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INDUCED SYSTEMIC RESISTANCE AND PROMOTION OF PLANT GROWTH IN OIL PALM SEEDLINGS BY ENDOPHYTIC *Trichoderma virens*

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ABSTRACT

Trichoderma *spp. have been widely used as a biological control agent for plant disease including basal stem rot (BSR) in oil palm.* Trichoderma *spp. control pathogens through mycoparasitism, antibiosis and triggering induced systemic resistance (ISR) in plants during the colonisation of plant roots, limiting the manifestation of the pathogenic fungi. In this study, we investigated the role of endophytic* Trichoderma virens *as a plant-growth promoter and its ability to prime the immune system of the host through ISR in oil palm. Mixed application of two endophytic isolates of* T. virens *7b and 159c was found to significantly enhance the growth and development of oil palm seedlings, in terms of height, girth and chlorophyll content compared to individual treatment. However, results showed that individual treatment of 7b and 159c was better in lignin biosynthesis. Plant defence-related enzyme activities; peroxidase, polyphenol oxidase, superoxide dismutase and phenylalanine lyase were prominently elevated in the leaves of oil palm seedlings upon treatment of the respective* T. virens *isolates via plant roots. This study demonstrated the triggering of ISR in oil palm seedlings via* T. virens *treatment and proving that the inoculation of* T. virens *isolates 7b and 159c were able to promote the vegetative growth and development of oil palm seedlings.*

Keywords: oil palm, endophytic *Trichoderma*, plant growth promoter, induced systemic resistance.

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INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is an important commodity crop in Malaysia that plays major roles in Malaysia economy. Malaysia as the second largest oil palm producer in the world is currently contributing 34% of world palm oil production in 2017 (Kushairi *et al*., 2018). Oil palm planted areas in Malaysia grew from approximately 640 000 ha in 1975 to 5.81 million hectares in 2017 (MPOB, 2018). However, oil palm industry is faced with a serious fungal disease known as basal stem rot (BSR), which is caused by *Ganoderma boninense* that results in substantial losses in oil palm production worldwide (Khairudin, 1990; Rao, 1990). This disease is lethal, not only to old oil palms but also to the younger one (Singh, 1991). The emergence of the first symptom of the disease in oil palm indicates extensive internal tissue decay and the application of disease control at this stage would be ineffective (Hushiarian *et al*., 2013).

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Application of chemical treatments usually used to treat or control BSR is impracticable and costly, therefore, biological method is among the options to control and to suppress the development of BSR disease. Studies by several researchers on biological control agents have shown that beneficial microorganism including bacteria, fungi and actinomycetes have the ability to control this disease (Susanto *et al*., 2005; Sundram *et al*., 2008). *Trichoderma* is known as a versatile biological control agent (BCA) on various plant diseases (Etebarian *et al*., 2000; Howell, 2003; Shoresh *et al*., 2005; Segarra *et al*., 2007; Schuster and Schmoll, 2010). *Trichoderma* as an endophyte has an added advantage in the ability to enhance plant development and act as a better biocontrol agent to a variety of host plants. This is mainly due to its ability in development and colonising in different healthy tissue of living plants including stem, leaves and roots. Ho *et al*. (2016) discovered that oil palm roots colonised with *Trichoderma* improved nutritional status of the host plants through mobilisation of nutrients. *Trichoderma* was also found to be effective in biocomposting of oil palm fibres, thus enhancing soil micronutrient, plant growth performance and crop yield (Siddique *et al*., 2017). Many studies have shown that *Trichoderma* is one of the most effective beneficial microorganisms for controlling plant diseases (Papavizas, 1985; Benítez *et al*., 2004; Harman *et al*., 2004; Al-Sadi *et al*., 2015; Sundram, 2013a,b; Sundram *et al*., 2016) Biocontrol mechanism of *Trichoderma* is primarily attributed by mycoparasitism in which it attaches to pathogenic fungi by physical interaction such as coiling and strangulation of the pathogen (Howell, 2003; Harman *et al*., 2004). Although *Trichoderma* is a common option for controlling plant diseases, their efficacies depend mainly on environmental conditions (Hadar, 1984; Benítez *et al*., 2004). Furthermore, biocontrol activity by *Trichoderma* is sometimes unpredictable due to inheritable resistance. Their biocontrol activity may have been contributed due to either mycoparasitism or antibiosis or by both mechanisms. In a previous study, Angel *et al*. (2016; 2018) identified secondary metabolites released from both *T. virens* 7b and 159c, which were responsible in suppressing *G. boninense* through antibiosis. As for the current study, the effect on the host was investigated focusing on the plant defence stimulation through the inoculation of *Trichoderma*.

