon transient expression in *N. benthamiana*, whereas it did not compromise the activity of other cell-death-inducing genes; this I2CD suppressing activity of *SIX6* did not allow the *Fol* to overcome I-2 resistance in tomato, suggesting that I-2-mediated resistance was independent from cell death (Gawehns *et al.,* 2014).

Lievens *et al.* (2009) reported other *SIX* genes in *F. oxysporum* isolates: *SIX6* and *SIX7* genes were found in *F. oxysporum* f. sp. *lilii, F. oxysporum* f. sp. *melonis* and *F. oxsporum* f. sp. *radicis-cucumerinum*.

Induction of S*IX1* expression is reported to start immediately upon penetration of the root cortex and requires living plant cells, but is not host specific and does not depend on morphological features of roots, since plant cells in culture can also induce *SIX1* expression (van der Doe *et al.,* 2008). van der Doe *et al.* (2008 concluded that *F. oxysporum* was able to distinguish between living and dead plant material, preventing unnecessary switches from a saprophytic to an infectious lifestyle.

1.5.2.3 *Fusarium identification*

Morphological characters are the basis for the identification of *F. oxysporum* at the species level, but classification into *forma specialis* requires pathogenicity tests (Baayen *et al*., 2000) which is time consuming. Furthermore, this traditional approach based on diagnostic symptoms is becoming increasingly tenuous because more than one *formae speciales* may infect a particular host, along with non-pathogenic strains, which are common soil and rhizosphere inhabitants (Edel *et al.,* 2011). Separation of *F. oxysporum* is difficult based on just observation because different isolates that fall under one umbrella of a *forma specialis* might be polyphyletic (O'Donnell *et al*., 1998; Baayen *et al.,* 2000; Cramer *et al.,* 2003), and isolates that belong to different *forma specialis* may share a common ancestor or can be monophyletic (Kistler, 1997). However, studies of genetic difference among *formae speciales* have been analysed using pathogenicity test, Vegetative Compatibility Group (VCG), chromosomal features, rDNA, mtDNA and other molecular markers (Puhalla, 1985; Appel and Gordon, 1995; O'Donnell *et al.,* 1998; Katan and Katan, 1988; Alves-Santos *et al.,* 1999). These novel techniques are important to provide accurate identification of the fungi, compared to the classical methods. Moreover, the classical methods have other key limitations since they rely on the ability of the fungus to be cultured. The classical identification process complicates timely disease management decisions.

Molecular tools have had a major impact on the identification of plant pathogens. In the last two decades, molecular techniques have been implemented due to their high sensitivity and accurate nature as compared to classical methods for

identification. Currently, a wide range of molecular diagnostic tools are being used to detect and identify plant pathogens at the DNA and RNA levels. This approach allows for early timely, reliable identification, and quantification of the pathogen. In addition, it also enables effective informed decisions to be made to implement the correct measures to contain and control the pathogen. Currently, fungal diagnostics via molecular methods has emerged as a possible answer to the problems associated with existing phenotypic identification systems (Mishra *et al*., 2003). There has been success in using fluorescent-based polymerase chain reaction assays for rapid and reliable identification of pathogenic *Fusaria* (Mishra *et al.,* 2003). Flood *et al.* (1992) showed that, based on genomic DNA restricted fragment length polymorphism (RFLPs), *Foe* isolates from Zaïre and Brazil consisted of two distinct populations. In addition, Dossa *et al.* (1991) reported five VCGs corresponding to isolates with geographic origins in West Africa (Benin, Cameroon, Ghana, Ivory Coast and Zaire). Furthermore, there has been the combination of molecular markers such as RAPDs and RFLP with the classical method (pathogenicity test) for identification of the *Foe* in soil samples of distinct palm groves in DR Congo as well as other West African countries such as Ghana, Ivory Coast and Nigeria (Flood *et al.,* 1990; Flood *et al.,* 2006; Rusli, 2012). In order to find a *Foe*-specific marker, the samples were investigated with the same probe, followed by a pathogenicity test. More recently, Datta *et al.* (2011) developed a simple sequence repeat (SSR) technique for the identification of fusarium in lentil (*Lens culinaris medikus* sub sp. *culinaris*).

1.6 *Management and control of fusarium wilt*

Fusarium wilt has no known cure and due to this reason, prevention and exclusion are critical to disease management. The most effective methods of controlling fusarium wilt are preventive measures and at the plant material selection level (Renard and de Franqueville, 1989).

Vascular wilt of oil palm normally affects young palms in plantations, so it is essential to intervene as early as possible to reduce the risk of contamination of young plants (Renard and Quillec, 1983; Renard and Ravisé, 1986). The most effective way of controlling infected sites in the plantation is to destroy infected palms, by burning and ensure that they are replaced with tolerant palms. Tolerant palms refer to oil palm plant materials that have been tested in the nursery and their performance confirmed in the field (Renard *et al*., 1972; Renard *et al.,* 1980; Renard and Meunier, 1983; Renard and de Franqueville, 1989). Several reports on tolerant progenies of oil palm have been published (Taquet *et al.,* 1985; Renard and Ravisé, 1986). Such progenies should be included in breeding programmes to obtain breeding lines in parts of the world where fusarium wilt is hampering oil palm production. Renard *et al.* (1972) and Renard and Ravisé (1986) developed a performance index (I) of a strain in relation to the mean percentage of wilt-stricken plants in the whole of one test, i.e. the i value for each strain (say strain A) is obtained by the formula (Renard *et al*., 1972) below:

$$I = \left(\frac{\%\ wilt\ of\ infected\ plants\ of\ strain\ A}{\%\ wilt\ of\ infected\ plants\ in\ all\ strains} \right) X\ 100$$

Oil palms strain with a low index are most resistant to wilt based on this formula.

The use of chemicals for treating infected plants is of little importance because there is no cure of the disease upon infection. At the CSIR-OPRI, Ghana, the treatment of seed nuts with chemicals before germination is done as a preventive measure (Afram, E., Council for Scientific and Industrial Research – Oil Palm Research Institute, Eastern Region, Ghana, West Africa, Personal communication, 2014). The implementation of good field management practices is of immense importance to the control and prevention of the disease. The most important of these management practices is the source of plant materials. Oil palm seedlings should be obtained from a nursery where records prove zero incidence of the disease. In addition, the field area around the base of the trunk of the oil palm should be kept free from pruned fronds or branches, which provide a suitable environment for pathogen growth. As such, pruned branches or fronds of oil palm should be collected and kept away from the base of palms in the field and burnt upon drying. Also, pruning of palms, if possible, should be minimized and tools used for pruning should be cleaned by disinfectant (ethanol). In addition, using municipal-yard waste or household waste as mulch on palms should be avoided. It is advisable to use manual pruning with the Malayan knife rather than chain saw

since it is difficult to disinfect the latter (Afram, E., Council for Scientific and Industrial Research – Oil Palm Research Institute, Eastern Region, Ghana, West Africa, Personal communication, 2014).

The use of suppressive soils for controlling *Fusarium oxysporum* f.sp. *elaeidis* (*Foe*) has been reported. According to Alabouvette *et al.* (1993), suppressive soils are sites where despite the presence of a virulent pathogen and susceptible host, disease does not develop, or the severity and spread of disease through the site is restricted. This presence of suppressive soils for fusarium wilt has been reported in many regions of the world. Although the suppression has generally been shown to be due to soil physical structure (type of soil, drainage condition, presence of montmorillonoid soils and pH), biological factors and nutritional status together with microbial composition (fungi, bacteria and Actinomycetes) are said to play a major role (Scher and Baker, 1982; Alabouvette *et al.,* 1996).

Integrated control of fusarium has also been reported (Mandeel and Baker, 1991). Different strains, C5 and C14, of fusarium were used to suppress *F. oxysporum* f. sp. *cucumerinum* and it was concluded that the antagonistic effect that caused suppression of the fusarium wilt disease was due to competition for nutrients between the pathogenic and the antagonistic *Fusarium* sp. Further reports indicated competition for infection sites (Schnieder, 1984) by the antagonistic and pathogenic strains.

The application of biological organisms as control agents has been reported in other fusarium species (Sivan and Chet, 1993; Sundaramoorthy and Balabaskar, 2013) as well as other soil borne pathogens (Morsy *et al.,* 2009; Subalpara *et al.,* 2009). The most successful of such biological control agents (BCA) is *Trichoderma,* which was first reported as in the early 1930s (Weindling, 1932), since then it has also been used for controlling other diseases (Bliss, 1951; Aluko and Hering, 1970; Elad and Kapat, 1999; Morsy *et al.,* 2009; Subalpara *et al.,* 2009). There have been several studies into the properties or mechanisms that make it possible to use *Trichoderma* species as an antagonistic organism against plant pathogens (Howell, 2003). These mechanisms are very important since it serves as the premise for developing highly effective and efficient control of *Foe*. *Trichoderma* spp. have been reported to use mechanisms such as mycoparasitism, antibiosis, enzyme production, competition and rhizosphere competence, and induction of defence responses in plants (Blis, 1951; Aluko and Hering, 1970; Lo *et al.*, 1998; Howell, 2003) as modes of antagonistic control of plant pathogens. The production of enzymes such as chitinases and/or glucanases by *Trichoderma harzianum* can destroy cell wall integrity of pathogens via engulfing and penetrating hyphae of the pathogen. This process suppresses the pathogen via breaking down polysaccharides, chitin and β-glucans (Howell, 2003) and eventually leads to cell wall degradation and subsequent death of the pathogen.

1.7 *Research objectives*

Though oil palm is important to Ghana and Ghana is one of the contributors to the production of palm oil, the problem of fusarium wilt disease in oil palm plantations still exist. Therefore, this study is being conducted in order to help Ghana find possible methods of controlling *Fusarium oxysporum* f.sp. *elaeidis* (*Foe*) and if possible to avoid further spread of the *Foe* as well as to find alternative methods to reduce losses in oil palm plantations as these loses have direct impact on palm oil yield. Overall, these are five main objectives in this PhD research:

A. The survey of fusarium wilt disease.

B. Determination of variability of *Foe*

C. The use of fast molecular identification and detection techniques.

D. Control of *Foe* growth and infection.

E. Ascertain the resistance of oil palm bred lines and optimize tests for resistant crosses.

CHAPTER TWO: PHYLOGENETICS

2.0 *Introduction*

Knowledge on the mode of spread and genetic diversity of *Foe* is imperative for the implementation of effective control and management strategies, to combat fusarium wilt disease. Currently in Ghana, the identification of fusarium wilt diseased oil palms is done via physical examination of the trees in the field which are quiet misleading. The physical examination of the trees for identifying diseased palms by surveys in the field are normally done on a symptom observation basis and as such always need the confirmation of the pathogen in the host by using molecular tools to ascertain whether palms showing symptoms are infected or not. There have been reports on the spread of *Foe* in soil or fields of oil palm plantations in West Africa (W/A) such as Ghana, Ivory Coast, Nigeria and other oil palm production countries with the exception of Malaysia. Fusarium wilt disease can exist in a latent form (i.e. infected symptomless palms), making the mode of disease spread and infection highly unpredictable when symptoms were not visible.

Although efforts to sexually reproduce *Fo* sp. have not as yet succeeded, Yun *et al.* (2000) reported the cloning of mating type (*MAT*) genes identified in three *Fusarium* complexes. Furthermore, Simon *et al.* (2011) provided new insights into the sexual structure and evolution of *Fusarium* species. Taylor *et al.* (1999b) reported that the whole genome as a unit is transmitted from one generation to the

next, with different parts of the genome sharing the same evolutionary history. They further reported that isolates within a *forma specialis* traditionally were predicted to be genetically similar and separate from those with different host specificity. In addition, O'Donnell *et al.* (1998) supported by Taylor *et al.* (1999a) proposed that gene genealogies could be used to trace the evolution of phenotypic characters such as host specificity within this species complex. However, genetic variability in *Fo* is related to several factors. Recently, it was proposed by Dufresne *et al.* (2011), that some genetic variability in *Fo* might be caused by movement of transposable elements.

The applications of both phenotypic and genotypic markers have been studied in different fusarium species such as *Fusarium oxysporum* f.sp. *cubense* (Visser *et al.,* 2010). Despite the fact that *Foe* is common in the African region, further research needs to be done to show the genetic variation that exists between isolates from different regions across the world. Tools that have been used for identifying variation in *Foe* include amplified fragmented length polymorphisms (AFLP), inter-simple sequence repeats (ISSR) or simple sequence repeats (SSR) markers. These molecular tools have been applied in different ways in the identification and study of the phylogeny or genetic variation of different strains of *F. oxysporum* (Baayen *et al.,* 2000; Bogale *et al.,* 2005; Silva *et al.,* 2014) and have proven to be highly effective.

The aim of the work work reported in this chapter was to elucidate genetic variation in *Foe* with the use of specific regions in the genome or genes such as the *TEF-1α* (Translation Elongation Factor) and *SIX* (Secreted In Xylem) as markers, in addition to the use of AFLPs, SSRs or ISSRs to distinguish isolates, which is of importance for control of the pathogen as well as for quarantine purposes.

2.1 *Amplified Fragment Length Polymorphism* (*AFLP*)

Amplified fragment length polymorphism (AFLP) is a PCR-based tool used in DNA fingerprinting which was developed in the early 1990s (Vos *et al.,* 1995). AFLP uses restriction enzymes to digest genomic DNA, followed by ligation of adaptors to the ends of the restriction fragments. In effect it is a combination of the PCR and RFLP techniques. In simple terms, AFLP analysis is based on selective amplification of DNA restriction fragments (Vos *et al.,* 1995). AFLP has been used in several studies for the analysis of fungal population structure (e.g. Majer *et al.*, 1998; DeScenzo *et al.,* 1999; Purwantara *et al.,* 2000; Zeller *et al.,* 2000). Specifically, evaluation of the genetic variation among pathogenic isolates of *Fo* using AFLP markers has been widely reported (Baayen *et al.,* 2000; Sivaramakrishan *et al.,* 2002; Groenewald *et al.,* 2006; Stewart *et al.,* 2006). The utility, reproducibility and efficiency of the AFLP technique led to its broader application in the analysis of population diversity and identification of pathogens (Baayen *et al*., 2000; Abd-Elsalam *et al.,* 2002a; Bao *et al.,* 2002; Kiprop *et al.,* 2002; Groenewald *et al.,* 2006). AFLP has been used for diversity studies to examine genetic relationships among isolates of *F. oxysporum* f. sp. *vasinfectum* (*Fov*) while two *Fusarium*

oxysporum f.sp. *cubense* (*Foc*) races 1 and 2 are based on unique AFLP patterns respectively (Gurjar *et al.,* 2009; Wang *et al.*, 2006; Abd-Elsalam *et al*., 2004). Mohmed *et al.* (2003) used AFLP analysis to study the genetic relationships within and between natural populations of five Egyptian *Fusarium* spp. A total of 80 polymorphic markers were recorded with just four primer combinations and an average of 20 % polymorphic markers were observed for combination of two primers. Furthermore, Belabid *et al.* (2004) collected thirty-two isolates of *F. oxysporum* f. sp. *lentis* (*Fol*) from wilted lentil plants from different sites in North-West Algeria and used molecular tools including AFLP for evaluating the genetic variation. There was no correlation with the geographic origin or aggressiveness of isolates, and it was suggested that the *Fol* isolates were derived from two genetically distinct clonal lineages.

The advantage of AFLP over other techniques is that multiple bands are derived from all over the genome, preventing over-interpretation or misinterpretation due to point mutations or single-locus recombination, which affect other genotypic characteristics (Mohmed *et al.,* 2003). The challenging factor with the application of this technique, however, is that it is costly and needs a level of expertise to be successful.

2.2 *Simple Sequence Repeats* (*SSR*) *and Inter-Simple Sequence Repeats* (*ISSR*)

Simple sequence repeats (SSR) are tandemly repeated DNA sequence units of 1 - 6 bp. SSRs have a wide random distribution throughout the genome of eukaryotes. As a result of slipped-strand mispairing during replication, variations exist in SSR length (Levinson and Gutman, 1987). According to Kumar *et al.* (2013) SSRs are highly polymorphic, multi allelic, co-dominant and highly reproducible. This makes SSRs ideal for molecular identification of genetic variation.

There are several reports on the application of SSRs in distinguishing between isolates of fusarium. Bogale *et al.* (2005) studied SSRs markers for species in the *Fo* complex showing genetic similarity at nine loci ranged from 0.003 to 0.895, revealing 71 alleles among 64 isolates, to enable the study of the relationships existing amongst *F. oxysporum* isolates. Britz *et al.* (2002) developed SSR markers in *Fusarium circinatum* but these markers are not useful in *Fo*. Similarly, molecular diversity in Indian isolates of *Fusarium oxysporum* f. sp. *lentis* with nine SSR primer pairs revealed 21 alleles with 2.33 alleles per primer and placed all the *Fol* isolates in two major groups which were separated by 27 % of genetic similarity (Datta *et al*., 2011). Datta and Lal (2013) showed that twelve SSR primers produced an average of 9.4 bands per primer and a total of 27 alleles with an average of 2.25 alleles per marker in the study of genetic diversity of *Fusarium udum* which causes fusarium wilt in pigeon pea (*Cajanus cajan*) across India.

Inter Simple Sequence Repeats (ISSR) technique is a PCR based method, which involves amplification of DNA segments present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite directions. This genotyping technique is usually based on 16 - 25 bp long sequences on which single ISSR primers work as both the forward and reverse primer. ISSR primers have been reported to be either anchored or unanchored (Meyer *et al.,* 1993; Gupta *et al.,* 1994; Wu *et al.,* 1994). They can also exist in di, tri, tetra or penta nucleotides. This technique is very reproducible.

ISSRs have been applied in discriminating three *F. oxysporum* f. sp. *lycopersici* races (*Fol*) to determine their origin (Baysal *et al.,* 2013). This study further reported additional and new information on this pathogen based on races and the dominant existence in the west Mediterranean region of Turkey. Further genetic diversity studies on *Fusarium oxysporum* f.sp. *cubense* (*Foc*) reported ten ISSR primers which generated more polymorphic bands and indicated the presence of wide genetic diversity among the *Foc* isolates of India and its polyphyletic nature (Thangavelu *et al*., 2012). Recently the genetic diversity of *Fusarium mangiferae* isolated from mango in China has been examined with ISSRs in which the isolates were grouped into five distinct clusters from the fourteen ISSR primers that produced an average of 5.14 bands per primer (Liu *et al*., 2014). Furthermore, Hannachi *et al*. (2015) reported on how ISSR markers revealed the genetic and phenotypic differences of *F. oxysporum* f. sp. *citri* isolated from sweet orange and

tangerine in the characterisation of these Tunisian strains. Their results showed that these isolates clustered into two groups for the virulent strains studied.

2.3 *Materials and methods*

2.3.1 *Sampling Locations*

Sampling was done randomly from matured oil palms in plantations from two major Regions in Ghana (Western and Eastern). In the Eastern Region, the Oil Palm Research Institute (OPRI) (Fig. 2.1) was the only plantation where samples were collected. Samples were taken from the commercial fields of OPRI and a few from other selected sites in the plantation which are not for commercial purposes. In the Western Region three different oil palm plantations, namely Norpalm, Benso Oil Palm Plantation (BOPP) and Twifo Oil Palm Plantation (TOPP) were the sampling sites, as shown in Fig. 2.2. No oil palms were showing symptoms of fusarium wilt in the fields at TOPP unlike at the other sites, where symptoms were present. Hence no samples were collected from TOPP. In addition to these samples collected from parts of Ghana (West Africa) two other Fusarium isolates, CBS 217.49 and CBS 783.83, were obtained from culture collections of the CBS-KNAW in the Netherlands. Import and maintenance of the strains was in accordance with UK-DEFRA regulations (Licence No. 103928/198432/4) with an additional two isolates F3 and 16F obtained from the University of Bath-UK. Originally, isolates 16F and F3 were from diseased palms from the Ivory Coast and the Democratic Republic of Congo (DRC) respectively. *Foe* 16F was previously used by Institut de Recherches

pour les Huiles et Ol_eagineux (IRHO) as their fusarium wilt screening isolate (Mepsted *et al*., 1994a). Isolate F3 was used in Zaire (now DRC) as a screening isolate (Mepsted *et al*., 1994b). In comparative studies, 16F was shown to be more aggressive than F3 (Mepsted *et al*., 1994a) and the two isolates belong to different VCGs 0141 and 014 respectively (Flood *et al*., 1992)

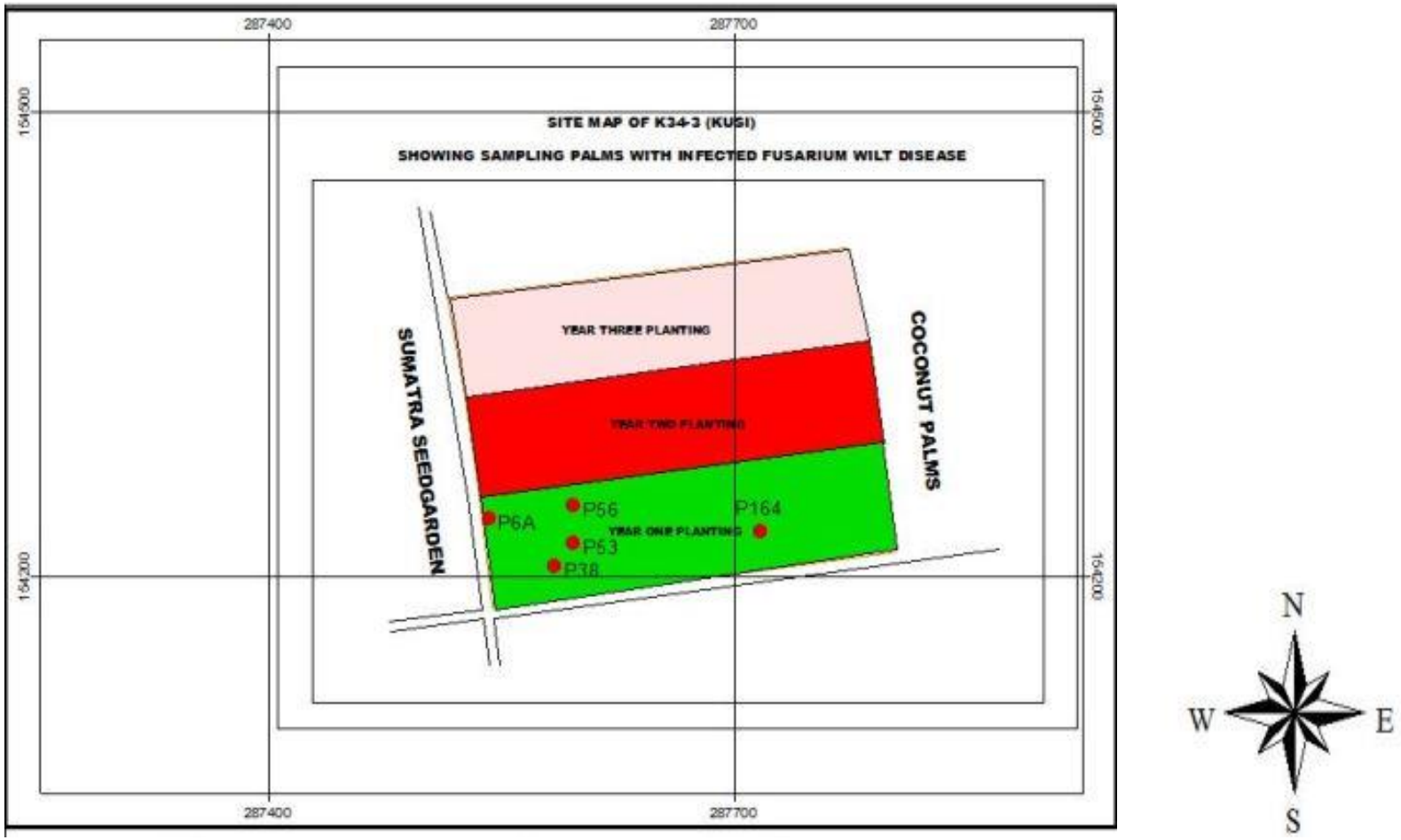


Figure. 2.1 Map showing Oil Palm Research Institute Plantation sampling sites

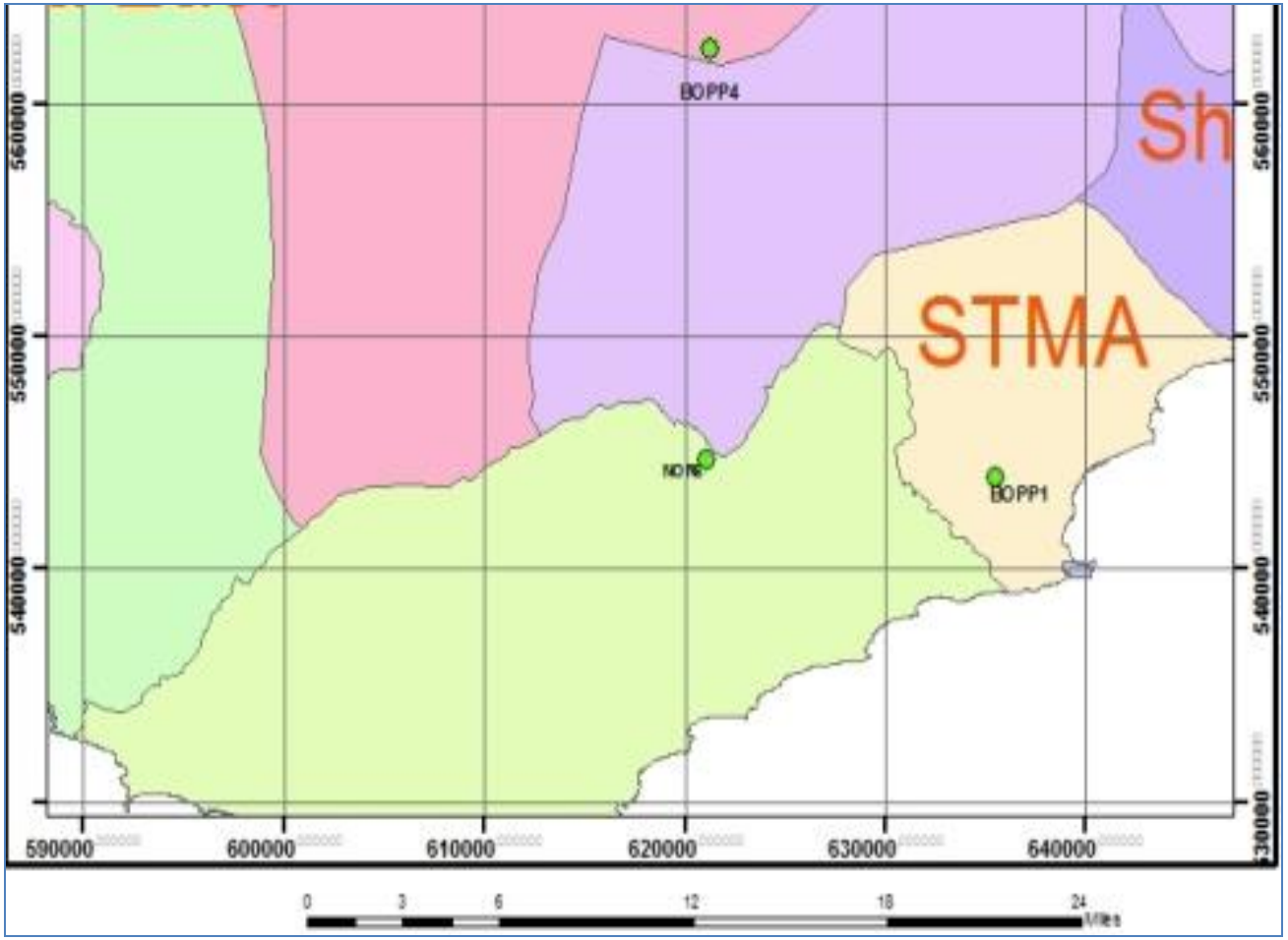


Figure. 2.2 Map showing Benso Oil Palm Plantations (BOPP) and NORPALM (NORP) sampling sites (green spots on map)

2.3.2 *Sampling procedure and culture of Foe isolates*

Two incremental borers (400 mm in length), one for sampling symptomatic, and one for symptomless palms were used to collect samples (Mepsted *et al.,* 1991; Cooper, 2011). Core samples (Fig. 2.3a) from the stem segment of matured oil palms showing symptoms of fusarium wilt and symptomless neighbouring oil palm trees in proximity (8 - 9 m apart) were randomly selected. The incremental borers were sterilized in absolute ethanol after every sample was collected, wiped with sterile cotton wool and used to drill into the stem part of the oil palm at three equidistant intervals (about 0.1 m) around the stem about 0.5 m from the base of the plant to increase the chance of getting to the vascular section of the palm. The stem core was removed and collected with sterile forceps. Some portions directly plated on-site on fusarium selective medium as shown in Appendix 1C (Papavizas, 1967) and the remaining core packed in aluminium foil with labels on each sample. This procedure was repeated for sampling symptomless palm trees neighbouring the sampled diseased palm trees. These collections were sent to the laboratory and sub-cultured on potato dextrose agar (PDA) (Appendix 1A) supplemented with antibiotics. In total, 28 samples (Table. 2.1) were collected from 28 trees. The selected isolates for the study are shown in Table. 2.2.

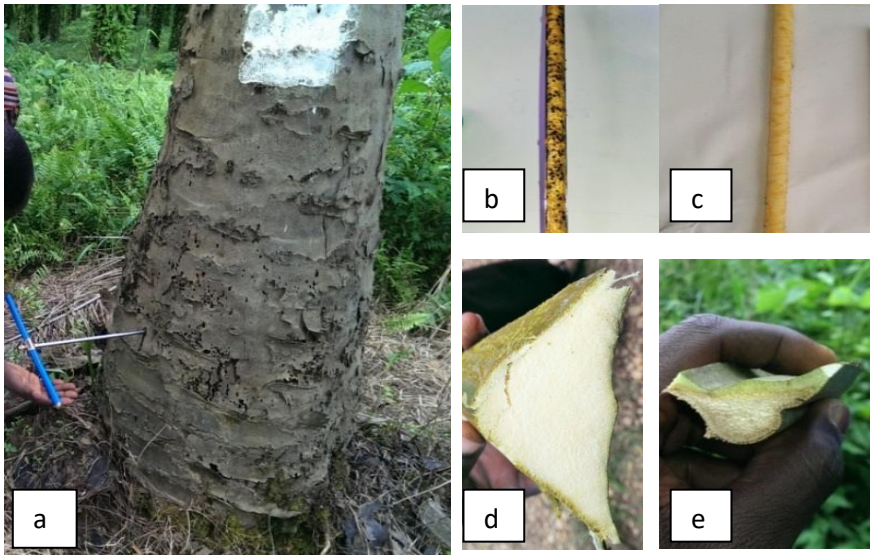


Figure. 2.3. Pictures showing **(a)** Drilling process with incremental stem borer in oil palm trunk **(b)** Drilled core (discoloured/brown/spotted) sample from trunk of diseased oil palm **(c)** Drilled core (clean) sample from trunk of a healthy oil palm **(d)** Healthy oil palm leaf fronds **(e)** Diseased oil palm blocked (brownish part) vascular section leaf fronds

Table. 2.1 List of all *Foe* sampled

Isolate Code	Source (Ex-host)	Origin
1	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
2	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
3	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
4	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
5	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
6	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
7	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
8	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
9	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
10	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
11	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
12	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
13	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
14	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
15	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
16	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
17	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
18	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
19	Oil palm vascular tissues of stem-direct Plating	Ghana-OPRI
N2	Oil palm vascular tissues of stem-direct Plating	Ghana-NORPALM
N3	Oil palm vascular tissues of stem-direct Plating	Ghana-NORPALM
N4	Oil palm vascular tissues of stem-direct Plating	Ghana-NORPALM
N5	Oil palm vascular tissues of stem-direct Plating	Ghana-NORPALM
N6	Oil palm vascular tissues of stem-direct Plating	Ghana-NORPALM
B2	Oil palm vascular tissues of stem-direct Plating	Ghana-BOPP
B3	Oil palm vascular tissues of stem-direct Plating	Ghana-BOPP
B4	Oil palm vascular tissues of stem-direct Plating	Ghana-BOPP
B5	Oil palm vascular tissues of stem-direct Plating	Ghana-BOPP

Table. 2.2 List of selected representative *Foe* and other fusarium isolates used for more extensive analyses in this study.

Fusarium species	Ex-host	Isolate code	Location/Source
F.oxysporum f. sp. *elaeidis*	Oil palm	IVORY COAST-16F	Ivory Coast
F.oxysporum f. sp. *elaeidis*	Oil palm	DR CONGO-F3	DR Congo
F.oxysporum f. sp. *elaeidis*	Oil palm	DR CONGO-Z/CBS 217.49	DR Congo
F.oxysporum f. sp. *elaeidis*	Oil palm	SURINAME-S/CBS 783.83	Suriname
F.oxysporum f. sp. *elaeidis*	Oil palm	GHANA NORPALM-N2	Ghana
F.oxysporum f. sp. *elaeidis*	Oil palm	GHANA NORPALM-N3	Ghana
F.oxysporum f. sp. *elaeidis*	Oil palm	GHANA NORPALM-N5	Ghana
F.oxysporum f. sp. *elaeidis*	Oil palm	GHANA BOPP-B4	Ghana
F.oxysporum f. sp. *elaeidis*	Oil palm	GHANA BOPP-B5	Ghana
F.oxysporum f. sp. *elaeidis*	Oil palm	GHANA OPRI-5	Ghana
F.oxysporum f. sp. *elaeidis*	Oil palm	GHANA OPRI-11	Ghana
F.oxysporum f. sp. *elaeidis*	Oil palm	GHANA OPRI-18	Ghana
F.oxysporum sp.	Tomato	Fus126B-3	ADAS
F.oxysporum sp.	Tomato	Foxy150-5	ADAS
Fusarium sp.	Tomato	Fus106-6	ADAS
F.oxysporum sp.	Tomato	Foxy sp-8	ADAS
F.oxysporum sp.	Tomato	Foxy2571-9	ADAS
F.oxysporum sp.	Tomato	Foxy sp-10	ADAS
Fusarium sp.	Tomato	Fus-11	ADAS
F.oxysporum sp.	Tomato	FoxyBX14/153b-A	UoN
F.oxysporum sp.	Tomato	FoxyBX14/153a-B	UoN
F.oxysporum f. sp. *lycopersici*	Tomato	Foxy.Forla-C	UoN
F.oxysporum f. sp. *lycopersici*	Tomato	Foxy.Forlb-D	UoN

2.3.3 *DNA Extraction*

Mycelium (50 - 100 mg) of *Foe* was collected from the PDA plates. Tissue disruption was carried out in 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 carried out using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol. *Foe* DNA was isolated from inculated soil samples in pots following the same procedure done for *Foe* mycelium using the DNeasy Plant Mini Kit (Qiagen) based on manufactrer's protocol.

2.3.4 *PCR*

Polymerase chain reaction (PCR) of various regions of the template DNA was performed using primer pairs *ITS*4, *SIX*1-14, *TEF* or cytochrome *P450*. PCR was carried out in 30 µl volumes consisting of 15 µl of master mix (MangoTaq™ DNA Polymerase), 1 µl (of 10 pmol / ul) of primer pairs mentioned above, 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 sec. The final extension was set at 72 °C for 5 min.

2.3.5 *Agarose gel electrophoresis*

PCR products were analysed by agarose gel electrophoresis in 1% w / v agarose (Fisher Scientific) in 1x TBE buffer (20% v / v 5x TBE stock: 0.4 M orthoboric acid, 0.3 M Tris-base and 0.5 M EDTA pH 8) with addition of 1 µl ethidium bromide (10 mg / ml) per 100 ml. DNA samples were loaded in Blue 6x loading dye (Promega) alongside a 100 bp DNA ladder (Promega). Electrophoresis was carried out at a constant voltage (typically 120 V) for approximately 30 min. The gel was visualised on a White/Ultraviolet transluminator (UVP) and the image captured using Canon G10 and Remote Camera DC software.

2.3.6 *Purification of PCR products*

PCR products were cleaned using the QIAquick PCR Cleanup kit (Qiagen) following the manufacturer's instruction and DNA concentration estimated by nano-drop and using a 1 kb ladder (Promega).

2.3.7 *Sequencing, identification of Fusarium isolates and data analysis*

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 ribosomal internal

transcribed spacer (*ITS*) regions (Rehner, 2001; Abd-Elsalam *et al.,* 2003) or the gene of interest (Appendix 4). The output from BLAST algorithms was used to query any unknown sequences against the database of all the fungal gene regions. MEGA6 software (Tamura *et al*., 2013) was used to edit and align the nucleotide data, after which the alignments were improved manually.

