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Evaluation of Malaysian soils for Potential Suppressiveness of Fusarium Wilt of Oil Palm Caused by *Fusarium oxysporum* f. sp. *elaeidis*

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Abstract

Vascular wilt disease caused by *Fusarium oxysporum* f. sp. *elaeidis* (*Foe*) causes a devastating disease of oil palm in West and Central Africa. However, this disease is a major threat to the Malaysian oil palm industry as it has not been reported in Southeast Asia, in spite of long term importation for breeding purposes of African seed and pollen, known to be often contaminated with *Foe*. This study shows that disease progress was substantially delayed/reduced in two non-autoclaved Malaysian soils compared to autoclaved Malaysian soils highlighting the possibility that *Foe*-suppressive soils in Malaysia might explain the non-appearance of this vascular disease there. This coincided with the limited presence of *Foe* in non-autoclaved soils compared to high presence of *Foe* in root (>145 cfu/g), bulb (>75 cfu/g), leaf 1 (>100 cfu/g) and leaf 3 (>60 cfu/g) of autoclaved soils. Population density of *Fusarium* spp. was also significantly greater in sterile soils compared to non-autoclaved soils. From this study, endophytes were also isolated from various parts of oil palm seedling inoculated with *Foe* and growing in Malaysian soils; showed degrees of antagonism towards *Foe* with 10 out of 15 isolates recording more than 50% inhibition.

Keywords: *Fusarium oxysporum;* Oil palm; Suppressive soil

Introduction

The oil palm has continued to gain in importance as a major crop in Malaysia during the last 20 years. Oil palm is a valuable economic crop and one of the most important oil tree species in tropical regions because of the high yield of raw materials it produces: palm oil and palm kernel oil. Fungal diseases of the oil palm can cause very serious losses in production of the CPO and crude palm kernel oil (CPKO). Because there are 25 years of productive life for oil palms, losses, especially if early on, come to several hundred thousand dollars per hectare [1]. The most important diseases are vascular wilt caused by *Fusarium oxysporum* f. sp. *elaeidis* (*Foe*) basal stem rot (*Ganoderma boninense*), red ring disease (*Rhadinaphelenchus cocophilus*), sudden wilt (*Phytomonas staheli*) and spear rot (unknown pathogen) [1,2]. Recently, the threat of bud rot disease caused by *Phytophthora palmivora* also has been documented in Latin America such as Colombia, Ecuador, Surinan and Brazil whereby this disease affecting the quality of the fresh fruit bunch [3]. In Malaysia, the most serious disease is BSR and it requires an urgent solution. Nevertheless, *F. oxysporum* f. sp. *elaeidis* is regarded as a major threat to the Malaysian oil palm industry, even though this disease has not yet been reported in Malaysia or in Southeast Asia [4].

It remains an anomaly that vascular wilt disease has not occurred or been reported in Malaysia. Previous study have reported contamination of oil palm pollen and seed by *F. oxysporum*, *F. solani* and several other fungi that are associated with oil palm diseases [5]. While, another previous study showed that oil palm progenies in Malaysia are highly susceptible to vascular wilt disease when artificially infected by *Foe*, with 75–90% of the palms infected [6]. 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 microflora that are antagonistic towards the disease-causing fungus [7] primarily Trichoderma and Gliocladium [8,9].

Previous report showed that isolates of *F. oxysporum* that were isolated from roots of healthy palms in Malaysia appear to be non-pathogenic [6]. Study also showed that inoculation of Malaysian non-pathogenic strains onto *Foe* inoculated seedling roots showed that infection by *Foe* was prevented by these non-pathogenic strains [10]. Thus, suggested competition between introduced pathogenic and native non-pathogenic isolates. Nevertheless, discovered that some strains caused mild symptoms in susceptible clones [11].

The application of endophytes as BCAs offers unique association with plants in a relatively uniform and protected environment when compared with the rhizosphere and rhizoplane, avoiding also the large scale soil applications with the possible adverse effects on the natural microbial community [12]. Nevertheless, the potential of endophytes as BCAs is still relatively unexplored. Most of the published studies refer to the use of endophytic bacteria, mainly belonging to the Pseudomonas and Bacillus genera, against different *ff.* spp. of *F. oxysporum*, also *V. dahliae*, *Rhizoctonia solani* and *Sclerotium rolfsii* [13-15]. One of the few studies about the application of an endophytic fungal BCA concern the use of *Verticillium albo-atrum* strain WCS850 against *Ophiostoma ulmi* infection in elm trees (*Ulmus americana* L.) [16]. This research was conducted to investigate the possible suppressive nature of Malaysian soils against *Foe* infection of oil palms and to isolate endophytes and test their possible antagonistic activities against *Foe*.

