Journal of Oil Palm Research Vol. 31 (2) June 2019 p. 321-330
DOI: https://doi.org/10.21894/jopr.2019.0009

A TOOL FOR MONITORING *Trichoderma* AND *Fusarium oxysporum* f.sp. *elaeidis* OIL PALM INTERACTIONS, USING CONSTITUTIVE AND INDUCIBLE GREEN FLUORESCENT PROTEIN (GFP) AND RED FLUORESCENT PROTEIN (DsRED) REPORTER SYSTEM

RUSLI, M H*; IDRIS, A S* and COOPER, R M**

ABSTRACT

Vascular wilt disease of oil palm is cause by Fusarium oxysporum *f.sp* elaeidis. *The pathogen normally invades intact roots of palms or wounds and colonises the xylem vessels where it causes water stress, and hormonal imbalance result in severe yield loss and possible palm death. This study attempted to visualise the stages of colonisation and penetration into roots by* Fusarium oxysporum *f.sp* elaeidis *expressing Red Flourescent Protein (DsRed) in susceptible oil palm line and it's interactions with Trichoderma TPP4 expressing Green Fluorescent Protein (GFP). Trichoderma TPP4 and* Fusarium oxysporum *f.sp* elaeidis *were successfully transformed using* Agrobacterium tumefaciens*-mediated transformation with both GFP and DsRed respectively using vectors pCAMDsRed and pCAMBgfp whereby this is the first report that* Fusarium oxysporum *f.sp.* elaeidis *has been genetically modified. Analysis showed that early colonisation of Foe hyphae on the surface of secondary roots while colonisation by* Trichoderma *was observed at early stages after inoculation and became denser with time.* Trichoderma *TPP4 also was seen coiling around the* Foe *when inoculated together showing potential mycoparasitisic action.*

Keywords: *Fusarium oxysporum* f.sp. *elaeidis (Foe), Trichoderma,* Green Fluorescent Protein (GFP), Red Fluorescent Protein (RFP).

Date received: 21 May 2018**; Sent for revision:** 21 May 2018**; Received in final form:** 18 October 2018**; Accepted:** 12 March 2019.

INTRODUCTION

Vascular wilt disease of oil palm is caused by *Fusarium oxysporum* f.sp. *elaeidis* (*Foe*) (Wardlaw, 1946). *Fusarium* vascular wilt is the most important disease of oil palm, endemic in western and central Africa including the Ivory Coast, Ghana, Benin, Nigeria, Cameroon and Congo Democratic Republic (Turner, 1981; Corley and Tinker, 2003; Cooper and Rusli, 2014). However, it remains an anomaly that vascular wilt disease has not occurred or been reported in Malaysia despite reported contamination of oil palm pollen and seed by *F. oxysporum*, *F. solani* and several other fungi that are associated with oil palm diseases (Flood *et al*., 1990). This is thought to be a result of other soil microflora that are antagonistic towards the disease-causing fungus (Mace *et al*., 1981) primarily *Trichoderma* and *Gliocladium* (Chet and Baker, 1981; Papavizas, 1985).

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* 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 BA2 7AY, United Kingdom.

Trichoderma spp. have been shown to be antagonistic against *Fusarium* species, with *T. viride* suppressing *Fusarium* wilt of chrysanthemum (Papavizas *et al.,* 1984). Different strains of *Trichoderma* acting as biological control agents for *Sclerotium cepivorum* (white rot of onion) and *Verticillium dahliae* also have been reported in various studies (Abd-el Moity and Shatla, 1981; Chet and Baker, 1981; Jordon and Tarr, 1978). They achieved this by using antibiotics or through mycoparasitic mechanism, although their effectiveness has been shown to vary (Harman *et al*., 2004). *Trichoderma* species readily colonise plant roots and some strains are rhizosphere-competent, 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*., 2004).

Whereas, *Foe* colonises the xylem vessels where it can become systemically distributed to all parts of the palm by conidia carried in the transpiration stream (Corley and Tinker, 2003; Mepsted *et al*., 1995). Infection of the xylem and production of microbial polysaccharides, enzymatic breakdown of vessel walls and host occluding defence responses causes water stress, and hormonal imbalance result in severe yield loss and possible palm death (Cooper, 2011; Mepsted *et al*., 1995).

However, the details of the interactions between *Trichoderma* and *Foe*, in relation to antagonistic relationships, are difficult to study. Therefore, methods such as *Agrobacterium tumefaciens*-mediated transformation of fungal plant pathogens to express Green Fluorescent Protein (GFP) and other fluorescent proteins have been used extensively to track fungal infection of host plants (Bourett *et al*., 2002). Zhong *et al*. (2007) successfully transformed *Trichoderma reesei* with *Agrobacterium tumefaciens* as an efficient tools for random insertional mutagenesis while a GFP tagged *T. atroviride* has been generated and monitored for biocontrol activity against *Rhizoctonia solani*, on plant surfaces (Lu *et al*., 2004).

Several other fluorescent markers have been introduced for dual-labelling systems with GFP which could help to follow colonisation of host plants by several microorganisms at the same time. Red Flourescent Protein (DsRed) is a GFP homologue that naturally occurs in Discosoma reef coral gene (Wall *et al*., 2000). Three filamentous ascomycete species have been transformed with DsRed and pathogenic strain of *F. oxysporum* f. sp. *lycopersici* (Fol8) expressing the DsRed2 gene (red) has been used to study the interaction between a non-pathogenic strain and a pathogenic strain, inoculated onto tomato roots in soil (Rodrigues *et al*., 2001; Czymmek *et al*., 2002; Nahalkova and Fatehi, 2003).