Phylogenetic analyses were conducted with MEGA6 on the combined data set of all sequences for all 28 isolates from the three main locations in Ghana. Unweighted parsimony analyses were performed. Clade stability was assessed by 1,000 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 clade stability. Sequences of other *Fusarium* spp. and f.sp. (Appendix 2) were obtained from the National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) to support the generation of the phylogenetic trees for the identification of the *Foe* in the study.

2.3.8 *Amplified Fragment Length Polymorphism* (*AFLP*) *assays*

Amplified fragment length polymorphism (AFLP) was used to investigate genetic diversity and variation of *Foe* isolates from Ghana, Ivory Coast, DR Congo and Suriname. Template preparation was carried out using an AFLP Core Regent Kit (Invitrogen), as suggested by the manufacturer, where genomic DNA isolated

(Section 2.3.3) was digested with restriction enzymes (*Eco*RI and *Mse*I). Fifteen microlitres of digested and undigested DNA was checked on a 1 % agarose gel before proceeding with the ligation steps. For pre-amplification, 5 µl of diluted ligation mixture, 1 µl (of 10 pmol / µl) each of *Eco*RI-universal primer and *Mse*I-universal primer and 18 µl sterile distilled water were added into Illustra™ PuReTaq Ready-To-Go PCR Beads (GE Healthcare, UK). Amplification conditions were 94 °C for 1 min followed by 10 cycles at 94 °C for 40 sec, 65 °C for 1 min and 72 °C for 1 min, then twenty five additional cycles at 94 °C for 40 sec, 56 °C for 1 min and 72 °C for 1 min. The amplified products were checked on 1 % agarose gel run at 100 volts for 60 min. Selective amplification was done using a mixture of 5 µl of diluted pre-amplification product, 1 µl of each selective primer and 18 µl of sterile distilled water and Illustra™ PuReTaq Ready-To-Go PCR Beads (GE Healthcare, UK). Primers tested were combinations of 3 *EcoR*I-primers and 13 *Mse*I-primers (Table. 2.3) with amplification conditions the same as for pre-amplification. Amplified products were checked on 2 % agarose gel run at 100 volts for 120 min. Fragment analysis was done for all the amplified products of the *Foe* isolates and was followed with further analysis with the CEQ software where the presence or absence as well as the size of fragments were detected. The CEQ software scored the presence or absence of bands as 1 or 0, respectively.

Table. 2.3. AFLP primer combinations (MseI + EcoR1)

MseI primers	EcoR1 primers		
	Eco-AA	Eco-TA	Eco-C
Mse-GC	Mse-GC+ Eco-AA	Mse-GC+ Eco-TA	Mse-GC+ Eco-C
Mse-CA	Mse-CA+ Eco-AA	Mse-CA+ Eco-TA	Mse-CA+ Eco-C
Mse-CC	Mse-CC+ Eco-AA	Mse-CC+ Eco-TA	Mse-CC+ Eco-C
Mse-TG	Mse-TG+ Eco-AA	Mse-TG+ Eco-TA	Mse-TG+ Eco-C
Mse-AG	Mse-AG+ Eco-AA	Mse-AG+ Eco-TA	Mse-AG+ Eco-C
Mse-TC	Mse-TC+ Eco-AA	Mse-TC+ Eco-TA	Mse-TC+ Eco-C
Mse-CG	Mse-CG+ Eco-AA	Mse-CG+ Eco-TA	Mse-CG+ Eco-C
Mse-TA	Mse-TA+ Eco-AA	Mse-TA+ Eco-TA	Mse-TA+ Eco-C
Mse-TT	Mse-TT+ Eco-AA	Mse-TT+ Eco-TA	Mse-TT+ Eco-C
Mse-GG	Mse-GG+ Eco-AA	Mse-GG+ Eco-TA	Mse-GG+ Eco-C
Mse-AC	Mse-AC+ Eco-AA	Mse-AC+ Eco-TA	Mse-AC+ Eco-C
Mse-CT	Mse-CT+ Eco-AA	Mse-CT+ Eco-TA	Mse-CT+ Eco-C
Mse-GT	Mse-GT+ Eco-AA	Mse-GT+ Eco-TA	Mse-GT+ Eco-C

2.3.9 *Simple Sequence Repeats* (SSR) *assay*

The use of Simple Sequence Repeats (SSR) marker was adopted for the study of the genetic variation in twenty-four (24) fusarium isolates which comprised twelve *Foe* isolates: Ghana (8), Suriname (1), Ivory Coast (1) and DR Congo (2) and twelve other fusarium isolates (Table. 2.2 in section 2.3.2). Amplification and resolution of SSR loci was performed with the labelled SSR primers and conditions described by Bogale *et al.* (2005). However, the thermocycling conditions were modified as: one cycle 95 °C for 5 min, one cycle of 35 °C for 1 min, 35 cycles 94 °C for 30 secs, 30 secs at varying annealing temperatures (Appendix 3) and extension at 72 °C for 10 min. Eight SSR primers in addition to one designed SSR primer were used for this work (Appendix 3).

2.4.0 *Inter Simple Sequence Repeats* (ISSR) *assay*

Inter Simple Sequence Repeats (ISSR) markers were used to demonstrate the genetic differences that exist in the *Foe* isolates collected and selected isolates of other *Fusarium* sp. Twenty primers were initially screened on 12 selected fusarium isolates to select the ideal primers. The thermocycling conditions were as described in section 2.3.4, but varying annealing temperatures (Appendix 3). After screening, primers that had reproducible and scorable polymorphic DNA products were selected for the study and used throughout the ISSR study.

2.5 *Results*

2.5.1 *Analysis of "housekeeping" genes and effector proteins*

The housekeeping genes of the pathogen were examined to ascertain whether they can be used as markers to distinguish isolates of the *Foe*. The ribosomal DNA (rDNA) regions, translation elongation factor (*TEF*) and the Internal transcribed spacers (*ITS*) genes were studied. The *ITS* and *TEF*-1α genes were identified in the *Foe* and non-*Foe* (Fig. 2.4, 2.5, 2.7 and 2.8) isolates used in this study. In the phylogenetic trees the *ITS* region clustered most *Foe* isolates together but two *Foe* isolates failed to cluster in the major group of *Foe* (Fig. 2.6). However, the other species of *Fusarium* were totally grouped as outliers in the phylogenetic tree (Fig. 2.6). The sequenced DNA of the two housekeeping genes *ITS* and *TEF*-1α in the *Foe* or non-*Foe* isolates were selected and aligned (Appendix 2A) (as described in section 2.3.7) and subsequently concatenated to generate the phylogenetic tree (Fig. 2.9) for eleven isolates (10 *Foe* and one non-*Foe*).

Effector proteins, specifically the Secreted In Xylem (*SIX*) genes, were amplified from the DNA (Fig. 2.10 - 2.13) of the selected *F. oxysporum* isolates (Table. 2.2). In general, *SIX8, SIX9* and *SIX11* genes amplified from most isolates and were used in further analyses, whereas the remaining *SIX* genes were recorded as been present (+) in some isolates but absent (-) in others (Table. 2.4). Fourteen *SIX* genes were examined. The effector genes *SIX2, SIX3, SIX4, SIX5, SIX6* and *SIX14* (Table. 2.4) were not identified in any of the *Foe* or non-*Foe* isolates. However, *SIX1* was present in only *Foe* DR Congo-F3 and *Fol*

(Foxy.Forla-C and Foxy.Forlb-D) whereas *SIX7* was present in *Foe* OPRI-18 and non-*Foe Fol* (Foxy.Forla-C and Foxy.Forlb-D).

Due to limited sequences of the *SIX* genes in all the isolates for this study, eleven isolates (10 *Foe* and a non-*Foe*) were selected to assess genetic variation. A concatenated phylogenetic tree (Fig. 2.14) was generated with sequences (Appendix 2B) of three *SIX* genes (*SIX8*, *SIX9* and *SIX11*) identified in the *Foe* or non-*Foe* isolates selected from 10 *Foe* and 1 non-*Foe* isolates. There was a major cluster of *Foe* isolates with three sub-clusters I, II and III, whereas the non-*Foe* was the outlier.

The concatenated phylogenetic trees for the effector proteins (Fig. 2.14) showed higher boostrapping values (100) than housekeeping genes (Fig. 2.9). The general phylogenetic tree generated where all the three effector genes and the two housekeeping genes were aligned together generated a major cluster A and an outlier (Fig. 2.15). This concatenated gene data set consisted of 1359 nucleotide characters from the five genes. Overall, bootstrap support increased when characters from the five gene regions were combined, particularly values at the nodes (Fig. 2.15). The combined gene data set dendogram suggested that isolates of *Foe* were in two separate sub-clusters where cluster B had only three isolates from Ghana (NORPALM-N2), Suriname (S) and DR Congo (Z) supported with higher bootstrap values of 100 and 50 (Fig. 2.15). Cluster A had all *Foe* isolates grouping closely. There were two sub-clusters in major cluster A (I and II). The only outlier was Foxy sp-10 which was distinct from the remaining isolates. Relatively higher bootstrap values supported the phylogenetic tree generated from the two rDNA genes and the three secreted effector proteins.

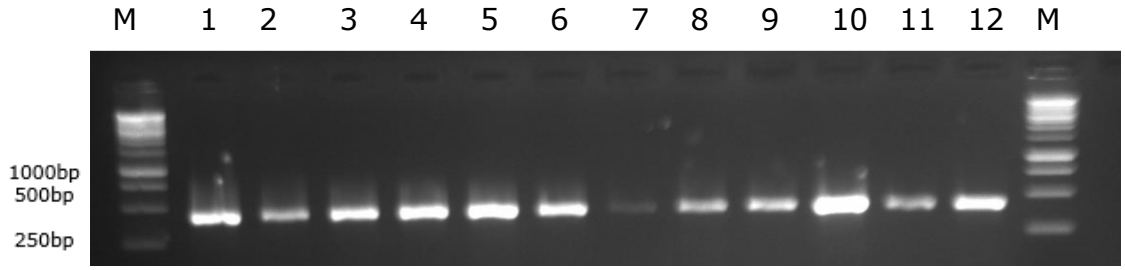


Figure. 2.4. Polymerase chain reaction amplification products from genomic DNA with primer *ITS*-Fu1f/*ITS*-Fu1r for *Foe* from Ivory Coast (Lane 1), DR Congo (Lanes 2 & 3), Suriname (Lane 4), Ghana Lanes 5,6,7,8,9,10,11 & 12). Marker (M) is 1 Kb ladder

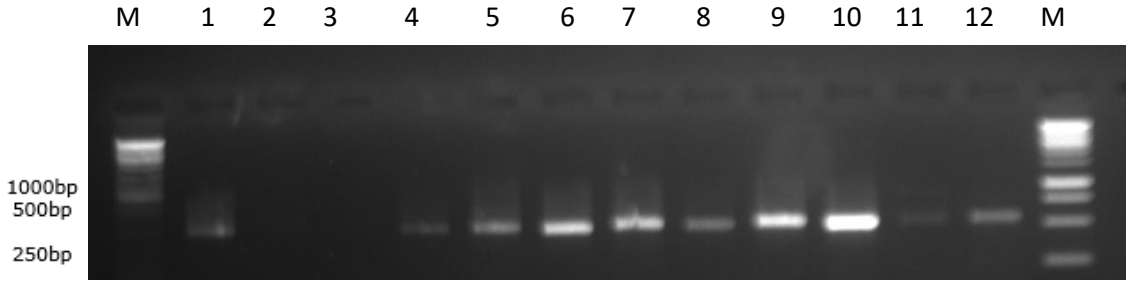


Figure. 2.5. Polymerase chain reaction amplification products from genomic DNA with primer *ITS*1 and *ITS*4 for *Foe* from Ivory Coast (Lane 1), DR Congo (Lanes 2 & 3), Suriname (Lane 4), Ghana Lanes 5,6,7,8,9,10,11 & 12). Marker (M) is 1 Kb ladder

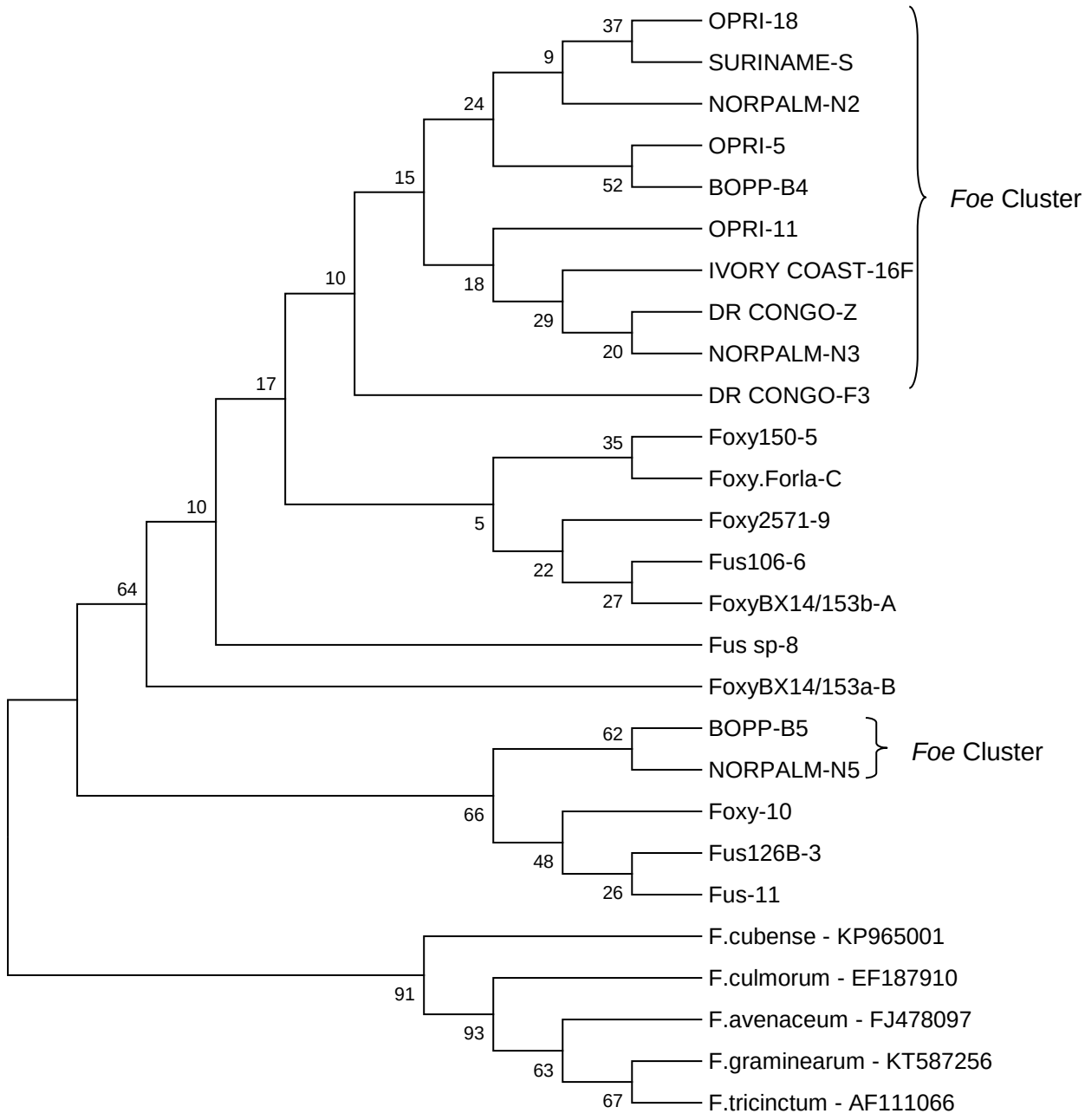


Figure. 2.6. Molecular Phylogenetic analysis by UPGMA statistical method with Maximum Composite Likelihood model tree of *Foe* isolates from oil palm, non-*Foe* from NCBI gene bank based on an alignment of *ITS* gene. Numbers in nodes represent bootstrap values from 1000 replicates.

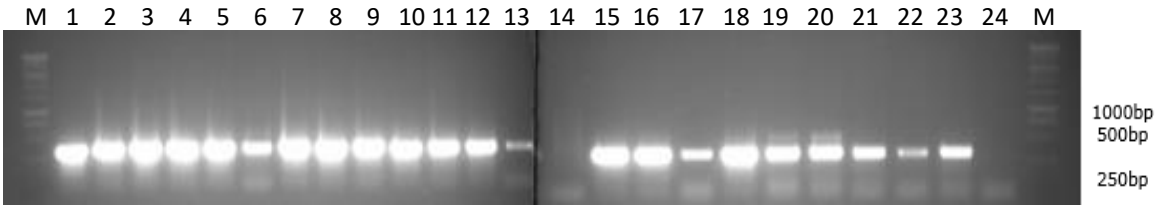


Figure. 2.7. Polymerase chain reaction amplification products from genomic DNA with primer pairs *ITS*4 for *Foe* from Ivory Coast (Lane 1), DR Congo (Lanes 2 & 3), Suriname (Lane 4), Ghana Lanes 5,6,7,8,9,10,11 & 12) and other *Fusarium* isolates (Lanes 13 to 24) not host specific to oil palm. Marker is 1 Kb ladder.

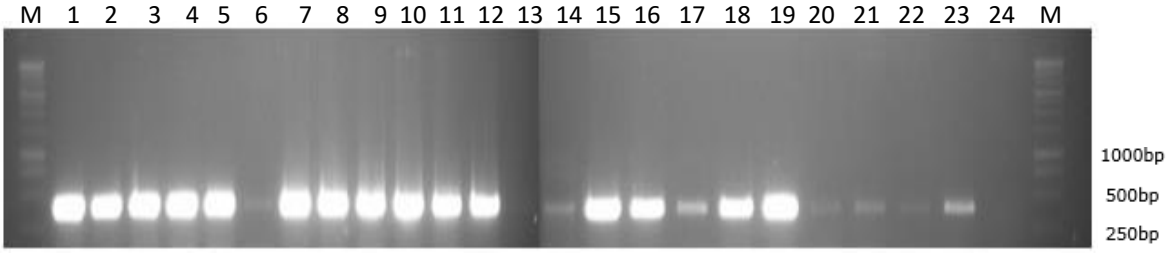


Figure. 2.8. Polymerase chain reaction amplification products from genomic DNA with primer pairs *TEF*-B2 for *Foe* from Ivory Coast (Lane 1), DR Congo (Lanes 2 & 3), Suriname (Lane 4), Ghana Lanes 5,6,7,8,9,10,11 & 12) and other *Fusarium* isolates (Lanes 13 to 24) not host specific to oil palm. Marker is 1 Kb ladder.

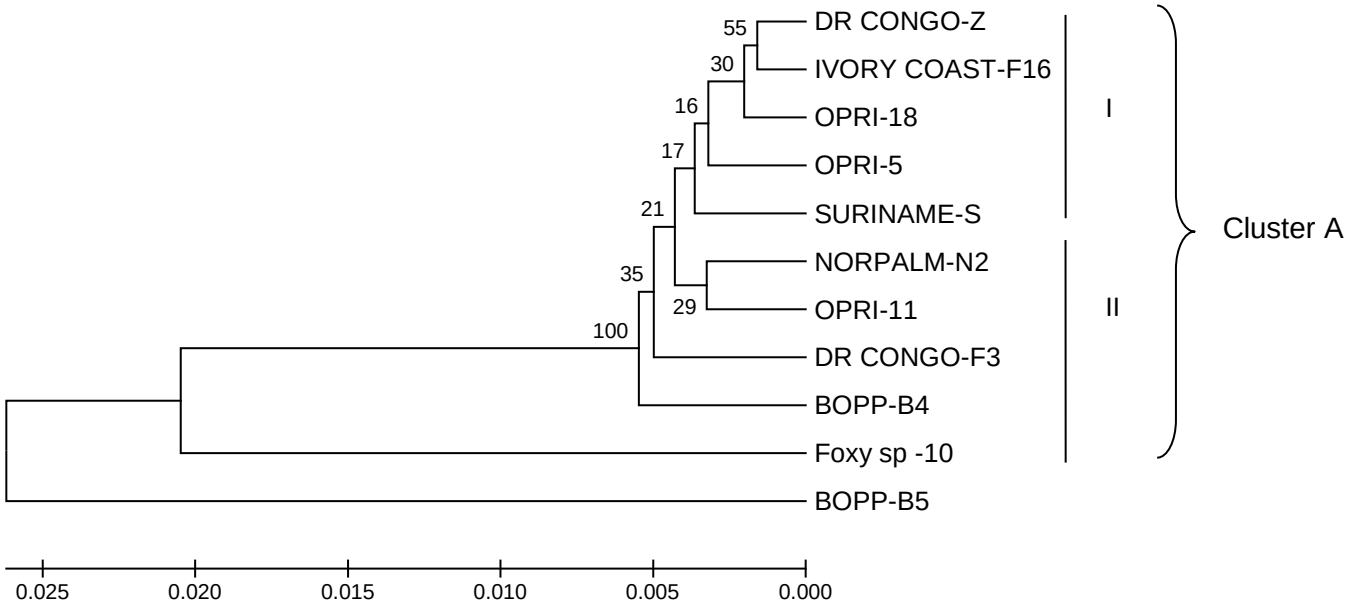


Figure. 2.9. Phylogenetic tree of the genetic relationships among 11 isolates (6 from Ghana, 1 from Ivory Coast, 2 from DR Congo, 1 from Suriname) of *Foe* and other *Fusarium* isolates (non-*Foe*) generated with the use of Unweighted Pair-Group Method with Arithmetic Average (UPGMA) based on a concatenated alignment of translation elongation factor-1a (*TEF-1a*) and *ITS*4. Numbers in the nodes represent bootstrap values from 1000 replicates.

Table. 2.4. Identified effector proteins in *Foe* and non-*Foe* isolates

Fusarium species	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*	IVORY COAST-16F	-	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum f. sp. *elaeidis*	DR CONGO-F3	+	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum f. sp. *elaeidis*	DR CONGO-Z	-	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum f. sp. *elaeidis*	SURINAME-S	-	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum f. sp. *elaeidis*	GHANA NORPALM-N2	-	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum f. sp. *elaeidis*	GHANA NORPALM-N3	-	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum f. sp. *elaeidis*	GHANA NORPALM-N5	-	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum f. sp. *elaeidis*	GHANA BOPP-B4	-	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum f. sp. *elaeidis*	GHANA BOPP-B5	-	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum f. sp. *elaeidis*	GHANA OPRI-5	-	-	-	-	-	-	+	+	-	+	+	-	-
F.oxysporum f. sp. *elaeidis*	GHANA OPRI-11	-	-	-	-	-	-	+	+	-	+	+	-	-

Fusarium species	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*	GHANA OPRI-18	-	-	-	-	-	+	+	-	+	-	-	-	-
F.oxysporum sp	Foxy sp -1	-	-	-	-	-	-	-	-	-	-	-	-	-
F.oxysporum sp	Fus126B-3	-	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum sp	Foxy150-5	-	-	-	-	-	-	+	+	-	+	-	-	-
Fusarium sp	Fus106-6	-	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum sp	Foxy sp-8	-	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum sp	Foxy2571-9	-	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum sp	Foxy sp-10	-	-	-	-	-	-	+	+	-	+	-	-	-
Fusarium sp	Fus-11	-	-	-	-	-	-	+	+	-	+	-	-	-
F.oxysporum sp	FoxyBX14/153b-A	-	-	-	-	-	-	+	-	-	-	-	-	-
F.oxysporum sp	FoxyBX14/153a-B	-	-	-	-	-	-	+	-	-	-	-	-	-
F.oxysporum f. sp. *lycopersici*	Foxy.Forla-C	+	-	-	-	-	+	+	-	+	-	+	-	-
F.oxysporum f. sp. *lycopersici*	Foxy.Forlb-D	+	-	-	-	-	+	+	-	+	-	+	-	-

+ = Present/detected - = Absent/not detected



Figure. 2.10. Polymerase chain reaction amplification products from genomic DNA with primer pairs *SIX1*F/*SIX1*R for *Foe* from Ivory Coast (Lane 1), DR Congo (Lanes 2 & 3), Suriname (Lane 4), Ghana Lanes 5,6,7,8,9,10,11 & 12) and other *Fusarium* isolates (Lanes 13 to 24) not host specific to oil palm. Marker is 1 Kb ladder

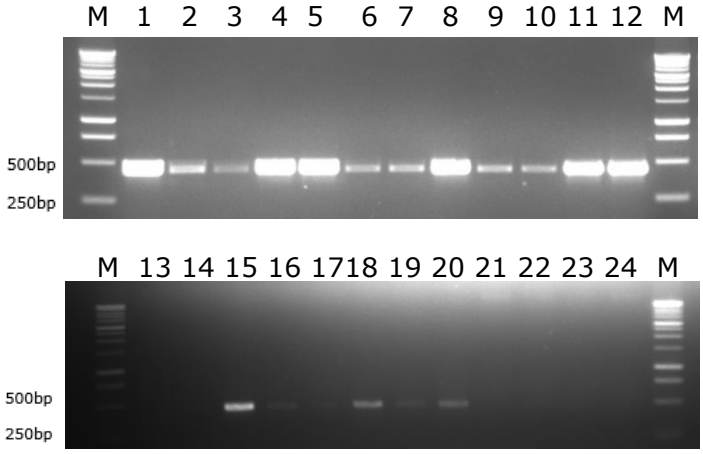


Figure. 2.11. Polymerase chain reaction amplification products from genomic DNA with primer pairs *SIX8*F/*SIX8*R for *Foe* from Ivory Coast (Lane 1), DR Congo (Lanes 2 & 3), Suriname (Lane 4), Ghana Lanes 5,6,7,8,9,10,11 & 12) and other *Fusarium* isolates (Lanes 13 to 24) not host specific to oil palm. Marker is 1 Kb ladder.

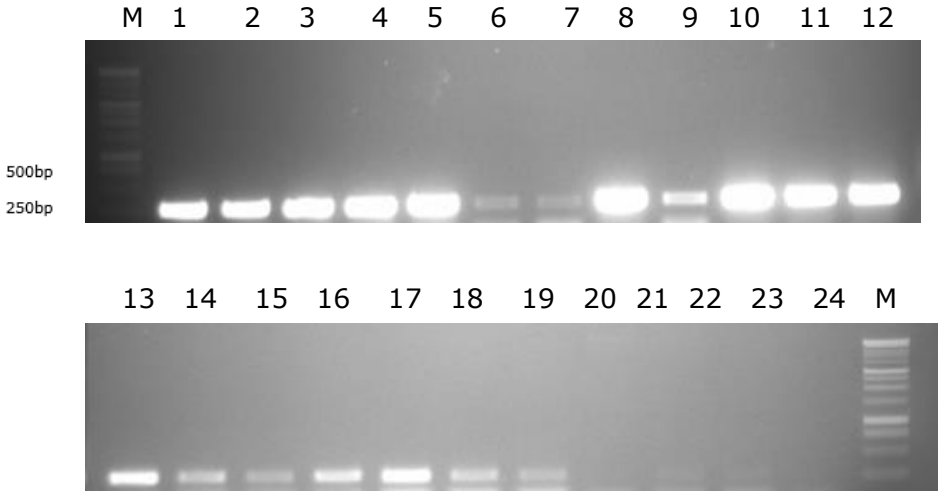


Figure. 2.12. Polymerase chain reaction amplification products from genomic DNA with primer pairs *SIX9*F/*SIX9*R for *Foe* from Ivory Coast (Lane 1), DR Congo (Lanes 2 & 3), Suriname (Lane 4), Ghana Lanes 5,6,7,8,9,10,11 & 12) and other *Fusarium* isolates (Lanes 13 to 24) not host specific to oil palm. Marker is 1 Kb ladder.

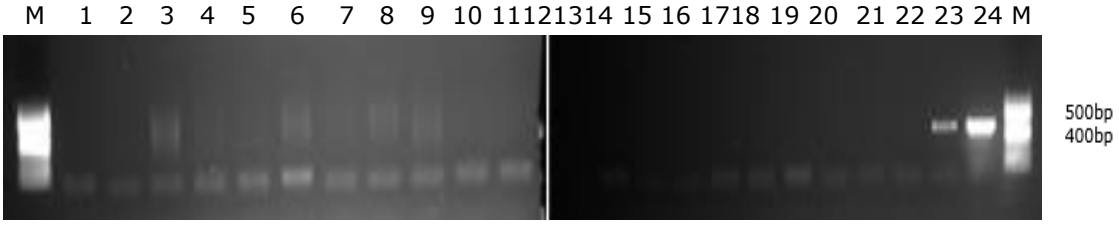


Figure. 2.13. Polymerase chain reaction amplification products from genomic DNA with primer pairs *SIX10*F/*SIX10*R for *Foe* from Ivory Coast (Lane 1), DR Congo (Lanes 2 & 3), Suriname (Lane 4), Ghana Lanes 5,6,7,8,9,10,11 & 12) and other *Fusarium* isolates (Lanes 13 to 24) not host specific to oil palm. Marker is 1 Kb ladder.

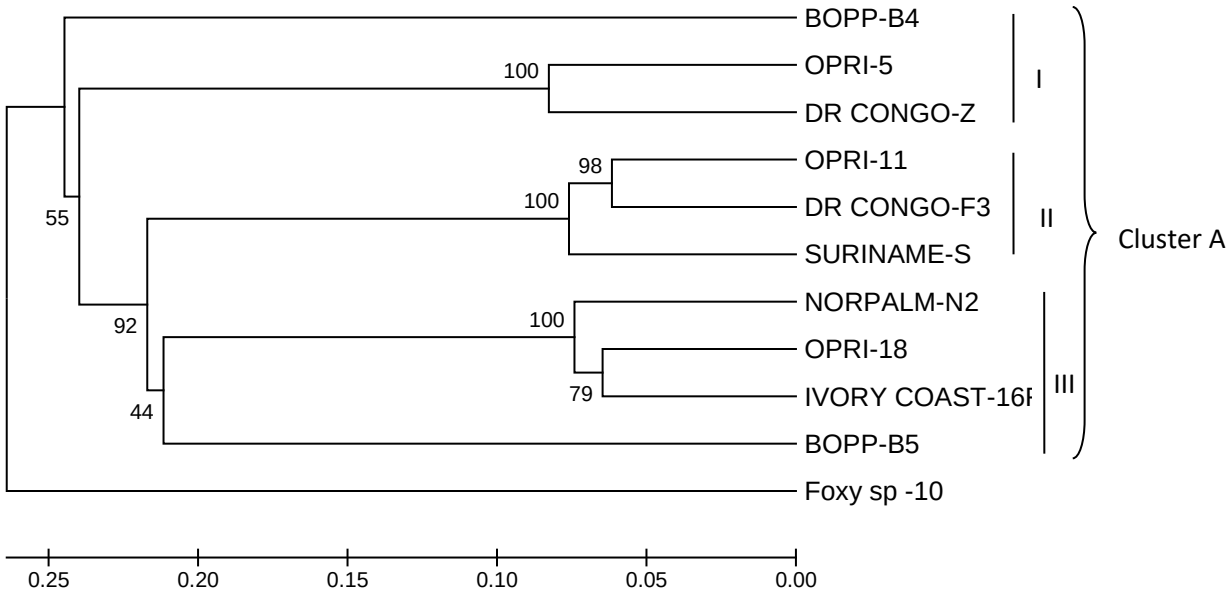


Figure. 2.14. Phylogenetic tree showing genetic relationships among 11 isolates (6 from Ghana, 1 from Ivory Coast, 2 from DR Congo, 1 from Suriname) of *Foe* and one other Fusarium isolate (non-*Foe*) generated with the Unweighted Pair-Group Method with Arithmetic Average (UPGMA) based on the concatenated sequences of three effector protein genes *SIX8*, *SIX9* and *SIX11*. Bootstrap values shown in the branches nodes.

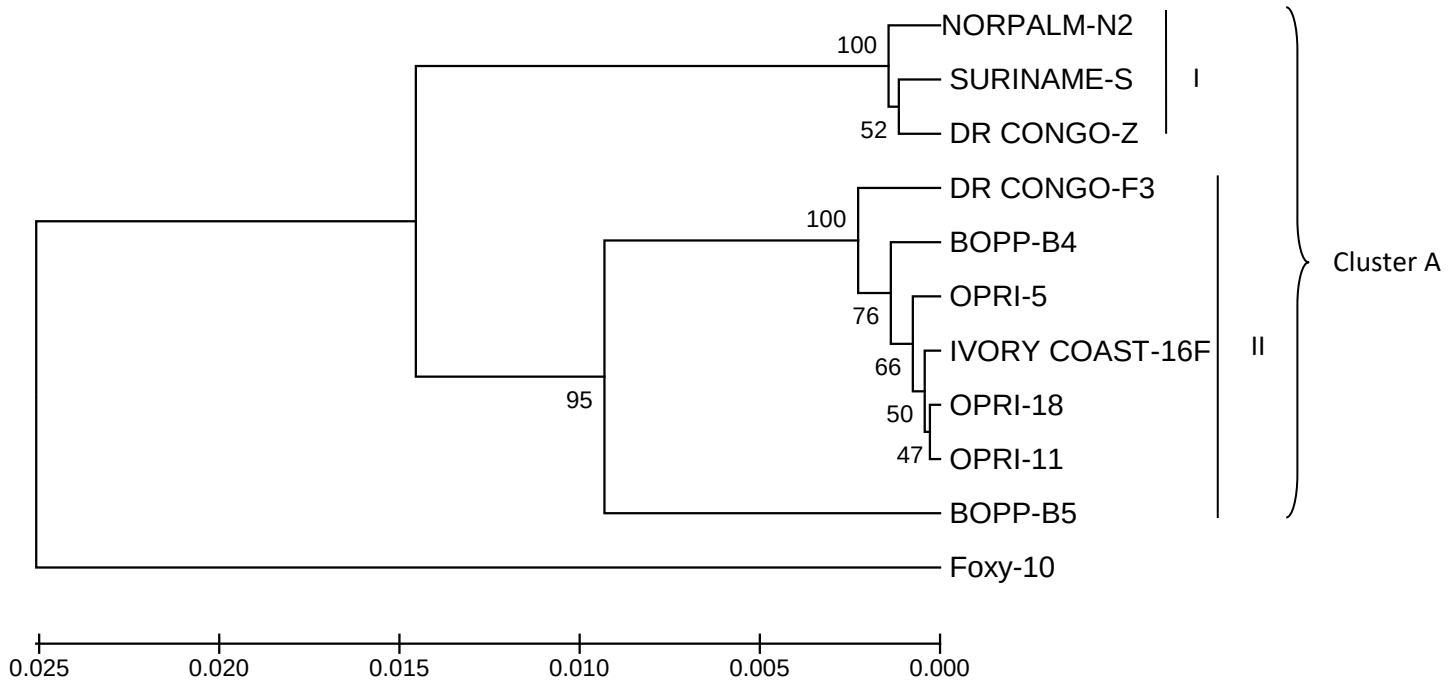


Figure. 2.15. Phylogenetic tree showing genetic relationships among 11 isolates (6 from Ghana, 1 from Ivory Coast, 2 from DR Congo, 1 from Suriname) of *Foe* and one other *Fusarium* isolate (non-*Foe*) generated with the Unweighted Pair-Group Method with Arithmetic Average (UPGMA) based on the concatenated sequences of three effector protein genes (*SIX8*, *SIX9*, *SIX11*) together with two housekeeping genes (Translation elongation factor-1a *TEF-1a*, Internal transcribed spacers *ITS*4). Bootstrap values shown in the branches nodes.

2.5.2 *Amplified fragment length polymorphism* (*AFLP*) *data analysis*

Data files were generated by the FreeTree AFLP analysis software. For analysis, the data generated with the FreeTree software (version 0.9.1.50) was analysed and subjected to distance or similarity matrix analysis using the Jacaard algorithm of the FreeTree software. Bootstrapping was done at 1000 for confidence support. The final phylogenetic tree for the data obtained from the FreeTree software was then analysed with the TreeView software (version 1.0.0.0).

The AFLP amplification of total genomic DNA from all 12 isolates of *Foe* produced two to nine bands. Through the selective amplification, AFLP fingerprints of high quality were achieved for each of the thirteen primer combinations (three EcoR1 primers: EcoAA, EcoTA, EcoC + thirteen Mse1: Mse-CC, TG, AG, TC, CG, TA, TT, GG, AC, CT, GT, CA, GC) primers.

AFLP fingerprinting of the three EcoR1 primer combinations with the thirteen Mse1 primers yielded a total of 4438 polymorphic bands (Table. 2.5) ranging from 50-1000 bp (Fig. 2.16), with an average of 113.79 polymorphic bands per primer combination (Table. 2.5). These data were then used to calculate genetic similarity between all the *Foe* isolates that were used in the study.

