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Population Dynamics of *Fusarium oxysporum* and *Trichoderma* spp. in Malaysian and Ghanaian Soils

M Hefni Rusli*; A Seman Idris* and Richard M Cooper**

ABSTRACT

Fusarium oxysporum *f. sp.* elaeidis (Foe) *is a pathogenic fungus that causes vascular wilt in oil palm. The disease is prevalent in African oil palm plantations, causing devastating losses. Oil palm seeds are used in global breeding programmes, and have recently been shown to be highly contaminated with Foe, with 3% of all Foe-infected seedlings developing* Fusarium *wilt during growth. Malaysia is the second largest oil palm producer in the world, and palms here are also susceptible to the disease, with Foe posing a potential major threat to the industry. Malaysia and other countries in Southeast (SE) Asia have previously imported seeds from Africa, yet remain unaffected by* Foe. *This is thought to be caused by the presence of antagonists, such as species of* Trichoderma *or other micro-organisms that give the soil its* Foe*-suppressive qualities. This study looks into the levels of* Foe *and* Trichoderma *in the soils from Ghana and Malaysia. From colony counts, Malaysian soil samples were shown to have a lower number of* Fusarium *species per gram, with 25% of these isolates revealed to belong to the species* F. oxysporum. *These soils were also found to have a higher number of* Trichoderma *species per gram of soil. Both these results were significantly different from those on the Ghanian soils (P=0.0450 and P=0.0003, respectively), and may be the reason for the absence of the disease in Malaysia.* Trichoderma *isolates from Ghana were tested in dual culture experiments, and it was found that* Foe *colony growth was inhibited by 7% to 32%, although the colonies were not killed in the process and no inhibition zones were seen. This indicates the potential, though unlikely, suppression quality of* Trichoderma *against* Foe *isolates which can be tested in a field setting. A comparison with Malaysian* Trichoderma *isolates may reveal significant differences in inhibition strength that may indicate* Trichoderma *as the antagonist behind* Foe *inhibition in Malaysia. This would strengthen the hypothesis that* Trichoderma *has potential as an antagonist against* Fusarium *wilt. This, among other inhibition studies, may provide a suitable candidate as a biological control agent in Ghana and Malaysia to prevent and control this devastating disease of oil palm.*

ABSTRAK

Penyakit layu vaskular yang disebabkan oleh Fusarium oxysporum *f. sp.* elaeidis (Foe) *merupakan penyakit sawit yang sangat serius di Afrika. Walau bagaimanapun, penyakit ini tidak pernah dilaporkan di Asia Tenggara, walaupun pengimportan biji benih dari Afrika sering dilakukan untuk tujuan pembiakbakaan. Malaysia ialah pengeluar minyak sawit yang kedua terbesar di dunia, dan* Foe *merupakan satu ancaman utama pada industri ini. Berdasarkan kajian yang lepas, kehadiran agen kawalan biologi seperti* Trichoderma *yang berpotensi merencatkan infeksi* Foe *merupakan antara salah satu faktor yang mungkin menyebabkan ketiadaan penyakit layu vaskular sawit di Malaysia dan Asia Tenggara. Kajian ini dijalankan untuk mengenalpasti dinamik populasi* Foe *dan* Trichoderma *dalam tanah di Malaysia dan Ghana. Berdasarkan keputusan yang diperoleh, kiraan koloni dari sampel tanah Malaysia ditunjukkan mempunyai bilangan spesies* Fusarium *yang lebih rendah per gram, dengan 25% isolat terdiri daripada spesies* F. oxysporum, *dan lebih banyak spesies* Trichoderma *bagi setiap gram tanah. Kedua-dua keputusan ini berbeza dengan signifikan (P = 0.0450 dan P = 0.0003, masing-masing) dibandingkan dengan keputusan daripada sampel tanah Ghana, dan boleh menjadi penyebab ketidakhadiran penyakit layu vaskular di Malaysia. Kajian* in vitro *menunjukkan* Trichoderma *yang diisolat daripada tanah Ghana boleh merencatkan pertumbuhan* Foe *sebanyak 7%-32%. Ini menunjukkan bahawa* Trichoderma *mempunyai potensi sebagai agen kawalan biologi terhadap* Foe.

Keywords: *Fusarium oxysporum*, *Trichoderma*, oil palm.

INTRODUCTION

Oil palm belongs to the palm family, Arecaceae, and grows mostly in the tropics. Vascular wilt of oil palm is caused by *Fusarium oxysporum* Schlechtend: Fr. f. sp. *elaeidis* (Flood, 2006), which will hereafter be

* Malaysian Palm Oil Board, 6, Persiaran Institusi, Bandar Baru Bangi, 43000 Kajang, Selangor, Malaysia.
E-mail: mohd.hefni@mpob.gov.my

** Department of Biology and Biochemistry, University of Bath, Bath BA27AY, United Kingdom.

referred to as *Foe*. The primary host for the fungus is the African oil palm (*Elaeis guineenis*), but the fungus is also pathogenic to the South American oil palm (*E. oleifera*) when artificially inoculated (Flood, 2006; Renard *et al*., 1980). *Fusarium* wilt has become a particularly major problem in Africa, where palms are grown in plantations (rather than for domestic use), with the close contact of the palms aiding in the spread of the disease. The fungus does not require prior injury to the plant to infect it, and can invade either wounded or unwounded roots, growing up the vascular elements using microconidia (Corley and Tinker, 2003). It is then transported throughout the plant via the transpiration system.