Plants recognise the presence of beneficial microorganisms by triggering defence mechanism including induce systemic resistance (ISR) which protects the plant against pathogen attack. This plant defense activation causes accumulation of enzymes that helps in pathogen and environmental stress protection. *Trichoderma* is also reported to trigger ISR in plant (Meyer *et al*., 1998). During the colonisation of plant root, *Trichoderma* will produce bioactive compounds and cell-wall degrading enzymes such as chitinase, glucanase, peroxidase (POX), polyphenol oxidase (PPO), superoxide dismutase (SOD) and/or phenylalanine ammonia lyase (PAL) (Harman *et al*., 2004). The accumulation of these compounds stimulates localised and systemic plant defence responses, limiting the growth of pathogenic fungi (Benítez *et al*., 2004; Djonovic *et al*., 2007). Previously, Sundram (2013a) isolated endophytic *Trichoderma* from oil palm roots. The isolates of *T. virens* 7b and 159c were identified as potential BCA against *G. boninense*. Nursery and field trials conducted by Sundram *et al*. (2016) has proven the ability of both isolates of *T. virens* in disease suppression on infected oil palm. Therefore, the present study was designed to investigate the vegetative growth enhancement of oil palm seedlings by application of both *Trichoderma* conidia spores. Additionally, the study also aims to determine the levels of inducible enzymes associated to ISR induction in oil palm triggered with the application of *T. virens* 7b and 159c.

MATERIALS AND METHODS

Inoculum Preparation

Endophytic *Trichoderma virens* isolates 7b and 159c were obtained from the *Ganoderma* and Disease Research for Oil Palm (GanoDrop) Unit, Malaysian Palm Oil Board (MPOB), Malaysia (Sundram, 2013a). Both *Trichoderma* isolates were subcultured on potato dextrose agar (PDA) and incubated at 28°C for seven days. Spore suspension of *T. virens* isolates 7b and 159c were prepared by washing the agar surface with distilled water and gently scrape the conidia spores with hockey stickglass rod (*Figure 1*). Spores were filtered using muslin cloth and suspended in sterile distilled water followed by adjustment of the concentration to $1x10^8$ conidia per ml.

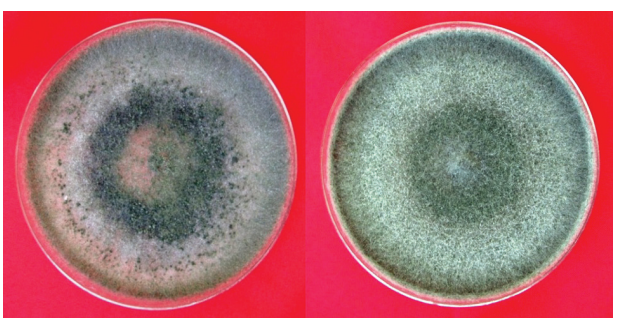

Figure 1. Trichoderma virens *isolates 7b (left) and 159c (right).* T. virens *7b shows a single green concentric ring cluster of conidia around the point of inoculation.* T. virens *159c has green and greenish to whitish heavier conidia density covering entire media with no distinct concentric rings.*