Therefore, the objectives of this experiment were to find out the potential port of entry for *Foe* to invade roots and attempting to facilitate the observation of *Trichoderma* and *Foe* hyphal interactions on the root surface. In this study, a GFP-expressing strain of *Trichoderma* (TPP4) was used to visualise the stages of fungal colonisation and penetration into roots and the development of *Foe* isolate expressing DsRed fluorescent protein in a susceptible oil palm line.

MATERIALS AND METHODS

Fusarium oxysporum f.sp. *elaeidis* (*Foe*) 16F Protoplast Preparation

Fungal protoplasts were obtained following the protocol described by Thirugnanasambandam *et al*. (2011) and Khang *et al.* (2006), with some modifications. The 5x10^6 spores/ml were inoculated into 200 ml of Potato Dextrose Broth (PDB) medium. After 14-15 hr incubation at 28ºC with shaking at 250 rpm, germlings were harvested by filtration through a 60 μm nylon mesh (Millipore) and washed thoroughly but carefully with an MgP solution. A sterile spatula was used to transfer germlings from the filter to sterile 50 ml Falcon tubes, containing 20 ml of MgP with 0.5% (w/v) Glucanex® (Novozyme) as the protoplasting enzyme. Mycelia were incubated in the enzyme solution for 45 min at 30ºC with slow agitation (60 rpm) and observed every 20 min under a microscope for sufficient numbers of protoplasts at 10^8 ml^{-1}. The sample was then filtered through a double layer of nylon filter and washed with two volumes of STC solution (1.2 M sorbitol, 10 mM Tris-Cl, pH 7.5, 50 mM $CaCl_2$), collecting the flow-through containing the protoplasts in pre-chilled ice-cold 50 ml centrifuge tubes. Filtrates were centrifuged at 4°C and 1500 g for 15 min to collect protoplasts, which were carefully re-suspended in 1 ml STC and counted. The protoplast suspension was adjusted to a final concentration of 2 x 10^8 protoplasts/ml and divided into 100 µl aliquots in Eppendorf tubes. Protoplast were either used immediately for transformation (for highest efficiencies), or 10% of polyethylene glycol (PEG) (v/v) and 1% Dimethyl (oxido) sulphur (DMSO, Merck) (v/v) were added for long-term storage at -80ºC.

Protoplast Transformation and *Agrobacterium*-mediated Transformation of Mycelial Fragments

Transformation of *Foe* 16F was performed as described by Khang *et al.* (2006), with some modifications. An *Agrobacterium* strain AGL1 containing the plasmids pCAMBDsRed (for DsRed expression) as used in Eckert *et al*. (2005) were supplied by Adrian Newton from The James Hutton Institute, Dundee, United Kingdom. The plasmid was originally produced at the Rothamsted Research, Harpenden, UK. *Agrobacterium* was grown in 5 ml Minimal Medium (MM) containing kanamycin (50 µg ml^{-1}) for two days at 28°C. Then, the bacterial cells were harvested by centrifugation at 16 000 g for 2 min, re-suspended and grown in Induction Medium containing kanamycin (50 µg ml^{-1}) at 28°C until the OD 600 reached 0.15 after 6 hr incubation at 200 rpm.

Foe 16F protoplasts (100 µl) were mixed with 100 µl of *A. tumefaciens* culture and spread onto nitrocellulose membrane (Whatman Cat. # 7141 104; 47 mm diameter; 0.45 µm pore size) placed on the co-cultivation medium. This mixture (200 µl per plate) was plated on a 0.45 µm pore, 45 mm diameter nitrocellulose filter (Whatman, Hillsboro, OR) and placed on co-cultivation medium (same as IM except that it contains 5 mM glucose instead of 10 mM glucose) in the presence and absence of 200 µM acetosyringone (AS). Following incubation at 25°C for 2 days, the filter was transferred to MM containing hygromycin B (75 µg ml^{-1}) as a selection agent for transformants and cefotaxime (200 µM) to kill the *A. tumefaciens* cells. Individual transformants were transferred into CDA amended with hygromycin B (75 µg ml^{-1}). Incubation at 28ºC was prolonged 3-5 days until the transformed colonies became clearly visible.

Preparation of *Trichoderma* TPP4 Spores

Before *Agrobacterium* cells had grown, *T. harzianum* spores from one week old cultures were harvested with 5 ml sterile water on potato dextrose agar. Spore suspensions were diluted with MM medium to 10^5–10^6 spores/ml.

Trichoderma TPP4 Transformation

One hundred microlitres of diluted spores were mixed with 100 µl *Agrobacterium* cells (OD660=0.6-0.8), and then the mixture was spread evenly on MM medium (200 µmol litre^{-1} AS) plates, and incubated at 27°C for two days. After two days, M-100 medium (containing 200 µg ml^{-1} hygromycin and 300 µg ml^{-1} Cefotaxime) was re-plated on the MM plates, and putative transformants were visible after 5-7 days.

Confocal Microscopy

Hygromycin-resistant fungal colonies were initially viewed using a Zeiss LSM 510 Meta confocal system with an Axiovert 200M microscope. Images for GFP fluorescence were collected using 488 nm line from the Argon laser with a 505-530 nm Band Pass filter and for RFP fluorescence, the 543 nm line from the HeNe laser with a 560 nm Long Pass filter was used. Unless otherwise stated, images are presented as maximum intensity projections and were assembled and edited using Adobe Photoshop CS version 8.0. Confocal observations were made with a confocal microscope Zeiss LSM 510 Meta confocal system with an Axiovert 200 M microscope. An EC Plan Neofluar 20 x 0.5 objective was used for most of the images.