The pathogen can spread from infected palms to surrounding healthy ones through the soil or via root contact with infected palm tissue. Thus, infection usually occurs in pairs or groups of palms, and this is the typical type of spread (Corley and Tinker, 2003; Renard and de Franqueville, 1989). Elongating roots most likely make contact with infected roots or debris that contain chlamydospores. Spore germination is then induced by root exudates, especially those from wounded roots, allowing for easier infection (Brown and Ogle, 1997). Oil palm seeds can also become infected, with around 50% found to be contaminated with *F. oxysporum*, while 3% of seedlings artificially infected with *Foe* will develop wilt (Flood *et al*., 1994), suggesting that seed transmission is a possibility and must be managed to limit the spread of the disease. Aerial transmission has also been suggested (Cooper and Rusli, 2014), and indeed plays a role in the dispersal of vascular wilts of other plant species.

This disease in oil palm was first described in the Democratic Republic of Congo (Wardlaw, 1946), and has since been reported in the Ivory Coast, Nigeria, Ghana and Cameroon (Flood, 2006), with outbreaks as far as Brazil and Ecuador (Renard and Franqueville, 1989; Van de Lande, 1984), showing the potential of the disease spreading trans-continentally. Therefore, despite the global shipment of palm oil seeds and their probable contamination, it is surprising that the disease has not yet been reported in SE Asia, as this is the area where most oil palm is grown. Many of the seeds are used in global breeding programmes (Flood *et al*., 1994), with countless quantities imported from Africa where the disease is prevalent. The climate of SE Asia is very similar to that of Africa, and should therefore be conducive to the disease. It has been found through artificial inoculation that palms grown in SE Asia are susceptible to *Foe* (Flood, 2006; Renard *et al*., 1980). Therefore, the absence of this disease is an anomaly, and believed to be due to the different soil properties of SE Asia and Africa.

Certain soil types are said to be '*Fusarium*-suppressive', meaning that even with a high population of infective *Fusarium* in the soil and the presence of susceptible hosts, the incidence of *Fusarium* wilt will be lower than in other soils. This is thought to be a result of other soil flora that are antagonistic towards the disease-causing fungus (Mace *et al*., 1981). Previous reports on suppressive soils have named the main contenders for suppression of *Fusarium* as *Trichoderma* and *Gliocladium* (Papavizas, 1985; Chet and Baker, 1980). *Trichoderma* is a genus of fungus containing 89 species, represented in soils and other organic matter collected at all latitudes (Samuels, 2006). Some species are more widespread than others, with *T. harzianum* being universal (Chaverri *et al*., 2003) and the most frequently found. Most *Trichoderma* strains, including the ones considered for biological (bio-) control purposes, have no sexual stage but instead produce only asexual spores. *Trichoderma* species readily colonise plant roots, and some strains are rhizosphere-competent, being able to grow on roots as they develop. Some *Trichoderma* have evolved numerous mechanisms enabling them to attack, parasitise and otherwise gain nutrition from other fungi (Harman *et al*., 2012). Different strains of *Trichoderma* control almost every pathogenic fungus for which control has been sought, including *Sclerotium cepivorum* (causing white rot of onion) and *Verticillium dahliae* (Abd-el Moity and Shatla, 1981; Chet and Baker, 1981; Jordon and Tarr, 1978). They achieve this by producing antibiotics or by being mycoparasitic although their effectiveness has been shown to vary (Harman *et al*., 2004). They also have mechanisms that can enhance plant and root growth [as have been reviewed by Harman *et al*. (2012)], and can have an important influence on the proteome and metabolism of plants. This is achieved either by inducing systemic resistance or through physiological changes resulting in plant defence mechanisms (Govindappa *et al*., 2010), with some strains being able to induce terpenoid phytoalexin defence compounds which strongly correlate with biocontrol (Howell *et al*., 2000). It has been shown that *Trichoderma* not only produces defence-inducing enzymes, but also triggers the production of these by the plant, including higher peroxidase and chitinase, while the concentration of phenols is also increased on occasion (Govindappa *et al*., 2010; Harman *et al*., 2004). Invasion by *Trichoderma* alone is able to elicit defence responses in the plant in this manner, and this response helps to suppress other infections.

Trichoderma spp. have also been shown to be antagonistic against *Fusarium* species, with *T. viride* being antagonistic towards *Fusarium* wilt of chrysanthemum (Papavizas *et al*., 1984), while isolates of *T. harzianum* have been shown to outcompete the *F. oxysporum* group of wilt-inducing pathogens for access to root exudates and cellulose (Lareen *et al*., 2016). Not only can other species such as *Trichoderma* be antagonistic to *Fusarium*, but

also the plants can be protected by non-pathogenic strains from the same genus (Flood, 2006). *Fusarium* wilt of banana was significantly reduced upon using non-pathogenic *Fusarium* strains in a greenhouse setting, although these strains were unable to protect the plants when field tested (Belgrove *et al*., 2011). Both non-pathogenic *F. oxysporum* (Fo47) and *Pseudomonas fluorescens* have been shown to suppress vascular wilts by either antagonising the *Fusarium* and/or inducing resistance in the plant (Duijff *et al*., 1998).

The aims of this study were to detect possible differences between soils from Africa and SE Asia, and relate these to the presence or absence of *Fusarium* wilt. Ghanaian soils were analysed to establish if they contained more *F. oxysporum* than Malaysian soils, and whether there was any evidence of Malaysian soils exhibiting greater suppression of *F. oxysporum* f. sp. *elaeidis* by the presence of *Trichoderma* species. This approach is facilitated by different selective media for *Fusarium* and *Trichoderma*, and may reveal the reason why *Fusarium* wilt of oil palm has not yet spread to SE Asia.

MATERIALS AND METHODS

Fungal Strains

The four single-spore isolates used in the study are listed in *Table 1*. They were stored at -20°C and used as PCR templates as positive or negative controls of *F. oxysporum*.

Soil Samples

Soil samples were taken from plantations in both Ghana and Malaysia as shown in *Table 2*. The Ghanaian plantation soil samples were collected from areas containing palms infected with *Foe*, while the Malaysian plantation soil samples were taken from areas that contained palms derived from seeds previously imported from Africa but had remained uninfected, making them good candidates for being *Fusarium*-suppressive.