A sequence matrix based on the proportion of fragments produced was used to establish the genetic relationship existing between these twelve *Foe*. The genetic similarity between *Foe* isolates DR CONGO-F3 and DR CONGO-Z was 65.21 % whereas IVORY COAST-16F and SURINAME-S was 78.72 %. This result was supported by the high bootstrap value of 100 (Fig. 2.17). Among the *Foe* isolates from Ghana, a higher relationship existed between BOPP-B4 and OPRI-18 with the highest (37.69 %) genetic similarity and bootstrap values of 100, respectively, between these two isolates compared with the remaining isolates. The least genetically related *Foe* isolates were both from Ghana (OPRI-11 and NORPALM-N2) but others had relatively low genetic similarities.

Though there were two major clusters (Fig. 2.17), cluster A included three of the Ghana *Foe* isolates (BOPP-B5, NORPALM-N3, and NORPALM-N5), cluster B had the majority (7) of the isolates (SURINAME-S, IVORY COAST-16F, DR CONGO-F3, DR CONGO-Z, BOPP-B4, OPRI-18, OPRI-5, OPRI-11 and NORPALM-N2). OPRI-5, OPRI-11 and NORPALM-N2 *Foe* isolates from Ghana were distinct and did not group under sub-clusters II or III of major cluster B. In all, there was one sub-cluster (I) for major cluster A with a significant bootstrap value of 88 supporting the genetic relationship existing between Ghana *Foe* isolates (NORPALM-N3 and NORPALM-N5). There were two sub-clusters (II and III) for cluster B which were both supported with significant (100) bootstrap values (Fig. 2.17). Generally, isolates from different geographical locations grouped into specific clusters being major or a sub-cluster (Fig. 2.17). The two main isolates from DR CONGO (F3 and Z) grouped in sub-cluster III, whereas sub-clusters I and II included only *Foe* isolates from Ghana.

Table. 2.5. AFLP result of the polymorphism generated bands among *Foe*

Primer combination	NB	NPB	% Polymorphic Bands
Eco-C + Mse-CC	134	134	100
Eco-C + Mse-TG	104	104	100
Eco-C + Mse-AG	129	129	100
Eco-C + Mse-TC	117	117	100
Eco-C + Mse-CG	97	97	100
Eco-C + Mse-TA	130	130	100
Eco-C + Mse-TT	122	122	100
Eco-C + Mse-GG	131	131	100
Eco-C + Mse-AC	127	127	100
Eco-C + Mse-CT	119	119	100
Eco-C + Mse-GT	95	95	100
Eco-C + Mse-CA	130	130	100
Eco-C + Mse-GC	98	98	100
Eco-TA + Mse-CC	89	89	100
Eco-TA + Mse-TG	105	105	100
Eco-TA + Mse-AG	122	122	100
Eco-TA + Mse-TC	101	101	100
Eco-TA + Mse-CG	94	94	100
Eco-TA + Mse-TA	114	114	100
Eco-TA + Mse-TT	125	125	100

Primer combination	NB	NPB	% Polymorphic Bands
Eco-TA + Mse-GG	144	144	100
Eco-TA + Mse-AC	162	162	100
Eco-TA + Mse-CT	114	114	100
Eco-TA + Mse-GT	117	117	100
Eco-TA + Mse-CA	77	77	100
Eco-TA + Mse-GC	108	108	100
Eco-AA + Mse-CC	117	117	100
Eco-AA + Mse-TG	125	125	100
Eco-AA + Mse-AG	90	90	100
Eco-AA + Mse-TC	110	110	100
Eco-AA + Mse-CG	104	104	100
Eco-AA + Mse-TA	130	130	100
Eco-AA + Mse-TT	114	114	100
Eco-AA + Mse-GG	101	101	100
Eco-AA + Mse-AC	103	103	100
Eco-AA + Mse-CT	112	112	100
Eco-AA + Mse-GT	120	120	100
Eco-AA + Mse-CA	99	99	100
Eco-AA + Mse-GC	108	108	100
Total	4438	4438	
Average	113.79	113.79	

NPB = Number of polymorphic bands NB = Number of bands

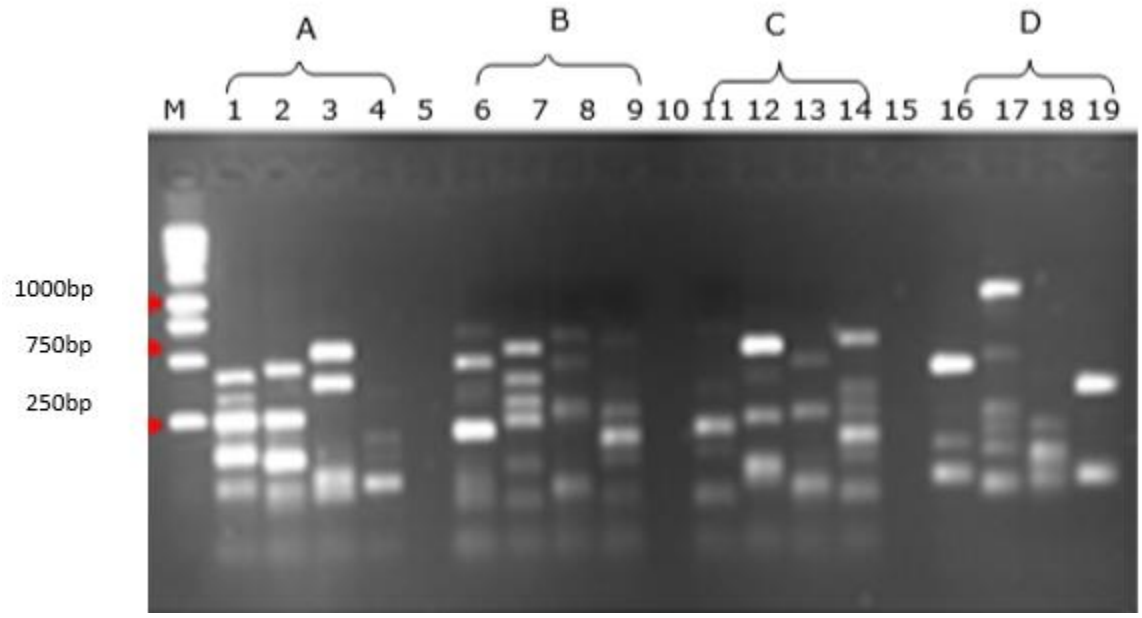


Figure. 2.16. AFLP fingerprinting profile of *Foe* isolates with EcoAA + MseTG (DR Congo: Lanes 1 & 2; Ivory Coast: Lane 3; Ghana: Lane 4) **(A)** ECcoAA + MseCC (DR Congo: Lanes 6 & 7; Ivory Coast: Lane 8; Ghana: Lane 9) **(B)** EcoAA + MseTC (DR Congo: Lanes 11 & 12; Ivory Coast: Lane 13; Ghana: Lane 14) **(C)** and EcoAA + MseAG (DR Congo: Lanes 16 & 17; Ivory Coast: Lane 18; Ghana: Lane 19) **(D)** primer combinations.

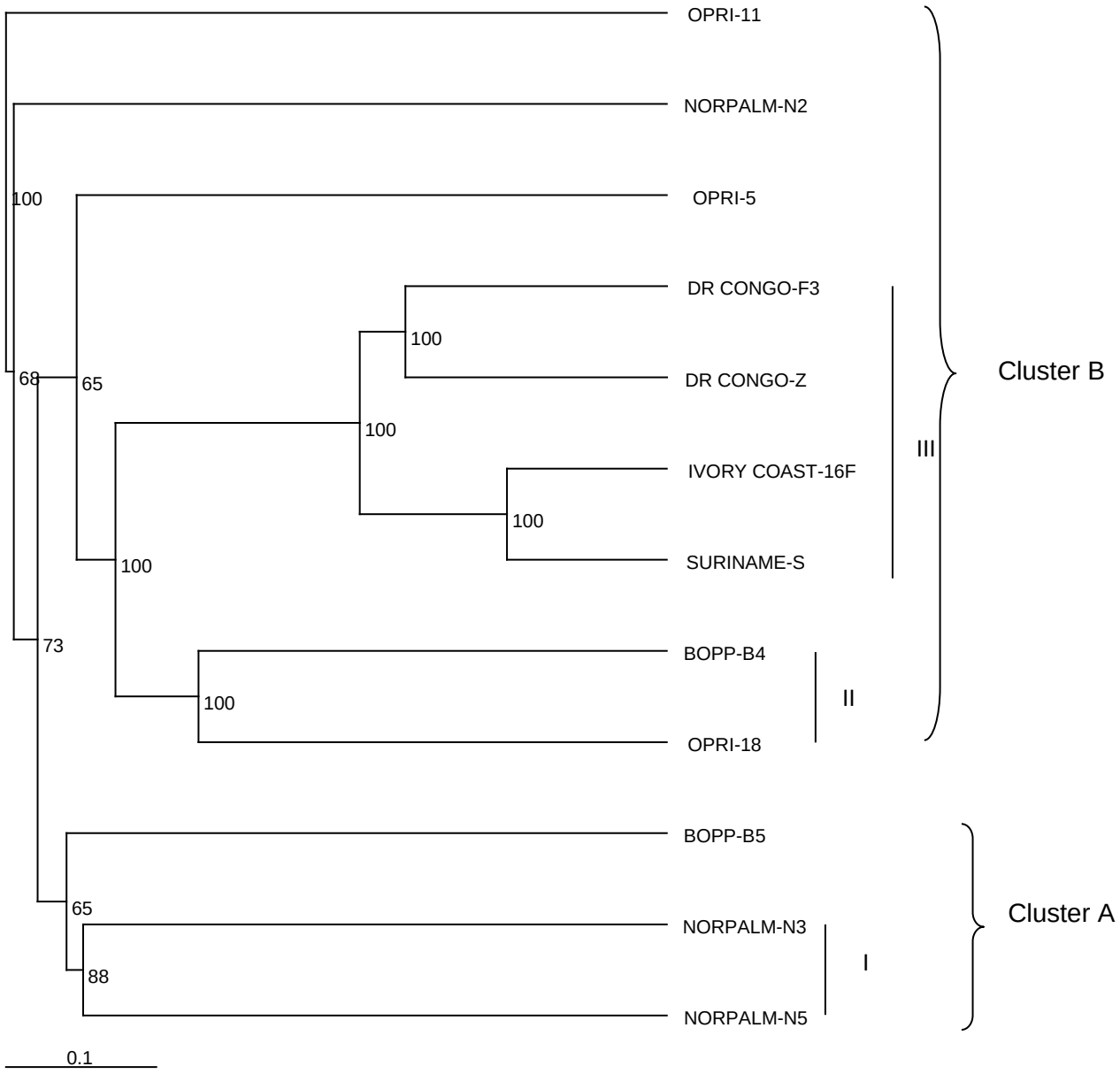


Figure. 2.17. Dendogram generated by Unweighted Pair-Group Method with Arithmetic (UPGMA) cluster of composite data set from AFLP assays showing similarity (B-Jaccard) between 12 *Foe* isolates [Ghana (8), Ivory Coast (1), Suriname (1), DR Congo (2)] for a combination of three EcoR1 with thirteen Mse1 primers yielding a total of 117 combinations. Bootstrapping was done for 1000 with bootstrap values shown in the nodes of the dendogram.

2.5.3 *Simple Sequence Repeats* (*SSR*) *analysis*

There were between two and five bands for the two sets of SSR primers that were effective for amplification of total genomic DNA from all twelve *Foe* and the twelve other fusaria isolates. Among these a number of polymorphic bands were generated with sizes ranging from 50 to 1000 bp. An example of the amplification reaction with primer SSR1 is presented in Fig. 2.18. A total of 8 amplified products were produced with an average of 4 bands per primer, with the total and average polymorphic bands having same values respectively (Table. 2.6). A sequence matrix based on the proportion of fragments produced was used to establish the relationship level between the twelve *Foe* and the other twelve *Fusarium* sp. and these data were used to calculate genetic similarity. The genetic similarity between all the isolates ranged from 12.5 to 100 %. The most genetically related (100 %) isolates were OPRI-11 and OPRI-18, with the genetic relationship supported by a significant bootstrap value of 59 (Fig. 2.19). This observation was similar to *Foe* isolates OPRI-5 and SURINAME-S which had a significant bootstrap value of 96, supporting the close genetic relationship. In addition, *Foe* isolates DR Congo-F3 and NORPALM-N5 also had a significant (66.67 %) genetic relationship supported with a bootstrap value of 55. The phylogenetic tree consisted of two main clusters: A and B. Cluster A contained most of the non-*Foe* isolates. Cluster B had two sub-clusters (I and II) with the majority of *Foe* isolates grouping in main cluster B though scattered with some few non-*Foe* in the sub-clusters.

Table. 2.6. Results on the polymorphic generated bands among *Foe* and other *Fusarium* (non-*Foe*) with SSR primers

Primer code	Primer sequence	Annealing temperature ^{O}C	Amplified bands		% Polymorphic band
			Total number of bands	Polymorphic bands	
6	GCCTTGGTTGTTGTC/GAA GCCTCCACTACCTCG	45	5	5	100
6A	ATATGCTTAAGTTCAGCG GGTGGAGGATGAGCTCGA TGATGAAG/CTAAGCCTG CTACACCCTCG	60	3	3	100
Total			8	8	
Average			4	4	

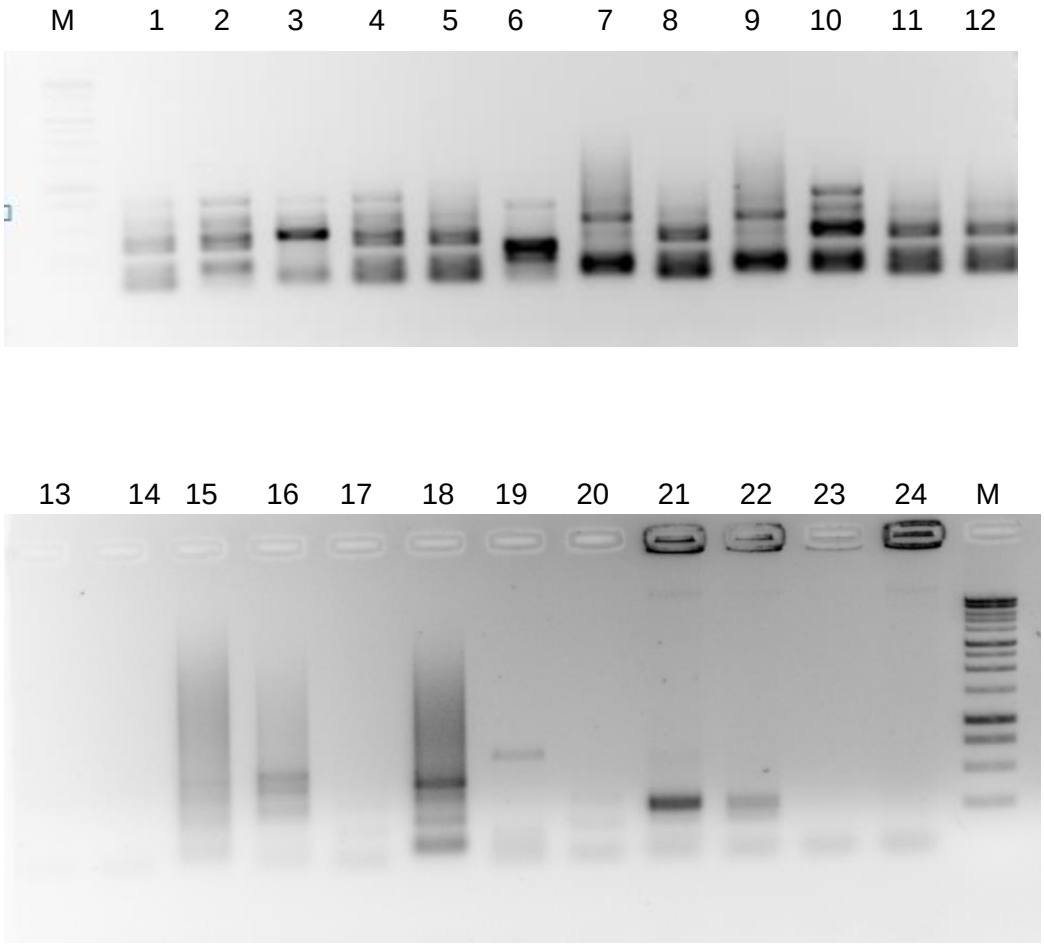


Figure. 2.18. Simple sequence repeats (SSR) amplification patterns obtained with a primer 6 and DNA of: *Foe* lanes 2-13 (16F, F3, Z, S, N2, N3, N5, B4, B5, 5, 11, 18) and other fusarium (non-*Foe*) isolates from lanes 14-24 (Foxy sp-1, Fus126B-3; Foxy150-5; Fus106-6; Foxy sp-8; Foxy2571-9; Foxy sp-10; Fus-11; FoxyBX14/153b-A; FoxyBX14/153a-B; Foxy.Forla-C; Foxy.Forlb-D).

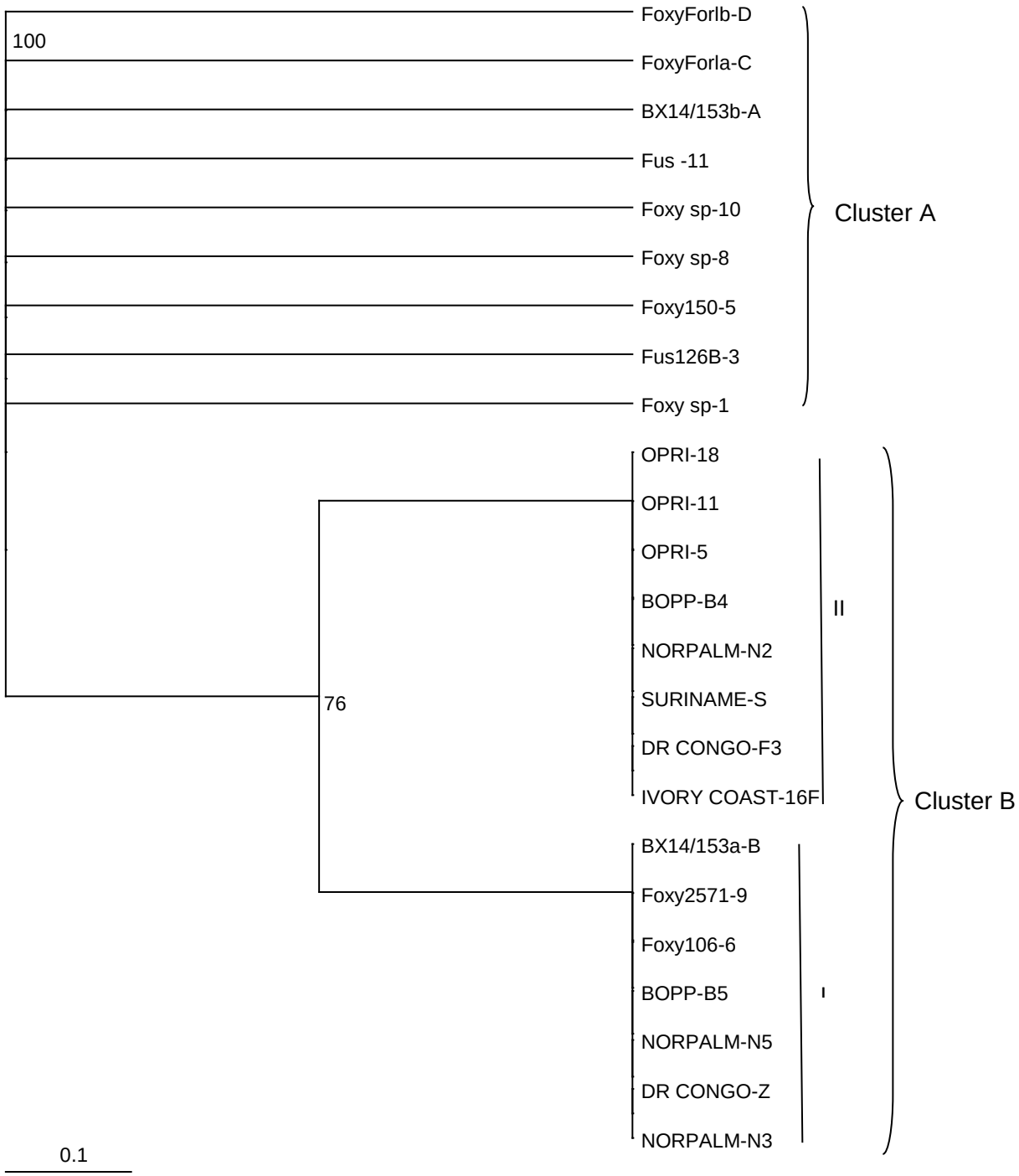


Figure. 2.19. Dendogram generated using statistical model Unweighted Pair Group Method with Arithmetic Average (UPGMA) cluster of composite data set from eight SSR-PCR assays for 12 *Foe* [Ghana (8), Ivory Coast (1), Suriname (1), DR Congo (2)] and 12 other Fusarium (non-*Foe*) isolates. Bootstrapping was done for 1000 with bootstrap values shown in the nodes of the dendogram. Only bootstrap values greater than 50 are shown.

2.5.4 *Inter-Simple Sequence Repeats* (*ISSR*) *analysis*

The ISSR amplification of total genomic DNA from the 24 isolates produced a number of polymorphic bands, ranging from two to six. The size of the amplified products ranged from 250 to 3500 bp. Thirty-three amplified products were produced with an average of 4.7 bands per primer (Table 2.7). The total polymorphic bands generated was 29 with average of 4.14 polymorphic bands per primer (Table 2.7). Among these, five of the markers generated 100 % polymorphism (Table 2.7). An example of the amplification reaction with primer F5 is presented in Fig. 2.20. A sequence matrix based on the proportion of fragments produced was used to establish the relationship level existing between the *Foe* with the non-*Foe* (other *Fusarium* sp.) isolates. These data were then used to calculate genetic similarity matrices. The genetic similarities between all the isolates ranged from 5 % to 46.67 *%. Foe* isolates Ivory Coast-16F, BOPP-B4, BOPP-B5, DR CONGO-Z, and SURINAME-S were the most dissimilar among all the *Foe* isolates that were used for this study. This conclusion was supported by the phylogenetic tree where these *Foe* isolates were outliers (Fig. 2.21). Comparatively, isolate FoxyBX14/153a-B was also the only outlier for the non-*Foe* isolates. Among the non-*Foe*, the most genetically similar (46.67 %) were Fus106-6 and Foxy150-5 with the highest genetic similarity value, whereas for the *Foe* isolates, the greatest genetically similarity (40 %) was between NORPLAM-N3 and DR CONGO-F3. The ISSR result was supported by the phylogenetic tree (Fig. 2.21) depicting the level of closeness in the same cluster. The remaining isolates were scattered in the phylogenetic tree with a majority having bootstrap values of less than 50 (not shown). Generally, there were three

main clusters in the phylogenetic tree generated from the seven (7) ISSR markers; Cluster A and B (Fig. 2.21). Cluster B had two sub-clusters (I to II) including majority of non-*Foe* isolates. Cluster A was a major cluster for only outliers, with most (6) of the *Foe* isolates falling in that part of the phylogenetic tree. Generally, the bootstrap values were relatively low (<50) for all the genetic relationships existing between the isolates.

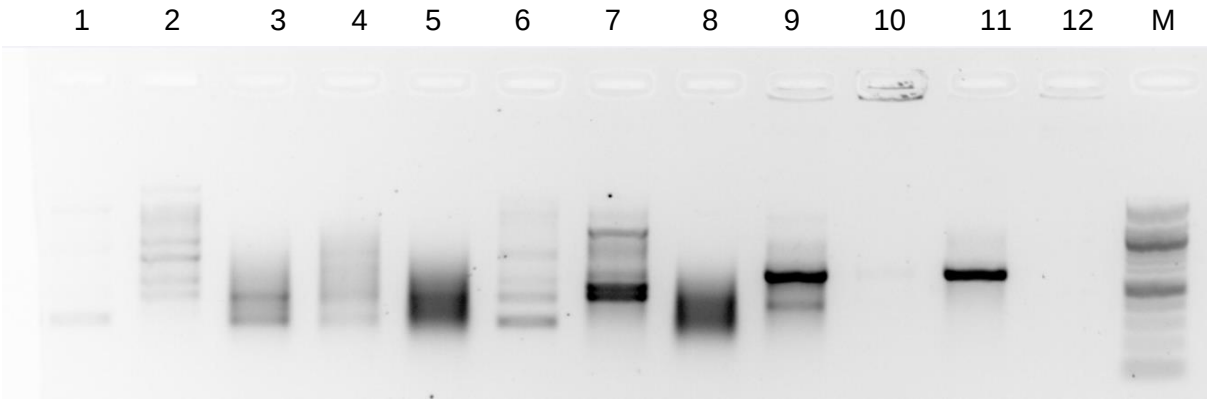


Figure. 2.20. Inter simple sequence repeats (ISSR) amplification patterns obtained with an ISSR primer F5 and DNA of: other fusaria isolates from lanes 1-12 (Foxy sp-1, Fus126B-3; Foxy150-5; Fus106-6; Foxy sp-8; Foxy2571-9; Foxy sp-10; Fus-11; FoxyBX14/153b-A; FoxyBX14/153a-B; Foxy.Forla-C; Foxy.Forlb-D) and lane Marker (M).

Table. 2.7. Results of the polymorphic generated bands among *Fusarium Foe* and other *Fusarium* (non-*Foe*) with ISSR primers

Primer code	Primer sequence	Annealing temperature ^{O}C	Amplified bands		% Polymorphic band
			Total number of bands	Number of polymorphic bands	
F2	$(AG)_8$	50	4	3	75
F3	$(GTG)_5$	50	5	5	100
F5	$(CAG)_5$	54	5	5	100
F7	$(ACC)_5$	54	6	6	100
F9	$(GA)_8C$	54	4	1	25
F12	$(AG)_8G$	54	6	6	100
F13	$(CA)_8T$	54	3	3	100
Total			33	29	
Average			4.71	4.14	85.71

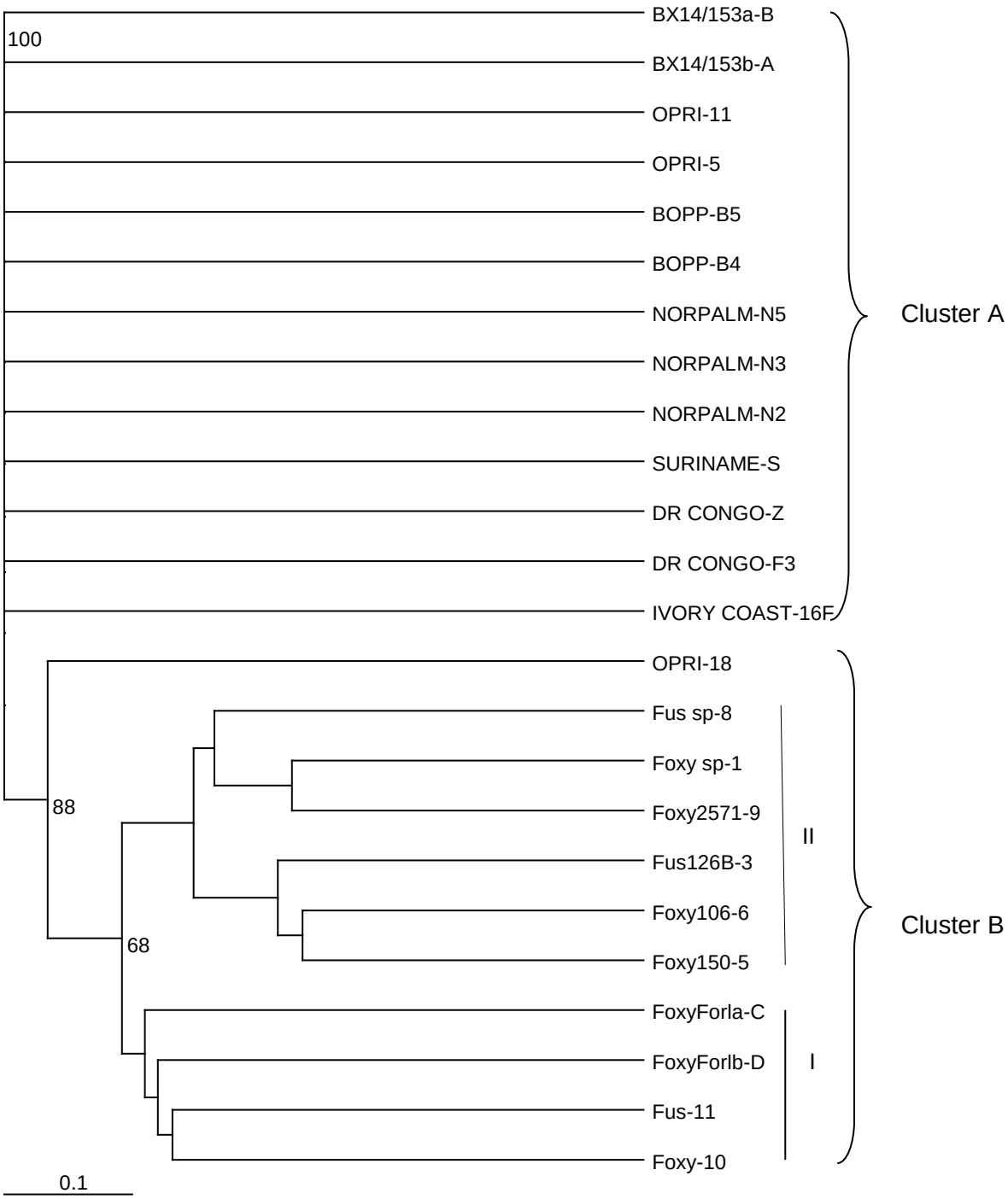


Figure. 2.21. Phylogenetic tree generated using Unweighted Pair-Group Method with Arithmetic Average (UPGMA) cluster of composite data set from eight *ISSR-PCR* assays for 12 *Foe* [Ghana (8), Ivory Coast (1), Suriname (1), DR Congo (2) isolates] and 12 non-*Foe*

2.6 *Discussion*

The ribosomal DNA (rDNA) genes are useful for identification of plant pathogens (Abd-Elsalam, 2003). Although DNA sequencing of some housekeeping genes such as the *ITS* is informative to distinguish species within the eukaryotes, it has proved to be ineffective for phylogenetic analysis in *Fusarium* sp. (O'Donnell, 1992; O'Donnell and Cigelnik, 1997). However, one of the frequently used locus for studying genetic variation within *Fusarium* species complexes is the *TEF*-1α (Geiser *et al.,* 2004; Laurence *et al.,* 2014; Laurence *et al.,* 2015; Pinaria *et al.,* 2015). In this study, we analysed two housekeeping genes, the *ITS* and *TEF*-1α in order to characterise *Foe* and non-*Foe* isolates. The phylogenetic tree (Fig. 2.14) for the two concatenated housekeeping genes could not differentiate the non-*Foe* isolate from the *Foe* isolate as they were all in one cluster, whereas the *Foe* isolate BOPP-B5 was an outlier. Similarly, *TEF*-1α phylogeny of *F. oxysporum* f. sp. *canariensis* from Australia and other international isolates of the same pathogen as well as other *formae speciales* were not monophyletic (Laurence *et al.,* 2015). The Australian isolates grouped in the same clade of outliers. In addition, the concatenation of the housekeeping genes did not group the *Foe* isolates by geographical locations. According to Lievens *et al.* (2009), diversity among *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f.sp *radices-lycopersici* based on the *TEF*-1α region is a result of exchange of genetic material between the *Fol* isolates that lead to switching of clades. Furthermore, the sexual stage of *F. oxysporum* is not known but functional mating types have been identified are expressed (Arie *et al.,* 2000; Yun *et al.,* 2000). The mating types of *Foe* were not established in this present study in contrast to the report by Arie *et al.*

(2000) and Yun *et al.* (2000), reliance on the *TEF*-1α gene for diversity studies of *F. oxysporum* f. sp. *cubense* was congruent with the phylogenetic tree determined using eight protein coding regions (or loci) (Laurence *et al.,* 2014). More over, neither *F. oxysporum* f. sp. *canariensis* of palm nor *F. oxysporum* f. sp. *vanilla* of vanilla plant were monophyletic based on the *TEF*-1a phylogeny (Laurence *et al.,* 2015; Pinaria *et al.* 2015). Similar results were observed in *F. oxysporum* f. sp. *vasinfectum and F. oxysporum* f. sp. *phaseoli* by Silva *et al.* (2014) based on two housekeeping genes, *EF*-1a and IGS. Similarly results to those reported for *F. oxysporum* f. sp. *vanilla, Foc, F. oxysporum* f. sp. *vasinfectum* and *F. oxysporum* f. sp. *phaseoli* were found in the current study of *Foe* isolates from the concatenated sequences of several housekeeping genes.

Initially it was thought that Secreted In Xylem (*SIX*) genes were unique to *F. oxysporum* f. sp. *lycopersici* (*Fol*)*,* but detection has been made in other *formae speciales* of banana (Fraser-Smith *et al.,* 2014). The phylogenetic tree (Fig. 2.14) generated from the sequences for the three *SIX* genes (*SIX8*, *SIX9* and *SIX11*) in this study clustered all *Foe* with the non-*Foe* isolate as the outlier. However, the *Foe* isolates were spread widely in the sub-clusters of major cluster A which suggest they are not monophyletic based on the *SIX8, 9, 11*. Schmidt *et al.* (2013) reported that the *SIX* genes are class II transposable elements (TEs) - enriched chromosomal sub-regions. The presence of TEs can cause genetic variation via mutation or re-organising of the genome of the organism (McDonald and Linde, 2002; Ma *et al.,* 2010). Transposable elements (TEs) have been further reported to be rich in the lineage specific (LS) chromosome where there are *SIX* genes (Rep *et al.,* 2004; Lievens *et al.,* 2009;

Gawehns *et al.,* 2014) for some *formae specilaes* of *Fusarium,* such as *lycopersici*, that differentiates them from the core genome (Ma *et al.,* 2013; Schmidt *et al.,* 2013). These characteristics of the *SIX* genes as investigated could have resulted in the failure of the clustering of the *Foe* isolates based on their geographical origin due to the genetic variations. A similar report was made on the inability of *SIX* genes to differentiate Australian *F. oxysporum* f. sp. *cubense* (*Foc*) isolates from other *Foc* isolates (Laurence *et al.,* 2015). Low sequence diversity among the *Foc* isolates based on the *SIX* genes suggested horizontal gene transfer mechanisms were operating (Ma *et al.,* 2010). The low diversity between and within *formae speciales* of *Fusarium* has been shown in 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 Does *et al.,* 2008; Thatcher *et al.,* 2012).

Some of *SIX* genes were not detected in the *Foe* isolates and similar absence of some *SIX* genes (*SIX2, SIX3, SIX4, SIX5, SIX7*, and *SIX10*) in collections of *F. oxysporum* populations were reported from Australia (Rocha *et al.,* 2015), supporting the view of Laurance *et al.* (2014) on the complexity of *forma specialis* of *Fusarium*. Similarly, *SIX1* was present in one *Foe* and two *Fol* isolates with the PCR based method. The biological function of *SIX* proteins remains largely unknown and their amino acid sequences show little or no homology with other known proteins or each other (Fraser-Smith *et al.,* 2014). However, *SIX* genes differentiated *Foc* race 4 from races 1 and 2, and further distinguished tropical and sub-tropical race 4 isolates based on the *SIX8* gene.

In the present study, it was possible to concatenate both the housekeeping genes and effector genes (*SIX8*, *SIX9*, *SIX11*, *ITS* and *TEF*-1α) for ten *Foe* isolates to generate a combined phylogenetic tree (Fig. 2.15). There were considerable genetic variations in the *Foe*, though there was a clustering of all *Foe* isolates away from the non-*Foe* isolate. A similar report on the concatenation of *TEF*-1α with other genes such as mitochondrial small subunit ribosomal RNA gene (mtSSU rDNA), RNA polymerase II second largest subunit (RPB2) and β-tubulin (TUB2) genes (Pinaria *et al.,* 2015; Taylor *et al.,* 2015) showed the genetic diversity in the *F. oxysporum* f. sp. *cepae* and *F. oxysporum* f. sp. *vanilla*e. Similar conclusions were made for the concatenated sequences for the rDNA genes and *SIX* genes in this current study.