Inoculation of the Transformants onto Roots of Oil Palm Seedlings and Microscopic Analysis

Preparation of *Foe* transformants was based on standard preparation of pathogen inoculum (Rusli *et al*., 2015). Fifty ml of 3 x 10^6 spores/ml of *Foe* 16F suspension were sprayed thoroughly onto washed roots of up-rooted plants. This was followed by inoculation of 50 ml *Trichoderma* GFP suspension at 3 x 10^6 spores/ml, three days after *Foe* 16F inoculation. Controls comprised of *Foe* alone and *Trichoderma* TPP4 alone. Roots were kept moist by spraying with sterile distilled water and enclosing in polythene.

Observations were made at 72 hr, 144 hr, and 216 hr after *Foe* inoculation on the seedlings. Three types of roots (primary, secondary and tertiary) were identified based on Purvis (1957) and Jourdan (1997) and removed, rinsed in sterile distilled water to wash away from the soil. The whole root was cut into sections and placed directly on glass slides and observed under the microscope, and the most interesting areas were observed by confocal laser microscopy.

RESULTS AND DISCUSSION

Trichoderma-Foe Interactions on Roots: Confocal Microscopy of Fungi Expressing Two Fluorescent Proteins, GFP and RFP

Trichoderma TPP4 and *Foe* 16F were successfully transformed using *A. tumefaciens*-mediated transformation with both GFP (*Figure 1*) and DsRed (*Figure 2*) using vectors pCAMDsRed and pCAMBgfp against the wild type. This is the first report that *Foe* has been genetically modified.

Colonies of *Trichoderma* GFP and *Foe* 16F RFP expression were observed under the fluorescence microscope in order to determine the positive transformants that expressed the fluorescent proteins. The fluorescent mycelia were then further examined under the confocal microscope to determine the emission spectra of each transformed isolate compared to the level of auto-fluorescence from the wild-type isolates. No auto-fluorescence was observed for either transformant.

In order to test the stability of expression in co-transformants, they were sub-cultured successively on PDA for *Trichoderma* TPP4 and CDA for *Foe* 16F without selection pressure. After 10 transfers they exhibited stable expression at 488 nm for GFP and 543 nm for RFP.

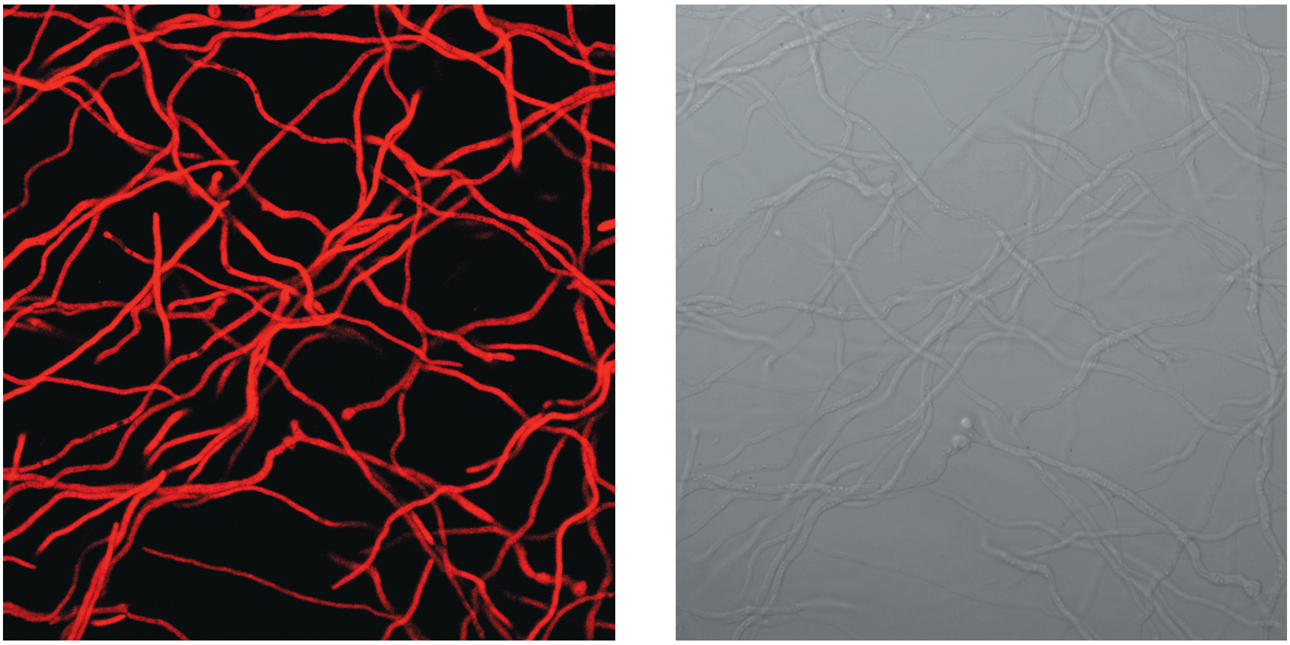

Figure 1. Fluorescence microscopy: Fusarium oxysporum *f.sp.* elaeidis (Foe) *16F Red Flourescent Protein (DsRed) visualised with tetramethylrhodamine isothiocyanate (TRITC) filter (left) compared with the* Foe *16F wild type (right).*