Materials and Methods

Collection and preparation of soils from Malaysian plantations

Soil samples were collected at three different locations in two

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Region	Location	Progeny Code	Origin	Date Planted	Soil type
MPOB UKM, Selangor, Malaysia	Bangi	D X D	Dumpy Avros D x D	1993	Munchong
Jengka, Pahang, Malaysia	Phase 6R PPPTR	BAK	Yangambi TxT	June 1973	Temang

Table 1: Locations and details of soil samplings in Peninsula Malaysia.

geographical regions in Malaysia 150 km distant. There were Bangi, Selangor in western Peninsular Malaysia and two locations in Tun Razak Agriculture Research Centre, Pahang in eastern Peninsular Malaysia. The origin and progenies and date of the oil palm planted in the area with soil series are indicated in Table 1. At each location, soil was taken within 2–3 m from each palm at a depth of 10-15 cm and later thoroughly mixed. Overall, five hundred kg of soil samples were collected from all locations and were shipped to the United Kingdom under strict FERA regulations.

Plant materials and maintenance

In Experiment 1, oil palm seedlings (standard oil palm genetic crosses, Serdang X La Me) were supplied by the Malaysian Palm Oil Board (MPOB), Malaysia and for Experiment 2, oil palm clones were supplied by Felda Agricultural Services Sdn. Bhd. from Malaysia. Once a month, palms were watered with liquid fertilizer (BHGS; 1 in 45 dilutions, containing N, P, K. in the ratio of 8:3:3 and trace elements). Compost pH ranged from 5.0 at the beginning of the experiment to 6.4 after 6 months. They were watered from below on alternate days.

Preparation of pathogen inoculum and standard inoculum procedure

Single spore isolates of *Foe* 16F was used in pathogenicity experiments. *Foe* 16F was previously used by Institut de Recherches pour les Huiles et Oléagineux (IRHO) as their Fusarium wilt screening isolate [17]. The pathogen inoculum was prepared according [18]. 10 mL of spore suspension was applied with a sterile syringe onto the soil surface around the base of each palm. The inoculum was then washed and watered in with sterile distilled water for 2 weeks. Un-inoculated plants served as controls. The palms were inoculated at 3 months age.

Suppressive soils inoculation studies

Experiments comprised of six treatments consisting of artificial inoculation with 10 mL of 3×10^6 spores/mL of *Foe* 16F into the soil mixture and double steam-sterilized (30 min at 121°C). *Foe* inoculated soil mixtures and mixed compost (Levingston F2+sand, Levingtons M2, Perlite in ratio 1:1:1) served as control. Un-inoculated mixed soils, double steam-sterilized mixed soils and mixed compost also served as control. Ten replicate of seedlings were used for each treatment.

Assessment of Disease Symptoms

Disease severity index

Symptoms were measured at 3 month intervals and wilt index adapted from Varghese and detailed by Rusli was used to score the disease severity in each treatment [6,18].

Plant height and dry weight

Plant height (cm) was measured from soil level to the apical, fully expanded leaf. The palms were washed twice with tap water before the aerial parts of plant dry weight (g) was determined following 72 h at 80°C in a drying oven to achieve constant weight.

Colonization of oil palm tissues

For qualitative re-isolation, fragments of plant materials (2.5 cm section of root, petiole or stem core sample) were surface sterilised in 2% (v/v) sodium hypochlorite for 10 min (5 min for tissue cores) before rinsing twice in sterile distilled water (SDW). The materials were then plated onto Fusarium Selective Medium [19] or Trichoderma Selective Medium [20] and incubated for 4 days at 28°C.

For quantitative re-isolation, 1 g of root, bulb or stem tissue was surface sterilised and washed as described above. The tissue was ground using a sterile pestle and mortar with 1 cm^3 of sterile sand and 9 ml of SDW. A 10-fold dilution series was prepared and 0.5 µl of the suspension was pipetted onto triplicate plates of FSM or TSM and spread with a disposable L-spreader. After incubation at 28°C for 4 d, colonies of *F. oxysporum* and Trichoderma were counted and the number of colony forming units (CFUs) per g fresh weight of palm tissue was calculated.