Measurements of Oil Palm Seedling Growth

Three months old oil palm seedlings were arranged in randomised complete block design (RCBD) with four treatments. Each treatment consisted of 10 seedlings and replicated for four times. Booster *T. virens* inoculi were applied with standard application to seedlings according to treatment. The 20 ml of $1x10^8$ conidia per ml of *T. virens* were inoculated to oil palm seedlings before transplant. After two weeks, the seedlings were transplanted into bigger polybags pre-inoculated with another 20 ml of $1x10^8$ conidia per ml of *T. virens*. Booster treatment was applied every two weeks for six times to allow colonisation of *T. virens* and seedlings were left undisturbed thereafter. Biometric parameters; height (cm), girth (cm), frond count and chlorophyll content were recorded to assess the vegetative effect of *T. virens* 7b and 159c application in oil palm seedlings. Height was measured from 1 cm above the soil level to the tip of the leaves. The girth of oil palm seedlings was recorded at the same height above ground using a vernier digital calliper. Chlorophyll content of oil palm seedlings was recorded on leaf two of each seedling using chlorophyll meter (SPAD-502 – Konica Minolta). All the parameters were recorded at monthly interval for six months. Fresh weight of root and shoot mass was also recorded at the end of experiment.

Quantification of Lignin in Roots of Oil Palm Seedlings

The ligninthioglycolic acid (LTGA) assay was carried out with oil palm roots to determine the potential lignification effect by the microbes in the oil palm seedlings. The experimental seedlings were harvested and each seedling was separated into shoot and roots. Lignin quantification in oil palm roots was determined using the LTGA assay as described by Doster and Bostock (1988) with minor modifications. The washed seedling roots of about 2 g were cut into 2 cm length and incubated in methanol for 48 hr. The roots were then dried overnight in a vacuum desiccator and weighed. Five ml of 2 N HCl containing 0.5 ml of TGA (Sigma) was added to the root samples and incubated in oven at 95°C for 4 hr. The acid was drained off and the samples were rinsed in 5 ml deionised water before treating in 5 ml of 0.5 N NaOH for 18 hr at room temperature. The NaOH extracts were transferred into 12 ml tubes, while the root samples were rinsed with 5 ml deionised water and decanted into the 12 ml tubes. The NaOH extracts were acidified with 1 ml of concentrated HCl and incubated at 95°C for 4 hr followed by centrifugation at 850 x g for 10 min at 4°C. The pellet was suspended in 2 ml of 0.5 NaOH and centrifuged again to remove any remaining undissolved matter. Absorbance of the supernatants was measured at 280 nm.

Plant Material and Sample Collection

Three months old oil palm seedlings (*Dura* x *Pisifera*) were planted in plastic pot trays and arranged in RCBD. The experiment was conducted in three replications for each treatment (T1, T2, T3, and T4) including *Trichoderma virens* isolates 7b and 159c and untreated control. The spore suspension of *Trichoderma virens* isolates 7b and 159c ($1x10^8$ spores ml^{-1}) were inoculated by pipetting into the plastic pot at 1 ml for each seedling. Seedlings applied with sterile distilled water were considered as untreated controls. Leaf samples were collected at 0, 12, 23, 36, 48 and 72 hr post inoculation and cleaned with tap water before storing at -80°C for further enzyme activity analysis.

Assays for POX, PPO and SOD Activities

One gram of washed leaf samples was homogenised in 10 ml of chilled 0.1 M phosphate buffer (pH 7) using pre-chilled mortar and pestle. The homogenate was filtered through muslin cloth and centrifuged at 11 800 x g for 15 min at 4°C. POX activity was carried out according to Chowdappa *et al*. (2013). A total of 0.05 ml of enzyme extract was mixed with 2.9 ml of 0.05 M phosphate buffer (pH 6.8), 0.1 ml freshly prepared 0.3% H_2O_2, and 0.05 ml freshly prepared pyrogallol. The reaction mixture was incubated at room temperature for 5 min and reaction was stopped by adding 0.5 ml of 5% (v/v) of H_2SO_4. Blanks were prepared using the reaction mixture without the enzyme extract and absorbance was read at 420 nm at 60 s interval for 3 min. In the PPO assay, the enzyme extract was mixed with 2.9 ml of 0.05 M citrate buffer (pH 6.8) and 0.1 ml of freshly prepared pyrogallol. The reaction mixture was incubated at room temperature for 5 min and absorbance was read at 450 nm at 60 s interval for 3 min. SOD activity was determined by adding 1.3 ml of enzyme extract to the assay mixture of 3 ml of 0.05 M of phosphate buffer (pH 7.8), 0.25 ml of 0.05 M EDTA and 0.65 ml of 0.065 M methionine. The reaction mixture was mixed with 1 ml of 20 μM of riboflavin and 0.1 ml of 150 μM of nitroblue tetrazolium chloride. Blanks were prepared using the reaction mixture without the extract and kept in the dark and absorbance was read at 560 nm.