Morphological characterisation of pathogens cannot fully ascertain genetic differences between isolates (Abdel-Satar *et al.,* 2003), so AFLP has been used to determine relatedness between genetic differences in closely related *Fusarium* species or other species (Kiprop *et al.,* 2002; Groenewald *et al.,* 2006; Gurjar *et al.,* 2009). This work examined the genetic variation between twelve *Foe* isolates representative of different locations in oil palm plantation sites in Ghana, Ivory Coast, DR Congo and Suriname. In general, the AFLP markers suggested a high level of genetic variation present in the *Foe* isolates, which may be associated with genetic drifts and mutations within fungal populations or the reproductive strategies of fungi (Lima *et al.,* 2009; Thangavelu *et al.,* 2012). Higher similarity existed in *Foe* isolates from DR Congo (F3 and Z), isolates from Ivory Coast and Suriname clustered closely. Mouyna *et al.* (1996) reported that *Foe* isolates from Ecuador and Brazil had the same RFLP fingerprinting patterns as some Ivorian *Foe* isolates. Flood *et al.* (1992) made similar assertions. It was suggested that

the *Foe* isolates from Brazil and Ecuador may have originated from Ivory Coast via seed transmission across borders or cover crop debris on the oil palm seeds (Flood, 2006). The link between *Foe* isolates from Brazil and Ecuador makes it likely that *Foe* isolates from Suriname may have also been transmitted from Brazil as these two countries share a border. Molecular fingerprinting of *F. oxysporum* f. sp. *passiflorae* (*Fop*) isolates specific to passion fruit, did not differentiate all isolates tested (Silva *et al.,* 2014) based on geographical origins, possibility due to the different AFLP markers used in both studies and the particular *Fo formae speciales* studied.

This is the first study to use AFLPs to characterise *Foe* isolates from Ghana. Ghanaian *Foe* isolates were are genetically diverged, as some of the isolates from the same plantations grouped in different sub / major clusters (Fig. 2.17). This result was reflected in the fingerprinting pattern as some *Foe* isolates from the same location in Ghana were not identical. In Ghana, the main source of oil palm seedling supply to growers in NORPALM or BOPP is OPRI (Afloe, G. Norplam Plantation site - Takoradi, Ghana, personal communication, 2014). However, the genetic variation between these *Foe* isolates from Ghana which did not groupe in a major cluster in this study could be attributed to the following hypothesis. According to Kistler (1997), though genetic variation can result from para-sexuality that occurs within heterokaryon fungi, there is no known or reported sexual stage of *F. oxysporum* (*Fo*). Fourier *et al.* (2009) demonstrated this feature through VCG studies in *Foc*. The genetic variation that existed in Ghanaian *Foe* isolates in this study, suggested a polyphyletic or paraphyletic nature. This finding supports the report on the complexity of the origin of pathogenic lineages from the characterisation of 89 strains representing known

genetic and pathogenic diversity in *8 formae speciales* associated with wilt diseases, roots and bulb rot (Baayen *et al.,* 2000). The ability of the AFLP markers to distinguish between *Foe* isolates shows it is useful as a technique for genetic variation studies.

Among all the SSR markers that were used to examine genetic variation study in the *Foe* isolates, two produced good discriminatory bands. The SSR markers did not produce many unique bands in the non-*Foe* isolates (Fig. 2.18) compared to the *Foe* isolates. The non-*Foe* isolates used for the comparative study with the *Foe* isolates were spread in the phylogenetic tree revealing the genetic variability. However, nine of the non-*Foe* isolates grouped together in one cluster of the phylogenetic tree.

Some of the *Foe* isolates were not in same major/sub – clusters (Fig. 2.19) or were spread along the phylogenetic tree, which did not correspond with the geographical origin of the isolates studied. The variation found in the *Foe* isolates from the same geographical origin may be due to the polyphyletic nature of the *Foe*. Polymorphism reported in several fungi has been suggested to be a direct effect of evolution (Sanders, 2002; Schardl and Craven, 2003). According to Mouyna *et al.* (1996), there is a higher diversification of the African *Foe,* leading these authors to conclude that Africa is a diversity area for the fungus. Costa *et al*. (2014) reported that SSR markers revealed the polyphyletic characteristics of 214 *Foc* isolates collected from different locations in Brazil. Similar results demonstrated in a study of Ethiopian *Fo* isolates, where other *formae speciales* studied with the *Fo* were spread widely across the three *Fo*

clades (Bogale *et al.,* 2006). It was concluded that the three lineages of Ethiopian *Fo* isolates did not correspond with the host or geographical location. Ma *et al.* (2010) showed that the presence of TEs in *Fusarium* sp. was common; this finding was supported by the presence of palm transposons in *Foe* from South American isolates (Mouyna *et al.,* 1996), which strongly suggest that the palm transposons were present in the *Foe* isolates used in this present study.

As a molecular tool, SSR is reported to reliable for characterizing other fungal species (Jarne *et al.,* 1996; Barnes *et al.,* 2005; Enjalbert *et al.,* 2005). However, the inability of the SSR markers to group *Foe* isolates based on geographical origin and also not totally differentiating non-*Foe* isolates from *Foe* in the phylogenetic tree generated could be due to several factors. Different *Fusarium* species have different frequencies of SSR types and repeat units (Li *et al.,* 2004; Garnica *et al.,* 2006; Lawson and Zhang, 2006) and the number of repeats is a good indicator of variability, as demonstrated in other organisms (Vigouroux *et al.,* 2002). According to Kim *et al.* (2008) and Levdansky *et al.* (2008), the tri and hexa-nucleotide repeats mostly dominate coding regions, whereas in the non-coding regions, di, tetra, and penta-nucleotide repeats are often found in abundance. It has been reported that longer repeats have high mutation rates and could, therefore, be less stable (Kruglyak *et al.,* 2000). In addition, random mutation followed by DNA polymerase slippages are mainly responsible for short microsatellite repeats (Kruglyak *et al.,* 2000). A genome wide study of *F. oxysporum* f. sp. revealed that microsatellite distribution was not random (Mahfooz *et al.,* 2012) which could have led to the discrepancy in the grouping or clustering of the non-*Foe* isolates together with the *Foe* isolates in this study.

Inter-simple sequence repeat (ISSR) markers have been widely used in diversity studies in other pathogens (Wang *et al.,* 2012; Shafiei-Astani *et al.,* 2015), DNA fingerprinting (Shen *et al.,* 2006) and phylogenetic analysis (Iruela *et al.,* 2002). The evaluation of genetic diversity of *Foe* isolates and non-*Foe* isolates for comparative reasons was successful in this study. There was a relative abundance of ISSR's in the non-*Foe* isolates compared with the *Foe* isolates (Fig. 2.20). This finding is reflected in the phylogenetic tree where the majority of the *Foe* isolates from Ghana, Ivory Coast, DR Congo or Suriname grouped in cluster A. Most of the non-*Foe* isolates grouped in one cluster. ISSR analysis showed similar polymorphic patterns among the non-*Foe* isolates and also failed to group *Foe* isolates based on their geographic sources. This problem emphasized the polyphyletic nature of the pathogen. Only two of the non-*Foe* isolates were spread in the phylogenetic tree (Fig. 2.21) as the majority were in cluster B or one of the sub-clusters. A similar observation was made for OPRI-18 which was further from the other *Foe* isolates, possibly due to poor quality amplification products as a result of the genomic DNA for the ISSR. Unlike the diversity study in the *Foe* isolates with ISSR's, *F. oxysporum* f. sp. *citri* yielded a homogenous population structure across Tunisia (Hannachi *et al.,* 2015). This difference may be due to the host specificity of citrus and the geographical location.

ISSR markers have been used to discriminate between individual fungal isolates (Haji-Allahverdipoor *et al.,* 2011) including *formae speciales* (Baysal *et al.,* 2013). There have been studies where ISSRs were more reliable and superior to RAPD markers in examining pathogenic isolates of *F. oxysporum* f. sp. *citri* in sweet orange and tangerine (Hannachi *et al.,* 2015). The superiority of the ISSRs was associated with the length of the markers, inducing more stringent

polymerase chain reaction conditions, as reported by Hannachi *et al.* (2015). However, in this study, the ISSR markers reliably discriminated between the non-*Foe* isolates compared with the *Foe* isolate due to the strong band patterns produced by the ISSR makers, especially F7 and F12. Baysal *et al.* (2013) reported the use of ISSR as an effective genetic variation study tool for *Fol* of tomato and *F. oxysporum* f. sp. *melongenae* (*Fom*) of eggplant. Thus, ISSR markers are useful for diversity assessment between isolates of *F. oxysporum* (Bayraktar and Dolar, 2008) but unreliable to discriminate within some *formae speciales* (e.g. *F. oxysporum* f. sp. *elaeidis*) from different locations, as shown in this current study. However, molecular fingerprinting of *Fol* with ISSR markers recently was successful and resulted in a wide genetic variation (Nirmaladevi *et al.,* 2016). This difference could be due to the use of different ISSR markers or different *Fusarium* forma speciales used.

The ISSR markers for fingerprinting developed in this study were useful for diversity assessment between *Foe* and non-*Foe* isolates, though there were challenges in the scoring for the presence or absence of polymorphic bands, as this process was done manually. This could have resulted in some of the non-*Foe* isolates such as BX14/153a-B and BX14/153b-A being grouped with the *Foe* isolates and OPRI-18 *Foe* isolate in the non-*Foe* isolates major cluster. Despite these challenges with ISSR, it has been reported to be a valuable molecular tool for fingerprinting and a useful alternative to single-locus or hybridization-based methods (Bornet and Branchard, 2001) and less costly (Ng and Tan, 2015) compared to molecular techniques such as RAPD and AFLP (Sudisha *et al.,* 2008; Qi *et al.,* 2009).

In this chapter, the use of housekeeping genes, effector genes, AFLPs, SSRs or ISSRs for examining the genetic variability in *Foe* has demonstrated that *Foe* isolates are not monophyletic. Overall, the *Foe* isolates did not cluster based on geographical origin for all the phylogenetic trees constructed based on the concatenated sequences of of all *SIX8, 9, 11* genes, housekeeping and effector genes as well as the phylogenetic trees for the AFLPs, SSRs or ISSRs in this work.

CHAPTER THREE: INOCULATION OF *FUSARIUM OXYSPORUM F. SP. ELAEDIS* (*FOE*) ON OIL PALM GENOTYPES

3.0 *Introduction*

There have been numerous techniques to assess and identify progenies of oil palm resistant to fusarium wilt disease via inoculation tests but success rates as yet have not been made known. Flood (2006) reported that resistant progenies are available now in the Ivory Coast. However, these fusarium wilt resistant oil palm progenies have not been validated in other oil palm growing countries.

A report by Martyn (2014) showed how resistant watermelon cultivars were bred in the USA through a pathogenicity test developed by Smith (1899) who characterized, named and finally established pathogenicity of *F. oxysporum* f. sp. *niveum* (*Fon*). With this foundation Orton (1907) developed a wilt resistant watermelon cultivar but this concept of developing resistant traits of watermelon is not well understood by the research community. However, gradually over the years, breeding efforts have improved for producing wilt-resistant cultivars. In tomato plant, resistant lines such as *Lycopercisi pimpinellifolium* have been identified to the *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) races 1 (Walker, 1971). Mes *et al.* (1999) identified tomato line (OT364) which has resistance to *Fol* race 2 but is suceptible to race 1. Resistance to *Fol* race 3 has been identified in accessions PI414773 and LA716 of *L. pennellii* (Bournival and Vallejos, 1991).

In another study on interaction between oil palm and *Foe*, a technique called petiole infusion (Mepsted *et al.,* 1995) was developed to screen oil palm plants for potential resistance to *Foe*. This process terminated the normal mode of destructive assessment of the oil palm crop during screening for *Foe* which adversely affected breeding. The petiole infusion technique is a non-destructive method of assessing resistant oil palm seedlings within few days. This method involves cutting the petiole of inculated oil palm and infusing water from one cut-end of the petiole to observe/assess if the water will be occluded from flowing to the other end of the cut petiole. However, one of the most difficult aspects of developing resistant crops with pathogenicity tests is to obtain and develop a breeding line that will be resistant to all strains of a particular pathogen across the world. This problem has become a conundrum to plant breeders and pathologist.

Pathogenicity tests usually involve inoculating the host plant with pathogens and identifying symptoms (Fraselle, 1951; Renard and Ravisé, 1986; Renard and de Franqueville, 1989). As such, the need to inoculate oil palm seedlings to examine or identify possible resistant oil palm seedlings.

3.1 *Materials and methods*

3.1.1 *Plant materials*

The progenies used in the inoculation tests were selected from those currently used in the plant breeding programme of the Council for Scientific and Industrial Research - Oil Palm Research Institute (CSIR-OPRI), Ghana. Some progeny

plants from the Malaysia Palm Oil Board (MPOB) were also included to broaden the scope of obtaining possible *Foe* resistant oil palm progenies. The oil palm types used for the pathogenicity tests obtained from Ghana were Dura (K8-1283D progeny lines) and Tenera (DXP) or 'commercial type', whereas from Malaysia the source was Tenera (DXP). All the oil palm seedlings were obtained using the assisted pollination method to yield these specific progenies.

Germinated seeds were transplanted to seed trays (9 cm) filled with compost (Compost 3) and maintained in a controlled glasshouse environment (30 ºC, 90 % RH and 12 hr photoperiod), with light (240 µ mol m^{-2} sec^{-1} photon flux density (PFD) (Fig. 3.1). RH in the glasshouse was reduced to 80 % after 2 months when the oil palm seedlings were transplanted into Soparco black pots (13 cm). Light levels were maintained between 800 and 500 µ mol m^{-2} sec^{-1} with a day length of 14 - 17 hr; humidity and temperature ranged from 60 to 80 % and 20 to 35 ºC, respectively. The seedlings were watered at the base at regular intervals when necessary. Conditions in the glasshouse were maintained throughout the experiments.

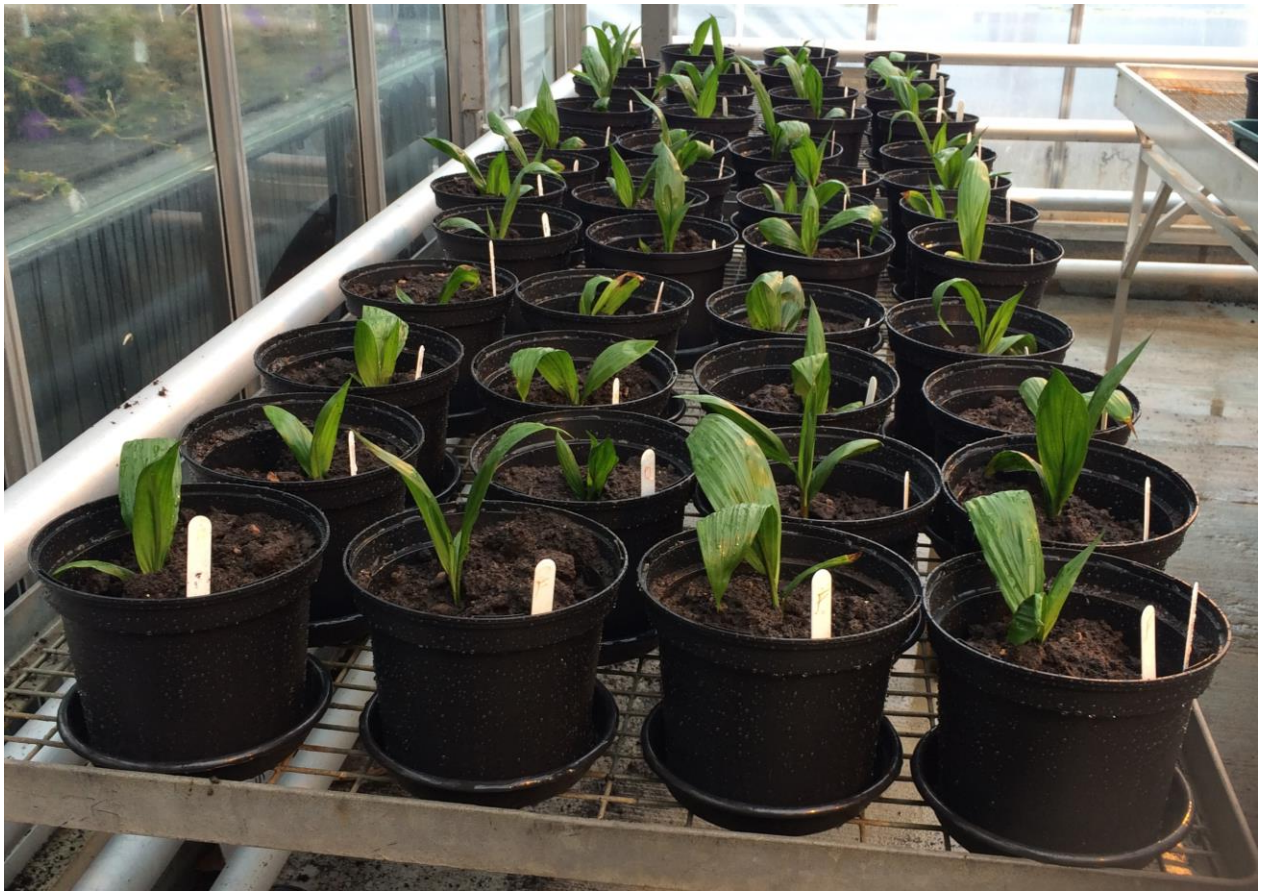


Figure. 3.1. Potted oil palm seedlings in the glass-house

3.1.2 *Preparation of inoculum*

Spores were isolated from BOPP-B5, OPRI-5, NORPALM-N5 (Fig. 3.2) or 16F and used in the pathogenicity experiments. Flood *et al*. (1993) confirmed the pathogenicity of the 16F isolate (from Ivory Coast) towards oil palm and hence this was selected as the reference isolate*. Foe* isolates were cultured on PDA and incubated at 29 ºC for 7 days.

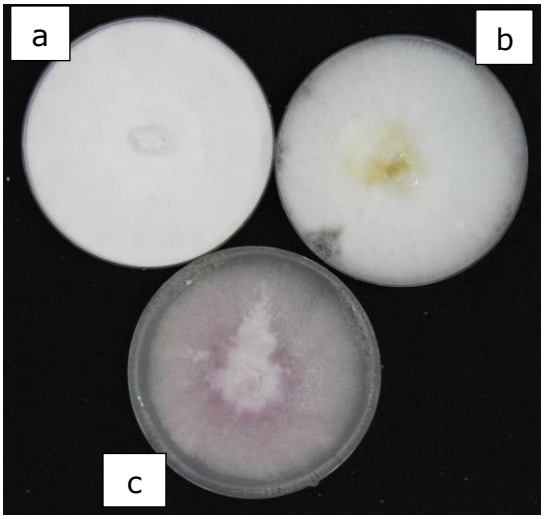


Figure. 3.2. Growth of *Foe* isolates collected from Ghana **(a)** OPRI **(b)** BOPP and **(c)** NORPALM.

The isolates were sub-cultured into 100 ml Czapek-Dox liquid medium (Appendix 1B) in 250 ml conical flasks for 3 days, at 150 rpm in an incubator at 29 ºC. The suspension was filtered through Miracloth (CALBIOCHEM) to remove mycelial fragments and the filtrate centrifuged at 670 x g for 10 min MSE Centaur 2. The spore pellet was gently re-suspended in 50 ml Falcon tubes with sterile distilled water and adjusted to 10^6 spores ml^{-1}. A Neubauer haemocytometer was used to counted the spore concentration (Fig. 3.3a), which was calculated using the formula:

$$\text{Counts/ml} = \frac{\text{Total spore counts}}{\text{No. of 4x4 grids counted}} \text{ x } 10^4 \text{ x sample dilution}$$

Spore viability was quantified in a 50 µl drop of 10^6 / ml of spore suspension on a glass slide incubated at 29 ºC for 6 – 7 hrs. When optimum numbers of germ tubes (Fig. 3.3. b) formed, the germination of the spores was stopped using a

drop of lactic acid cotton blue. Percentage germination was calculated using the formula:

$$\text{Percentage germination (\%)} = \frac{\text{No. of germinated spores per field view}}{\text{Total No. of spores per field view}} X\ 100$$



Figure. 3.3. Microscope images **(a)** micro/macro-conidia and **(b)** germinated spores of *Foe*.

3.1.2.1 *Foe inoculation methods*

Inoulation of 3_X10^6 or 12_X10^6 *Foe* spore suspension was carried out using a sterile syringe in three different treatments to oil palm seedlings at 3 months old as described below;

1. 12_X10^6 spores ml^{-1} *Foe* suspension poured directly onto the soil surface around the base of each palm (Fig. 3.4a).
2. The roots of the oil palm seedlings were soaked in 3_X10^6 spores ml^{-1} *Foe* suspension spore (Fig. 3.4b) overnight with slight wounding around the roots.

3. The roots of the oil palm seedlings were then soaked in 3_X10^6 spores ml^{-1} *Foe* suspension (Fig. 3.4b) overnight without wounding.

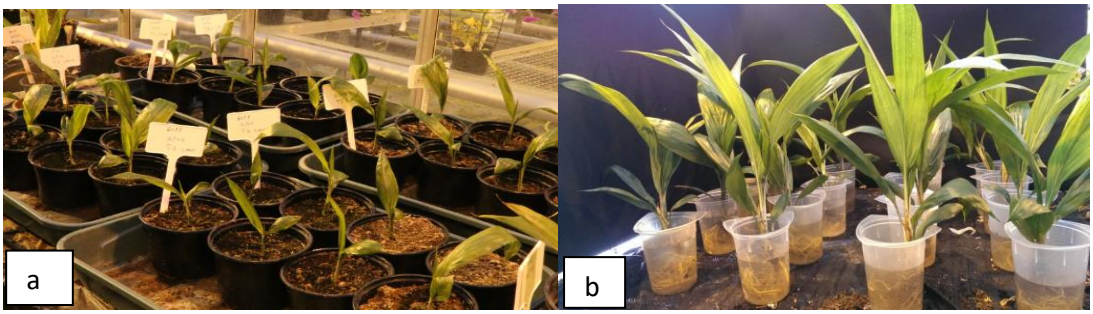


Figure. 3.4. Picture of oil palm seedlings **(a)** already potted with soil at the base to be inoculated and **(b)** removed from pots with roots washed and soaked in spore suspension in beakers.

3.1.3 *Screening of inoculated oil palm seedlings*

Oil palm seedlings that showed symptoms of fusarium wilt were randomly sampled from the glasshouse. The roots were assessed for discolouration and the tissues from the root or stem aseptically cultured on a PDA supplemented with antibiotics (penicillin 300µl/0.5l and streptomycin 250µl/0.5l) as described in section 2.3.2. The DNA was isolated (as described in section 2.4.2) from the root or stem/leaf and assessed for presence or absence of *Foe* using the appropriate primer pairs for *ITS*, *TEF*-1α or *SIX8* genes as described in section 2.3.4, and via the Loop Mediated Isothermal Amplification (LAMP) technique, described in chapter four. Statistical analysis of variance (ANOVA) was done with GenStat 16th Edition.

3.2 *Results*

Tables 3.1 and 3.2 show the results from the three different inoculation treatments used to test resistance test of palm plants to *Foe*. The Ghanaian oil palm progeny line K-1213D was subjected to only three of the *Foe* isolates (OPRI-5, BOPP-B5, 16F). In all the pathogenicity assay experiments for this study, the 16F *Foe* isolate was used as the reference isolate.

Oil palms inoculated via direct application with $12x10^6$ *Foe* suspension onto the base of the potted plants took 10 months to show symptoms, when inoculated with *Foe* OPRI-5 and 16F, whereas those inoculated with BOPP-B5 *Foe* showed symptoms at 11 months. However, ANOVA showed that there was no significant difference ($P>0.05$) between the *Foe* isolates time of appearance of symptoms in the Ghana K-1213D oil palm progenies (Appendix 4A). Malaysian oil palm seedlings that had either minor wounds or no wounds on the roots and were soaked in $3x10^6$ *Foe* spores overnight before potting also showed symptoms at 4 and 7 months respectively (Fig. 3.5). However, the ANOVA showed that there were no significant differences ($P>0.05$) among the *Foe* isolates in the time of first symptoms for the Malaysia oil palm progeny (DXP) (Appendix 4B). The nature of discolouration of the roots as a result of infection by *Foe* are shown in Fig. 3.6b and c. Roots were tested for the presence of *Foe* by plating on PDA supplemented with antibiotics described in section 3.1.3 (Fig. 3.6a and b), and DNA isolated from the mycelia for PCR. PCR was done to test *Foe* for in randomly sampled leaves of seedlings showing symptoms after inoculation though the result was not shown. None of the oil palm seedlings used as controls for the pathogenicity assesment showed symptoms and none tested positive for *Foe* by

re-isolationo of *ITS* or *SIX8* genes (Figs. 3.8 and 3.9). Amplification products were confirmed as *Foe* via Blast of the sequences. The potted soil in which the plants were inoculated were tested for the presence of the *Foe* (Fig. 3.10) with the DNA isolation protocol as described in Chapter 2 section 2.3.3. *Foe* was identified in all the soil pots that were inoculated under glass house conditions.

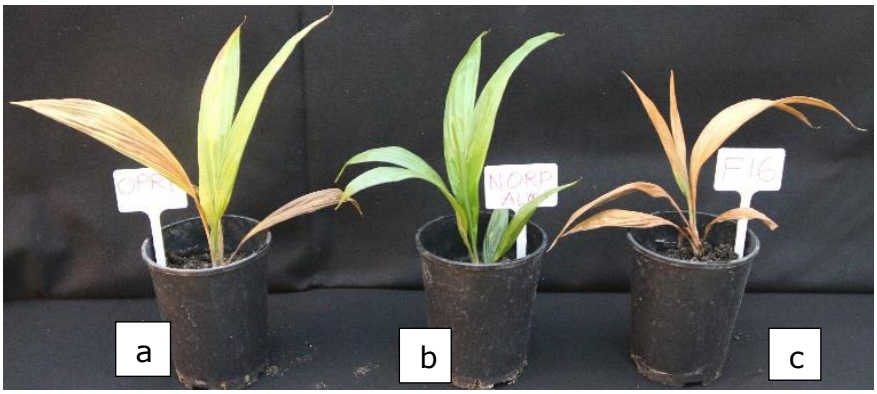


Figure. 3.5. Oil palm seedlings showing symptoms of fusarium wilt disease after inoculation with *Foe* from **(a)** OPRI-Ghana **(b)** NORPALM-Ghana and **(c)** 16F-Ivory Coast.



Figure. 3.6. Oil palm roots **(a)** healthy (control treatment) or *Foe* inoculated **(b)** mildly discoloured **(c)** severely discoloured.

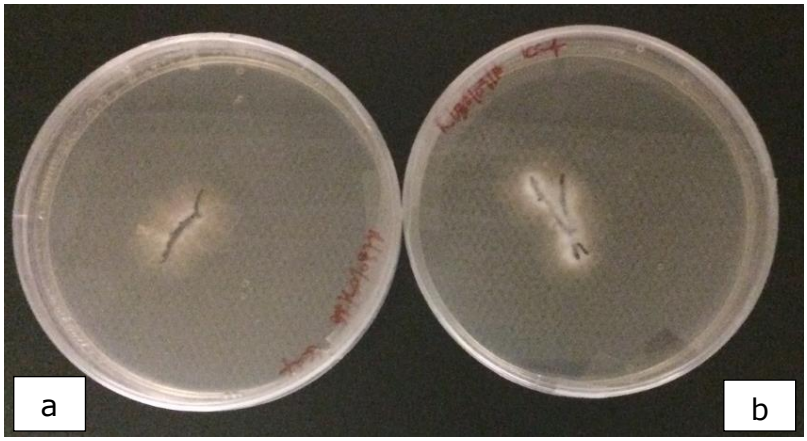


Figure. 3.7. Growth of *Foe* **(a)** OPRI and **(b)** BOPP isolated from oil palm roots

Table. 3.1. *Foe* infected Malaysian oil palm seedlings via root wounding or no-wounding inoculation mechanisms

Oil palm source	Inoculation method	*Foe* isolates inoculated			
		OPRI	BOPP	NORPALM	16F
Malaysia (DXP)	W	+	+	+	+
	W	+	+	+	+
	W	+	+	+	+
Malaysia (DXP)	NW	+	+	+	+
	NW	+	+	+	+
	NW	+	+	+	+

Each *Foe* isolate was replicated three times per treatment. There was no significance difference (P>0.05) between both inoculation methods for time of appearance of symptoms since inoculation among the four *Foe* isolates according to Fisher's protected least significant difference test.

Selected oil palm seedlings inoculated via soaking wounded roots and non-wounded roots in $3x10^6$ in spore suspension

NW = Non – wounded roots

W = Wounded roots

+ = Infected seedlings

Table. 3.2. *Foe* infected Ghanaian oil palm seedlings via direct inoculation method

Oil palm source	Inoculation method	*Foe* isolate inoculated		
		OPRI	BOPP	F16
Ghana	D	+	+	+
(DXD)	D	+	+	+
K-1213D	D	+	+	+

Each *Foe* isolate was replicated three times per treatment. There was no significance difference (P>0.05) for time of appearance of symptoms since inoculation among three *Foe* isolates according to Fisher's protected least significant difference test.

Selected oil palm seedlings inoculated direct application (i.e. $12x10^6$ *Foe* spore suspension poured unto the base of potted oil palm seedlings in glass house)

D = Direct application of spores

+ = Infected seedlings

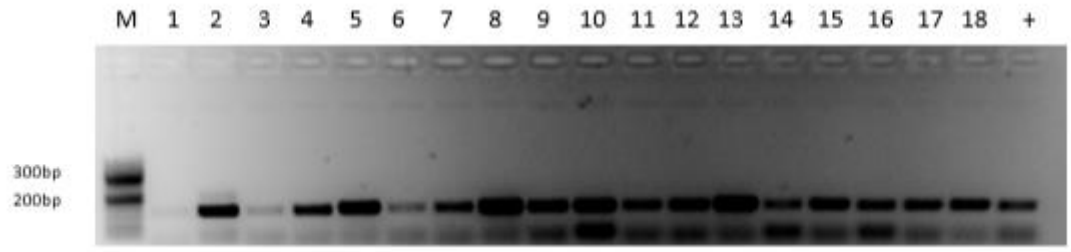


Figure. 3.8. Polymerase chain reaction amplification products from genomic DNA isolated from inoculated Ghana oil palm progeny (K8-1213D) after 9 months post inoculation with primer pairs *ITS4*F/*ITS4*R. *Foe* isolates; OPRI-5 from roots (lanes: 1-3), BOPP-B5 from roots (lanes: 4-6), 16F from roots (lanes: 7-9), OPRI-5 from stems (lanes: 10-12), BOPP-B5 from stems (lanes: 13-15), 16F from stems (lanes: 16-18) and positive control (+) Marker is 100 bp ladder.

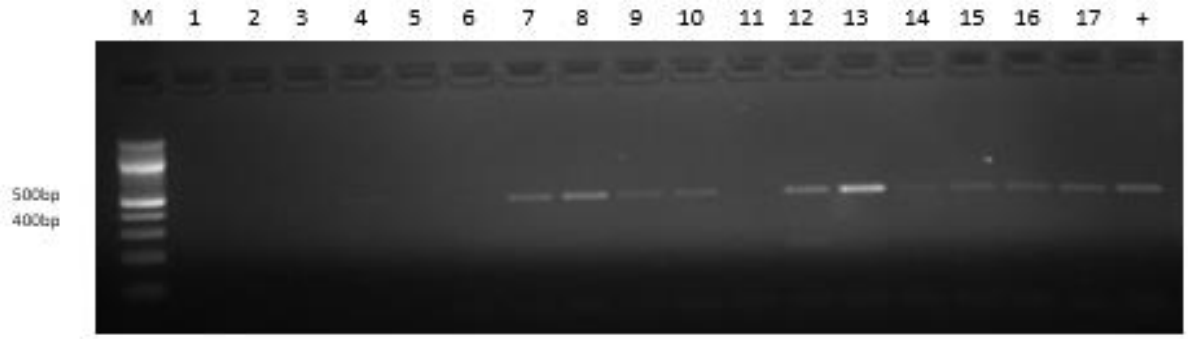


Figure. 3.9. Polymerase chain reaction amplification products from genomic DNA isolated from inoculated oil palm progeny (K8-1213D) from Ghana after 9 months post inoculation swith primer pairs *SIX8*F/*SIX8*R. *Foe* isolates; OPRI-5 from roots (lanes: 1-3), BOPP-B5 from roots (lanes: 4-6), 16F from roots (lanes: 7-9), OPRI-5 from stems (lanes: 10-12), BOPP-B5 from stems (lanes: 13-15), 16F from stems (lanes: 16 & 17) and positive control (+) Marker is 100 bp ladder.

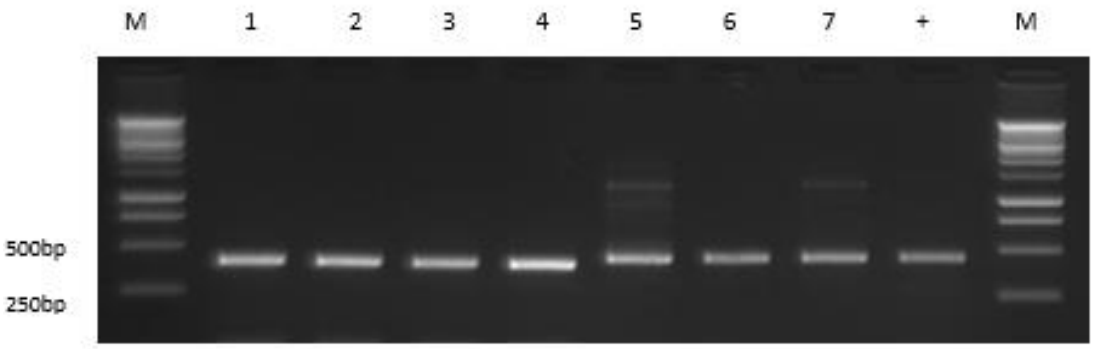


Figure. 3.10. Polymerase chain reaction amplification products from genomic DNA isolated from *Foe* inoculated soil in which oil palm seedlings were potted after 9 months post inoculation with primer pairs *EF5*F/*EB5*R (16F: lane 1, OPRI-5: lane 2, BOPP-B5: lane 3, NORPALM-N5: lane 4); *SIX8*F/*SIX8*R (16F: lane 5, OPRI-5: lane 6, BOPP-B5: lane 7) and positive control (+). Marker is 100 bp ladder.

3.3 *Discussion of Foe inoculation*

The oil palm-infecting *Foe* has not been classified into races (Gordon and Martyn, 1997) unlike the banana pathogen *F. oxysporum* f. sp. *cubense* (Li *et al.,* 2015). Some research on the virulence between *Foe* isolates and possible variation in susceptibility in host (oil palm) has been done (Flood *et al.,* 1989; Flood, 2006; Cooper, 2011; Rusli *et al.,* 2015) despite the process being laborious and time (Mouyna *et al.,* 1996). The test for resistance to fusarium wilt in several crops such as banana (Li *et al.,* 2015) and tomato (Scott and Jones, 1989) has been achieved via inoculation tests. However potential resistance of oil palm to *Foe* in Ghana has not previously been studied in detail.

Several factors including environment can significantly affect resistance of the host (Smith and Hamill, 1999) and according to Ploetz *et al.* (1999) and Hwang and Ko (2004), field evaluations are more reliable than greenhouse tests. However, field studies also have the potential for disease escape as a result of irregular inoculum distribution or specific local edaphic conditions (Ribeiro *et al.,* 2011). In this current study, the pathogenicity tests were conducted in the glasshouse. All the oil palm progenies or genotypes from Ghana and Malaysia were susceptible to *Foe* (Tables. 3.1 and 3.2). Recent work by Rusli *et al.* (2015) and a previous study by Duran-Gasselin *et al.* (2000) showed that all four oil palm genotypes from Malaysia were susceptible to *Foe,* confirming that, although fusarium wilt disease is not a problem in Malaysia (Ho *et al.,* 1985), the oil palm genotypes grown were susceptible to the disease. However, reasons such as the nature of the soil conditions (Olivain *et al.,* 2006) have presumably

supported the non-prevalence of the disease in Malaysia. In Africa, climatic conditions, such as rainfall patterns, are suggested to support the prevailance of the *Foe* (Turner, 1981).

There was yellowing of the leaves of the infected oil palm seedlings (Fig. 3.5) which is one prominent symptom of fusarium wilt disease (Flood *et al.,* 1993; Flood, 2006). *Foe* was detected in oil palm seedling roots or stems and PCR based detection confirmed the presence of the pathogen with the Secreted in Xylem (*SIX8*) gene or internal transcribed spacer gene primers (Figs. 3.8 and 3.9). The *SIX8* and *ITS*4/*ITS*1 genes both detected *Foe* in the inoculated palms. *Foe* (Fig. 3.10) was also detected in the soils in which the plants were inoculated under glass-house conditions to confirm and make the pathogenicity test complete, as it is known that *Foe* is soil borne (Flood, 2006; Rusli *et al.,* 2015).