TABLE 2. LIST OF PLANTATIONS SUPPLYING SOIL SAMPLES.

Plantation	Code	Source
Benso Oil Palm Plantation	BOPP	Benso, Ghana
Twifo Oil Palm Plantation	TOPP	Twifo Praso, Ghana
Ghana Oil Palm Development Corporation	GOPDC	Kwaebibirem, Ghana
Norwegian Palm Ghana Limited	NORPALM	Prestea, Ghana
Felda Holdings Bhd	FELDA	Jengka, Pahang, Malaysia
Malaysian Oil Palm Board	MPOB/ BANGI	Bangi, Selangor, Malaysia

Note: The six plantations represented plantations from both ghana and malaysia, and are referred to by their code names from here on.

Media and soil dilution plating

In order to analyse the differences in the soil properties between samples from SE Asia and Africa, soil samples from infected plantations in Ghana, and uninfected plantations from Malaysia were used as representatives. Soil samples were cultured onto *Fusarium*-selective medium (FSM) and *Trichoderma*-selective medium (TSM) in order to determine the numbers of viable spores. Subcultures of both sets of isolates were grown onto Potato dextrose agar (PDA). All cultures were incubated at 25°C for up to a week. Isolates were identified by the colour, size and shape of their colonies, and the colony forming units (CFU) of *Fusarium* and *Trichoderma* were determined by counting the number of colonies on the spread plates and converting them into CFU per gram of soil (CFU g^{-1}).

These were compared to see if they provided an explanation for the presence of *Foe* in Ghana

TABLE 1. LIST OF *Fusarium* spp. AND SELECTED ISOLATES ISOLATES OF *F. oxysporum* SPECIES WILL GIVE A POSITIVE RESULT IN PCR USING *F. oxysporum*-SPECIFIC PRIMERS

Genus	Species	f. sp	Code	Origin
Fusarium	*oxysporum*	*elaeidis*	*Foe* 16F	Ivory coast
Fusarium	*oxysporum*	*elaeidis*	Y1 original	Was 4A093, Yaligimba, Zaire
Fusarium	*oxysporum*	*lycopersici*	*For* LA	Tomato
Sclerotinia	*sclerotiorum*	-	Isolate L3	Lettuce (W Sussex), University of Warwick

and its absence in Malaysia. *Fusarium* colonies were analysed using a *F. oxysporum* DNA probe to examine the proportion of *F. oxysporum* isolates, the species that the *Fusarium isolates* belong to.

Isolates of *Trichoderma* were tested as potential antagonists, with isolates from Ghanaian soils used in dual culture experiments to test for inhibitory effects they may have against *Foe.* Similar experiments on Malaysian soils would have been more insightful, both alone and as a comparison, but were not done due to time constraints.

Liquid Cultures for DNA Extraction

A cork borer was used to add four mycelium discs (5 mm) of each *Fusarium* isolate subculture to conical flasks of 100 ml FSM liquid medium, and the cultures incubated overnight at 25°C and at 180 rpm. Alternatively, a colony (or scraping of mycelia) was initially added to 10 μl of water, and then heated to 98°C for 10-15 min, before being utilised as a PCR template.

DNA Analysis

The *Fusarium* cultures were identified using PCR reactions, utilising DNA probes specific for *F. oxysporum.*

Dual Culture Experiments

Inhibition by *Trichoderma* isolates against a selected *F. oxysporum* f. sp. *elaeidis* isolate (16F) of known pathogenicity was assessed in dual cultures on PDA. The different timing of inoculations was based on the relative growth rates of *Foe* and *Trichoderma* isolates. Percentage inhibition of colony growth was calculated using colony diameter [adapted from Etebarian *et al*. (2005)], using the formula (a –b)/a*100, where a is the diameter of the uninhibited colony, and b is the diameter under *Trichoderma* inhibition. Diameters were measured to the nearest mm, and calculated using the mean of 3 random diameters (Etebarian *et al*., 2005). *Foe* 16F with no antagonist was used as a control, and used to represent 100% growth.

In order to determine whether the antagonist was able to overgrow or parasitise *Foe*, mycelia were taken at the point of interaction and placed on FSM to be observed after seven days.

Statistical Analysis of Data

A normality test was performed, and revealed the data to be non-normally distributed. The non-parametric Mann-Whitney U test was conducted to analyse the difference between the *Fusarium* and *Trichoderma* CFU counts from soils of the two countries. Statistical analysis was carried out using the software Minitab 16.0.

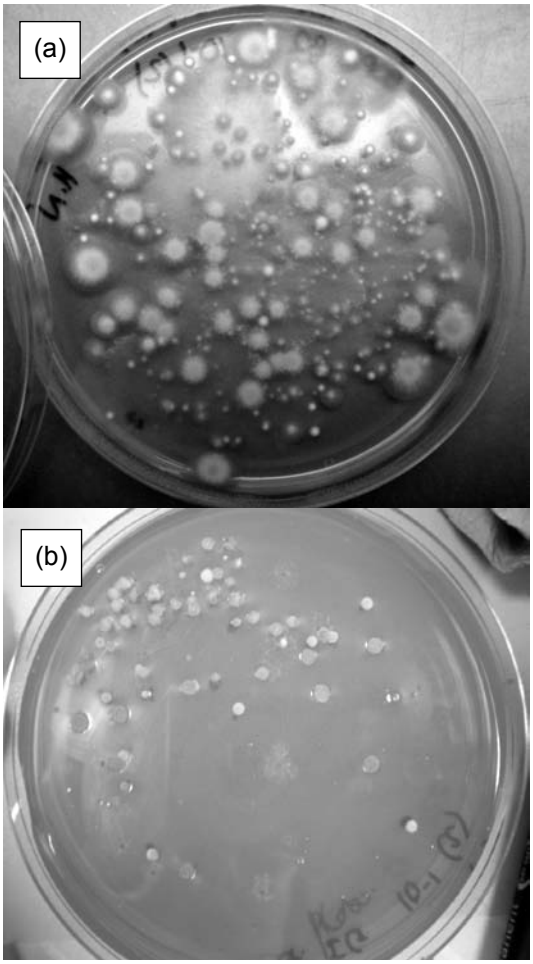

Figure 1. Representative plates with spread soil. (a) Fusarium *colonies on FSM spread plate, (b)* Trichoderma *colonies on TSM spread plate. Plates had been incubated at 25°C for up to one week.*

RESULTS

Typical plates where soil samples from Ghana and Malaysia had been spread onto selective media (FSM and TSM) are shown in *Figure 1*. The number of colonies present were counted and then converted into CFU g^{-1}.