In vitro evaluation of antagonistic activity of the endophytes against *Foe* 16F

In this study, putative endophytes were carefully isolated from the roots and lower stems of healthy oil palms planted in two soil types from Malaysia as described in Table 1 and a control comprising compost only were used in this study. Segments of the root system from healthy plants were sterilized with 0.5% of sodium hypochlorite and isolated onto Potato Dextrose Agar (PDA), Czapek Dox Agar (CDA) and Luria Broth agar (LB). The endophytes isolated after one week of growth selected for *in vitro* evaluation based on their morphologically similarly to *Fusarium* spp. and *Trichoderma* spp. and through PCR identification (data not shown).

Results

The effect of Malaysian soils on Fusarium wilt development

In the first experiment using standard crossing seedlings, greater disease severity based on visual symptoms occurred in autoclaved soils and compost than in untreated soils (Figure 1). Disease severity for plants grown in autoclaved soils and compost progressed rapidly after 15 weeks p.i. in contrast to inoculated plants in Temang and Munchong

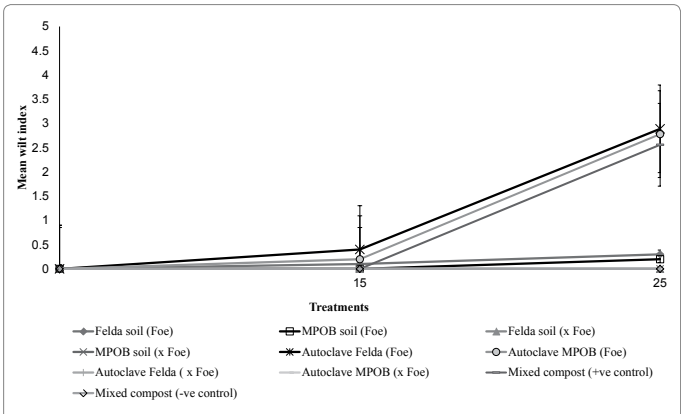

Figure 1: Wilt symptom development in oil palms inoculated with Foe 16F in sterile and non-sterile Malaysian soils.

*Different letters denote a significance ($p<0.05$) between soil treatments. This data was analyzed by Tukey HSD

soils, which showed less prominent symptoms and slower disease development. No symptoms occurred in control treatments.

However, experiment using clones grown in Temang non-autoclaved soil showed significant disease severity, thus contrasting with results showed in Experiment 1 whereby an average of 0.3 DSI recorded in non-autoclaved Temang soil compared to only Experiment 2 using clones with an average of 3 DSI. Nevertheless, less prominent symptoms were recorded in Munchong non-autoclaved soil where only 1.5 DSI was recorded. Table 2 also shows that there were significant disease incidences in autoclaved soil for Temang and Munchong soils.

Quantitative re-isolation of *Foe* from root, bulb, leaf 1, leaf 3 and leaf 7 at 25 weeks p.i.

Based on quantitative re-isolation from roots, *Foe* 16F was present abundantly in inoculated autoclaved Munchong soil, inoculated autoclaved Temang soils and inoculated mixed compost, with statistically similar quantities (Table 3). Reflecting the populations in soils as found in various treatments above, the results also showed the population density of *Foe* 16F in bulb, leaf 1, leaf 3 and leaf 7 in inoculated autoclaved soils were significantly higher than inoculated, non-autoclaved soils. One exception was in bulbs from inoculated autoclaved and non-autoclaved Munchong soils. It is evident that these population differences reflect the disease severity symptoms showed in Figure 1.

Table 4 also showed the present of *Foe* 16F in oil palm clones was significantly higher in inoculated autoclaved Munchong and Temang soils bulb, leaf 1, leaf 3 and leaf 7. The results also showed *Foe* 16F present abundantly in inoculated non-autoclaved Temang soils compared to inoculated non-autoclaved Munchong soil with population density of *Foe* 16F was recorded six times higher in inoculated Temang soil.