The differences in time to symptoms showing as a result of *Foe* infection in the oil palm seedlings could be associated with the methods or procedures of inoculating the oil palm seedlings, such as wounding and soaking of the roots in the spore suspension. These treatments could have hastened infection of the host, compared with the application of the inoculum directly onto the base of potted plants in soil (Fig. 3.4a). The length of time for the appearance of symptoms following direct application of spore into the soil of potted plants was between 10 – 11 months. However, in other studies, results were obtained at the nursery in 6 months post-inoculation before the seedlings were sent to the field (Corley and Tinker, 2003). However, Corley and Tinker (2003) further reported that the inoculation test was performed 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. In this study, time to appearance for syptoms in the seedlings inoculated with BOPP-B5 *Foe* isolate from Ghana, was late to the host compared to *Foe* isolates 16F, OPRI-5 and NORPALM-N5. Slow growth of mycelia from the BOPP-B5 isolate on PDA was observed compared with the other isolates suggested that this isolate may also grow slowly in the host. The BOPP-B5 isolate was different from the other isolates based on the phylogenetic trees, particularly for the housekeeping and *SIX* genes (Chapter two). The response was an exception in this work to and gene expression studies may explain the process further. Rusli *et al.* (2015) investigated gene expression levels of the *Foe* F3 and 16F isolates in the host-oil palm progeny PK-5463 roots. Rusli *et al.* (2015) reported partial resistance of Malaysian oil palm progeny PK-5463 inoculated with F3 *Foe* isolate. It is unclear why the seedlings for the inoculation test in the current study showed no partial resistance to the *Foe.* A contributory factor may be that, the *Foe* isolates used in this study originated from West Africa, as opposed to those used by Rusli *et al.* (2015) which came from different regions in Africa. The environmental conditions under which the study was done may also have influenced the results.

None of the oil palm progenies showed any resistance to the four *Foe* isolates used in the inoculations. The time difference for to cause symptoms in the host via direct inoculation, without wounding the root differed, indicating the complex nature of the *Foe* pathogen. The standard practice for identifying resistant oil palm progeny would be to screen different oil palm progenies rather than assuming that one *Foe* isolate is enough to be used as a representative of all *Foe* isolates.

Mechanisms of resistance in oil palm to fusarium wilt are still being investigated but resistance of wilt in oil palm is not well understood, based on the resistance (*R*) genes reported by Meunier *et al.* (1979). However, several reasons have been suggested for this problem, including resistance being controlled by simple inheritance via segregation (Renard *et al.,* 1993), by only two genes (de Franqueville and de Greef, 1988) or controlled polygenically (Cochard *et al.,* 2005). Suggestions of accumulation of unidentified antifungal compounds in the xylem vessels of petioles in resistant clones in comparison to a susceptible clone were made (Mepsted *et al.,* 1995). This finding supported the work of Cooper *et al.* (1986) who revealed that tyloses (i.e. outgrowth of parenchyma cells that bulge through the circular bordered pits of vessels members and block water movement) occluded infected fungal vessels compared to uninfected oil palm seedlings as a result of the presence of the *Foe* pathogen.

Colonization of oil palm roots by *Foe* (Fig. 3.4) was demonstrated by the presence of *SIX8* genes. The *SIX8* was detected in the roots of the inoculated and infected oil palm seedlings (Fig. 3.9). Roots of *Foe* infected oil palm seedlings were discoloured compared to the roots of control plants. Similar results were reported by Rusli *et al.* (2015) although in palms older than the ones used in this current study. Though *Foe* was detected in the leaves of some of the oil palm genotypes that showed severe yellowing, the pathogen was not detected consistently. This result agrees with the study of Rusli *et al.* (2015) who showed the pathogen could be detected in some leaf tissues of some oil palm progenies from Malaysia. They provided a comprehensive report on the effect of the fusarium wilt on the oil palm height, dry weight accumulation and also assessed disease severity index and defence related genes by spraying of

Foe suspension on the roots of the oil palm to study the host-pathogen interaction. In this current study, the roots of the oil palm were soaked in the *Foe* suspension overnight (Fig. 3.4b) which also gave symptoms in the oil palm genotypes. The detection of *Foe* in the stems of infected oil palm plants was also positive in most samples for the current study.

Effector proteins such as the *SIX* genes have been examined in some *F. oxysporum* f. sp. and associated with their ability to cause infection in the host (Rep *et al.,* 2005; Van der Does *et al.,* 2009; Laurence *et al.,* 2015; Rocha *et al.,* 2016; Taylor *et al.,* 2015). However, the ability of the four *Foe* isolates (BOPP-B5, OPRI-5, NORPALM-N5 and 16F) to cause infection in oil palm could not be linked to the presence or absence of the any of the *SIX* genes. The identified *SIX* genes common to all four *Foe* isolates used in the inoculation test were *SIX8*, *SIX9* and *SIX11* (Table. 2.4). However, *Foe* OPRI-5 also had *SIX12* which was absent in the remaining three *Foe* isolates used here (Table. 2.4), but all the four *Foe* isolates were pathogenic to the oil palm progenies. This finding contrast with *F. oxysporum* f. sp. *lycopersici* (*Fol*) which was reported to depend on *SIX1* gene for full pathogenicity (Rep *et al.,* 2005). In the current study, PCR showed that *SIX1* was absent in the *Foe* isolates used for the inoculation test, but could cause infection in the oil palm plants. Although effector proteins may play a role in pathogenicity of *Foe*, they cannot currently be linked with the ability or inability of a *Foe* isolate to successfully invade the host and cause infection. Similar results showed that *SIX* genes could not be associated with ability of the *F. oxysporum* f. sp. *cepae* (*Foc*) to cause infection in onion, as *SIX1-14* genes were either present or absent in both pathogenic and non-pathogenic isolates (Taylor *et al.,* 2015).

CHAPTER FOUR: DEVELOPMENT OF MOLECULAR DIAGNOSTICS

4.0 *Introduction*

An important step for efficient crop disease management is early and accurate diagnosis, since control measures are effective when introduced at an early stage of disease development. Disease diagnosis based solely on symptoms is problematic since the disease under study may be in an advanced stage in the host plant before symptoms first appear. Furthermore, the expression of symptoms in the host can be misleading because of the high level of variability in expression. All aspects of plant disease epidemiology, from studies of disease spread to estimation of yield loss or disease relationships, require the ability to correctly identify the pathogen through diagnosis (McCartney *et al.,* 2003).

Several techniques have been employed for the diagnosis of plant pathogens including immunological methods, DNA or RNA probe technology and Polymerase Chain Reaction (PCR) amplification of nucleic acid sequences (McCartney *et al.,* 2003), including multiplex PCR (Hamelin *et al.,* 1996; Chiocchetti *et al.,* 1999; de Haan *et al.,* 2000), Real Time-PCR (RT-PCR) or quantitative PCR (Schena *et al*., 2004; Lievens *et al*., 2006; Edel *et al.*, 2011). More recently, the loop mediated isothermal amplification (LAMP) technique has been developed as an alternative highly reliable approach for molecular diagnostics (Notomi *et al.,* 2000; Ayukawa *et al.,* 2016).

Currently, the detection of plant pathogens is a dynamic and rapidly evolving research area where established protocols are often modified or replaced shortly after having been developed (Saikia and Kadoo, 2010). Accurate and routine pathogen detection ideally requires high levels of specificity, sensitivity, reliability and speed (Kaneko *et al.,* 2007; Mori and Ntomi, 2009). Lopez *et al.* (2003) reported that specificity can be defined as the ability to detect the pathogen in the absence of false positives and negatives, whilst sensitivity relates to the lowest number of pathogens reliably detected per assay or sample. According to Lievens *et al.* (2008), pathogen quantification is also becoming more important and reliable, because it serves as a foundation for establishing the extent of at which intervention is required to limit or prevent losses.

Molecular methods such as PCR assays have been frequently applied for identification and detection of *Fusarium* plant pathogens (Parry and Nicholson, 1996; Schilling *et al.,* 1996; Yoder and Christianson, 1998; Möller *et al*., 1999; Wilson *et al*., 2004; Klemsdal and Elen, 2006). As *Foe* is known to survive and remain latent in soil for many years, a detection method of high sensitivity, specificity and reliability is of value to make an informed decision in its control. Whilst PCR could be used for this, the aim in this project was also to compare the more recently developed LAMP technology as a detection and diagnostic technique for *Foe*.

4.1 Loop Mediated Isothermal Amplification (LAMP)

Diagnostic testing of plants showing symptoms of infection may often need to be carried out in the laboratory and this normally requires bulky samples being sent to the laboratory. Attempts were made ealier to develop real-time PCR equipment for use in the field (Schaad *et al.,* 2002; Mavrodieva *et al.,* 2004; Tomlinson *et al.*, 2007). However, the use of the LAMP technique to support on-site (field) detection of diseased plants has been demonstrated (Ayukawa *et al.,* 2016).

LAMP is a rapid amplification method employing a strand displacing *Bacillus stearothermophilus* (*Bst*) DNA polymerase and 4 – 6 primers, two of which are 'fold back' primers (Notomi *et al.,* 2000; Nagamine *et al.,* 2001; Kimura *et al.,* 2011) which form stem-loop motifs with self-priming capability (Fig. 4.1). The primers used are two sets, the internal primers and external primers. Subsequent studies have found the use of additional 'loop primers', which bind to the loop structures, can greatly reduce the reaction times (Nagamine *et al.,* 2002) resulting in a total of 6 primers. The 60 – 65 ^{o}C reaction temperature combined with a minimum of 4 primers makes LAMP a highly specific reaction - the high level of specificity results from the requirement for primers to bind up to eight regions of the target sequence. This process results in an amplification scheme where the priming sequence is copied with each round of replication and remains tethered to the previous amplicon resulting in a concatenated product of alternating sense / anti-sense repeats of varied length. A large number of

amplicons results which can be used for further studies in detection (Bekele *et al.*, 2011).

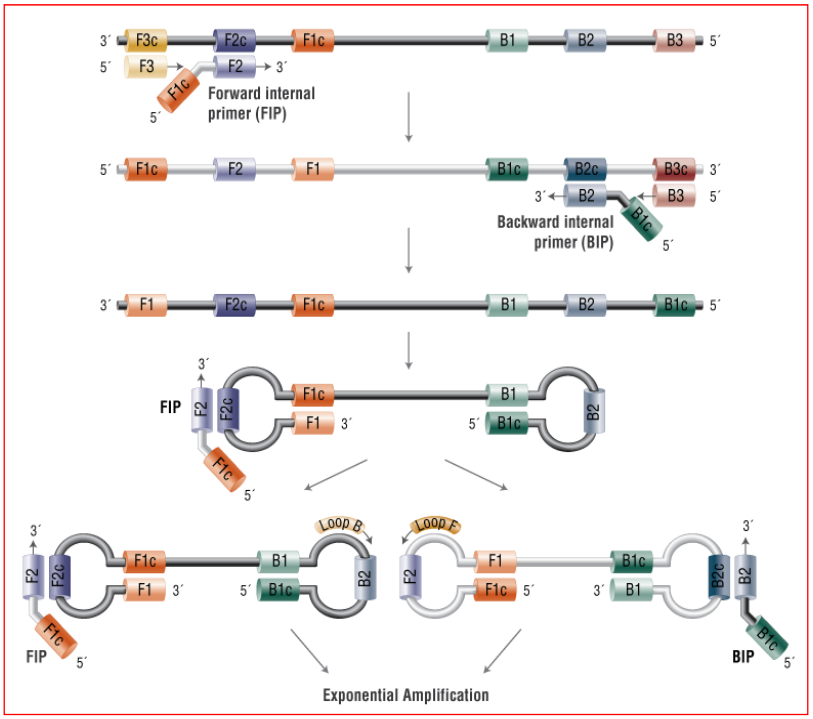


Figure. 4.1 Loop-mediated Isothermal Amplification (LAMP) uses 4 - 6 primers recognizing 6 - 8 distinct regions of target DNA. A strand-displacing DNA polymerase initiates synthesis and 2 of the primers form loop structures to facilitate subsequent rounds of amplification (Source:https://www.neb.com/applications/dna-amplification-and-pcr/isothermal-amplification).

LAMP is one of the most well established methods for isothermal amplification of nucleic acids to date. The technique has been used as a molecular tool for the detection of several plant pathogens over recent years (Fukuta *et al.,* 2004; Nie, 2005; Tomlinson *et al.*, 2007; Tomlinson

and Boonham, 2008) including fungi (Lucas *et al.,* 2010; Niessen and Vorgel, 2010; Sun *et al.,* 2010). Several reports have been published on the LAMP technique for the detection of *Fusarium* spp. (Niessen and Vogel, 2010; Abd-Elsalam *et al.,* 2011; Denschlag *et al.,* 2012; Niessen, 2013; Ghosh *et al.,* 2015; Ayukawa *et al.,* 2016). Recently Ayukawa *et al.* (2016) applied a LAMP assay for detection and differentiation of *F. oxysporum* f.sp. *lycopersici* (*Fol*) race 1 isolates based on the *SIX4* and *SIX5* genes using three primer sets. These genes are present only in race 1 of *Fol*. The method could detect *Fol* race 1 in infected tomato stems without DNA isolation. Niessen and Vogel (2010) developed a LAMP assay for the specific detection of *F. graminearum* (*Fg*) based on the *gaoA* gene (galactose oxidase) of the fungus. Amplification of DNA during the reaction was indirectly detected *in situ* using calcein fluorescence as a marker without electrophoretic analysis which is time-consuming. The assay was optimized for rapidity, specificity, and sensitivity and was shown to detect less than 2 pg of purified target DNA per reaction within 30 min. Within 132 fungal species tested, exclusively DNA isolated from cultures of *Fg* (lineages 1 - 9) resulted in a fluorescent signal after amplification with the LAMP assay. The usefulness of the analysis was demonstrated using fungal cultures by direct analysis of surface scrapings from agar cultures, direct testing of single infected barley grains, and detection of *Fg* in total genomic DNA isolated from bulk samples of ground wheat grains.

LAMP assays have also been used for quantifying plant pathogens. Zhang *et al.* (2013) demonstrated the use of RT-LAMP in detecting the *F. oxysporum* f. sp. *cubense* (*Foc*). The assay could detect approximately 0.4 pg / µl plasmid DNA of

Foc in inoculated soil without. It was confirmed that the LAMP assay could quantify genomic DNA of *Foc*-TR4 collected in South China via tests for both artificially and naturally infected soil samples.

The sensitivity of the LAMP technique has also been reported by Almasi *et al.* (2013), who demonstrated that even though both PCR and LAMP assays could successfully detect positive infected samples of tomato, considering the time, safety, cost and simplicity, the latter technique was superior overall. Interestingly, they further demonstrated that LAMP reactions could successfully detect *Fol* without DNA purification (direct-LAMP). In addition, the real-time application with the Optigene-system (Optigene, UK) has several advantages such as easy mobility or portability of the detection device.

4.2 *Materials and methods*

4.2.1 *LAMP primer design*

Foe genomic DNA was isolated as described in Chapter two section 2.3.3. More *Fusarium* isolates were used to increase the knowledge on the study of the *SIX* genes and housekeeping genes. *Foe* DNA was isolated and amplified by PCR described in Chapter two section 2.33 and 2.3.4. These PCR products were sequenced as shown Chapter two section 2.3.7. PrimerExplorer V4 software was used to design LAMP primers from the *Foe* isolate sequences amplified. The LAMP primers designed were; forward inner primer (FIP), backward inner primer (BIP), backward outer primer (B1), forward outer primer (F1), forward loop primer (F2) and backward loop primer (B2). LAMP primers designed were based on six genes, namely, cytochrome *P450* and the effector genes Secreted in Xylem (*SIX*) *SIX1*, *SIX8*, *SIX10*, *SIX12* and *SIX13* (Appendix 5).

4.2.2 *Loop Mediated Isothermal Amplification* (*LAMP*) *assay*

Foe detection was performed by preparing the LAMP primer mix which consisted of 152 µl sterile distilled water with concentrations of 10 µM each of the F1, B1, F2, B2 primers and 2 µM each of the FIP, BIP primers. Master mix for eight reactions consisted of 23 µl LAMP primer mix, 46 µl sterile distilled water and 115 µl Optigene master mix (dNTPs, *Bst* DNA polymerase and $MgCl_2$). Twenty µl of the reaction mix was dispensed into each of the eight LAMP tubes and 1 µl genomic DNA added. The maximum time set up for the assay to detect *Foe* was set at 30 min for all the reactions for each primer set. LAMP tests were also

performed randomly on inoculated infected oil palm roots in the glasshouse to confirm the sensitivity and specificity of the entire assay on some selected or targeted genes.

Table. 4.1. List of *Foe* and non-*Foe* isolates selected for the LAMP study

Fusarium species	Isolate code	Location/Source
F. oxysporum f. sp. *elaeidis*	IVORY COAST-16F	Ivory Coast
F. oxysporum f. sp. *elaeidis*	DR CONGO-F3	DR Congo
F. oxysporum f. sp. *elaeidis*	DR CONGO-Z/CBS217.49	DR Congo
F. oxysporum f. sp. *elaeidis*	SURINAME-S/CBS783.83	Suriname
F. oxysporum f. sp. *elaeidis*	GHANA NORPALM-N2	Ghana
F. oxysporum f. sp. *elaeidis*	GHANA NORPALM-N3	Ghana
F. oxysporum f. sp. *elaeidis*	GHANA NORPALM-N5	Ghana
F. oxysporum f. sp. *elaeidis*	GHANA BOPP-B4	Ghana
F. oxysporum f. sp. *elaeidis*	GHANA BOPP-B5	Ghana
F. oxysporum f. sp. *elaeidis*	GHANA OPRI-5	Ghana
F. oxysporum f. sp. *elaeidis*	GHANA OPRI-11	Ghana
F. oxysporum f. sp. *elaeidis*	GHANA OPRI-18	Ghana
F. oxysporum sp.	Fus126B-3	ADAS
F. oxysporum sp.	Foxy150-5	ADAS
F. oxysporum sp.	Foxy sp-8	ADAS
F. oxysporum sp.	Foxy2571-9	ADAS
F. oxysporum sp.	Foxy sp-10	ADAS
Fusarium sp.	Fus-11	ADAS
F. oxysporum sp.	FoxyBX14/153b-A	ADAS
F. oxysporum sp.	FoxyBX14/153a-B	ADAS
F. oxysporum f. sp. *lycopersici*	Foxy.Forla-C	UoN
F. oxysporum f. sp. *lycopersici*	Foxy.Forlb-D	UoN
Fusarium solani 1	F1	UoN
Fusarium solani 2	F2	UoN

Fusarium species	Isolate code	Location/Source
FORL A	F3	UoN
FORL B	F4	UoN
F. oxysporum BX15/4a	F5	UoN
F. oxysporum BX15/4b	F6	UoN
F. oxysporum BX15/4c	F7	UoN
F. oxysporum f. sp. *cubense* (FOC2A)	F8	UoN
F. oxysporum f. sp. *cubense* (FOC2B)	F9	UoN
F. oxysporum BX14/153a	F10	UoN
F. oxysporum BX14/153b	F11	UoN
F. oxysporum BX14/153c	F12	UoN
F. oxysporum BX14/152a	F13	UoN
F. oxysporum BX14/128a	F14	UoN
F. oxysporum BX14/168d	F17	UoN
F. oxysporum f. sp. *lycopersici* race 1	F18	Alison Jackson (UK)
F. oxysporum f. sp. *lycopersici* race 2	F19	Alison Jackson (UK)
F. oxysporum f. sp. *radices lycopersici*	F20	Alison Jackson (UK)

4.3 *Results*

The LAMP data for determining the presence or absence and the detection time of the genomic DNA of the *Foe* and non-*Foe* isolates are shown in Tables 4.2 - 4.6. The amplification and derivative curves generated confirmed the specificity of the products amplified (example is shown in Fig. 4.2). Generally, amplification was observed at 65 ^{0}C. There were four *SIX* genes (*SIX1*, *SIX8*, *SIX10* or *SIX13*) identified in both the *Foe* and non-*Foe* used here. Only *SIX12* was not detected in any of the isolates, either *Foe* or non - *Foe* (Table. 4.2). Collectively *SIX1* was detected in only four *Foe* isolates; F3 and Z from DR Congo, S from Suriname and N2 from Ghana-Norpalm (Table. 4.3) among 40 isolates used. However, *SIX1* was not detected in the remaining eight *Foe* (16F, N3, N5, B4, B5, 5, 11, 18) isolates. The *SIX8* gene was detected in all *Foe* and some non-*Foe* isolates via LAMP (Table. 4.4). *SIX10 and SIX13* (Table. 4.5) were also specific for detecting *F. oxysporum* f. sp. *lycopersici* isolates Foxy.Forla-C and Foxy.Forla-D, but were not detected in the *Foe* isolates (Table. 4.3).

The threshold time (detection time) varied among all the genes (*SIX* or cytochrome *P450*) that were used (Tables 4.2 – 4.6). The time for detection for all the effector protein genes was between 4:00 to 29:15 min. The *SIX1* gene was specific (Fig. 4.3a; b) to some *Foe* isolates and discriminated between *Foe* and non-*Foe* isolates in the study with a detection time at 22:30 min (Table 4.3). Though the *SIX8* gene could not distinguish *Foe* isolates from non-*Foe*, it could be used to detect the

presence of the *Foe* within 4:00 min (Table 4.4) whereas in the non-*Foe* the earliest time for detection was 9:00 min (Table 4.4). The two specific LAMP primers for *SIX10* and *SIX13* genes could amplify the Fola-C and Forlb-D isolates within 13:45 min and 8:00 min (Table 4.5). The *P450* gene was detected at 6:45 min (Table 4.6). All the LAMP primers designed, either for cytochrome *P450* or the *SIX* genes (*SIX1*, *SIX8*, *SIX10*, *SIX13*) gave positive reactions with the genomic DNA at a detection time below 30 min.

The LAMP primers were randomly tested on the sampled roots of oil palm seedlings that showed symptoms of *Foe* infection to confirm the sensitivity of the assay for on-site detection, and these assays were positive with the *SIX8* gene primer set (Fig. 4.4a; b). The detection time was at 9:45 min for *Foe* detection.

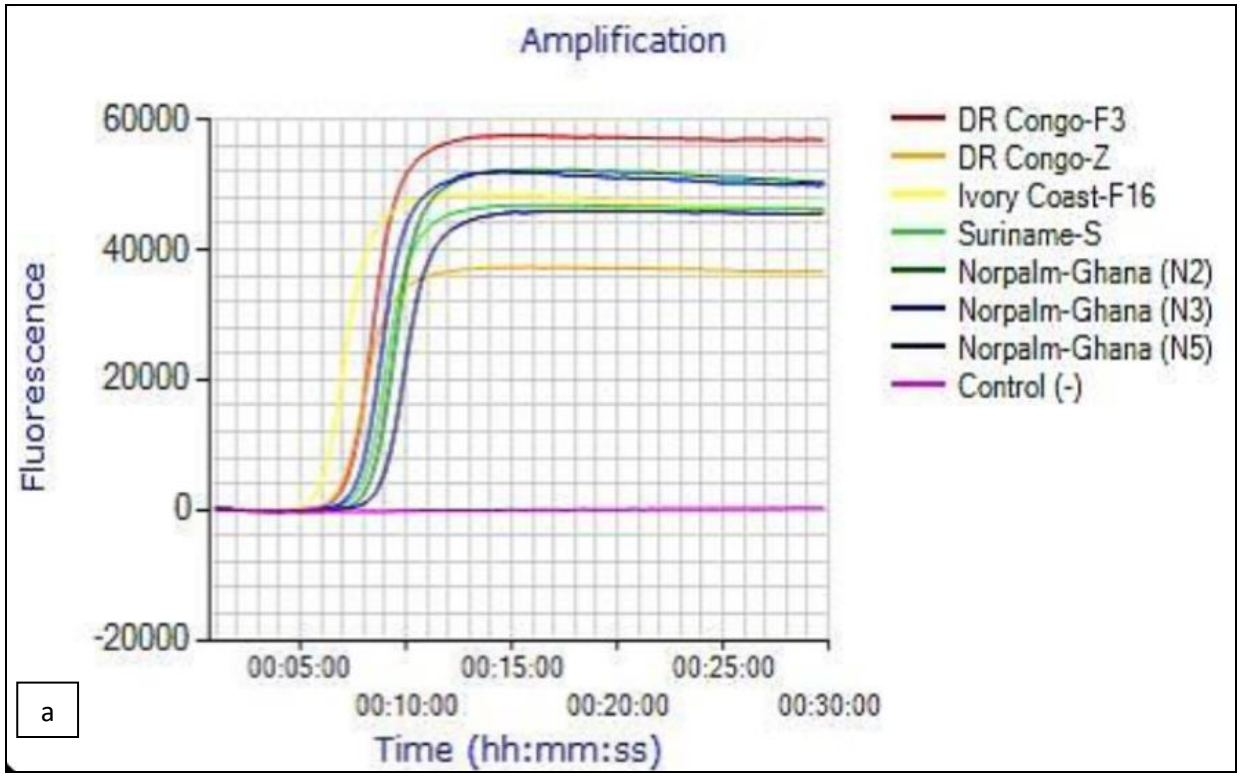


Figure. 4.2. (a) Amplification curve of seven isolates of *Foe* from DR Congo, Ivory Coast, Suriname and Ghana showing specific time for detection with LAMP primer *P450* cytochrome gene

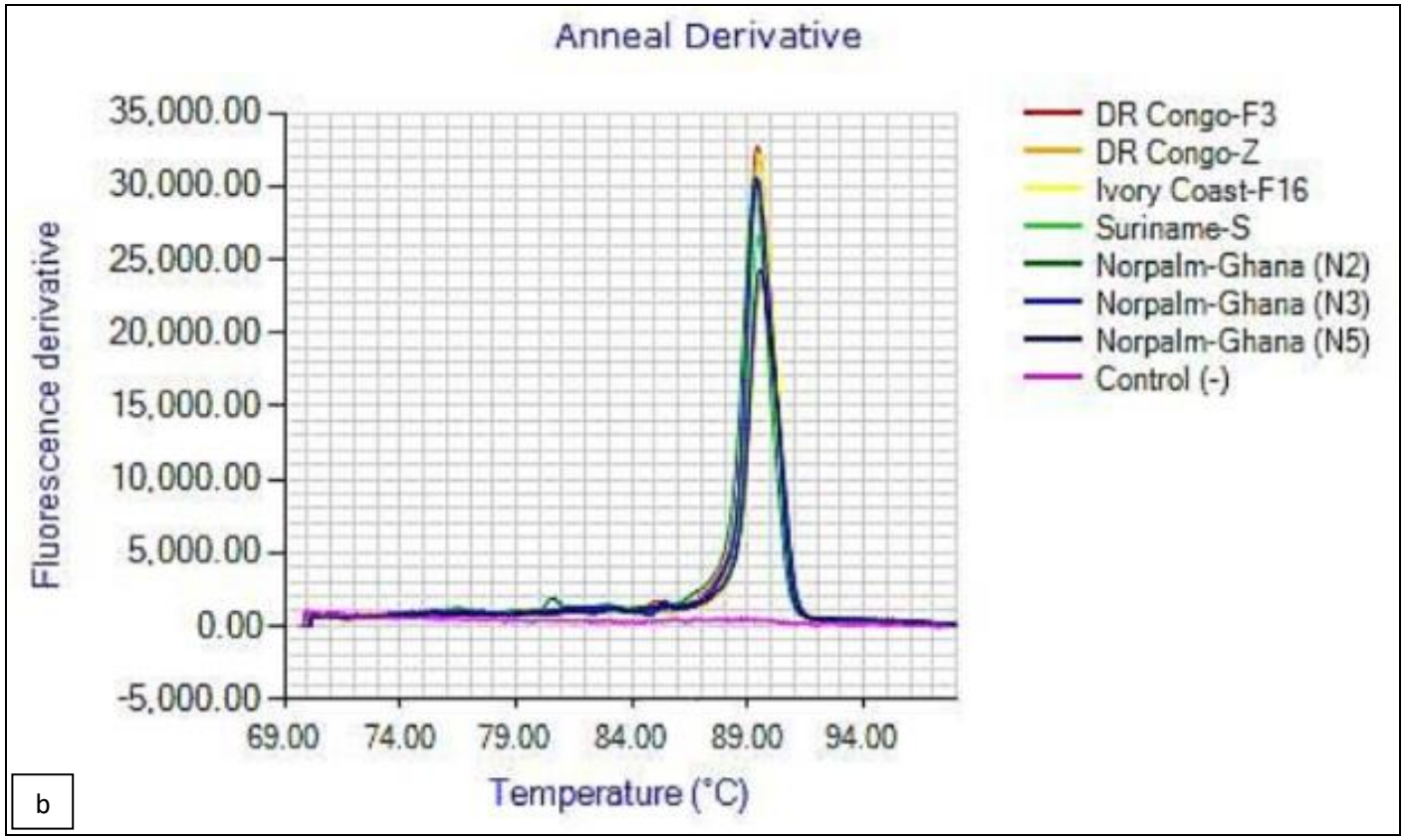


Figure. 4.2. (b) Annealing curve of seven isolates of *Foe* from DR Congo, Ivory Coast, Suriname and Ghana showing specific time for detection with LAMP primer cytochrome *P450* gene.

Table. 4.2. Presence or Absence of Secreted in Xylem genes in *Foe* and other selected fusarium isolates via LAMP technique

Fusarium oxysporum isolates	Ex-Host	Isolate code	Pathogenicity	*Secreted In Xylem (SIX) genes*				
				1	8	10	12	13
F. oxysporum f. sp. *elaeidis*	Oil palm	IVORY COAST-16F	P	–	+	-	-	-
F. oxysporum f. sp. *elaeidis*	Oil palm	DR CONGO-F3	P	+	+	-	-	-
F. oxysporum f. sp. *elaeidis*	Oil palm	DR CONGO-Z	P	+	+	-	-	-
F. oxysporum f. sp. *elaeidis*	Oil palm	SURINAME-S	P	+	+	-	-	-
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA NORPALM-N2	P	+	+	-	-	-
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA NORPALM-N3	P	-	+	-	-	-
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA NORPALM-N5	P	-	+	-	-	-
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA BOPP-B4	P	-	+	-	-	-
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA BOPP-B5	P	-	+	-	-	-
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA OPRI-5	P	-	+	-	-	-
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA OPRI-11	P	-	+	-	-	-
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA OPRI-18	P	-	+	-	-	-
F. oxysporum sp.	Tomato	Fus126B-3	NH	-	+	-	-	-
F. oxysporum sp.	Tomato	Foxy150-5	NH	-	+	-	-	-
F. oxysporum sp.	Tomato	Foxy sp-8	NH	-	+	-	-	-
F. oxysporum sp.	Tomato	Foxy sp-10	NH	-	+	-	-	-
Fusarium sp.	Tomato	Fus-11	NH	-	+	-	-	-
F. oxysporum sp.	Tomato	FoxyBX14/153b-A	NH	-	+	-	-	-
F. oxysporum sp.	Tomato	FoxyBX14/153a-B	NH	-	+	-	-	-
F. oxysporum f. sp. *lycopersici*	Tomato	Foxy.Forla-C	NH	-	+	+	-	+
F. oxysporum f. sp. *lycopersici*	Tomato	Foxy.Forlb-D	NH	-	+	+	-	+
Fusarium solani 1	Soil	F1	NH	-	NT	NT	NT	NT
Fusarium solani 2	Soil	F2	NH	-	NT	NT	NT	NT

Fusarium oxysporum isolates	Ex-Host	Isolate code	Pathogenicity	*Secreted In Xylem (SIX) gene*				
				1	8	10	12	13
FORL A	Tomato	F3	NH	-	NT	NT	NT	NT
FORL B	Tomato	F4	NH	-	NT	NT	NT	NT
F. oxysporum BX15/4a	Tomato	F5	NH	-	NT	NT	NT	NT
F. oxysporum BX15/4b	Tomato	F6	NH	-	NT	NT	NT	NT
F. oxysporum BX15/4c	Tomato	F7	NH	-	NT	NT	NT	NT
F. oxysporum f. sp. *cubense* (FOC2A)	Tomato	F8	NH	-	NT	NT	NT	NT
F. oxysporum f. sp. *cubense* (FOC2B)	Banana	F9	NH	-	NT	NT	NT	NT
F. oxysporum BX14/153a	Tomato	F10	NH	-	NT	NT	NT	NT
F. oxysporum BX14/153b	Tomato	F11	NH	-	NT	NT	NT	NT
F. oxysporum BX14/153c	Tomato	F12	NH	-	NT	NT	NT	NT
F. oxysporum BX14/152a	Tomato	F13	NH	-	NT	NT	NT	NT
F. oxysporum BX14/128a	Tomato	F14	NH	-	NT	NT	NT	NT
F. oxysporum BX14/168d	Tomato	F17	NH	-	NT	NT	NT	NT
F. oxysporum f. sp. *lycopersici* race 1	Tomato	F18	NH	-	NT	NT	NT	NT
F. oxysporum f. sp. *lycopersici* race 2	Tomato	F19	NH	-	NT	NT	NT	NT
F. oxysporum f. sp. *radices lycopersici*	Tomato	F20	NH	-	NT	NT	NT	NT

P = Pathogenic NH = Not oil palm host NT = Not Tested +/- = Presence or absence of gene

Table. 4.3. LAMP results for threshold time (detection time) for *SIX1* gene in *Foe* isolates

Foe	Ex-Host	Isolate code	*Secreted In Xylem (SIX1)*	
			Amplification Time (mm:ss)	Annealing Temperature (^{O}C)
F. oxysporum f. sp. *elaeidis*	Oil palm	DR CONGO-F3	22:30	89.52
F. oxysporum f. sp. *elaeidis*	Oil palm	DR CONGO-Z	29:15	89.09
F. oxysporum f. sp. *elaeidis*	Oil palm	SURINAME-S	22:30	89.94
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA NORPALM-N2	29:15	89.26

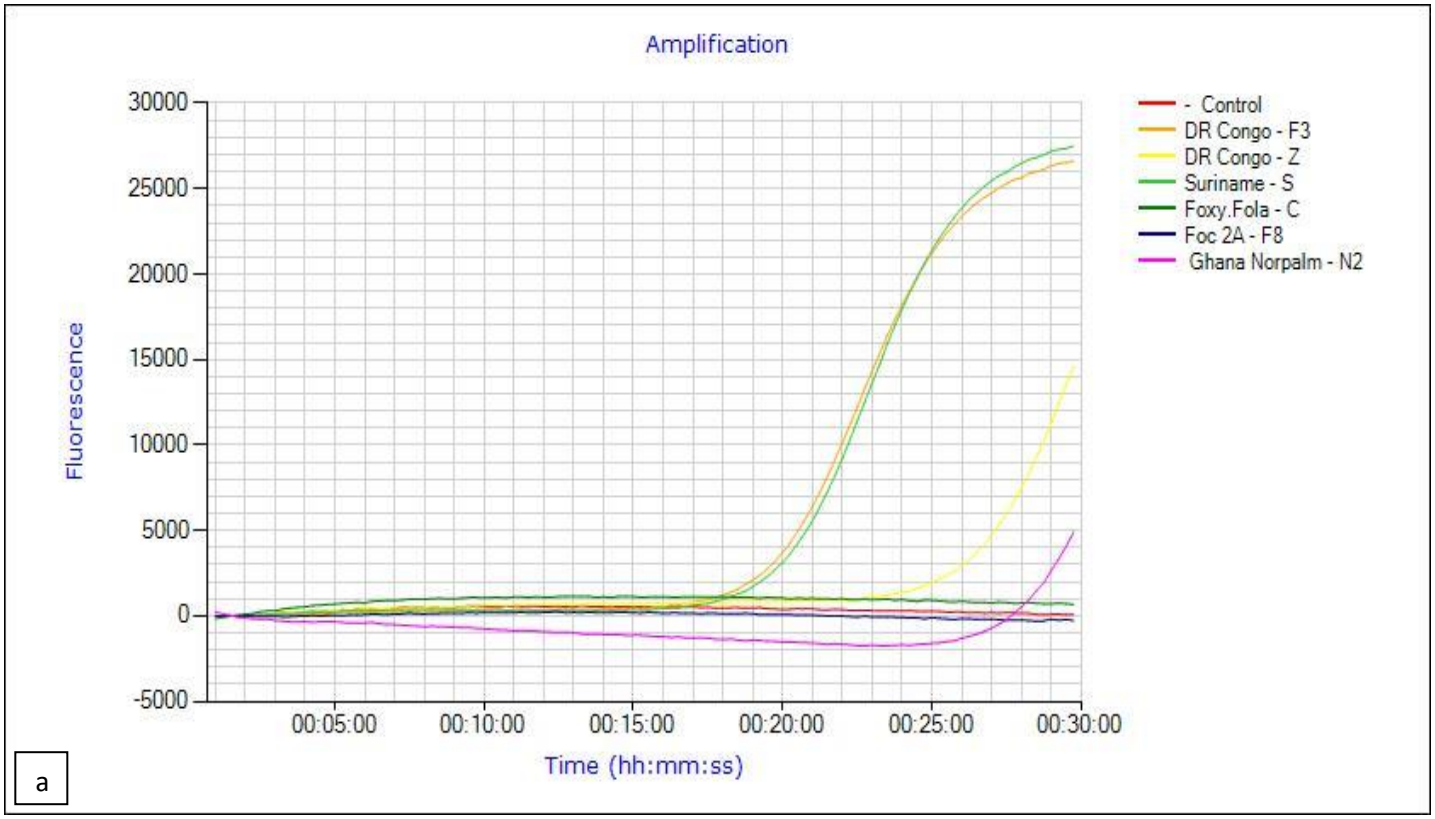


Figure. 4.3. (a) Amplification curve showing the specificity of *SIX1* gene LAMP primer set on four *Foe* from DR Congo, Ivory Coast, Suriname and Ghana and other fusarium isolates (non-*Foe* isolates).