Comparative Analysis of *Fusarium* Species in Malaysian and Ghanaian Plantation Soils

CFU g^{-1} counts are depicted as scatter plots, first, for each plantation (*Figure 2*), and then also for each country (*Figure 3*) as a general comparison of the soil property differences.

Counts from the soil samples showed that all the samples from the Ghanaian plantations had similarities, despite a wide range in CFU g^{-1} counts. They tended to have the majority of their counts

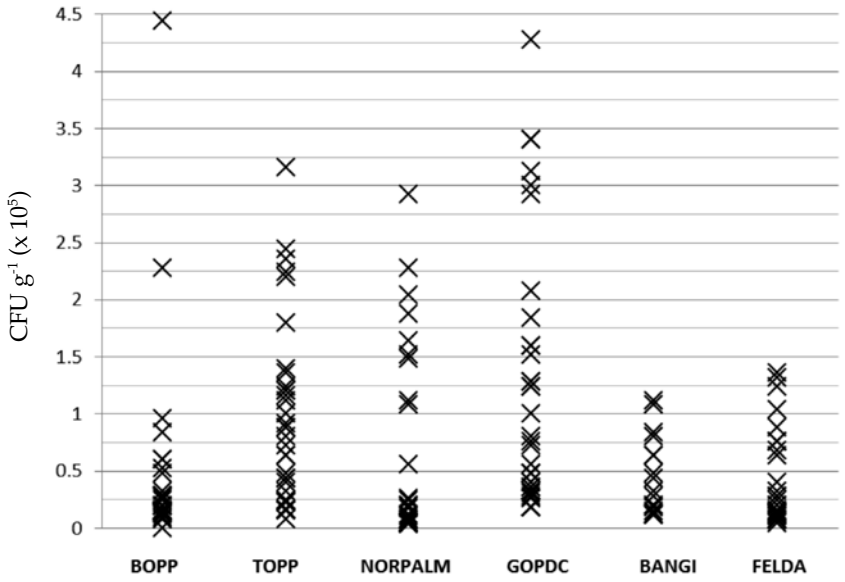

Figure 2. Fusarium *colony counts from each plantation. Counts are represented as CFU g^{-1} and are shown in thousands, with every replicate within the counting range used (2-60 colonies per plate). BOPP to GOPDC were samples from plantations in Ghana, and BANGI and FELDA were samples from plantations in Malaysia.*

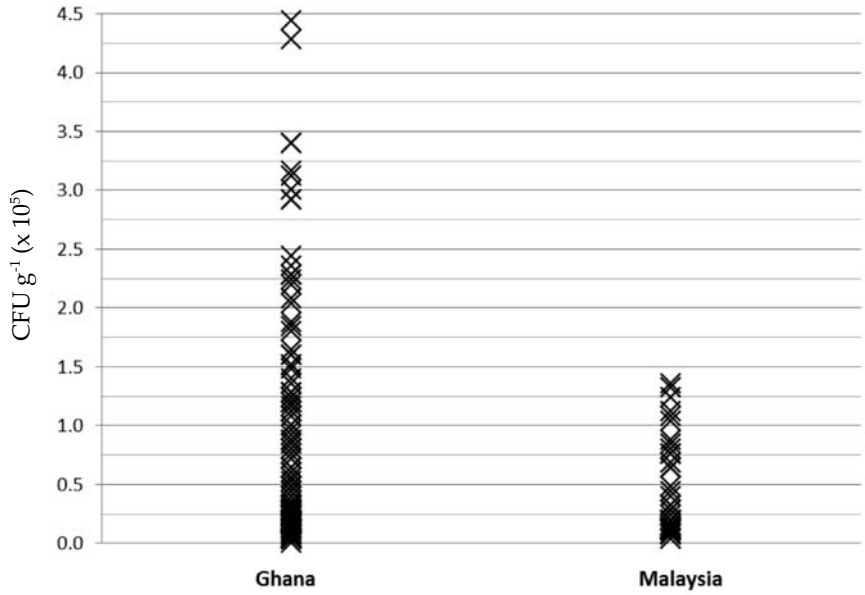

Figure 3. Fusarium *colony counts from Ghanaian and Malaysian soils.*

below 2 x 10^5 CFU g^{-1}, with each plantation having an even scattering of higher counts, some of which ranged up to 4.5 x 10^5 CFU g^{-1}. Soils from the two Malaysian plantations showed similarities to one another, with a smaller distribution of counts that were at the lower end of the Ghanaian sample range, and with all counts below 1.5 x 10^5 CFU g^{-1}. The similarity of the samples from plantations in the same country shows that the counts were reliable representations, and that the results were not skewed by any sample count from a particular plantation.

Counts are represented as CFU g^{-1} and shown in thousands, with every replicate within the counting range used (2-60 colonies per plate). The Ghanaian sample counts were from BOPP, TOPP, NORPALM and GOPDC plantations, and the Malaysian sample counts were from BANGI and FELDA plantations.