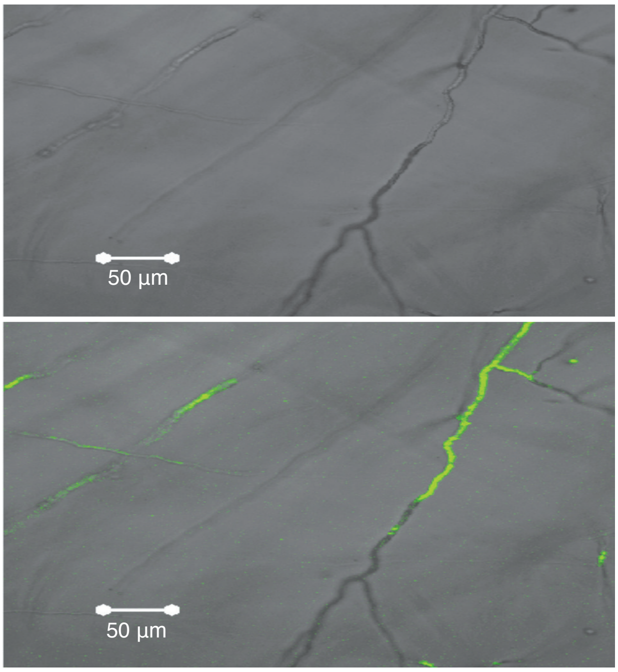

Figure 2. Transformant strain of Trichoderma *TPP4 showing the fungus constitutively expressing Green Fluorescent Protein (bottom) and wild type (top).*

In vivo Examination of Oil Palm-root Colonisation by *Trichoderma* TPP4 and *Foe* 16F Using Confocal Laser Scanning Microscopy (CLSM)

In view of the reported timing of invasion of roots by *F. oxysporum* ff. spp. and *Trichoderma* spp., observations were made at 72 hr, 144 hr and 216 hr after inoculations (Olivain *et al.*, 2006; Chacon *et al.*, 2007). Seventy-two hours after inoculation, 1 cm of secondary roots, tertiary and quaternary roots (Purvis, 1957; Jourdan, 1997) were observed. Several patches of *Foe* hyphae were observed colonising on the surface of secondary roots (*Figure* 3). The hyphae form a network growing along the borders between root epidermal cells and also across the cells. The colonisation patterns observed here are similar to the previous study whereby *F. oxyspourm* f.sp. *melonis* colonised the root in three days (Zvirin *et al.*, 2010). At this time, *Foe* was observed mostly at the base of the secondary roots.

However, no root penetration into the epidermal layers by *Foe* 16F was observed through Z-stack series of images at different root depths. Colonisation became more intense 144 hr after inoculation with extensive mycelial coverage on a pneumatode (*Figure* 4). However, there were occasional swollen hyphae representing possible penetration sides (*Figure* 5). Swollen hyphal structures were also observed during *Foe* colonisation of the root tip 144 hr post infection (h p.i.) (*Figure* 6). Lagopodi *et al.* (2002) also reported a direct pentration of epidermal cells by *F. oxysporum* f.sp. *radicis-lycopersici* hyphae that became swollen at the penetration site but there were no evidence of formation of appressorium-like structures during the penetration.

At 216 h p.i., *Foe* 16F produced thickened hyphae on the root surface and within cells of the oil palm root epidermis and cortex. *Foe* 16F forms a network of hyphae that grow and fill all the junctions of the epidermal cells and it was recorded that the development of this hyphal network is faster and richer at the secondary root region (*Figure* 7).

Foe 16F pattern suggests that the primary infection sites are at random positions on the root

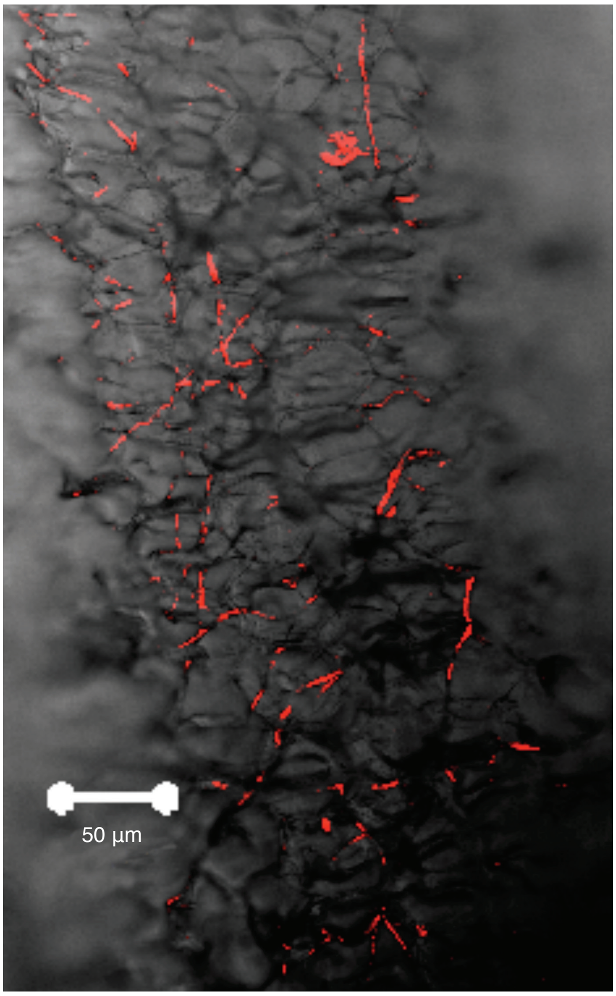

Figure 3. Colonisation of Fusarium oxysporum *f.sp.* elaeidis *on oil palm root surface 72 hr post infection (h p.i.).*