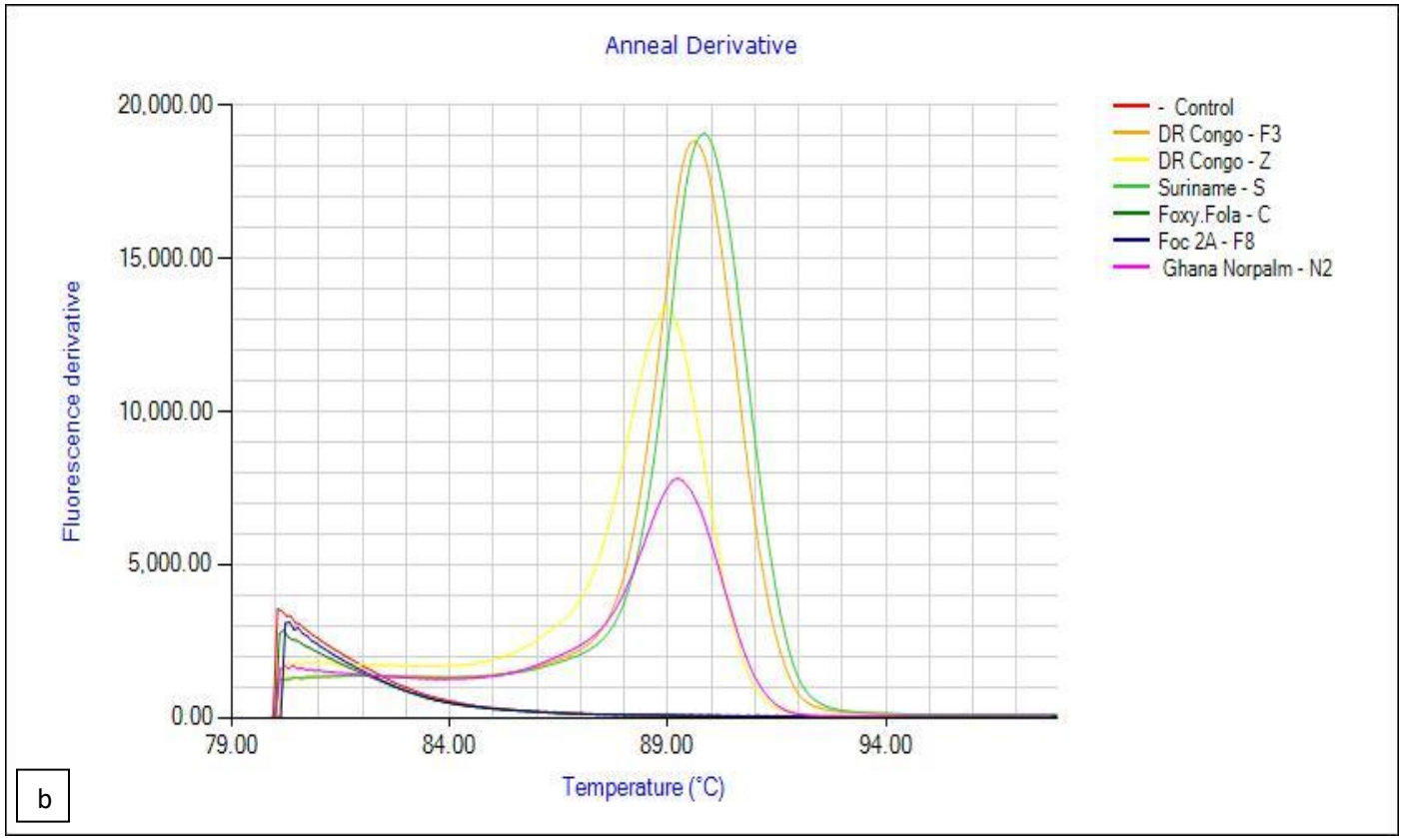


Figure. 4.3. (b) Annealing curve showing the specificity of *SIX1* gene LAMP primer set on four *Foe* from DR Congo, Ivory Coast, Suriname and Ghana and other fusarium isolates (non-*Foe* isolates).

Table. 4.4. LAMP results for threshold time (detection time) for *SIX8* gene in *Foe* and non-*Foe* isolates

Fusarium oxysporum isolates	Ex-Host	Isolate code	*Secreted In Xylem (SIX8)*	
			Amplification Time (mm:ss)	Annealing Temperature (^{O}C)
F. oxysporum f. sp. *elaeidis*	Oil palm	IVORY COAST-16F	13:45	87.75
F. oxysporum f. sp. *elaeidis*	Oil palm	DR CONGO-F3	24:15	87.50
F. oxysporum f. sp. *elaeidis*	Oil palm	DR CONGO-Z	24:15	87.50
F. oxysporum f. sp. *elaeidis*	Oil palm	SURINAME-S	10:00	87.67
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA NORPALM-N2	9:00	87.77
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA NORPALM-N3	18:15	87.02
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA NORPALM-N5	26:30	87.28
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA NORPALM-B4	12:30	87.49
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA BOPP-B5	21:45	87.90
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA OPRI-5	4:00	82.10
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA OPRI-11	10:15	87.67
F. oxysporum f. sp. *elaeidis*	Oil palm	GHANA OPRI-18	9:00	87.58

Fusarium oxysporum isolates	Ex-Host	Isolate code	*Secreted In Xylem (SIX8)*	
			Amplification Time (mm:ss)	Annealing Temperature (^{O}C)
F. oxysporum sp.	Tomato	Foxy sp-8	26:30	87.28
F. oxysporum sp.	Tomato	Foxy sp-10	17:15	87.68
Fusarium sp.	Tomato	Fus-11	12:45	87.66
F. oxysporum f. sp. *lycopersici*	Tomato	Foxy.Forla-C	15:15	87.66
F. oxysporum f. sp. *lycopersici*	Tomato	Foxy.Forlb-D	11:00	87.77

Table. 4.5. LAMP results for threshold time (detectioin time) for *SIX10* and *SIX13* genes in *Fusarium oxysporum* f.sp. *lycopersici* isolates

Non-*Foe* isolates	Ex-Host	Isolate code	*Secreted In Xylem (SIX13)*		*Secreted In Xylem (SIX10)*	
			Amplification Time (mm:ss)	Annealing Temperature (^{O}C)	Amplification Time (mm:ss)	Annealing Temperature (^{O}C)
F. oxysporum f. sp. *lycopersici*	Tomato	Foxy.Forla-C	11:45	87.83	24:15	85.16
F. oxysporum f. sp. *lycopersici*	Tomato	Foxy.Forlb-D	8:00	87.81	13.45	85.70

Table. 4.6. LAMP results for detected cytochrome *P450* gene in isolates of *Foe* and other *Fusarium* isolates

Fusarium oxysporum isolates	Ex-Host	Isolate code	Cytochrome *P450* gene	
			Amplification Time (mm:ss)	Annealing Temperature (^{O}C)
F. oxysporum f.sp. *elaeidis*	Oil palm	IVORY COAST-16F	11:15	87.73
F. oxysporum f.sp. *elaeidis*	Oil palm	DR CONGO-F3	9:15	87.67
F. oxysporum f.sp. *elaeidis*	Oil palm	DR CONGO-Z	6:45	87.67
F. oxysporum f.sp. *elaeidis*	Oil palm	SURINAME-S	7:30	87.58
F. oxysporum f.sp. *elaeidis*	Oil palm	GHANA NORPALM-N2	6:45	87.67
F. oxysporum f.sp. *elaeidis*	Oil palm	GHANA NORPALM-N3	14:45	86.99
F. oxysporum f.sp. *elaeidis*	Oil palm	GHANA NORPALM-N5	13:30	87.71
F. oxysporum f.sp. *elaeidis*	Oil palm	GHANA BOPP-B4	7:30	87.65
F. oxysporum f.sp. *elaeidis*	Oil palm	GHANA BOPP-B5	6:00	82.07
F. oxysporum f.sp. *elaeidis*	Oil palm	GHANA OPRI-11	7:30	87.68
F. oxysporum f.sp. *elaeidis*	Oil palm	GHANA OPRI-18	6:30	87.58
F. oxysporum sp	unknown	Foxy150-5	8:15	87.30
F. oxysporum sp	Tomato	FoxyBX14/153b-A	16:00	87.38
F. oxysporum sp	Tomato	FoxyBX14/153a-B	26:30	87.27
F. oxysporum f.sp. *lycopersici*	Tomato	Foxy.Forla-C	11:15	87.58
F. oxysporum f.sp. *lycopersici*	Tomato	Foxy.Forlb-D	8:15	87.72

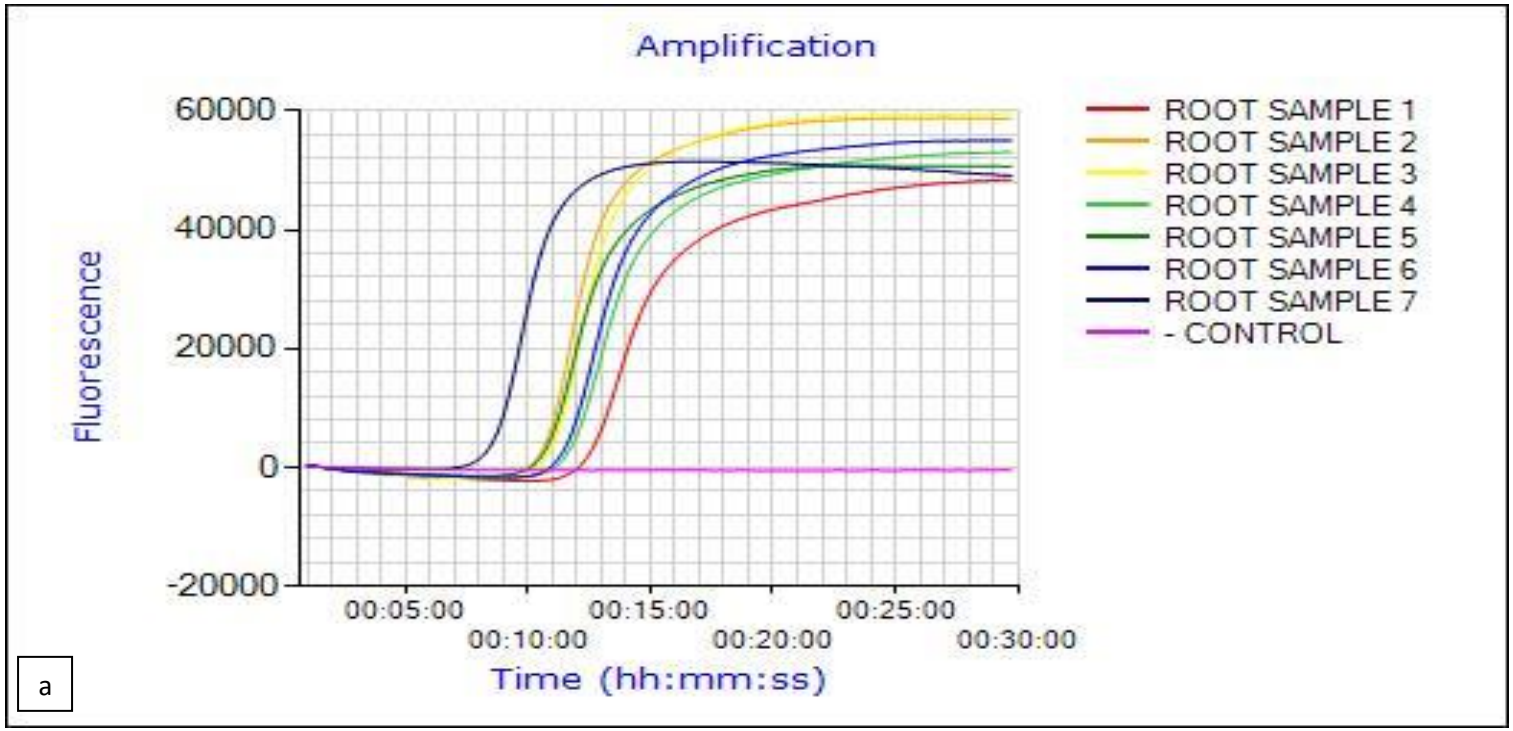


Figure. 4.4. (a**)** Direct detection of *Foe* with *SIX8* via LAMP assay in root samples of infected oil palm showing the amplification curves

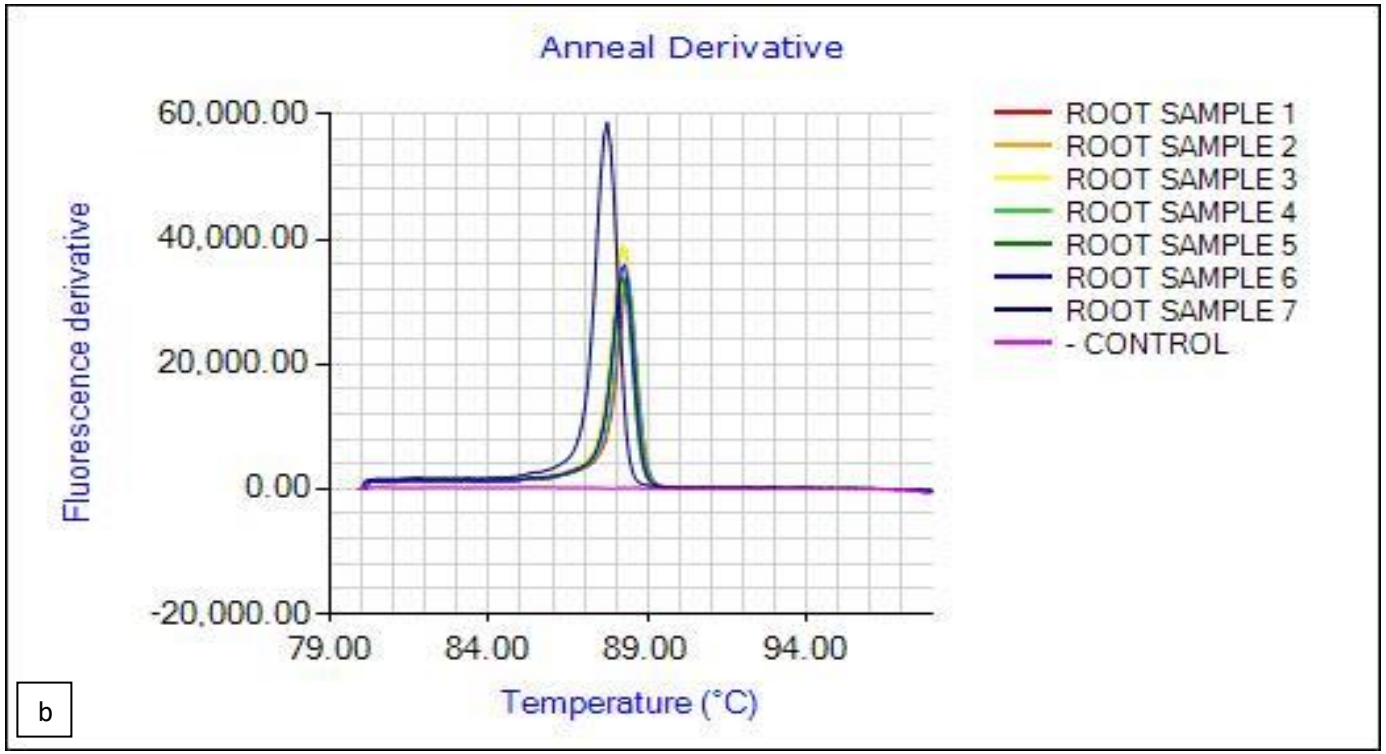


Figure. 4.4. (b) Direct detection of *Foe* with *SIX8* via LAMP assay in root samples of infected oil palm showing the annealing curves.

4.4 *Discussion*

Although there are reports on developed Loop Mediated Isothermal Amplification (LAMP) assays for detecting several *formae speciales* of *Fusarium* (Abd-Elsalam *et al.,* 2011; Ghosh *et al.,* 2015; Ayukawa *et al.,* 2016) and other plant pathogens (Villari *et al.,* 2013), this is the first LAMP research for the detection of *Foe* isolates from Ghana, Ivory Coast, Suriname and DR Congo.

Cytochromes *P450* (P450s) constitute a large superfamily of heme-containing monooxygenases (Nazmul *et al.,* 2010). They have several functions such as the synthesis of secondary metabolites and cell wall degrading enzymes (Nazmul *et al.* 2010). CWDE have been identified in *Foe* during its infection process (Cooper, 2011) in addition, the presence of cytrochrome *P450* is linked with pathogenicity of as demonstrated in *Fusarim oxysporum* f.sp. *vasinfectum* (*Fov*) (Lopez-Berges *et al.,* 2010) as such there was the need to examine the *Foe* isolates for the presence or absence of cytochrome *P450* in this present work.

There was positive detection using the cytochrome *P450* primers based LAMP assay. The cytochrome *P450* gene was detected in all *Foe* isolates tested as well in five of the non-*Foe* isolates (Table. 4.6). *P450* genes are distributed widely in many organisms (Nazmul *et al.,* 2010) and has been associated with pathogenicity in some fusarium such as the *F. oxysporum* f. sp. *cubense* (*Foc*) (Sutherland *et al.,* 2013). There is also wide variation in the *P450* gene which is attributed to a

common ancestor branching into various organisms (Gotoh 1993; Nelson *et al.*, 1993; Lewis *et al.,* 1998). Furthermore, the *P450* gene has been associated with specific metabolic pathways such as detoxification of xenobiotics, synthesis of secondary metabolites and molecular functional diversities (Demain and Fang, 2000; Ortiz-de-Montellano, 2005). According to Nelson (1999), such divergence that there is a driving force in evolution to develop strategies for survival in an organism. Research on the scale of diversity of the *P450* gene has been reported (Deng *et al.,* 2007; Doddapaneni *et al.*, 2005; Intikhab *et al.*, 2007; Park *et al.,* 2008). The level of *P450* differences such as the copy numbers or gene families, varies significantly across Kingdoms, phyla and species (Nazmul *et al.,* 2010) and this could be the possible reason why *P450* was present or absent in some of the *Foe* and the non-*Foe* isolates.

The time for detecting the *P450* genes varied in the isolates that tested positive (Table. 4.6). The variation in time of detection or amplification may be based on the differences in the genomic DNA concentrations of the *Foe* that resulted during the DNA isolation process. Generally, the cytochrome *P450* gene designed for LAMP diagnostics failed to distinguish *Foe* isolates from non-*Foe* isolates in the study. However, detection was rapid though not specific.

The secreted in xylem (*SIX*) effector proteins, are small, secreted and well known to be cysteine-rich, first identified in the xylem sap of tomato infected with *Fol* (Houterman *et al.,* 2007; Schmidt *et al.,* 2016). The LAMP technique has been designed for the detection of several *SIX* genes in different *Foc* to distinguish

between *Foc* and other plant pathogenic fungi (Ghosh *et al.,* 2015; Ayukawa *et al.,* 2016). The detection of *Fol* (Villari *et al.*, 2013) has been achieved based on the 28S rRNA regions (Almasi *et al.,* 2013) but this technique did not distinguish between races of *Fol*. The detection of *SIX* genes in *Foe* isolates and other *Fusarium* sp. in the current study was partially successful with the LAMP assays developed (Tables. 4.2 – 4.6).

The only specific *SIX* gene LAMP primer that distinguished *Foe* from non-*Foe* isolates was *SIX1* (Table. 4.3). However, only four *Foe* isolates, specifically from DR Congo (F3 and Z), Suriname (S) and Ghana (Norpalm-N2), tested positive with *SIX1*. The specificity of the *SIX1* primer was assessed via observation of the annealing derivative curve and the melting temperatures to distinguish between non-specific, specific and false-amplifications (Villari *et al.,* 2013). The discrimination power of the *SIX1* gene between the *Foe* and non-*Foe* isolates can be attributed to the *SIX1* LAMP primer designed from the sequence of the F3 *Foe* isolate during the study. Initially the F3 sequence of the *SIX1* region was obtained from products amplified with the *SIX1* primer via PCR and subsequently the *SIX1* LAMP primer designed from the PCR product of the F3 *Foe* isolate. In addition, the presence of transposable elements (TEs) in sites where *SIX* genes are localised is a factor in the diversity (Lievens *et al.,* 2009; Gawehns *et al.,* 2014) between the *Foe* isolates based on the *SIX1* gene.

The LAMP assay using the *SIX8* gene was positive for all *Foe* isolates and five non-*Foe* isolates (Table. 4.4). This results further explain the high level of diversity and

complexity of the *F. oxysporum* complex (Laurence *et al.,* 2014) and the divergence that exists in the *SIX* gene regions (Hatta *et al.,* 2002). The *SIX* gene LAMP primers that could detect only non-*Foe* isolates were *SIX10* and *SIX13* (Tables 4.5 and 4.6), possibly because the *SIX10* and *SIX13* LAMP primers were designed from the sequence of the Foxy, Forlb-D or the *Fol* isolates used in this study. The presence or absence of some of the *SIX* genes in the *Foe* and non-*Foe* isolates also is congruent with a recent study that shows fourteen *SIX* genes have been identified and most share similarities with each other or with other fungi (Williams *et al.,* 2016).

The LAMP assays for the *SIX* genes were rapid for *Foe* detetion compared to PCR and the threshold time varied from one isolate to the other and also differed among *SIX* genes. These differences may be due to the concentrations of the genomic DNA used in the and the presence of inhibitors, which may have influenced the time of amplification. Genetic variation amongst the isolates also may have contributed to the differences in detection time.

The sensitivity of the *SIX8* LAMP primers did not directly detect the *Foe* isolates 16F, N5, B5 and OPRI-5 in artificially inoculated soil, but detected the *Foe* extracted from artificially inoculated soil with DNA kits. However, Ayukawa *et al.* (2016) developed a LAMP assay to directly detect *SIX4* or *SIX5* in the *Fol* race 1 isolate artificially inoculated soil. Similarly, Peng *et al.* (2014) also reported on LAMP as an effective tool for detecting *Foc* race 4 isolate in soils. The LAMP assay used here directly detected *Foe* in the roots of inoculated infected oil palm seedlings in the

glasshouse (Fig. 4.3) with the *SIX8* primer set. Ayukawa *et al.* (2016) also reported a non-destructive method of detecting *Fol* race 1 in the stems of infected tomato without DNA isolation.

The LAMP primers developed in this study, either the cytochrome *P450* or the *SIX1, SIX8, SIX10, SIX13* genes, were extremely rapid and sensitive (Kaneko *et al.,* 2007), and less prone to inhibitors (Mori and Notomi, 2009) in the diagnosis of *Foe* infection and/or non-*Foe* isolates. However, as yet the assays lack the specificity required to discriminate between *Foe* and other fusarium species, so additional genes and primers need to be investigated in the future to develop an assay that could detect all isolates of *Foe* and no other fungi. Currently, the method has the potential for use to screen for *Foe* oil palm seedlings at nursery and soils in plantation prior to transferring oil palm seedlings to the field for planting. LAMP can support field or on-site diagnostics (Ghosh *et al.,* 2015) since it can rapidly detect the *Foe* pathogen compared to other molecular tools such as PCR-based methods, for instance; multiplex and real-time PCR (Steyn *et al.,* 2008). If rapid LAMP-based diagnostics in the field were to indicate the absence of *Foe* and related *Fusarium* species, then no further testing would be required and the site could be regarded as clean. However, if the LAMP technique did detect the potential presence of *Foe* at a site, samples could then be sent to the laboratory for further PCR-based testing to confirm whether the Fusarium present is *Foe* or a non-pathogenic *Fusarium* species.

CHAPTER FIVE: SURVEY AND CONTROL OF *FUSARIUM OXYSPORUM* F. SP. *ELAEIDIS* (*FOE*)

5.0 *Introduction*

Surveying for the occurence of fusarium wilt disease in oil palm plantations of Ghana is very pertinent due to the global significance of the crop. Cooper and Rusli (2014) established the spread of *Foe* in some oil palm plantations of Ghana, showing that the pathogen was soil borne. Furthermore, another medium through which the *Foe* can spread in oil palm plantations is contamination of the oil palm seed from both outside of the seed (Locke and Colhoun, 1973) and on the kernel surface (Flood *et al.,* 1990). *Foe* has globally, including Brazil and Ecuador; spread between these two countries was confirmed via RFLPs that in addition showed similar patterns with *Foe* from the Ivory Coast (Mouyna *et al.,* 1996).

Application of biological control has been reported (e.g. Howell, 2003; Rose *et al.,* 2003; Harman *et al.,* 2004; Sundaramoorthy and Balabaskar, 2013; Rashmi *et al.,* 2016). Some biological controls, such as the use of *Trichoderma* isolates to control other fusarium species, has been demonstrated (Weindling, 1932; Elad *et al.,* 1980). However, attempts to develop effective control of *Foe* has seen mixed results. Treatment of oil palm seeds with chemicals failed to eradicate *Foe* (Flood *et al.,* 1999; Mepsted, 1992). The use of fungicides is expensive for large oil palm plantations (Renard and Ravisé, 1986) though some success has been reported

with chemical control (Mohammadreza *et al.,* 2015). A chemical treatment which involved a 5 min soaking of plantlets in 15 ppm alkyl mercury solution as a preventive condition in the pre-nursery gave promising results, but according to Ravisé (1965), the chemical is toxic to oil palms. Hence there is a need to use different chemicals that are not toxic to control *Foe* or a biological control that is less costly and more environmentally friendly.

The exchange of oil palm germplasm which is normally via pollen collection has made it easier for plant breeders to rapidly carry oil palm progenies across borders. However, there is a risk that the oil palm pollen is a carrier of the *Foe*. The need for control of *Foe* in seed of oil palm has been noted (Cooper, 2009) as the seed may also serve as a carrier (Flood, 2006). This problem needs controlling and eradicating in oil palm pollen, since this is a potential source of *Foe* spread.

5.1 *Materials and methods*

5.1.1 *Sampling in oil palm plantation sites*

The random sampling of *Foe* in infected (symptomatic) or healthy (symptomless) palms during the survey was carried out in four oil palm plantation sites as described in chapter two section 2.3.2. These sites were Twifo Oil Palm plantation (TOPP), Benso Oil palm plantation (BOPP) and Norpalm (all three in the Western Region of Ghana) and the Oil Palm Research Institute of the Council for Scientific and Industrial Research (OPRI- CSIR), in the Eastern Region of Ghana. All the oil palm trees in these four plantations were Dura x Pisifera (DxP) progeny. In the study, infected oil palms were identified in three sites but not at TOPP. Over five hundred oil palms were observed in the four different sites. Core or tissue samples were taken from 28 oil palm trees randomly as described in Chapter two section 2.3.2. From OPRI-CSIR site tissue samples were collected from twelve palms with symptoms and seven without symptoms. Sampling was made randomly from three palms with symptoms and three without symptoms at the Norpalm plantation. However, one sample from tissue with symptoms from Norpalm was discarded because of contamination after re-isolation and plating. Finally, tissue samples from two symptomless and two symptomatic palms were collected from BOPP. The source of the seedlings for each plantation was also recorded.

5.1.2 *Biological control of Foe*

5.1.2.1 *Identification of biological control agents* (*BCA*)

Thirteen strains of *Trichoderma* were obtained from the University of Nottingham culture collection and DNA extracted. Of these, 10 were amplified (Fig. 5.1) via PCR (described in chapter two section 2.3.4) and their identities confirmed (Table 5.1) as shown in chapter two section 2.3.7. Three speices: *Trichoderma cerinum* (T1), *Trichoderma atroviride* (T2) and *Trichoderma harzianum* (T3) were then selected for the study.

5.1.2.2 *Evaluation of BCA activity through dual culture*

Four (4) isolates of *Foe*, BOPP-B5, OPRI-5, NORPALM-N2 and 16F were selected to represent the various locations where pathogenicity of *Foe* was established. In order to evaluate the activity of the three *Trichoderma* isolates (T1, T2 and T3) against the four pathogenic isolates of *Foe*:

1. *Trichoderma* sp. and *Foe* isolate disc (0.5 cm) were obtained from one-week old culture on PDA. The 0.5 cm discs of the *Trichoderma* sp. and *Foe* isolate were paired as dual cultures with each disc 1 cm from the edge of the petri dish on PDA the same day (Fig. 5.2).

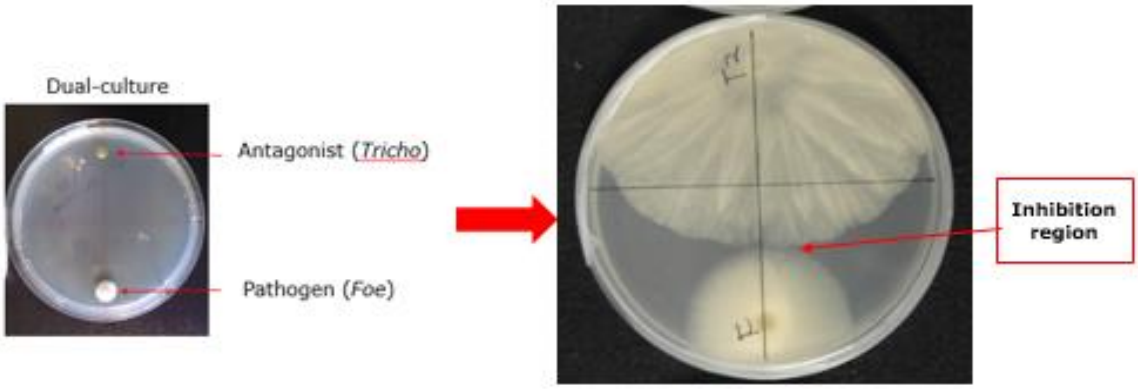


Figure. 5.1. Dual culture of *Trichoderma atroviride* (T2) in antagonism tests against *Foe* isolates: Zone of inhibition is visible between the two organisms.

2. Individual *Trichoderma* sp. or *Foe* isolate was cultured on PDA without pairing *Trichoderma* sp. and *Foe* isolate in plate to serve as the control treatment. This was to assess the growth of only *Trichoderma* sp. or *Foe* isolate on PDA.

The experimental set-up (Table 5.2) was replicated four times. All treatments were incubated at 28 ^{0}C for one-week and measurements of radial growth of the pathogen (*Foe*) and antagonist (*Trichoderma*-T1, T2 and T3) recorded. The percentage inhibition (PI) of *Foe* was calculated using the formula:

$$I= (TC-TT/TC) X 100$$

Where; I= percentage inhibition of pathogen growth by antagonist, TT= Radial growth of *Trichoderma* in treatments (i.e. dual culture of *Trichoderma* with *Foe*) and TC= Radial growth of control (antagonist or *Trichoderma* in monoculture). The averages of the percentage inhibitions over the *Foe* or the radial growth of the

mycelia of the *Foe* were subjected to analysis of variance (ANOVA) with GenStat 16th Edition statistical software.

Table. 5.1. Experimental set up matrix for dual culture of *Foe* and *Trichoderma* species

Foe isolates (Code)	Antagonist (*Trichoderma* sp.)			
	Control (*Foe*)	*Trichoderma cerinum* (T1)	*Trichoderma atroviride* (T2)	*Trichoderma harzianum* (T3)
BOPP (B)	B	B+T1	B+T2	B+T3
NORP (N)	N	N+T1	N+T2	N+T3
OPRI (O)	O	O+T1	O+T2	O+T3
16F (F)	F	F+T1	F+T2	F+T3

Each treatment was replicated four times

5.1.3 *Chemical control of Foe*

5.1.3.1 *Chemical control treatment of Foe*

Three different concentrations (8000 ppm, 16000 ppm or 24000 ppm) of sodium silicate pentahydrate were used to treat *Foe* under *in vitro* conditions. Potato dextrose agar (PDA) was supplemented with the concentrations of the chemical. Each Petri dish contained 25 ml of PDA on which the *Foe* was inoculated under aseptic conditions and incubated at 28 – 30 ^{0}C. Each treatment was replicated four times for *Foe* isolates BOPP-B5, OPRI-5, NORPALM-N5 or 16F. The diameter of

mycelial growth was recorded after 7 days incubation. The data were analysed with Genstat 16th Edition for mean separation and ANOVA.

5.1.4 *Control of Foe spores in oil palm pollen via ultra violet light (UV)*

5.1.4.1 *Pollen viability test*

The pollen of five different progenies of oil palm (Table. 5.2) were collected from CSIR-OPRI, Ghana to ascertain the viability and fertilization ability prior to treatment with different doses of ultra violet (UV) light. The fluorescein diacetate - reaction (FDA-R) test was used to assess pollen viability. BK buffer S15 MOPS (pH 7.5 Appendix 9) was prepared fresh to 1 ml. One microlitre of FDA stock solution (2 mg FDA / ml of acetone stored at -20 °C) was added. A drop of the FDA-buffer mixture was placed on a slide previously cleaned with absolute ethanol. A needle was dipped into the pollen grains and added to the drop of FDA-buffer mixture on the slide. A coverslip was placed on top of the slide and the preparation viewed with Leica DM 5000B optical microscope using a fluorescence filter block for blue excitation (wavelength = 495 nm). Viable pollen grains showed fluorescence (Fig. 5.2) whereas non-fluorescing grains were non-viable (Bio-protocol, 2011).

Table. 5.2. List of oil palm pollen source

Oil palm variety	Source	Code
Dura (D)	OPRI-GHANA	1680
Dura (D)	OPRI-GHANA	899
Dura (D)	OPRI-GHANA	1750
Dura (D)	OPRI-GHANA	1968
Dura (D)	OPRI-GHANA	1960

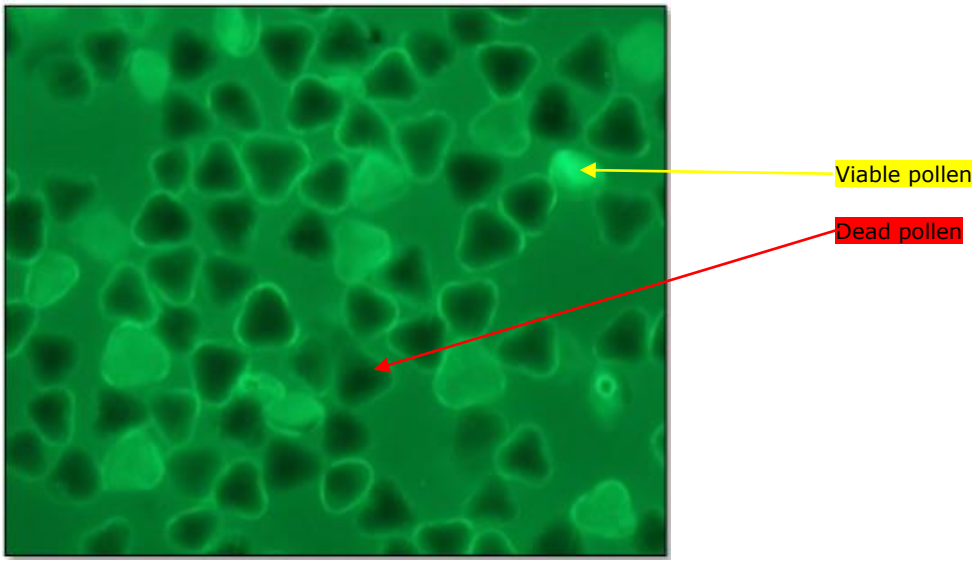


Figure. 5.2. Oil palm pollen stained with FDA (Magnification 40X Light Microscope)

5.1.4.2 *Pollen germination test*

The fertilization capacity of the pollen was determined and established with different combinations of boric acid and sucrose in a BK buffer (Appendix 9). One genotype (Dura sample ID 1960, Ghana) was selected for the trials to develop the optimum protocol for the germination test. Pollen stored at -20 °C was added to 500 µl each of the germination media taken in small culture dishes and incubated at

28 ^{0}C for 2 hrs. Treatment E (Table. 5.3), with 500 µl of 5 % sucrose solution with 500 ppm boric acid showed maximum germination (Fig. 5.3), hence this was selected and used throughout the work on pollen germination. The number of pollen tubes against the total pollen grains for 5 microscopic fields were recorded.