Similarly, *Figure 3* shows that the samples from Malaysian plantations had a narrower range of colony counts, with lower values (all under 1.5 x 10^5 CFU g^{-1}). It also shows clearly that Ghanaian soil samples as a whole had a much wider spread of

counts, with higher CFU g^{-1} values and no obvious anomalies. These differences were shown to be significant (P= 0.0450), with Ghanaian soil samples having a significantly higher number of *Fusarium* CFU per gram of soil.

The Mann-Whitney-U test showed that the counts on the soils between the two countries were statistically different (P=0.0450), indicating that the soils from Ghana had a significantly higher population of *Fusarium* colonies than the soils from Malaysia.

Comparative Analysis of *Trichoderma* Species in Malaysian and Ghanaian Plantation Soils

The same method was undertaken with the soil samples but diluted and plated onto TSM instead to identify the number of *Trichoderma* colonies present, as these could be potential antagonists to *Fusarium* species. The CFU g^{-1} counts are depicted as scatter plots, first for each plantation (*Figure 4*), and then for each country (*Figure 5*) for general comparisons.

Counts are represented as CFU g^{-1} and are shown in thousands, with every replicate within the counting range used (2-60 colonies per plate). BOPP to GOPDC were samples from plantations in Ghana, and BANGI and FELDA were samples from plantations in Malaysia.

Trichoderma counts showed a similar trend across all plantations, with the majority of the counts being below 5 x 10^4 CFU g^{-1}. Samples from the Ghanaian plantations had this majority of low *Trichoderma* counts, with most of these samples displaying a few higher counts which were evenly distributed, with no obvious anomalies. Samples from the plantations from Malaysia had a similar pattern to the Ghanaian soil counts, but with fewer samples having 5 x 10^4 or higher counts. The samples from BANGI, however, had potential anomalies that could skew the results, with counts of nearly 3 x 10^5 CFU g^{-1}. These three samples are three dilutions from a single soil sample.

Counts are shown in thousands, with every replicate within the counting range used (2-60 colonies per plate). The Ghana sample counts were from BOPP, TOPP, NORPALM and GOPDC plantations, and the Malaysian sample counts were from BANGI and FELDA plantations.

Overall, soils from both countries had the majority of their samples displaying low *Trichoderma* colony counts, with the few higher counts evenly distributed for most of the plantations. The Ghanaian soils had fewer counts above 5 x 10^4, but this was likely due to the larger number of samples used. The Malaysian soils had potential anomalous results, although this was harder to tell with the smaller number of samples collected. However, the difference in *Trichoderma* colony counts between the soils from the two countries was shown to be statistically significant (p=0.0003, *Table 5*), with