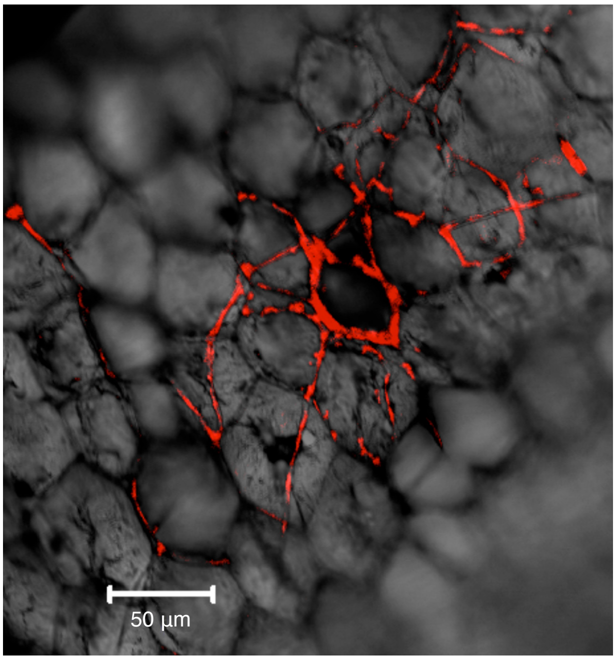

Figure 4. Extensive colonisation pattern of Fusarium oxysporum *f.sp.* elaeidis *expressing the Red Flourescent Protein 2 (DsRed2) gene (red) in palm root epidermis and cortex of newly formed root tissue 144 hr post infection (h p.i.).*

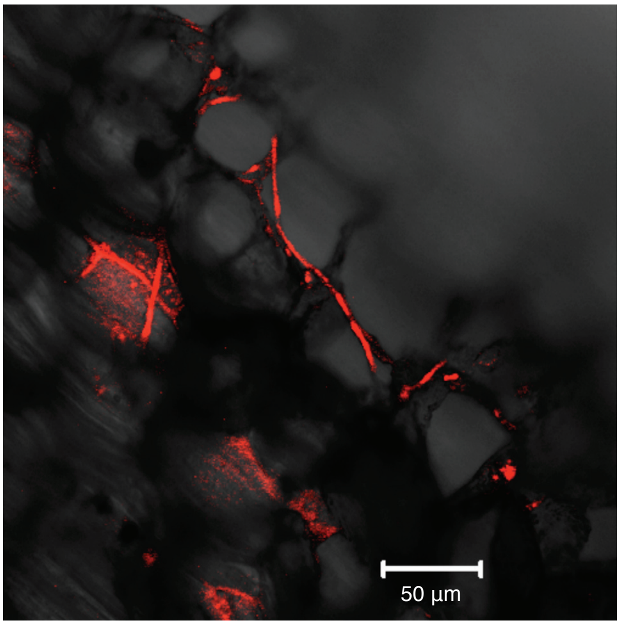

Figure 5. Hyphal growth of Fusarium oxysporum *f.sp.* elaeidis *16F in intercellular spaces along and across junctions of root epidermal cells 144 hr post infection (h p.i.).*

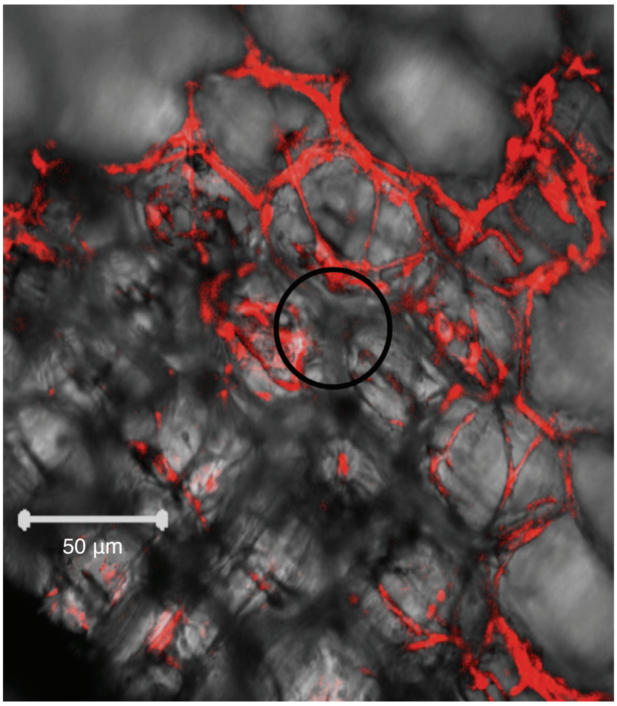

Figure 6. Swollen hyphae on the root tip surface of the oil palm root (circle).

(and not just from the tip of a secondary root or from the damaged cortical tissue since the fungus is probably able to penetrate the cells directly (Locke and Colhoun, 1973). The same infection pattern was also observed by Lagopodi *et al*. (2002).