Table. 5.3. Establishing the optimum medium for better germination of pollen in different combinations of boric acid and sucrose

	Treatments	Not germinated	Germinated pollen	% Germination
A	10% 0ppm	21	34	61.82
B	10% 500ppm	30	16	34.78
C	10% 1000ppm	35	29	45.31
D	5% 0ppm	31	39	55.71
E	5% 500ppm	12	81	87.10
F	5% 1000ppm	37	10	21.28

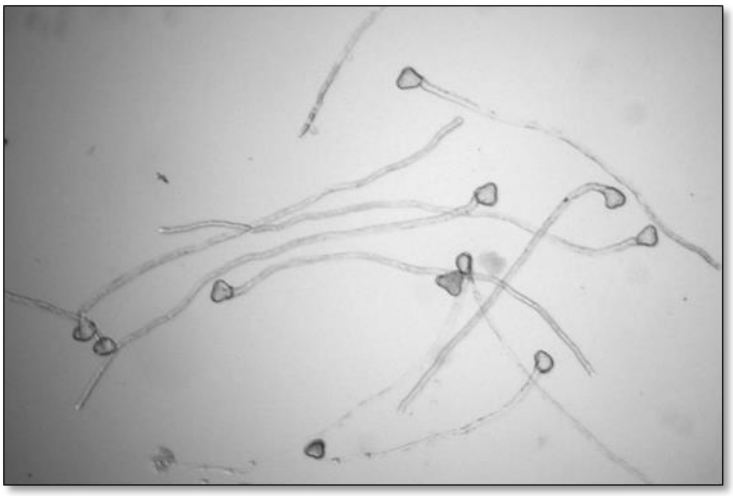


Figure. 5.3. Pollen germination in 5 % sucrose 500 ppm boric acid media (Magnification 40X Light microscope)

5.1.4.3 *UV treatment of Foe spores*

The germination of the *Foe* spores (Chapter 3 section 3.1.2) was repeated for the four *Foe* isolates 16F, BOPP-B5, NORPALM-N5 and OPRI-5. The isolated *Foe* spores (1×10^6) in suspension were dried collected onto Whatman® filter paper (0.2 µm) by using a vaccum pump to sieve the spores via the filter paper. The spores were trapped on the filter paper and dried on the filter paper cealed in a Petri dish covered overnight under room temperature. Two different sources of UV (Conventional and High Intensity Pulsed Polychromatic) were used for the study at three doses each. The conventional UV source (UV-C) was a U-shaped amalgam UV source (UVI 12OU2G11 CP15/469) obtained from Hőnle, A.G., Gräfelfing, Germany. The peak emission of the UV-C was at 254 nm. The U-shaped amalgam UV source (UVI 12OU2G11 CP15/469) was housed within an anodised aluminium parabolic reflector. The intensity was measured with a portable radiometer (Model UVX, UVP Instruments, Cambridge) fitted with a 254 nm sensor. The High Intensity Pulsed Polychromatic source (XENON LH-840 16" ozone free B lamp) was powered and controlled by a RT-847 cabinet and RC-802 controller, supplied by Lambda Photometrics (Harpenden, Herts). The High Intensity Pulsed Polychromatic source produced 505 J of energy per pulse with pulse width of 360 µs at 3.2 pulses per second and the spectral emissions of the source were between 240 and 1050 nm.

The UV doses applied on the pollen or *Foe* with the conventional UV source were 2.5 KJ, 5 KJ, 10 KJ, 20 KJ, 40 KJ or 60 KJ. Each treatment was replicated three times. The equivalent dose of the conventional doses for the High Intensity Pulsed

Polychromatic source were 10 pulses, 20 pulses, 40 pulses, 80 pulses, 160 pulses or 240 pulses calculated with the formula below:

$$D = I \times T$$

Where: D = dose measured in Joules (J), I = intensity measured in Watts (W), T = time measured in seconds (s)

Knowing that intensity was equivalent to 0.002 KJs^{-1} at 2000 μWcm^{-2}, the various calculations were done to know the time of exposure of the *Foe* spores or pollen to the conventional or High Intensity Pulsed Polychromatic UV source (Table 5.4). Each treatment was replicated three times and the means of the percentage pollen or spore germination calculated. ANOVA was performed with the GenStat 16th Edition to assess if there was any significant differences among the treatments.

Table. 5.4. Conversion table for UV dose from Conventional UV or Pulse UV source used to treat oil palm pollen and *Foe* spores

UV Dose/Treatments (KJ)		Exposure time (s) of pollen or *Foe* spores to Conventional UV source (C)	Exposure time (s) of pollen or *Foe* spores to Pulse UV source (P)
T1	Control	0	0
T2	2.5	125	10
T3	5	250	20
T4	10	480	40
T5	20	960	80
T6	40	1920	160
T7	60	2880	240

5.2 *Results*

5.2.1 Assessment of *Foe* in asymptomatic and diseased oil palms

The numbers of oil palms sampled that were either symptomatic of *Foe* infection or symptomless are show in Table 5.5. All the oil palms sampled were infected though some were symptomless from all the three locations (BOPP, NORPALM, OPRI) in Ghana. A sub-view (Fig. 5.4) of two oil palm plantations in Ghana where sampling took place shows the picture of how oil palm plantations suffer from *Foe* infection. However, Twifo oil palm plantation (TOPP) had no plants with physical symptoms so no sampling was performed.

Table.5.5. Assessment of *Foe* infection in healthy and diseased oil palms in three sampling sites

Sample Location	Symptomatic oil palm	Symptomless oil palm	Total number of oil palm	Total infected oil palm
BOPP	2	2	4	**4**
NORPALM	2	3	5	**5**
OPRI	12	7	19	**19**



Figure. 5.4. Oil palm trees showing symptoms of *Fusarium oxyporum* f.sp. *elaeidis* infection in NORPALM oil palm plantation, Ghana.

5.2.2 *Biological control of Foe*

The *Trichoderma* isolates were successfully amplified (Fig. 5.5) and identified. The results for the effect of three *Trichoderma* spp. (T1, T2, T3) on the radial growth of the four *Foe* (16F, OPRI-5, NORPALM-N5 or BOPP-B5) isolates are shown in Table 5.7. *T. harzianum* (T3) caused the highest percentage inhibition (PI) (example in Fig. 5.6) over all the *Foe* isolates tested compared with *T. cerinum* (T1) and *T. atroviride* (T2). The lowest recorded PI of *Foe* was by *T. cerinum* (T1). Among the four *Foe* isolates, NORPALM had the lowest PI values (45.31 and 48.44 %) in dual cultures with *T. cerinum* (T1) and *T. atroviride* (T2) respectively, whereas all three *Trichoderma* strains caused a 100 % PI against the BOPP isolate. ANOVA (Appendix 6) showed significant differences (P>0.05) between the PI means of the antagonists (T1, T2, T3) against the four *Foe* isolates (Table. 5.7).

Of all the three *Foe* isolates tested, OPRI had the highest (6.63 cm) radial mycelial growth in the control treatments (Fig. 5.6). None of the *Foe* isolates was inhibited more than 50 % in dual cultures (Table 5.7). There were significant differences (P>0.05) in the radial growth of *Foe* mycelia in the dual culture (Appendix 7). The interaction between the mycelia of the pathogen (*Foe*) and the antagonists (*Trichoderma* strains) were also examined under the Leica DM 5000B microscope as shown in Fig. 5.8. The confrontational zones in the dual cultures (Fig. 5.8) showed hyphae of *T. harzianum* encoiling around those of *Foe*.

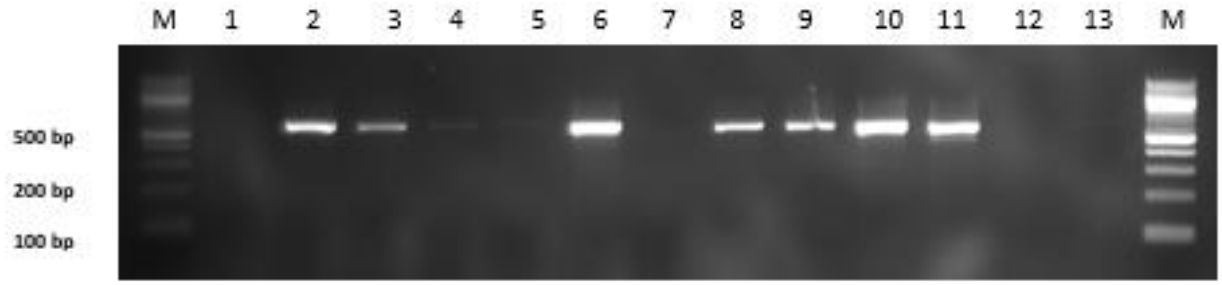


Figure. 5.5. PCR amplification products from *Trichoderma* spp. genomic DNA with primer pairs *ITS4/ITS1* (lane 1-13).

Table. 5.6. List of *Trichoderma* species identified as potential Biological Control Agents (BCA)

Trichoderma species Codes	Identified *Trichoderma* species	Colony colour
2	*Trichoderma cerinum*	Green
3	*Trichoderma atroviride*	Green
4	*Trichoderma cerinum*	Green
5	*Trichoderma harzianum*	Dark green
6	*Trichoderma harzianum*	Dark green
8	*Trichoderma harzianum*	Dark green
9	*Trichoderma cerinum*	Green
10	*Trichoderma cerinum*	Green
11	*Trichoderma cerinum*	Green

Table. 5.7. Effect of three *Trichoderma* species antagonist on mycelial growth of four *Foe* Isolates

Foe isolates	*Trichoderma* species (Antagonist)	Radial growth of *Foe* mycelium (cm)	Percentage Inhibition (%) over *Foe*
OPRI	T1	0.90^{bc}	86.43^{cd}
	T2	1.93^{de}	70.85^{bc}
	T3	0.13^{a}	97.99^{d}
	C	6.63^{g}	0.00
BOPP	T1	0.00^{a}	100^{d}
	T2	0.00^{a}	100^{d}
	T3	0.00^{a}	100^{d}
	C	2.17^{e}	0.00
NORP	T1	2.20^{e}	48.44^{a}
	T2	2.30^{e}	45.31^{a}
	T3	1.40^{cd}	67.19^{b}
	C	4.27^{f}	0.00
16F	T1	0.17^{ab}	97.58^{d}
	T2	2.33^{e}	66.18^{b}
	T3	0.10^{a}	98.55^{d}
	C	6.90^{g}	0.00

Each treatment was replicated three times. Mean values in the same column followed by the same superscripts are not significantly different (P>0.05) according to GenStat (Fisher's protected least significant difference test).

Where: T1= *Trichoderma cerinum,* T2= *Trichoderma atroviride,* T3= *Trichoderma harzianum,* C= Control (only *Foe*: OPRI, BOPP, NORP or 16F) in the absence of antagonist (T1, T2, T3)

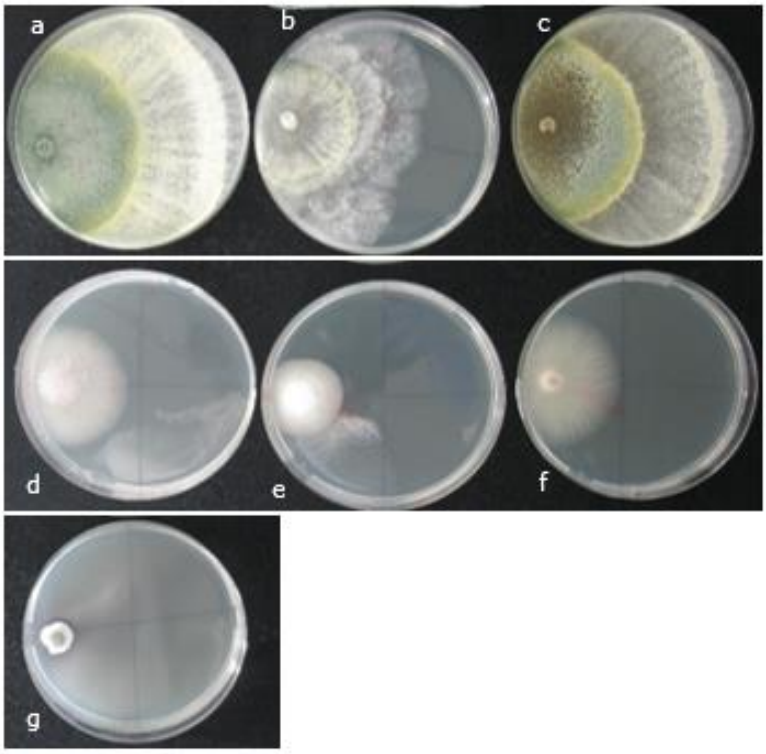


Figure. 5.6. Growth of control treatments of Antagonists **(a)** *Trichoderma cerinum* (T1) **(b)** *Trichoderma atroviride* (T2) **(c)** *Trichoderma harzianum* (T3) and *Foe* isolates from **(d)** OPRI **(e)** NORPALM **(f)** 16F **(g)** BOPP on PDA after 5 days of incubation.

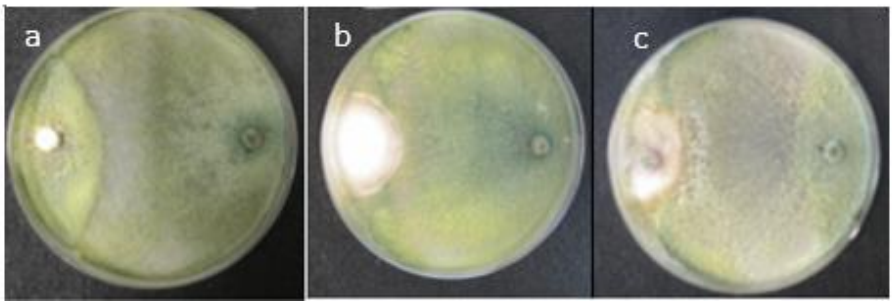


Figure. 5.7. Dual cultures of *Trichoderma harzianum* (T3) acting as (disc on the right) against *Foe* (disc on the left) isolates from **(a)** BOPP **(b)** NORPALM and **(c)** OPRI on PDA after 7 days incubation.

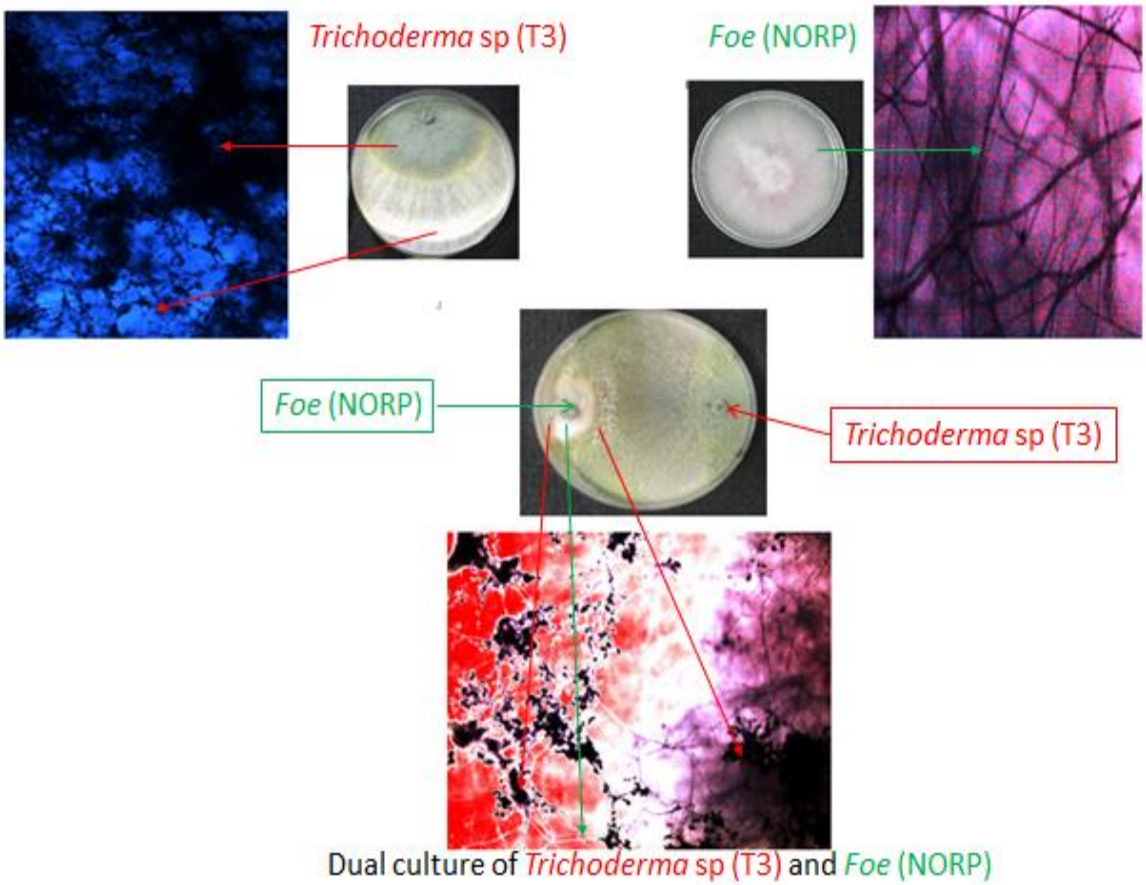


Figure. 5.8. Mycelial growth of the antagonist *Trichoderma harzianum* (red arrows) against *Foe* isolates from NORP (green arrows) on PDA. Dual cultures show coiling *Trichoderma harzianum* hyphae (red arrowed zone) around *Foe*-NORP (green arrowed zone).

5.2.3 *Chemical control of Foe*

The effects of sodium silicate pentahydrate on mycelial growth of the four *Foe* isolates are shown in Table 5.8. At the lowest concentration of 8000 ppm, the *Foe* 16F and OPRI-5 grew on the PDA (Fig. 5.9a; b), whereas BOPP and NORPALM could not (Fig. 5.9c; d). The OPRI *Foe* isolate had the highest (5.6 cm) diameter growth, with 16F on PDA media supplemented with the lowest 8000 ppm sodium silicate pentahydrate. However, 16F on the control PDA plates grew most rapidly (8.5 cm). The remaining concentrations 16000 ppm or 24000 ppm inhibited the growth of all the four *Foe* isolates. ANOVA (Appendix 8) showed that there was a significant ($P>0.05$) difference in radial growth for each *Foe* isolate on the different concentrations of sodium silicate.

Table.5.8 Effect of increasing concentrations of sodium silicate penta-hydrate on mycelial growth of four *Foe* isolates after 7 days incubation on PDA

Foe isolates	Mycelia growth diameter (cm)			
	Control	TI	TII	TIII
BOPP	2.2^{Aa}	0.0^{Ab}	0.0^{Ab}	0.0^{Ab}
NORP	6.2^{Ba}	0.0^{Bb}	0.0^{Ab}	0.0^{Ab}
OPRI	7.2^{Ca}	5.6^{Ab}	0.0^{Ac}	0.0^{Ac}
16F	8.5^{Da}	3.9^{Cb}	0.0^{Ac}	0.0^{Ac}

Each treatment was replicated four times. Mean values in the same column followed by the same Capital letters (superscripts) are not significantly different (P>0.05) according to Fisher's protected least significant difference test. Mean values in the same row followed by the same small lower-case letters are not significantly different (P>0.05) to Fisher's protected least significant difference test.

Where: TI= 8000 ppm (4g / 0.5 lt), TII= 16000 ppm (8g / 0.5 lt), TIII= 24000 ppm (12g / 0.5 lt)

Control= No supplemented sodium silicate pentahydrate

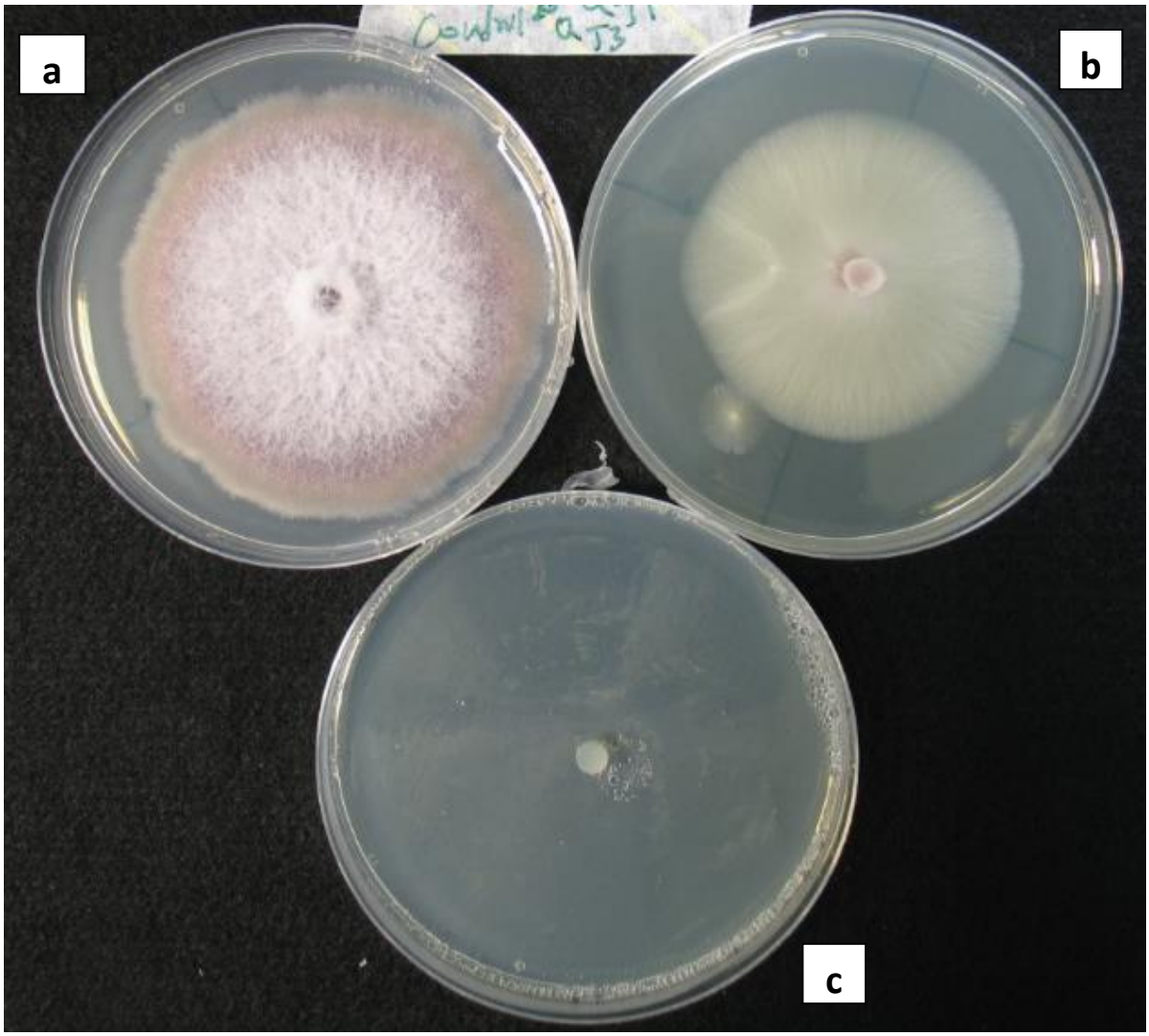


Figure. 5.9. Mycelial growth of *Foe* OPRI isolate on PDA **(a)** Control **(b)** supplemented with 8000 ppm sodium silicate pentahydrate **(c)** supplemented with 24000 ppm sodium silicate pentahydrate

5.2.4 Effects of *UV treatment on Foe spores and oil palm pollen*

The lethal dose for *Foe* spores with either a High Intensity Pulsed Polychromatic or conventional UV sources was 60 KJ. This dose from either source rendered the pollen dead (Figure 5.10). The other doses of 2.5 KJ, 5 KJ, 10 KJ, 20 KJ or 40 KJ reduced growth of *Foe* on PDA but did not kill the pathogen. At 20 KJ or 40 KJ doses from either the High Intensity Pulsed Polychromatic or conventional UV source, the pollen did not germinate (data not shown). ANOVA showed significant difference among the treatments ($P>0.05$) (Appendix 10). Generally, the higher the dose of UV from either source, the lower the percentage germination of *Foe*.

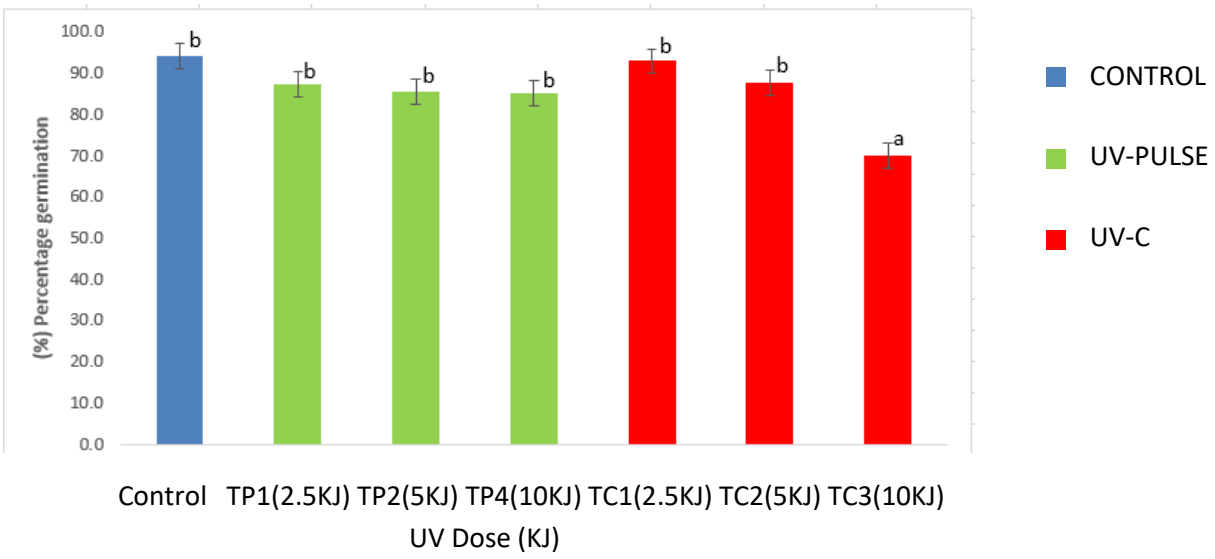


Figure. 5.10. Effect of different doses of UV from High Intensity Pulsed Polychromatic or Conventional source on the germination of freeze-dried oil palm pollen. Bars represents standard error (SE). Same letters on bars show treatment not significantly different from each other. Percentage germination of the pollen at TC3 (10KJ) UV dose treatment was significantly different (P>0.05) according to Fisher's protected least significant difference test.

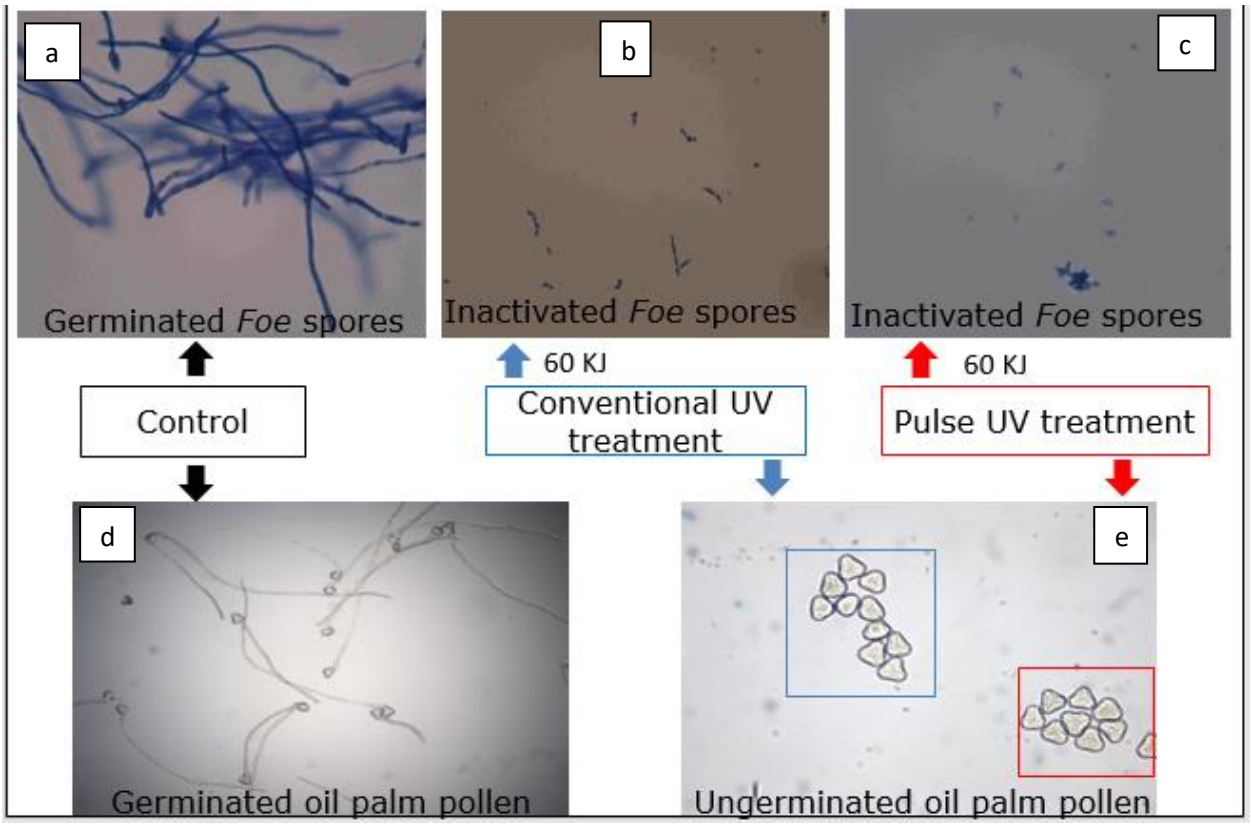


Figure. 5.11. Germinating spores of *Foe* **(a)** control **(b)** inactivated (non-germinated) at 60 KJ UV from conventional source **(c)** inactivated (ungerminated) at 60 KJ UV from pulse source; pollen **(d)** germinated control with no UV exposure and **(e)** ungerminated at 60 KJ UV from conventional source (in blue rectangle) or High Intensity Pulsed Polychromatic source (in red rectangle).

5.3 *Discussion*

The survey of *Foe* in plantations was assessed in all the four oil palm plantation sites based in Ghana, West Africa: Benso Oil Palm Plantation-BOPP, Norpalm and Twifo Oil Palm Plantation-TOPP and Council for Scientific and Industrial Research-Oil Palm Research Institute (CSIR-OPRI). Among these sites, CSIR-OPRI is located in the Eastern Region of Ghana, whereas the remaining three sites are in the Western Region.

The only site without fusarium wilt disease symptoms was TOPP. This finding confirms work of other researchers who visited the site about six years earlier (Afram, E., Council for Scientific and Industrial Research – Oil Palm Research Institute, Eastern Region, Ghana, West Africa, personal communication, 2014). There can be, difficulties in finding *Foe* symptoms in African palm plantations (Cochard *et al.*, 2005). There are also limited outbreaks in other countries, such as Ecuador (Renard and de Franqueville, 1989) and Brazil (Van de Lande, 1984). However, there was serious *Foe* infection on oil palm trees in Norpalm (Fig. 5.11a), compared to OPRI and BOPP. In Ghana, the major supplier of oil palm seedlings to Norpalm and BOPP is OPRI, which may be the main factor for mass infection in the three sites BOPP, Norpalm and OPRI. The spread of *Foe* between OPRI, BOPP or Norpalm could also be through infected seed-nuts as there have been reports of seed nuts contaminated with *Foe* (Locke and Colhoun, 1973; Flood *et al.,* 1990; Flood, 2006).

Fusarium wilt disease has been reported in oil palm plantations in OPRI (Tagoe, 2002) as well as in other parts of Ghana (Flood, 2006). The plantation where infected palms were observed in this study occurred in pairs (Corley and Tinker 2003) or within sampling areas of OPRI plantations. Furthermore, the infection in Norpalm was throughout the entire plantation where sampling was done (Fig. 5.4a). In the oil palm plantation sites in the current study, infection was high and common between palms growing close to each other (neighbour trees), and these were missing or dead palms. A similar observation was made by Prendergast (1957). However, BOPP had most palms infected in the plantations in pairs but randomly located unlike in OPRI or Norpalm. This finding strongly supports the report of Renard and de Franqueville (1989) that *Foe* spreads in soil via root contact and infected dead oil palm debris. Dumortier *et al.* (1992) also reported on the movement of the *Foe* in soil which is congruent with this current study. The observation of the latent or symptomless form of *Foe* infection in oil palm (Renard and de Franqueville, 1989) was reflected this work, as there was 100 % infection in all symptomless palms close or neighbouring symptomatic palms, strongly suggesting the root-root infection in oil palm plantations in Ghana.

The most effective method of controlling *Fusarium oxysporum* and its associated diseases is via the use of resistant cultivars (Fravel *et al.,* 2003). However, in the wilt resistant varieties of oil palm, absence of there is the need to find alternatives. *Trichoderma* species are well known saprophetic fungi common in the soil or the rhizosphere which are parasitic on soil borne plant pathogens (Lorito *et al.,* 1994).

Three species of *Trichoderma* were tested as potential biological control agents (BCA) against in this study.

Several important actions or mechanisms for BCAs have been reported including mycoparasitism, which is well established in *T. harzianum* and *T. atroviride* (Druzhinina *et al.,* 2011; Kubicek *et al.,* 2011), antibiosis, competition for nutrients, and enzyme activity such as chitinases or glucanases (Baek *et al.,* 1999; Howell, 2003; Alabouvette *et al.,* 2009; Rashmi *et al.,* 2016) and rhizosphere competence or root colonoization (Carvalho *et al.,* 2011a) and these lead to high levels of antagonisms (Lorito *et al.,* 1998). *T. harzianum* (T3) was the most effective strain to suppress growth of all the *Foe* isolates tested. As such, *Trichoderma harzianum* is a potential useful biocontrol agent. *T. harzianum* has been used successfully in the control of *F. oxysporum* f. sp. *phaseoli* in seed treatment of beans and compared to other strains of *Trichoderma* (Carvalho *et al.,* 2011b; Carvalho *et al.,* 2014). However, seed treatment of beans resulted in percentage inhibition (PI) values three times lower than in this study against *Foe* isolates. A recent report on the application of mutagenic *T. harzianum* and *T. atroviride* as biological control agent against *Sclerotium rolfsii* showed that *T. harzianum* could effectively controlling the pathogen (Rashmi *et al.,* 2016), with a PI value twice that recorded in this study.

In the current study, the *Foe* isolate BOPP-B5 was the slowest growing compared to the other three *Foe* isolates, and growth was totally inhibited by all the *Trichoderma*

sp. Though *T. atroviride* caused the lowest PI in the biocontrol study, it fully suppressed *Foe* BOPP-B5, which suggests it could be for biocontrol purposes.

The ability of *Trichoderma* spp. to penetrate hyphae of upon interaction pathogen has been demonstrated (Harman *et al.,* 2004). Howell (2003) showed that the hyphae of *Trichoderma* sp. could degrade the glucans and chitin which are important components of fungal cell-walls. Moran-Diez *et al.* (2009) and Brotman *et al.* (2008) further investigated that the secretion of an expansin-like protein with cellulose binding modules and endopolygalacturonase by *Trichoderma* sp. supported the penetration of host roots. The mechanism of hyphal penetration was evident in this study, as there was hyphal encoiling of the *Foe* pathogen by the antagonist (*Trichoderma* sp.), occured leading to the suppression or inhibition of the pathogens (*Foe*) growth, as the *Foe* mycelia degraded with time.

Trichoderma sp. have been used as products for long term control of fungal pathogen in the field and are also environmentally safer (Ha, 2010) compared to chemical approaches (Baker and Paulitz, 1996; Anand and Jayarama, 2009). In addition, 60 % of the worlds registered biofungicides are based on *Trichoderma* sp. (Verma *et al.,* 2007). For example, in India, some 250 *Trichoderma* based products are available for application in the field (Singh *et al.,* 2009). As such, there is potential for the use of *T. cerinum, T. atroviride* or *T. harzianum* as biocontrol agents in oil palm seed germination and in plantations, to control fusarium wilt disease or the causal organism.