Population of *Fusarium* spp. in soils

After 25 weeks of inoculation, the population density of *Fusarium* spp. were significantly greater in sterile soils with 288.5 cfu/g isolated from autoclaved Munchong soil, with around half that population in autoclaved Temang soil (127 cfu/g) and mixed compost (128 cfu/g) (Table 5). The lowest density was found in Munchong soil inoculated with *Foe* 16F at 67 cfu/g and followed by the inoculated Temang soil at 99 cfu/g.

In oil palm clones, the population of *Fusarium* spp. were also recorded significantly greater in sterile soils with 216.4 cfu/g from autoclaved Munchong soil and autoclaved Temang soil at 98.5 cfu/g followed by mixed compost at 116 cfu/g (Table 6). Nevertheless, in resonance with the disease severity showed in inoculate non-autoclaved

Treatment	Autoclaved soil				Non-Autoclaved soil					
	Munchong		*Temang*		*Munchong*		*Temang*		*Mixed compost*	
	Foe	*X Foe*	*Foe*	*X Foe*	*Foe*	*X Foe*	*Foe*	*X Foe*	*Foe*	*X Foe*
Average DSI	4.6a	0d	3.1	0	1.5bc	0d	3	0	3.1b	0d

All data were the means obtained test carried out in duplicates and data analyzed by Tukey HSD. Different letters denote a significance ($p<0.05$) between treatments

Table 2: Disease severity incidence for wilt symptom development in oil palms inoculated with *Foe* 16F in sterile and non-sterile Malaysian soils.

Treatment	Root	Bulb	leaf 1	leaf 3	leaf 7
Temang soil (*Foe*)	40c	120b	560c	100d	2.5de
Temang soil (x *Foe*)	10d	5d	2f	0e	0e
Autoclaved Temang soil (*Foe*)	145ab	230a	1500a	338b	4.2d
Autoclaved Temang soil (x *Foe*)	5d	3d	0f	0e	0e
Munchong soil (*Foe*)	145ab	85c	83e	101d	12.5c
Munchong soil (x *Foe*)	3d	3d	0f	0e	0e
Autoclaved Munchong soil (*Foe*)	195a	75c	1000b	200c	25b
Autoclaved Munchong soil (x *Foe*)	0d	12d	0f	0e	0e
Mixed compost (x *Foe*)	0d	0d	0f	0e	0e
Mixed compost (*Foe*)	140b	230a	129d	821a	125a

CFU isolated from root, bulb, leaf 1, leaf 3 and leaf 7 wereanalyzed by Tukey HSD. Different letters in the lowercase denote a significance difference ($p \leq 0.05$) between the treatments (columns)

Table 3: Quantitative re-isolation of *Foe 16F* from root, bulb, leaf 1, leaf 3 and leaf 7 25 weeks post inoculations.

Treatment	Root	Bulb	leaf 1	leaf 3	leaf 7
Temang soil (*Foe*)	180a	150a	220a	110a	30b
Temang soil (x *Foe*)	5c	0d	0d	0d	0d
Autoclaved Temang soil (*Foe*)	165a	180a	180a	140a	8c
Autoclaved Temang soil (x *Foe*)	3bc	3cd	0d	0d	0d
Munchong soil (*Foe*)	30b	15c	20c	10c	5c
Munchong soil (x *Foe*)	15b	0d	0d	0d	0d
Autoclaved Munchong soil (*Foe*)	212a	90b	100b	61b	19b
Autoclaved Munchong soil (x *Foe*)	14b	0d	0d	0d	0d
Mixed compost (x *Foe*)	0c	0d	0d	0d	0d
Mixed compost (*Foe*)	149a	180a	116b	158a	117a

All data were the means obtained test carried out in duplicates and data analyzed by Tukey HSD. Different letters denote a significance ($p<0.05$) between treatments

Table 4: Quantitative re-isolation of *Foe 16F* from root, bulb, leaf 1, leaf 3 and leaf 7 25 weeks post inoculations.

Temang soil, the population of *Fusarium* spp. was recorded highly 130 cfu/g. Lower density of *Fusarium* spp. was found in Munchong soil inoculated at 63.4 cfu/g.