Assay for Phenylalanine Ammonia Lyase (PAL) Activities in Oil Palm Leaves

PAL was determined using the method described by Kováčik and Klejdus (2012). The 0.3 g of leaf samples were homogenised in 2 ml of 0.1 M sodium borate buffer (pH 8.8) using cold mortar

and pestle and the homogenates were immediately centrifuged at 12 000 x g for 15 min at 4°C. Reaction mixtures consisting of 350 µl of enzyme extracts and 500 µl of 0.1 M sodium borate buffer were pre-incubated at 40°C for 5 min. The 300 µl of 0.005 M L-phenylalanine was added to the mixture to start the reaction and allowed to incubate for 1 hr at 40°C, followed by addition of 50 µl of 5 N HCl to stop the reaction. The samples were analysed at absorbance of 275 nm. Blanks were prepared using the reaction mixture without the enzyme extracts and L-phenylalanine.

RESULTS AND DISCUSSION

Effect of *Trichoderma virens* Treatment on Oil palm Seedlings Growth

The application of *T. virens* isolates 7b and 159c has significantly increased the height and girth of oil palm seedlings (*Figure 2*) as compared to the untreated seedlings (T1) observed on the sixth month. The increment of height and girth were generally slow at the beginning of the experiment for individual and mixed treatments but increased significantly compared to control after the fourth month of observation. This might due to the adaptability of *T. virens* in colonising the plant roots from the beginning of inoculation. Mixed application of both *T. virens* isolates (T4) to oil palm seedlings showed the highest height (67.38 cm) compared to other individual treatments. However, no significant difference was noted between treatments. As for girth of the seedlings, they were significantly different from each treatment and mixed application was recorded as highest (42.38 mm) increment at the sixth month of observation. *Trichoderma* has been widely known for their ability in growth promotion for large number of plants including vegetables and forestry crops (Steward and Hill, 2014). Steward and Hill (2014) also addressed that flowering and shoot growth of plant has positive effect after inoculation with *Trichoderma*. Application of both *T. virens* isolates to oil palm seedlings may have contributed to increased production of growth factors and phytohormones by both isolates thus assisting to an effective growth