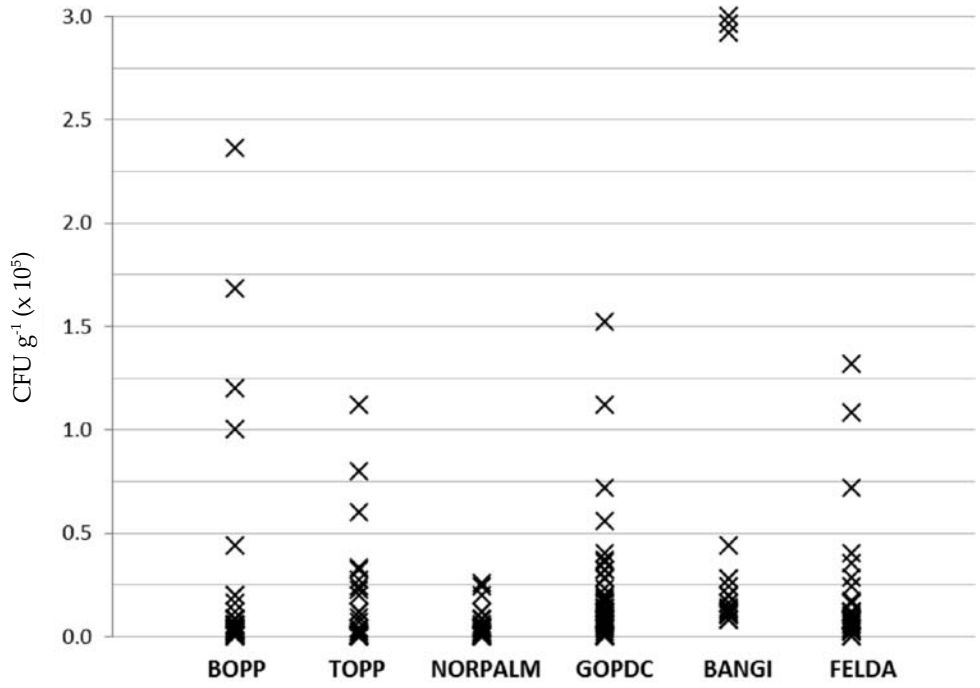

Figure 4. Trichoderma *colony counts from each plantation.*

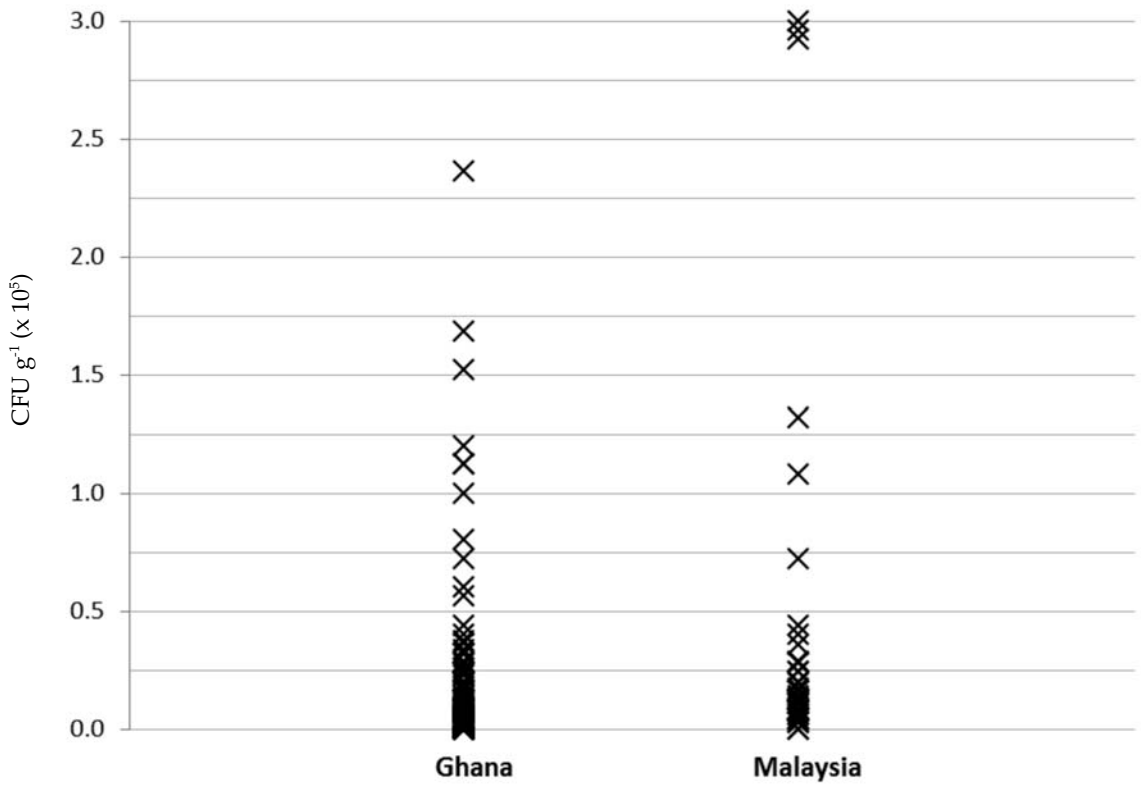

Figure 5. Trichoderma *colony counts from Ghanaian and Malaysian soils.*

samples from Malaysia having higher *Trichoderma* counts. As mentioned previously, the three high colony counts from Malaysia could be skewing these results, as all three were replicates from the same soil sample. However, without including these data, the difference was still seen to be significant as the median was not altered much by their removal. The Mann-Whitney-U test showed that the two sets of *Trichoderma* counts in the soils from Ghana and Malaysia were statistically different (P= 0.0003), revealing that Malaysian soils had a significantly higher population of *Trichoderma* colonies than the Ghanaian soils.

PCR using Specific Probes to Identify *F. oxysporum* Colonies

Colonies from spread plates were taken at random (one isolate from each soil sample) and subcultured onto PDA plates. These were used to make liquid cultures of the isolates so that they could be used as DNA templates in PCR and tested with a *F. oxysporum*-specific DNA probe.

Representative PCR images are shown *in Figures 6* and *7*, with bands showing a positive result for the presence of *F. oxysporum*. Of the 40 colonies that were subcultured, 32 were identified by morphology as *Fusarium* isolates. Of these 32, eight were shown by PCR (*Figures 6* and *7*) to be *F. oxysporum* using *F. oxysporum*-specific probes, giving a proportion of 25%.

Positive bands from *Figures 6* and *7* indicate the isolates that tested positive for *F. oxysporum*, with the absence of a band indicating a negative result. These were used to calculate the proportion of the *Fusarium* colonies picked from the FSM plates that were of the species *F. oxysporum* (*Table 3*).

Examination of *Trichoderma* Isolates for Antagonism towards *F. oxysporum* f. sp. *elaeidis*

Trichoderma isolates from Ghana were tested for their antagonistic ability against an isolate of *Foe* (*Foe 16F*). Fourteen isolates of *Trichoderma* were used in dual culture experiments and any antagonistic effects were noted after 8 days as shown in *Table 3*. Percentages were worked out using colony diameter (a mean of 3 measurements).

To determine the specific effect of the *Trichoderma*, mycelia were taken from the edge of the *Foe* isolate where it met the *Trichoderma* isolate and were subcultured onto FSM plates to establish whether the *Trichoderma* had fungicidal or fungistatic properties. All subcultures grew normally, showing that the fungus had not been killed during its interaction with *Trichoderma*. A representative photograph of a typical dual culture plate is shown in *Figure 8*.

Foe 16F is situated on the left side of the plate, with a *Trichoderma* isolate taken from GOPDC S20

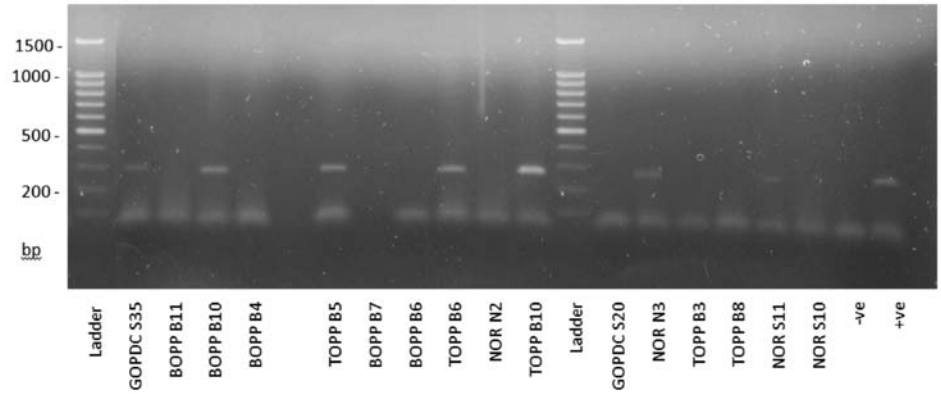

Figure 6. PCR amplifications using F. oxysporum*-specific primers. Strong positive bands at 280 bp are seen in six of the samples, with a faint band also present in the NOR S11 sample. The negative control was the* S. sclerotiorum *isolate L3 and the positive control was* Foe *16F. These were run alongside a 100 bp molecular DNA ladder for comparison.*