In vitro evaluation of antagonistic activity of the endophytes against *Foe* 16F

Antagonism was evident with most of the endophytes selected examined through the dual culture study where various reactions were recorded. Growth of *Foe* was inhibited by other *F. oxysporum* isolates (identified through *F. oxysporum* specific primers) and Trichoderma isolates and an inhibition zone was observed for one Trichoderma isolate (palm root 9) whereas contact between *Foe* and the other *F. oxysporum* and *Trichoderma* isolates occurred in all other cases. The growth inhibition of *Foe* isolate at 7 d after incubation (Table 7) revealed that a palm root 6 (*Trichoderma* sp.) caused greatest growth inhibition at >64% followed by palm stem 6 isolate with 60% inhibition. Other isolates in order of suppressiveness were from palm stem 16, palm root 4, palm root 7, palm root 8 and palm stem 17 with similar statistically similar levels of inhibition. *Foe* was least inhibited by an isolate from palm root [19] at 20% growth reduction.

Treatment	*Foe 16F* population in the soil (cfu/g)
Temang soil (*Foe*)	99c
Temang soil (x *Foe*)	34e
Autoclaved Temang soil (*Foe*)	127b
Autoclaved Temang soil (x *Foe*)	25.2ef
Munchong soil (*Foe*)	67d
Munchong soil (x *Foe*)	43.5e
Autoclaved Munchong soil (*Foe*)	288.5a
Autoclaved Munchong soil (x *Foe*)	17f
Mixed compost (*Foe*)	128b
Mixed compost (x *Foe*)	28.5f

Data of population of *Foe* 16F in soils were analyzed by Tukey HSD. Different letters in lowercase denote a significant difference ($p<0.05$) between the treatments. Initial inoculum was 3×10^6 spores/ml. *Foe*=Inoculated with Foe; x *Foe*=Not inoculated with *Foe*. *Mixed compost=mixture of* levingtons F2+sand, Levingtons M2, Perlite in ratio 1:1:1. Ten plants were used in every treatments

Table 5: Populations of Fusarium in soils after 25 weeks.

Treatment	Autoclaved soil				Non-Autoclaved soil					
	Munchong		*Temang*		*Munchong*		*Temang*		*Mixed compost*	
	Foe	*X Foe*	*Foe*	*X Foe*	*Foe*	*X Foe*	*Foe*	*X Foe*	*Foe*	*X Foe*
Average DSI	216.4a	14.2d	98.5	9.1	63.4c	22.8d	130	17.2	116b	19.5d

Data of population of *Foe* 16F in soils were analyzed by Tukey HSD. Different letters in lowercase denote a significant difference ($p<0.05$) between the treatments. Initial inoculum was 3×10^6 spores/ml. *Foe*=Inoculated with Foe; x *Foe*=Not inoculated with *Foe*. *Mixed compost=mixture of* levingtons F2+sand, Levingtons M2, Perlite in ratio 1:1:1. Ten plants were used in every treatments

Table 6: Populations of Fusarium in soils after 25 weeks

Endophytes samples		PIRG (mean)
Tomato roots 11	*Trichoderma* sp.	48.9^F
Palm root 9	*Trichoderma* sp.	40.0^G
Palm stem 3	*Fusarium* sp.	51.1^E
Palm root 4	*F. oxysporum*	53.3^D
Palm root 19	*Fusarium* sp.	20.0^J
Palm root 10	*Fusarium*	35.5^H
Palm root 7	*F. oxysporum*	53.3^D
Palm stem 23	*F. oxysporum*	51.1^E
Palm stem 15	*F. oxysporum*	55.6^C
Palm stem 13	*Fusarium* sp.	22.2^I
Palm stem 24	*Fusarium* sp.	51.1^E
Palm stem 6	*Trichoderma* sp.	60.0^B
Palm root 8	*Fusarium* sp.	53.3^D
Palm root 6	*Trichoderma* sp.	64.4^A
Palm stem 12	*Fusarium* sp.	53.3^D

All data were the means obtained from three sets of tests carried out in duplicates and data analyzed by Tukey HSD. Different letters in the uppercase denote a significance ($p<0.05$) between endophytes treatments. Each treatment represent by five plants. PIRG=Percentage Inhibition of Radial Growth

Table 7: Antifungal activity of 15 endophytes isolates against *Foe* 16F.