Trichoderma TPP4 hyphae were evident colonising the secondary root surface and had established in between epidermal cell after 72 h p.i. (*Figure 8*). However, the fluorescence intensity of

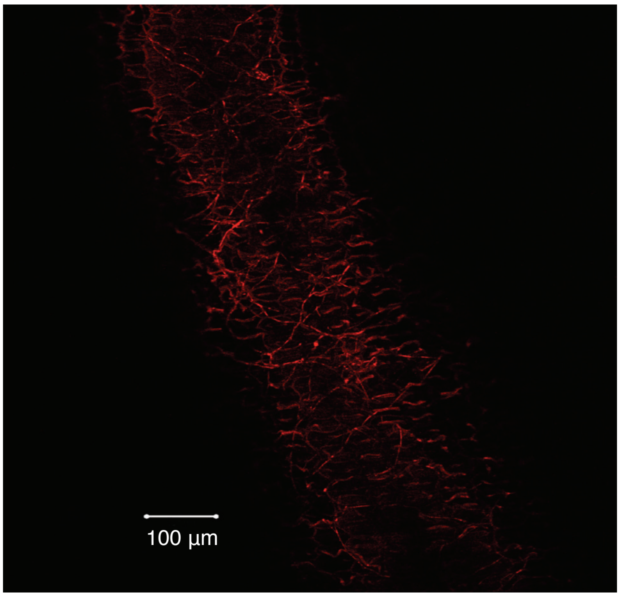

Figure 7. More extensive and thickened colonisation of F. oxysporum *f.sp.* elaeidis *was observed at 216 hr post infection (h p.i.).*

no disruption was observed to the host cell wall, in contrast to reports for various host-pathogen interactions (Roncero *et al*., 2003; Talbot, 2003).

Interactions between *Trichoderma* TPP4 and *Foe* 16F

T. harzianum possesses a recognition and response mechanism to competing fungi as it can sense the presence of a competing fungus by detecting the oligosaccharide products of the hydrolysis of cell-wall polymers (Mutawila *et al*., 2011; Harman *et al.*, 2004). In this study, *Trichoderma* TPP4 and *Foe* 16F were inoculated together onto roots in an attempt to unravel the interaction between this potential biocontrol agent and pathogen *in situ*. Both *Trichoderma* TPP4 and *Foe* 16F were observed colonising randomly in separate areas 72 h p.i. (data not shown). The same pattern occurred again at 144 hr on secondary, tertiary and quaternary roots. *Trichoderma* TPP4 was seen coiling around and attached together to *Foe* 16F mycelium on the oil palm root surface 216 h p.i. (*Figure 11*). *Foe* 16F mycelium was observed advancing along the borders between root epidermal cells. The *Trichoderma* TPP4 hyphae were more concentrated in regions of the root surface colonised by *Foe* 16F mycelium. This finding was supported by previous study whereby the inhibition of *Fusarium* wilt of tomato by *T. harzianum* where they showed lysis of pathogenic mycelium due to overgrowth and penetration by hyphal pegs and coiling produced by *T. harzianum*. Coiling of antagonistic hyphae around hyphae of *Fusarium* and lysis was also reported by many other workers (Elad *et al*., 1980; Kumar and Dubey, 2001).

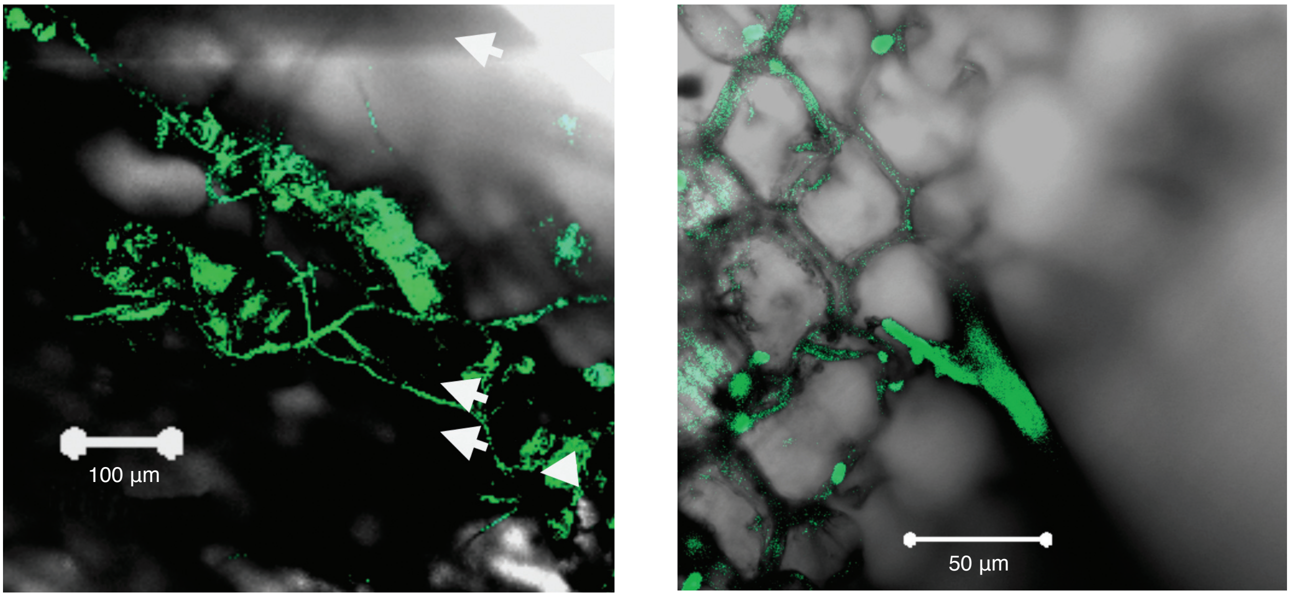

Figure 8. Trichoderma *TPP4 hyphae colonising the secondary root surface (left) and producing swollen tips (arrow) during the interaction.*

Trichoderma TPP4 became fainter in older hyphae. Root tissue also exhibited auto-fluorescence and interfered during observations. *Trichoderma* TPP4 mycelium became a more dense network at 144 h p.i. There was no preferential growth pattern as hyphae developed along and across the intercellular junctions (*Figure 9*).