There are many factors that could negatively affect the performance of *Trichoderma* sp. as an antagonist. According to Howell (2003), the mechanisms employed by the *Trichoderma* sp. to biologically control pathogens such as enzyme and antibiotic production, are affected by the substrate as well as pH, moisture and temperature conditions of the soil in which they are grown. Thus, the presence of some factors in natural soil (e.g. microflora) may negatively influence or reduce the potential efficacy of *Trichoderma* sp. by metabolizing the enzymatic or antibiotic products of the biocontrol agents. As a result, the BCA will not produce its full suppressive effects on the pathogens (Howell, 2003). In addition, Howell (2003) reported that some of the most effective biocontrol strains of *Trichoderma* do not have good storage qualities or function well in temperature and moisture conditions where pathogens flourish. *Trichoderma* spp. are high prolific secretors of secondary metabolites (Reino *et al.,* 2008; Kubicek *et al.,* 2011), and genome analyses suggest that these secondary metabolites are not necessarily produced under laboratory conditions (Kubicek *et al.,* 2011; Mukherjee *et al.,* 2012). For instance, *T. atroviride* used to suppress the *Foe* isolate in this study has been reported to produce volatile metabolites such as 6-pentyl-2H-pyran-2-one (6-PP) which plays a role in *Trichoderma*-plant and *Trichoderma*-fungal interactions (Vinale *et al.,* 2009; El-Hassan *et al.,* 2008).

Differences in the *Trichoderma* strains has been demonstrated. Druzhinina *et al.* (2011) showed that some *Trichoderma* sp. such as *T. stromaticum*, *T. amazonicum*, *T. evansii*, *T. martiale*, *T. taxi* and *T. theobromicola* can live in plant hosts as endophytes. These *Trichoderma* species, though different from the species

used in this current study, induced resistance to disease (or pathogen invasion) in a host-plant (Chaverri *et al.,* 2011; Druzhinina *et al*., 2011), as well as to abiotic stress (Bailey *et al.,* 2006; Bae *et al.,* 2009). A genome-wide expression study of *Trichoderma virens*, *T*. *asperellum*, *T. harzianum* and *T. atroviride* was carried out (Druzhinina *et al.,* 2011; Kubicek *et al.,* 2011). Genome sizes of 36.1 Mb for *T. atroviride* (11,863 gene models) and 40.98 Mb for *T. harzianum*, (14,095 gene models) were found. The differences might be partially responsible for differences seen in suppressive nature of the *Trichoderma* species as potential biological control agents for *Foe* but probably were not since the genome sizes of the three *Trichoderma* species used for this work were not studied.

The chemical treatment of *Fusarium oxysporum* has been shown (Flood *et al.,* 1999; Rose *et al.,* 2003; Mukhtar, 2007; Mohammadreza *et al.,* 2015) though it is costly for larger plantations (Renard and Ravisé, 1986) and there are mixed results in terms of efficacy and environmental effects of fungicides. There have been few attempts to eradicate or control via chemical methods (Mepsted, 1992; Flood *et al.,* 1999). However, according to Datnoff *et al.* (2007), one possible alternative for chemically controlling plant pathogens is with silicon, since it considerably reduced the severity of diseases in other crops. Silicon is the second most abundant element in the earth is crust and present in living organisms as amorphous silica (SiO_2nH_2O) and to a lesser extent, soluble silicic acid $Si(OH)_4$ (Fawe *et al.,* 1998). The presence of silicon caused vegetative growth and biomass increase in some plants (Agarie *et al.,* 1998) and the application of silicon to control fungal diseases in crops such as cucumber (Cherif *et al.,* 1992), peach fruit (Biggs *et al.,* 1997), muskmelon and

zucchini squash (Menzies *et al.,* 1992) has been reported. This information leads to the testing of sodium silicate penta-hydrate ($Na_2O_3Si.5H_2O$) to control *Foe* under *in vitro* conditions.

The concentrations of sodium silicate pentahydrate tested on the *Foe* isolates in this study were mostly effective. The *Foe* isolates OPRI-5 collected from Ghana and 16F from Ivory Coast were not totally inhibited from growth in the media supplemented with 8000 ppm sodium silicate penta-hydrate. However, the two *Foe* isolates, BOPP-B5 or NORP-N5 were completely inhibited at the same concentration. Comparing the the same two *Foe* (OPRI-5 and 16F) isolates that grew in the presence of 8000 ppm sodium silicate penta-hydrate, the isolate from Ghana OPRI-5, had the highest mycelial growth. These observations demonstrate variations in tolerance of the four *Foe* isolates to the chemical treatment (de Franqueville, 1991; Mepsted *et al.,* 1994; Paul, 1995). Increased concentrations of sodium silicate penta-hydrate in the growth media totally inhibited all the *Foe* isolates used in the study. Thus, at higher concentrations (above 8000 ppm) the chemical significantly suppressed *Foe* under *in vitro* conditions. Similar results were achieved with the application of sodium silicate penta-hydrate at different concentrations to inhibit the pathogen in laboratory conditions (Mohammadreza *et al.,* 2015). The sodium silicate penta-hydrate treatment reduced or suppressed mycelial growth of *Fusarium oxysporum* f.sp. *meloni* in melon at concentrations of 200 ppm, 400 ppm or 800 ppm *in vitro*. However, in this current study, the *Foe* mycelia grew in similar concentrations but at a slower rate during the trials for the optimum concentrations though not presented in the results. Hence, higher concentrations were applied in

this current study inhibit the *Foe*. Mohammadreza *et al.* (2015) also demonstrated the effectiveness of the application of sodium silicate to control fusarium wilt in melon fields. They established that the presence of the chemical in the soil could increase pH above 7.0 which this reduced the spread of *Fusarium oxysporum* f.sp. *meloni* in the melon field, whereas the low salinity (exchangeable cations – EC) was beneficial for the melon plants.

The presence of silicate can induce resistance in some crops such as rice against blast (Kim *et al.,* 2002) and was also suggested to be a plant defence activator in melon (Mohammadreza *et al.,* 2015). The application of other chemicals that contain Si, such as potassium silicate, can inhibit the mycelial growth of *F. oxysporum*, *F. solani*, *Phytophthora cinnamomi*, *P. capsici* and *Pythium* F-group and *Verticillium fungicola* (Kaiser *et al.,* 2005).

Other chemical fungicides including prochloraz, carbendazim, thiram, toclofos-methyl, hymexazol, azoxystrobin and carboxin have shown inhibitory activities against *F. oxysporum* under *in vitro* conditions (Song *et al.,* 2004). In addition, *in vitro* and *in vivo* experiments showed that prochloraz was more effective compared to bromuconazole in the control of tomato fusarium wilt (Amini and Sidovich, 2010). Furthermore, Christian *et al.* (2007) showed that five fungicides, benomyl (1 mg / l), dodine (50 mg / l), Manzate (100 mg / l), Copper sulphate (200 mg / l) and thiabendazole (4 mg / l) inhibited *Fusarium roseum* under *in vitro* conditions.

Although sodium silicate penta-hydrate could fully inhibit growth *Foe in vitro* in this study, there is a need to test the chemical under field conditions to ascertain the level of success. The most economical method to apply the silicate is to soak oil palm seed nuts silicate water solutions prior to germination. However, Mohammadreza *et al.* (2015) strongly established the success of the application of sodium silicate in field conditions after testing under *in vitro* conditions to control fusarium in melon, suggesting that the application of sodium silicate penta-hydrate may be a potential option as a chemical method of controlling fusarium wilt in oil palm plantations and nurseries.

The transmission of *Foe* across countries has been reported to be possibly via pollen as was reported by Flood *et al.* (1990), who showed that up to 40,000 c.f.u *F. oxysporum* / g could be isolated from half of the thirty samples of commercial freeze-dried oil palm pollen tested. They further showed that the isolated *Foe* were pathogenic on oil palm seedlings. Though there has reportedly been an effort to clean oil palm pollen of *Foe* spores via treatment with a wide range of fungicides in organic solvent carriers, this method failed to produce complete decontamination (Mepsted, 1992). In this study, the use of UV as a potential tool to clean *Foe* from in oil palm pollen was considered, since pollen is now used to exchange germplasm between countries that grow oil palm.

The ultraviolet spectrum is divided into three wavelength intervals: UV-A (315–400 nm), UV-B (280–315 nm), and UV-C (100–280 nm) (Coblentz, 1932). In this study, the UV-C and High Intensity Pulsed Polychromatic UV sources were employed. The

lethal dose for *Foe* isolates was established for both the High Intensity Pulsed Polychromatic and the Conventional UV sources. The effect of UV on fungi has been demonstrated previously (Gregory, 1973; Liyanage *et al.*, 1982; Atlas and Bartha, 1998; Henson *et al.,* 1999). A report on the direct exposure of fusarium spores to UV-C the emphasized that the carotenoids could not shield the pathogen from the lethal activity of UV-C (Asthana and Tuveson, 1992). The carotenoids produced by these spores, however, had the ability to reduce the effect of UV-A and UV-B on fusarium. The presence of carotenoids in fungi spores has also been reported to reduce production of glutathione that can inactivate the toxic reactive oxygen species induced by UV radiation (Miller *et al.,* 2004; Soriani *et al.,* 2009; Xie *et al.,* 2012; Avalos and Limón 2015). In this current study, the *Foe* spores were isolated from pinkish cultures, a sign of pigmentation or carotenoid production. Although *Foe* produced pinkish cultures which suggest carotenoid may be present though not tested in this work, however, *Foe* spores were damaged by the highest UV dose from both sources. Damage to DNA induced by UV radiation depends on the wavelength of the incident photons (Kielbassa *et al*., 1997; Douki *et al*., 2003; Schuch *et al.,* 2009; Cadet *et al.,* 2012; Karentz, 2015) absorbed by the DNA of the *Foe* spores. Therefore, the higher the dose, the greater the probability of damage to the DNA of the *Foe*.

The lower dose from both sources of UV on the *Foe* slowed spores germination. This The *Foe* is may have undergone nucleotide excision repair (NER) and photo-reactivation (PR), which are very important mechanisms in repairing UV-induced damage to DNA (Goldman and Kafer, 2004; Chelico *et al.,* 2005; Chelico *et al.,*

2006; Bayram *et al.,* 2008; Berrocal-Tito *et al.*, 2007; Chelico and Khachatourians, 2008; Fang and St. Leger, 2012). Conidia exposure to sub-lethal doses of UV radiation reduces conidial germination speed and subsequently affects virulence, because virulence is positively correlated to germination speed of the conidia (Paul *et al.,* 1997; Nascimento *et al.*, 2010). This makes the control of only the *Foe* with UV a potential alternative though the pollen could not survive under the UV dose lethal to *Foe* isolates.

Viability of the oil palm pollen reduced with increasing UV dose from both sources. Previous research has shown that pollen has a characteristic of reduced viability upon exposure to UV (Sophie and Thomas, 1996; van de Staaij *et al.,* 1997; Torabinejad *et al.,* 1998; Feng *et al.,* 2000; Zhang *et al.,* 2014). Exposure to UV can yield different effects that a reduced in the viability of the pollen and given zero germination at higher UV doses. According to Wang (1976) germicidal UV, which falls in the same category as UV-C in the current study, induces photochemical damage to DNA because of the covalent bonds formed between adjacent nucleotides when these nucleotides absorb the UV. This effect have caused damage to the oil palm pollen as a result of failure to undergo photo-reactivation (Fang and St. Leger, 2012) to repair the damaged DNA at higher UV doses.

There is limited knowledge on controlling fungi effectively using UV-C (Levitin *et al.,* 2001) and High Intensity Pulsed Polychromatic UV. Hence other methods need to be explored to eradicate *Foe* in pollen of oil palm. When this is achieved, it would

serve as a means of controlling *Foe* transmission from one country to the other and would affirmatively impact germplasm exchange.

CHAPTER SIX: GENERAL DISCUSSION

This research provides important information about fusarium wilt disease in oil palm plantations and the genetic relationships existing between the *Foe* that originate from different geographical locations (West Africa, Central Africa and South America). In addition, the current research has revealed that the fusarium wilt pathogen is also a threat to the Malaysian oil palm industry as Malaysian progenies of the host were susceptible to the pathogen. Furthermore, this research work is the first to develop the Loop Mediated Isothermal Amplification (LAMP) technique for diagnostics, based on primers that target the Secreted in Xylem (*SIX*) genes in *Foe* and *Fusarium oxysporum* f.sp. *lycopercisi* (*Fol*) isolates. More so, the presence or absence of different effector protein (*SIX*) genes in *Foe* was established in the current study. Finally, UV, chemical and biological strategies was used as potential methods to control *Foe*. This research sought to enhance and strengthen knowledge to handle the challenges that fusarium wilt disease has posed to the oil palm industry in Ghana and across the world.

6.1 *Phylogenetics*

The housekeeping genes (*TEF-1α* and *ITS*) and effector proteins, specifically the *SIX* genes, were used as molecular markers to distinguish *Foe* from non- *Foe* isolates and to determine the genetic diversity among the *Foe* based on geographical sources of isolates. The study showed that the housekeeping genes

could not fully differentiate *Foe* from non-*Foe* isolates. However, the concatenation of gene sequences based on the three *SIX* genes (*SIX8, SIX9* and *SIX11*) and the two housekeeping genes (*TEF-1α* and *ITS*) distinguished *Foe* isolates from non-*Foe,* but did not cluster *Foe* isolates based on their origins. The study of the *SIX* genes as molecular markers can be used to distinguish organisms based on pathogenicity, as reported in *F. oxysporum* f.sp. *lycopersici* race 1 (*Fol*) isolates (van der Does *et al.,* 2008; Lievens *et al.,* 2009).

Amplified fragment length polymorphism (AFLP) analyses of the *Foe* isolates from the four geographical locations revealed further genetic information about the pathogen. Overall, the AFLP markers showed that the twelve *Foe* isolates were not monophyletic. The AFLP results were significant for research on interactions between oil palm and *Foe* since the twelve *Foe* isolates from Ghana, Ivory Coast, DR Congo and Suriname examined varied genetically, showing the diversity of *Foe* isolates. The AFLP markers in this study had good discriminatory power to distinguish *Foe* isolates. In general, AFLP was a powerful tool for the genetic variation study of *Foe* isolates from Ghana, Ivory Coast, DR Congo and Suriname, as previously shown in *F. oxysporum* f.sp. *cubense* (*Foc*) (Groenewald *et al.,* 2006). The AFLP markers for the study can also serve as potential target gene regions to support understanding between *Foe* – oil palm interactions.

The application of the simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) markers to either discriminate isolates of *Foe* from different locations or differentiate the *Foe* from non-*Foe* isolates also suggested a polyphyletic nature

of the *Foe* isolates based on the geographical origin. However, the ISSR markers only showed moderate discrimination between the non-*Foe* isolates in the current study, due to the strong banding patterns produced among them compared to the *Foe* isolates.

The separation of *Foe* into various clusters in the phylogenetic trees generated for the rDNA genes (*TEF* and *ITS*) and effector protein (*SIX*) genes used as markers, as well as other molecular tools including AFLPs, SSRs and ISSRs can also help in screening for resistance to the pathogen. This work, conducted for the first time for *Foe* in oil palm, is important in developing possible management methods via inter transfer of planting materials between oil palm growing countries to better understand the polyphyletic characteristic of *Foe.* Understanding the diversity of this pathogen is an additional value to fusarium wilt research allowing the identification of future *Foe* races.

6.1 *Pathogenicity of Fusarium oxysporum* f. sp. *elaeidis* (*Foe*) *in oil palm genotypes*

Foe is important to the oil palm industry in Ghana and abroad due to the importance of the crop. The screening for resistant oil palm progenies from Ghana and Malaysia with four *Foe* isolates, BOPP-B5, NORPALM-N5, OPRI-5 or 16F revealed the virulence of the pathogen. The screening tested if widening the genetic base of oil palm progenies could be used to combat fusarium wilt disease. The

progenies from Ghana, K-1213D and the commercial type Tenera (T), as well as Malaysian oil palm Tenera (T) progeny lines were all susceptible to the four *Foe* isolates. This finding confirmed that although the *Foe* isolates from Ghana were from three different locations within the country, the oil palm germplasm appeared to be equally susceptible to all isolates. Hence there is the need to invest in future screening programmes via pathogenicity assays to identify potential resistant lines of the oil palm. Malaysian oil palm plantations do not currently suffer from fusarium wilt. However, this study has shown that oil palm progeny from Malaysia were susceptible to the pathogen. There is a clear need to implement good quarantine strategies in Malaysia and Ghana as germplasm are normally exchanged between countries as a way of expanding the oil palm industry across the globe.

6.3 *Development of molecular diagnostics*

Rapid molecular diagnostics and detection tools are important for timely intervention to prevent the spread and infection of *Foe* across borders and within parts of a country. This study developed the first host or genus specific primers for *Foe* and *Fusarium* oxysporum (non-*Foe*) respectively for use with Loop Mediated Isothermal Amplification (LAMP). The primers for the LAMP diagnostics were designed based on sequences of cytochrome *P450* and Secreted in Xylem (*SIX*) genes regions of *Foe.*

The *SIX1* LAMP primers distinguished *Foe* isolates from non-*Foe* isolates. Secreted in Xylem gene (*SIX1*) could be used to detect *Foe* isolates from DR Congo, Suriname and one from Norpalm-Ghana, whereas the *SIX10* and *SIX13* were highly specific for *Fusarium oxysporum* f.sp. *lycopercisi* (*Fol*) for tomato. *SIX8* primers detected any of the *Fusaria* isolates, irrespective of the host. Similarly, the cytochrome *P450* gene could not discriminate between *Fusarium* isolates used here. All the detection results were produced within 30 minutes. The LAMP primers for the *SIX* genes showed that the regions were conserved in *Foe* isolates from other countries. The LAMP primers for diagnosing *Foe* or non-*Foe* isolates in this study were sensitive (Kaneko *et al.,* 2007), robust, highly reproducible, consistent and provided rapid diagnostics (Ghosh *et al.,* 2015) for on-site detection as the method was tested in root samples, as well as genomic DNA from inoculated oil palm seedlings and *Foe* mycelium. This LAMP method of diagnostics is extremely useful for the quarantine and control of *Foe,* hence there is a need to disseminate this technique with stakeholders, researchers and possible users.

6.4 *Survey and control of Fusarium oxysporum f. sp. elaeidis* (*Foe*)

There have been few previous surveys on the occurrence of fusarium wilt disease in oil palm plantations of Ghana. The current survey revealed that in the oil palm plantations of BOPP, OPRI and Norpalm, all the neighbouring healthy looking (symptomless) oil palm trees in the field *Foe* infected (symptomatic) oil palm trees tested positive for presence of *Foe.* This spread of *Foe* in oil palm plantations of

Ghana was confirmed (Flood, 2006). It is suggested that all the diseased palms should be fell and removed, as proposed by Moureau (1952), to avoid further infection in the three oil palm sites of Ghana where the survey was undertaken.

Three different mechanisms for controlling the selected four *Foe* isolates, BOPP-B5, NORP-N5, OPRI-5 and 16F under *in vitro* conditions were tested in the current study. In the first method three species of *Trichoderma* were used aspotential BCAs. The most effective or suppressive the *Foe* was *Trichoderma harzianum.* However, *T. cerinum* and *T. atroviride* also inhibited the *Foe* substantially.

Sodium silicate penta-hydrate inhibited the growth of *Foe* at concentrations > 8000 ppm under *in vitro* conditions. The two concentrations 16000 ppm (8g / 0.5 lt) and 24000 ppm (12g / 0.5 lt) totally inhibited all the four *Foe* isolates used in the study.

The use of UV was killed conidia of *Foe.* Exposure of *Foe* to two sources of UV: High Intensity Pulsed Polychromatic or conventional UV showed that doses of 60 KJ rendered *Foe* spores inactive. Attempts made to clean oil palm pollen of *Foe* were not successful, as the lethal dose of 60 KJ for *Foe* to the pollen. However, a potential mechanism of applying UV from two different UV sources as been established. This UV method can be useful for treating oil palm seed nuts during germination and also to treat seed nuts imported or exported as germplasm.

6.5 Future research recommendations and conclusions

Although the current study of *Foe* has made progress with respect to the survey of *Foe* in oil palm plantations, genetic variations of *Foe*, pathogenicity of *Foe* to host plants, development of a specific LAMP diagnostic for *Foe*, and application of biological agents and High Intensity Pulsed Polychromatic or conventional UV to inhibit *Foe*, further research is required to complement the objectives achieved in the present study. These include:

1. Understanding *Foe* diversity among different isolates at the whole genome level via next - generation sequencing. This will better establish the understanding of mechanisms underpinning *Foe* pathogenicity and to further develop novel control methods.
2. Gene expression analysis in *Foe* - infected oil palms and dissection of plant responses to infection for the potential development of resistant plants when possible resistant progeny lines are identified. This could be done via plant transformation of oil palm seedlings by introducing resistance genes that may be identified during gene expression analysis. This will assist molecular breeding for resistant oil palm genotypes.
3. Next - generation sequencing of the biological control agents - *Trichoderma* sp. genome used in this study and collection of more *Trichoderma* spp. in Ghana for further research. This is important as the *Trichoderma* spp. in Ghana may have adapted to the environmental conditions already so will be used in its natural environment as opposed to *Trichoderma* spp. used in the

United Kingdom for this present work. Next-generation sequencing of all these *Trichoderma* spp. may ascertain or identify the specific suppressive genes to *Foe* as well as *Foe-Trichoderma* interaction mechanisms.

4. The application of LAMP to screen oil palm seedlings at the nursery and field soil samples randomly. This will help curb the spread of *Foe* and if possible LAMP applications extended to other plant pathogens of importance.
5. Field studies with sodium silicate penta-hydrate to ascertain the viability of the method as a technique to control the disease globally.

These research aspects can be addressed to ensure that *Foe* and its host are well studied and to ensure that one of the key challenges to the oil palm industry, fusarium wilt disease, is overcome.

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APPENDICES

Appendix 1A

Potato Dextrose Agar (PDA)

Components	Quantity
PDA powder	58.5 g
Sterilised distilled water	1000 ml

Appendix 1B

Czapek Dox Liquid (CDOX) Medium

Components	Quantity
CDOX powder	33.4 g
Sterilised distilled water	1000 ml
Cassamino acid	2.3 g

Appendix 1C

PCNB selective medium

Ingredient	1 L	500 ml
Part 1		
distilled water	1 L	500 ml
Difco peptone	15 g	7.5 g
K_2HPO_4	1 g	0.5 g
$MgSO_4 \cdot 7\ H_2O$	0.5 g	0.25 g
agar	15 g	7.5 g
PCNB (Terraclor 75 % WP)(pentacloronitrobenzene)	0.5 g	0.25 g
Part 2		
streptomycin sulfate	0.1 g	0.05 g
chlorotetracycline HCl	.05 g	0.025 g
oxgall (Bile Bovine)	0.5 g	0.25 g

Appendix 2

List of other selected *Fusarium* sp isolates from gene NCBI gene bank

Name	NCBI Code	Reference(s)
Fusarium graminearum	KT587256	Kruthika, H.S., Bharathi, T.R. and Prakash,H.S.(unpublished)
Fusarium avenaceum	FJ478097	Jiang,J., Sun,Y., Ju,X., Cao,X., Yin,T., Miao,Q. and Li,C.(Unpublished)
Fusarium tricinctum	AF111066	Yli-Mattila *et al.* (2002)
Fusarium oxysporum f. cubense	KP965001-	Taylor *et al.* (2015)
Fusarium culmorum	EF187910	Di Maiuta,N. and Schwarzentruber, P. (Unpublished)

Appendix 2A

Foe or non-*Foe c*oncatenated housekeeping genes sequences

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>DR_CONGO_Z
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Appendix 2B

Foe and non-*Foe* concatenated *SIX8, SIX9* and *SIX11* sequences

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Appendix 3

List of primers in the study

Primers	Sequence 5′-3′	Annealing Temperature (^{0}C)	Target region	Reference
ITSkaf/ITSkar	ACCCCTAAACT GTTTCTATATG/ ACGAGGGTTTT ACTACTAC	60	*ITS*	This study
ITS1/ITS4	TCCGTAGGTGA ACCTGCGG/TC CTCCGCTTATT GATATGC	58	*ITS*	Abd-Elsalam *et al.* (2003)
ITS-Fuf/ITS-Fur	CAACTCCCAAA CCCCTGTGA/G CGACGATTACC AGTAACGA	58	*ITS*	Abd-Elsalam *et al.* (2003)
ITS-Fu1f/ITS-Fu1r	ACAACTCATAA CCCTGTGAACA T/CAGAAGTTG GGTGTTTTACG G	58	*ITS*	Abd-Elsalam *et al.* (2003)
ITS4AF/ITS4AR	ACCCCTAAACT CTGTTTCTATAT G/ACGAGGGTT TTACTACTACG C	60	*ITS*	This study
ITS4BF/ITS4BR	CCCCTAAACTC TGTTTCTATATG T/TACTACGCTA TGGAAGCTCG	60	*ITS*	This study
TEFB2F/TEFB2R	TGACAACATAT GCTTACCCCC/ GGGTCGTTCTT GGAGTCAC	60	*EF-1α*	This study
EF5F/EF5R	GCACTTACCCC CGCCACTT/CTC GAACTTCCAGA GAGCAATATC	60	*EF-1α*	This study

ND =
Not determined

SIX1F/SIXR	GTATCCCTCCG GATTTTGAGC/A ATAGAGCCTGC AAAGCATG	60	*SIX1*	Taylor *et al*. (2015)
SIX2F/SIX2R	CAACGCCGTTT GAATAAGCA/T CTATCCGCTTTC TTCTCTC	ND	*SIX2*	Taylor *et al*. (2015)
SIX3F/SIXR	CCAGCCAGAAG GCCAGTTT/GG CAATTAACCAC TCTGCC	ND	*SIX3*	Taylor *et al*. (2015)
SIX4F/SIX4R	TCAGGCTTCAC TTAGCATAC/GC CGACCGAAAAA CCCTAA	ND	*SIX4*	Taylor *et al*. (2015)
SIX5F/SIX5R	ACACGCTCTAC TACTCTTCA/GA AAACCTCAACG CGGCAAA	ND	*SIX5*	Taylor *et al*. (2015)
SIX6F/SIX6R	CTCTCCTGAAC CATCAACTT/CA AGACCAGGTGT AGGCATT	ND	*SIX6*	Taylor *et al*. (2015)
SIX7F/SIX7R	CATCTTTTCGCC GACTTGGT/CTT AGCACCCTTGA GTAACT	ND	*SIX7*	Taylor *et al*. (2015)
SIX8F/SIX8R	CCTTATCCCTAT CGGGGCCT/AC CAAGCCGCCAC TATTGA	60	*SIX8*	This study
SIX9F/SIX9R	GGGAAGTGCTC GTTCATCTTG/G AGGAGGCAAGT TCAGGAA	60	*SIX9*	This study
SIX10F/SIX10R	GTTAGCAACTG CGAGACACTAG AA/AGCAACTTC CTTCCTCTTAAC TAGC	64	*SIX10*	This study
SIX11F/SIX11R	TGAACCAGATC ATCGACAAA/C AGAGTACACTC TCCAACCT	60	*SIX11*	This study

Primers	Sequence 5'-3'	Annealing Temperature (^{0}C)	Target region	Reference
	GATCCTCCACC TCCACCTC			*t al.* (2005)
SIX13F/SIX12 F	CCTTCATCATC GACAGTACAAC G/ATCAAACCC GTAACTCAGCT CC	ND	*SIX13*	Taylor *et al*. (2015)
SIX14F/SIX14 R	ATAAAGTGCGA CTGGACTTCTG CC/ACCCCCATC CACATTCCTAA GCGA	ND	*SIX14*	Taylor *et al*. (2015)
SIX1F/SIX1R	GTATCCCTCCG GATTTTGAGC/A ATAGAGCCTGA AAGCATG	60	*SIX1*	This study
SIX8AF/SIX8A R	TCCCTATCGGG GCCTAATCC/AT AAAGCCTCGAC CAAGCCG	58	*SIX8*	This study
SIX8BF/SIX8B R	CCTTATCCCTAT CGGGGCCT/AC CAAGCCGCCAC TATTGA	58	*SIX8*	This study
SIX8CF/SIX8C R	GGGGCCTAATC CACACAACA/A CCAAGCCGCCA CTATTGAA	58	*SIX8*	This study
SIX9AF/SIX9A R	AAGTGCTCGTT CATCTTGG/CAA GTTCAGGAATG TACCAGG	58	*SIX9*	This study
SIX9BF/SIX9B R	GGGAAGTGCTC GTTCATCTTG/G AGGAGGCAAGT TCAGGAATG	58	*SIX9*	This study

ND = Not determined

Primers	Sequence 5'-3'	Annealing Temperature (^{0}C)	Target region	Reference
SIX11AF/SIX11AR	ACAATAACCAC CGCCAGAATG/ ACCCGGTGTGA CACAAAACA	ND	*SIX11*	This study
FitsMB13 F/MB13R	ATATGCTTAAG TTCAGCGGGT GGAGGATGAG CTCGATGAAG/ CTAAGCCTGCT ACACCCTCG	ND	SSR	Bogale *et al.* (2005)
23SMB2 F/ MB2R	TTCGCCTTTCC CTCACGGTACT TGCTGTGTATG GATGGATGG/C ATGGTCGATAG CTTGTCTCAG	ND	SSR	Bogale *et al.* (2005)
23SMB5 F/MB5R	TTCGCCTTTCC CTCACGGTACT ACTTGGAGGA AATGGGCTTC/ GGATGGCGTT TAATAAATCTG G	ND	SSR	Bogale *et al.* (2005)
23SMB9 F/MB9R	TTCGCCTTTCC CTCACGGTACT TGGCTGGGAT ACTGTGTAATT G/TTAGCTTCA GAGCCCTTTG G	ND	SSR	Bogale *et al.* (2005)
23SMB10 F/CMB10R	TTCGCCTTTCC CTCACGGTACT TATCGAGTCCG GCTTCCAGAA/ TTGCAATTACC TCCGATACCAC	ND	SSR	Bogale *et al.* (2005)
FitsMB11 F/MB11R	ATATGCTTAAG TTCAGCGGGT GTGGACGAAC ACCTGCATC/A	ND	SSR	Bogale *e*

FitsMB13 F/MB13R	ATATGCTTAAG TTCAGCGGGT GGAGGATGAG CTCGATGAAG CTAAGCCTGCT ACACCCTCG	ND	SSR	Bogale *et al.* (2005)
FitsMB14 F/MB14R	ATATGCTTAAGT TCAGCGGGTCG TCTCTGAACCAC CTTCATC/TTCCT CCGTCCATCCTG AC	ND	SSR	Bogale *et al.* (2005)
FitsMB17 F/ MB17R	ATATGCTTAAG TTCAGCGGGT ACTGATTCACC GATCCTTGG/G CTGGCCTGACT TGTTATCG	ND	SSR	Bogale *et al.* (2005)
FitsMB14 F/MB14R	ATATGCTTAAG TTCAGCGGGT CGTCTCTGAAC CACCTTCATC/ TTCCTCCGTCC ATCCTGAC	ND	SSR	Bogale *et al.* (2005)
FitsMB17F/ MB17R	ATATGCTTAAG TTCAGCGGGT ACTGATTCACC GATCCTTGG/G CTGGCCTGACT TGTTATCG	ND	SSR	Bogale *et al.* (2005)
F2	$(AG)_8$	50	ISSR	Haung, C-H. PhD. Thesis (2009)
F3	$(GTG)_5$	50	ISSR	Batista *et al*. (2008)
F5	$(CAG)_5$	54	ISSR	Bornet *et al.* (2004)
F7	$(ACC)_5$	54	ISSR	Chen *et al.* (2010)
F9	$(GA)_8C$	54	ISSR	Debnath *et al.* (2007)
F12	$(AG)_8G$	54	ISSR	Debnath *et al.* 2007
F13	$(CA)_8T$	54	ISSR	Sharma *et al.* 2009

ND = Not determined

Appendix 4A

ANOVA for pathogenicity test for direct application of spores (inoculation) on potted Ghana oil palm plants with three *Foe* isolates

Variate: Month of infection

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolates	2	0.0000	0.0000	0.00	1.000
Residual	15	4.5000	0.3000		
Total	17	4.5000			

Appendix 4B

ANOVA for pathogenicity test for two inoculation mechanisms on Malaysian oil palm with four *Foe* isolates

Variate: Month_of_infection

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolates	3	0.000	0.000	0.00	1.000
Residual	20	54.000	2.700		
Total	23	54.000			

Appendix 5

List of primers for LAMP assay

Genes	Primer	Sequence (3'-5')
SIX1	F3	TCGCATTGTCAACCTTGG
	B3	AGTAGACTTGTCACTGGTAGG
	FIP(F1c+F2)	AGTCGCTCGAAAGCCTGTGCCAATGACATGCTCCAGG
	BIP(B1c+B2)	GACTAGGGAACTCCAATGTGCGGCATTCCTGGTTCTGTGG
	LoopF	TTCAACGCAGAGACCATGAA
	LoopB	GCAGGCTCAATGACCAGA
SIX8	F3	CGTACCACATGTGAGAGC
	B3	CCTTGTCGAACGGTAAGTAAT
	FIP(F1c+F2)	GGTGTGGCGTTTCAGGCATACCATCCTCAACGTAAGAGTG
	BIP(B1c+B2)	CACCTGTTATGCAGGCGAGTAATTGACGCTGCTTCTAATGTAA
	LoopF	ACAGGATGCTATCTCACTGC
	LoopB	CACTGGTGTCGCTGTGAT
SIX10	F3	GCCATTCTCTGGATCTGTG
	B3	GACCATGAGGTGAATAGTCG
	FIP(F1c+F2)	TCCTCTGAAAGCGTTGTAACCGCCACTTGTTCGTTCAGGTT
	BIP(B1c+B2)	CAGTACTCCGCATCGACTCGGTAGATTGGTCTGCCGTTC
	LoopF	GATCCGAGATCGAAGATCCTG
	LoopB	TTGGTGTCTACGTTGATACTGG
SIX13	F3	GCCTTCAACGAAGAGGAG
	B3	ATCGTTGTGCGTTTGAGT
	FIP(F1c+F2)	CGTCGTCTTGACGCTTCTGTGTAGAGCGAACAGAGCATAC
	BIP(B1c+B2)	CACCCTAACGGACCTTGCCCGTTGCATGAACTATCTTCATC
	LoopF	CTGCTGCTTCCGAGACTAG
	LoopB	GGGAGGACGTCTTTATGTTGAT
P450	F3	TGAACAATACATTTCCACCGA
	B3	CTCACTCTCCTCACTATCGT
	FIP(F1c+F2)	CAGATCCAGAGCGAAGCGAGGGTTGCGATGAGGCTAT
	BIP(B1c+B2)	GTCAGATGAGTGTGAACGCCTTTAATATCCGCCTTCAGCCTA
	LoopF	ATCTCTTTGGGAGTCATAACGG
	LoopB	TACCATAGTTGATCGCCGTG

Appendix 6

ANOVA for the percentage inhibition (PI) of *Trichoderma* strains over *Foe*

Variate PI

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolates	11	14483.52	1316.68	13.52	<.001
Residual	24	2337.65	97.40		
Total	35	16821.17			

Appendix 7

ANOVA for Radial growth or diametre of mycelia of *Foe* in dual culture

Variate: Diametre

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Foe	15	224.7533	14.9836	72.07	<.001
Residual	32	6.6533	0.2079		
Total	47	231.4067			

Appendix 8

ANOVA for the effect of different levels of chemical (silicate pentahydrate) on the mycelia growth of *Fusarium oxysporum* f.sp. *elaeidis* (*Foe*)

Variate: Diametre

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
STRAIN	3	78.17855	26.05952	431.24	<.001
TREATMENT	3	387.53449	129.17816	2137.66	<.001
STRAIN.TREATMENT	9	106.10191	11.78910	195.09	<.001
Residual	48	2.90063	0.06043		
Total	63	574.71559			

Appendix 9

BK buffer S15 MOPS (pH 7.5) protocol

$Ca(NO_3)_2.4H_2O$ (MW 236)	30 mg/L (0.127 mM)
$MgSO_4.7H_2O$ (MW 246.5)	20 mg/L (0.081 mM)
KNO_3 (MW 101)	10 mg/L (0.1 mM)
Sucrose	15%
MOPS (MW209)	10 mM (pH 7.5)
Stored at -20 °C	

Appendix 10

ANOVA for Pollen germination percentage

Variate: %_Germination

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatments	6	1138.08	189.68	6.98	0.001
Residual	14	380.34	27.17		
Total	20	1518.42			