Discussion

Soils suppressive to diseases induced by many soilborne fungi, bacteria and nematodes such as root rot and wilt diseases induced by *Aphanomyces euteiches*, *Cylindrocladium* sp., *F. oxysporum*, *Gaeumannomyces graminis*, *Pythium* spp., *Phytophthora* spp., *Rhizoctonia solani*, *Ralstonia solanacearum*, *Streptomyces scabies, Verticillium dahliae* and *Thielaviopsis basicola* have been well documented [21,22]. Previous studies have repeatedly associated non-pathogenic *F. oxysporum* and fluorescent *Pseudomonas* spp. to be involved in suppression of fusarium wilts in naturally occurring, disease suppressive soils [23]. Therefore the explanation as to why Malaysia has not yet attained the disease is likely to revolve around the soil properties, in particular the microflora.

Competition for carbon between pathogenic and non-pathogenic *F. oxysporum* is one of the main modes of action of biocontrol as some non-pathogenic strains were more competitive for a carbon source than others [24]. Direct competition between two strains of *F. oxysporum* within the vessel of the host plant could also play roles in suppressive soil as non-pathogenic strains of *F. oxysporum* were able to reduce the colonization of the carnation *Dianthus caryophyllus* stem by the *F. oxysporum* f. sp. *dianthi*, resulting in a decrease in disease severity [25]. Moreover, microbes such as Mitsuaria and Burkholderia could also be reducing fungal and oomycete plant pathogen growth *in vitro* and reducing disease severity in infected tomato and soybean seedlings [26].

This study shows that greater disease severity occurred in autoclaved soils and compost than in non-autoclaved soils. This coincided with an approximate four-fold greater population density of *Foe* 16F in autoclaved MPOB soil than in MPOB non-autoclaved. Previous study has shown that high inoculum level of pathogen could led to an increase in the percentage of diseased plants in conducive soil [27]. This study also found out that the amount of *Foe* in root, bulb, leaf 1 and leaf 3 were also significantly higher in the autoclaved soils compared to the non-autoclaved soils. Previous study also isolated 26 colonies per g soil dry wt of mainly *F. oxysporum* from *F. oxysporum* f. sp. *apii* suppressive soil compared to 104 colonies in conducive soil [28]. Similar intereactions were reported by Flood et al. [11] and Rusli et al. [18]. On other note, high disease severity shown by clones in non-autoclaved Temang soil in Experiment 2 could be related to the low presence of putative endophytes in roots, lower stems and petioles of the seedlings.

These provide evidence to suggest microbial diversity in Malaysian soil might play some significant role in disease suppression of the vascular wilt disease. Microbiota in a "rich" soil has been reported generally to reduce the severity of attack by many soilborne plant pathogens [29].

There are certain non-pathogenic bacteria and fungi capable of living within plant tissues without causing harm, and these endophytes can sometimes result in beneficial effects to the host plant [30]. Therefore, isolation of endophytic microorganisms and screening for their biocontrol ability are a common strategy that relies on the available microbial biodiversity [31]. Beneficial effects against disease might result from direct antimicrobial activity of from indirect effects on host defences [32]. Competition for nutrients in soil is certainly one of the modes of action in many biocontrol agents such as *Trichoderma* spp. [33]. Recently, Trichoderma isolates have been reported as being able to act as endophytic plant symbionts [34]. Endophytes isolated from various parts of oil palm seedling inoculated with *Foe* and growing in Malaysian soils showed degrees of antagonism towards *Foe* with six isolates recording more than 50% inhibition. These endophytes were identified as *F. oxysporum* and *Trichoderma* spp. based on their morphological characteristic and molecular identification.

Malaysia has many other diseases that infect oil palm that could also be present as potential suppressors of *Foe*, or inducers of plant resistance [35]. An example of this 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 Africa, but in lower amounts, so if it did have the ability to suppress Fusarium, it could help explain the disease epidemiology of *Foe* between the two countries [36]. Nevertheless, the likelihood of Malaysia having Fusarium suppressive soils must be re-tested, as glasshouse trial could not prove this hypothesis. Results from these experiments could serve to (a) explain why *Foe* has not established in Malaysia, (b) provide the industry with confidence or conversely keep it on its guard in terms of the possibility of a future *Foe* problem, (c) suppressive soils may identify antagonists, competitors and/or endophytes which might be exploited in sustainable disease control strategies. However, this experiment has a flaw whereby there is no ideal control as the most suitable control would be to get some soil type from non- infested areas in Africa (for example Ghana) as wider soil surveys need to be done in order to obtain concrete results on the potential occurrence of Fusarium suppressive soil in Malaysia.

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