The hyphal network progressed further after 216 h p.i. whereby, the newly emerged root was found to be colonised heavily by *Trichoderma* TPP4 (*Figure 10*). TPP4 mycelium also was found at the tip of the lateral root advancing in between the intercellular spaces and dense colonisation was also evident in the cortex. In this study, *Trichoderma* TPP4 growth was observed mainly inside the epidermal cells and

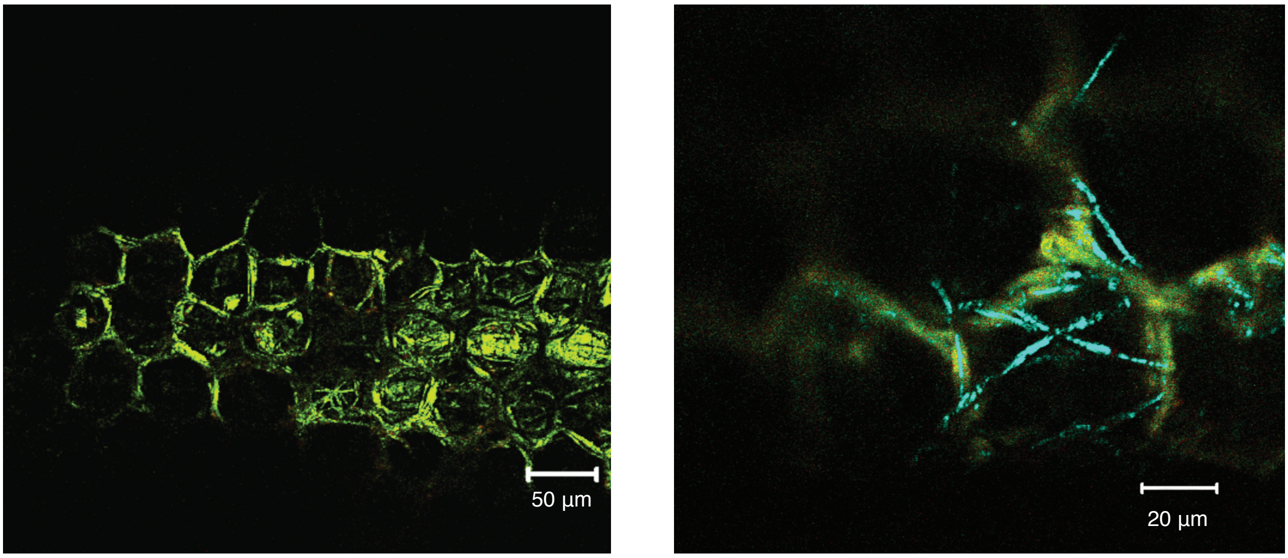

Figure 9. Extensive colonisation of Trichoderma *TTP4 inside oil palm root (left) and outside (right) the secondary root surface after 144 hr post infection (h p.i.).*

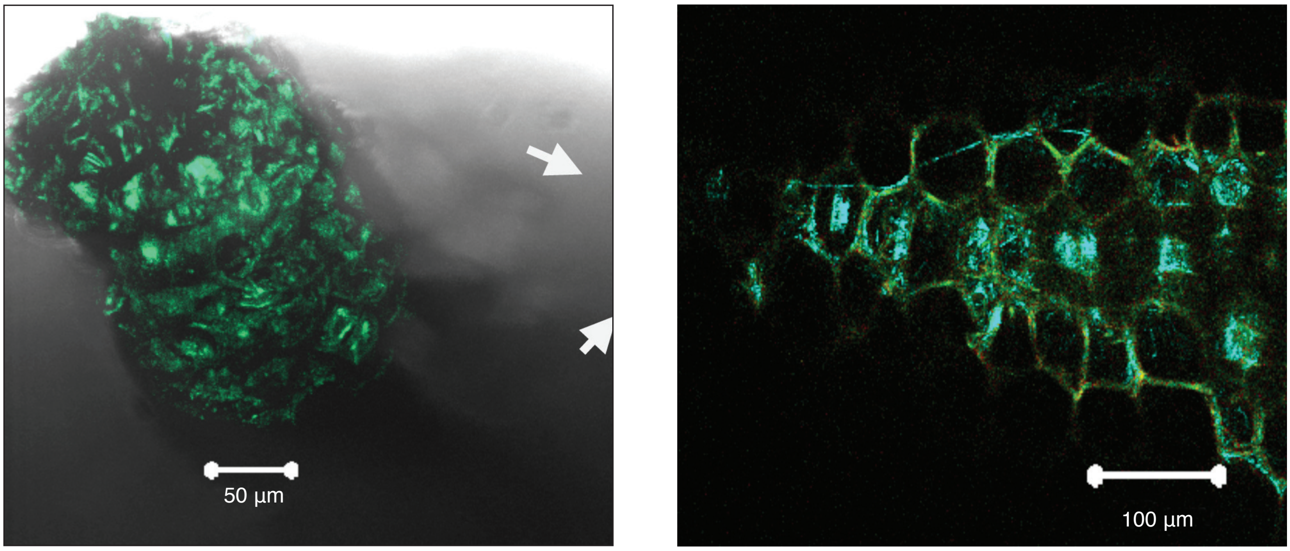

Figure 10. A pneumatode fully colonised by Trichoderma *TPP4 216 hr post infection (h p.i.) (left) and* Trichoderma *TPP4 mycelium was observed advancing and colonising the cell junction (arrow right).*