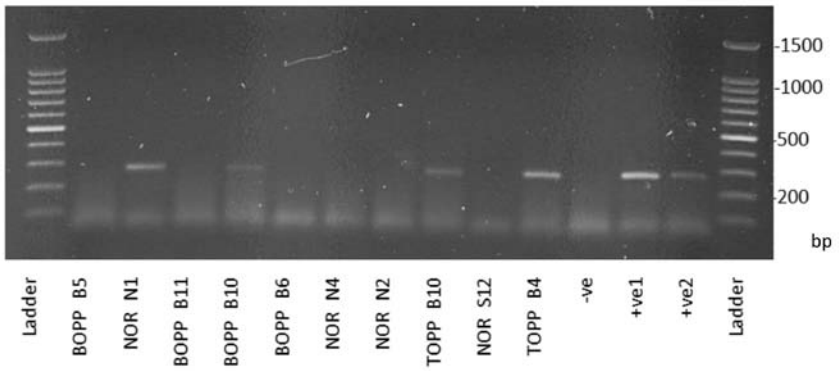

Figure 7. PCR amplifications using F. oxysporum*-specific primers. Strong positive bands of 280 bp are seen in four of the samples. The negative control was the* S. sclerotiorum *isolate L3 and the positive controls were for LA (+ve1) and* Foe *16F (+ve2). These were run alongside a 100 bp molecular DNA ladder for comparison.*

TABLE 3. GROWTH INHIBITION OF *Foe* 16F BY *Trichoderma* ISOLATES FROM DIFFERENT SOIL SAMPLES ON DUAL CULTURE PLATES

***Trichoderma* isolate (soil sample)**	***Foe* mean diameter (cm)**	***Foe* growth inhibition (%)**	**Rank ***
BOPP B2	8.40	6.7	13
BOPP B4	6.70	25.6	9
BOPP B8	6.10	32.2	1
BOPP B9	6.17	31.4	8
BOPP B10	6.26	30.4	4
BOPP B11	6.10	32.2	1
TOPP B3	6.63	26.3	3
TOPP B6	7.27	19.2	10
TOPP B9	6.30	30.0	5
NORPALM N1	6.40	28.9	6
GOPDC IB	8.39	6.8	14
GOPDC IJ	7.47	17.0	11
GOPDC S20	6.40	28.9	6
GOPDC S34	8.17	9.2	12
Control (no antagonist)	9	0	0

Note: Diameters were the means of 3 measured diameters, and rounded off to 2 decimal places.
*1 = most inhibited, 14 = least inhibited

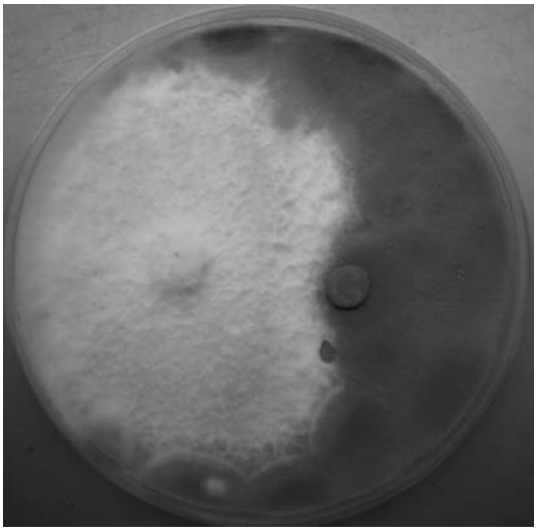

Figure 8. A typical dual culture plate.

soil on the right. Mycelial discs were spaced 3 cm apart, and inhibition was calculated using the diameter of the *Foe* colony compared to that of the uninhibited control.

Overall, the Ghanaian soil samples contained a higher *Fusarium* count (P=0.0450) and a lower *Trichoderma* count (P=0.0003) than the Malaysian samples. This may explain why Malaysia is still *Foe*-free. Of the 40 *Fusarium* colonies tested through PCR, 25% of them were revealed to be *F. oxysporum*. Through the dual cultures, *Foe* appeared to be slightly inhibited by the *Trichoderma* isolates (by 6.2 to 32.2%), but was not killed by any of them, as shown from the re-growth of the mycelia and the lack of inhibition zones.

DISCUSSION

The Presence of *Fusarium* Wilt of Oil Palm in Ghana but Not Malaysia

The Ghanaian soil samples were shown to contain a significantly higher number of *Fusarium* colonies per gram than the Malaysian (P= 0.0077), with median *Fusarium* counts of 4×10^4 and 2.8×10^4 CFU g^{-1}, respectively. Having less *Fusarium* (and potentially less *Foe*) in Malaysia could be a possible reason why the disease has not yet established itself here. This method is good for analysing the amount of *Fusarium* as a whole, but does not necessarily show the presence of *Foe*, and is therefore limited in its analysis. Therefore, this is no more than a small insight into the reason behind the disease levels rather than a conclusive answer. The counts (0 to 4.5×10^5 CFU g^{-1}) from our experiments, however, are similar to those of a previous study in which fungal counts ranged from 1×10^3 to 8×10^5 CFU g^{-1} (Magnoli *et al*., 1999). For further identification, 40 *Fusarium* colonies were picked at random from the Ghanaian spread plates, one from each of the soil samples. These were tested for *F. oxysporum* using species-specific primers, narrowing down the species classification of the colonies grown. It was found that at least 25% of the *Fusarium* colonies were *F. oxysporum*. This is limited, as the testing was not specifically for *Foe*; however, it was more accurate than using FSM plates, in which case the species was studied rather than just the genus. These colonies should be tested using the newly invented *Foe*-specific primers (Rusli, 2012) so that the proportion of *Foe* in the soil can be determined. This method should also be adopted for the *Fusarium* colonies isolated from Malaysia to provide a better comparison and a more accurate view of any difference between the amounts of *Foe* in soil samples from the two countries. If no *Foe* is present in Malaysia, then this will be the reason for the absence of *Fusarium* wilt of oil palm here, whereas if it is found in similar quantities to that of the soils from Ghana, then it can be deduced that either an antagonist or an inducer of plant resistance is present to prevent the disease from establishing.

Antagonistic Properties of the Soil May Suppress *Foe*

Inhibitory properties may also be present in the soil as many soils have previously been shown to contain suppressive qualities, including against *Fusarium* species (Alabouvette, 1986). Malaysia has many other diseases that infect oil palm which could also be potential suppressors of *Foe*, or inducers of plant resistance (Holliday, 1980). A possible candidate for this phenomenon could be members of the *Ganoderma* species. *G. boninense* causes basal stem rot (BSR) of oil palm, a very serious disease in Malaysia and Indonesia causing severe losses. It is also found in Ghana, but at lower infection rates. Thus, if it does have the ability to suppress *Fusarium*, this could help explain the difference in disease epidemiology of *Foe* between the two countries (Idris *et al*., 2004). Many other micro-organisms could also potentially be suppressive, competing for carbon, nitrogen and iron which is shown to be one mechanism for the biocontrol or suppression of *Fusarium* wilt in several systems, including by non-pathogenic *Fusarium* and *Trichoderma* species (Whipps, 2001). *Trichoderma viride* for example, has been shown to be effective against *Fusarium* wilt of chrysanthemum (Papavizas *et al*., 1984). *Trichoderma* is therefore a good potential candidate against *Foe* as many species have antagonistic properties against a wide range of pathogens, including *F. oxysporum* and other *Fusarium* wilts.

For the above reasons, *Trichoderma* was chosen as a likely potential antagonist for this study. Again, soil dilutions were plated out, but this time onto TSM. Malaysian soils were found to

have a significantly higher *Trichoderma* count than Ghanaian (P= 0.0001), with median counts of 12 000 and 6000, respectively. The higher numbers of this potential antagonist could be a reason why there is no *Fusarium* wilt of oil palm in Malaysia. There were potential anomalous results from a Malaysian soil sample that had high *Trichoderma* counts (ca. 30 000 CFU g^{-1}) which should be taken into account as they may have skewed the sample. However the Mann-Whitney U test provided a significant result even without including these results, as the median was not largely affected by their removal. The apparent anomalies could be due to the lower sample size that was taken from Malaysia (10 instead of 40); thus, taking more samples may have improved the results and demonstrated whether or not these replicates were truly representative.

Trichoderma isolates were tested individually on dual culture plates to observe whether they had any inhibitory properties against *Foe*. Traditional dual culture experiments take only one diameter into account when calculating the percentage of growth inhibition, which could be deemed to be unreliable due to the constraints of the petri dish. Thus, a more accurate method was followed, in which inhibition was calculated using three random diameter measurements (similar to the method of Etebarian *et al*., 2005). The different *Trichoderma* isolates had different growth rates so may have had an effect on the outcome. The results show that the *Foe* isolate could be inhibited up to 32.2% compared to the isolate with uninhibited growth, suggesting that *Trichoderma* species could potentially play a role in reducing the levels of *Foe*. However, this was subsequently shown to be unlikely because there was a lack of fungicidal properties as shown by the re-growth of subcultured *Foe* mycelia, and the absence of *Foe* colonies being over-run. These *Trichoderma* isolates were not expected to be overly antagonistic as they were isolated from Ghana where the disease is prevalent, and where the soil did not appear to be suppressive enough to prevent the disease. Instead, the inhibition of *Foe* growth in these experiments was more likely due to direct competition for nutrients with the *Trichoderma* isolate occupying otherwise available space, and *Foe* being unable to outcompete it. The absence of inhibition halos proved the absence of antibiosis. In some cases, *Foe* dominated the plate, covering around 95% of the surface area, and with one *Trichoderma* isolate being overgrown by *Foe*. These *Trichoderma* isolates are therefore unlikely to have an effect in the soil, but further tests could be undertaken on the palms to observe the inhibition of *Fusarium* in a field setting, at the same time studying population densities, and comparing them to isolates from Malaysia where the soils are more likely to be suppressive. If the Malaysian *Trichoderma* isolates have significantly stronger antagonistic effects against *Foe*, then this could be deduced as a possible reason for the absence of the vascular wilt disease in Malaysia. It would also be more beneficial to observe the dual culture plates daily to study how the antagonism reaction occurred over time.

CONCLUSION

The study found that soil samples from Ghana contained higher populations of *Fusarium* than Malaysian soil samples (P=0.0450), with 25% of these colonies revealed to be *F. oxysporum* species. This proportion of *Foe* isolates can be tested further in PCR with the *Foe*-specific primers (Rusli, 2012) to provide a better insight into the precise amounts of the disease-causing agent. The results also show that Ghanaian soils had lower populations of *Trichoderma* (P=0.0003) which is a potential antagonist of *Foe*, being able to inhibit *Foe* by up to 32%, but not kill it.

For a more accurate insight into the soil differences between the two countries, more soil samples are required to provide more precise *Fusarium* and *Trichoderma* counts, as well as more reliable comparisons of the soil properties. *Trichoderma* isolates from Malaysia should also be used in dual cultures to compare their results to those of the Ghanaian dual cultures, so as to determine the relative inhibitory strengths of the isolates from the two countries. Such an approach will better evaluate whether *Trichoderma* has potential as a biocontrol agent for the prevention of *Foe* infection of oil palm. Other species or combinations could also be tested to identify an agent or a mixture that could be used in Ghana and Malaysia to control and prevent this devastating disease that is jeopardising the oil palm industry.

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