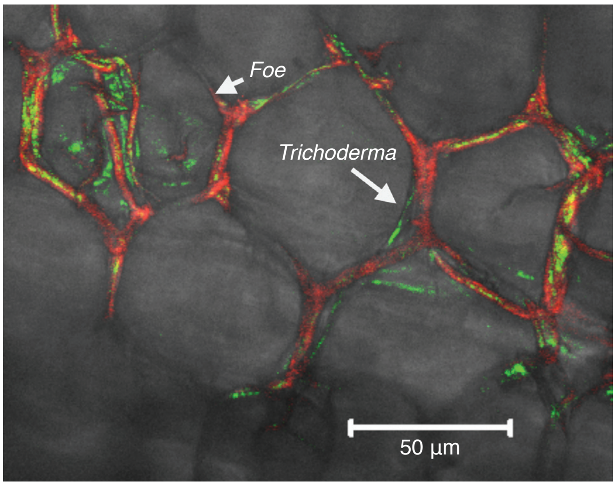

Figure 11. Fungal interaction between Trichoderma *TPP4 (GFP-tagged) and* Fusarium oxysporum *f.sp.* elaeidis *16F (RFP-tagged) on oil palm root 216 hr post infections (h p.i.).*

CONCLUSION

Understanding plant-pathogen interactions can be practically important as it could provide a fundamental basis for the development of the pathogen inside the host. In this study, a RFP-expressing strain of *Foe* 16F was successfully transformed to visualise the initial stages of fungal invasion of a susceptible oil palm and likewise the GFP gene was used to transform *Trichoderma* isolate TPP4 to show root invasion and interaction with *Foe*. Chacon *et al*. (2007) transformed *T. harzianum* with the GFP label in order to study its ability to colonise the oil palm root system during the early stages. A GFP-expressing strain of *F. oxysporum* f.sp. *melonis* (*Fom*) was used to visualise infection of a susceptible melon cultivar (Zvirin *et al*., 2010). One of our aims in this study was to find the port of entry for *Foe*, but unfortunately we were unable to identify where exactly the penetration occurred. Olivain and Alabouvette (1999) reported that penetration events occurred as early as 24 h p.i when they studied early interactions between tomato and pathogenic *vs*. non-pathogenic GUS-expressing *F. oxysporum* strains. As the hyphal network became denser over time (144 h p.i. and 216 h p.i.) there was no evidence of formation of appressorium-like structures. However, swollen hyphae were observed sporadically, which could represent possible penetration sites.

Salerno *et al*. (2000) reported that *F. oxysporum* sometimes formed an ill-defined appressorium-like structure before infection of epidermal tissue without causing any damage. In another study, Salerno *et al*. (2004) showed hyphal penetration by appressorium-like structures produced by *F. oxysporum* directly through the outer epidermal cell of *Eucalyptus viminalis* roots. In the current study, only swollen hyphal structures were observed during *Foe* colonisation on root tips after 144 h p.i. Lu *et al*. (2004) reported that before epidermal cells of banana roots were penetrated by *F. oxysporum* f.sp. *cubense* (race 4), hyphae became swollen at the penetration sites and then entered epidermal cells through what appeared to be a narrow penetration pore by means of a constriction that returned to its normal size once inside the epidermal cell. Swollen hyphal tips described as papilla (Chacon *et al*., 2007) were recorded during the fungus-host interaction. Previous studies by Lu *et al*. (2004) indicated that papilla formation can occur due to environmental factors other than contact with host fungi; alternatively, exudates released from the host mycelium could also diffuse and induce distant papilla formation in *Trichoderma*.

Zvirin *et al*. (2010) did not find any visible penetration structures produced by *F. oxysporum* f.sp. *melonis* (*Fom*), but observed the mycelium forcing itself through narrow openings that were apparently digested in cell walls of melon. In other studies, Rodriguez-Galvez and Mendgen (1995) reported typically thinner penetration hyphae of *F*. oxysporum passing through cotton roots pores produced by lysing host walls. It was observed here that the *Foe* hyphae form a small complex network growing along the borders between root epidermal cells and also across the cells. Salerno *et al*. (2004) demonstrated *F. oxysporum* hyphae grew along the junction between epidermal cells and Lagopodi *et al*. (2002) reported preferable colonisation sites of *F. oxysporum* f.sp. *radices-lycopersici* on the root surface at the junctions between epidermal cells, where the fungus attaches its growing hyphae soon after approaching via the root hairs.

This study showed that *Trichoderma* TPP4 was able to colonise the outside and inside of secondary, tertiary and quaternary roots. More concentrated *Trichoderma* TPP4 hyphae were observed in the regions of the root surface where colonised by *Foe*. *Trichoderma* TPP4 shows potential as a biological control agent of *Foe* as it was seen coiling around and attached together to *Foe* hyphae outside epidermal cells. Inbar *et al*. (1996) showed that hyphae of *T. harzianum* strain BAFC Cult. No. 72 coiling along *Sclerotinia sclerotiorum* hyphae in co-culture. Ojha and Chatterjee (2011) also observed the inhibition of *Fusarium* wilt of tomato by *T. harzianum* where they showed lysis of pathogenic mycelium due to overgrowth and penetration by hyphal pegs and coiling produced by *T. harzianum*. Coiling of antagonistic hyphae around hyphae of *Fusarium* and lysis was also reported by many others (Elad *et al*., 1980; Kumar and Dubey, 2001). Dubey *et al*. (2007) reported that *T. viride* and *T. harzianum* were able to reduce mycelial growth of *F. oxyporum* f.sp. *ciceris* as well as enhancing seed germination, root and shoot length, and decreasing wilt incidence of chickpea under greenhouse conditions. *T. asperellum* was also reported to inhibit *Gibberella fujikuroi* growth by Watanabe *et al*. (2007).

ACKNOWLEDGEMENT

This research was supported by MPOB with a studentship to Mohd Hefni Rusli. The authors wish to thank the Director-General of MPOB for permission to publish this article.

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