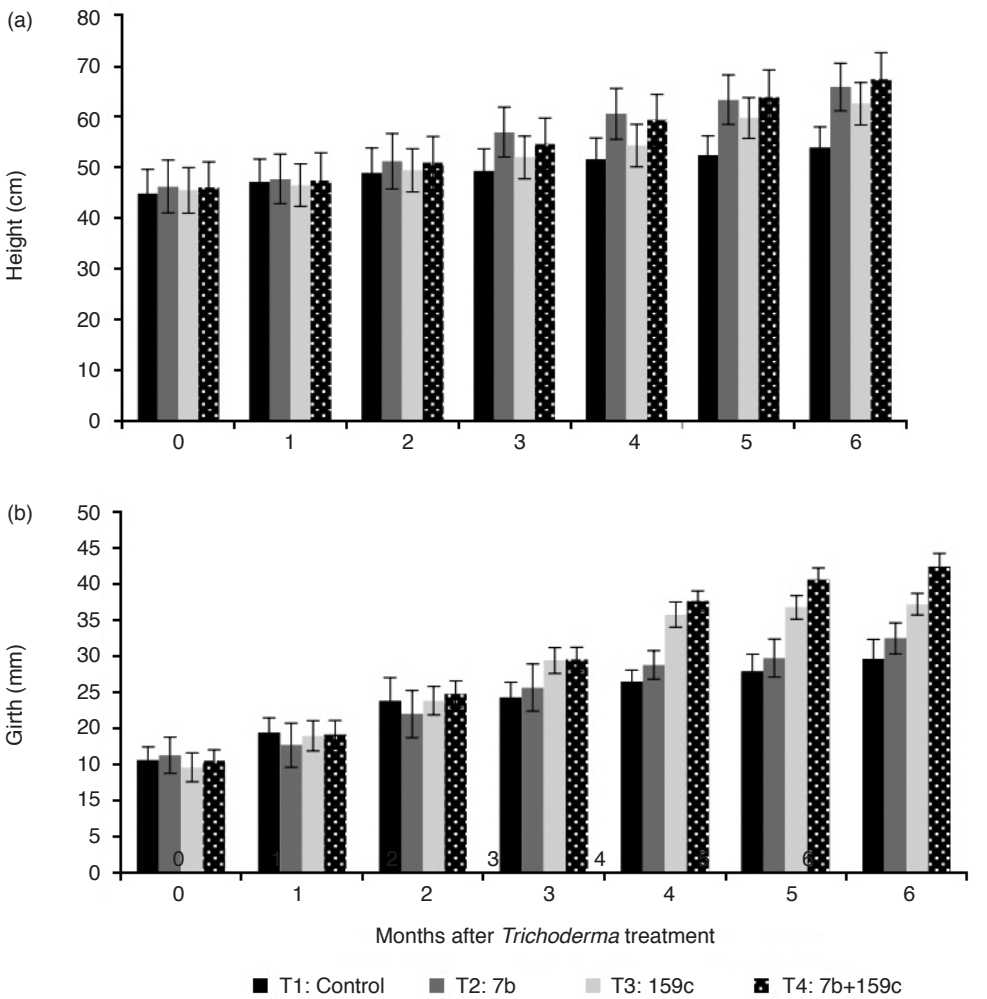

Figure 2. Effect of Trichoderma virens *on plant height (a) and girth (b) of oil palm seedlings at different growth stages. Values are means of four replicates; vertical bars indicate standard error; 7b* - T. virens*; 159c* - T. virens.

and development of seedlings (Chowdappa *et al*., 2013). From *Figure 3*, similar observation was also recorded in frond count of the seedlings in the mixed application of *T. virens* isolates 7b and 159c (T4) which it recorded the highest increment at sixth month observation. Control seedlings in T1 recorded the lowest increment of oil palm seedlings frond count among all the treatments. However, single application of *T. virens* in T3 had almost the same frond count value as the mixed application of *T. virens* isolates in T4 after sixth month observation with the value of 11.8 and 11.725 units respectively. The frond count of oil palm seedlings appear to show a steady increase but there were no significant increase between treatments and control. Similar responses were recorded in the study by Sundram (2013c) whereby mycorrhizal application showed differences in height, girth and leaf area but not in the frond count of the oil palm seedlings. Responses of oil palm seedlings to *T. virens* isolates may vary with concentration of growth factors exhibited by each *T. virens* isolates (Contreras-Cornejo *et al*., 2009). Chlorophyll is a biochemical parameters for plants that indicates the photosynthetic capacity, health and nutrient status of plants (Filella and Penuelas, 1994). From *Figure 4*, chlorophyll content in T4 recorded 59.14 compared to control plants at 38.672. Although the lowest chlorophyll content was recorded in control plants (T1), it was treatment T2 that gave the lowest chlorophyll content when compared among the *Trichoderma* treatments (T3 and T4). However, the chlorophyll content of oil palm seedlings decreased on the fourth month of observation. This could be influenced by certain factors. Plants are known to adjust their chlorophyll content for adaptability to a given environment and optimise photosynthesis (Li *et al*., 2018). Climate plays an important role in regulating chlorophyll content of certain plant. Decrease in chlorophyll content in the fourth month for all treatments may be caused by the change in climate. Too high or too low temperature can cause enzyme reaction

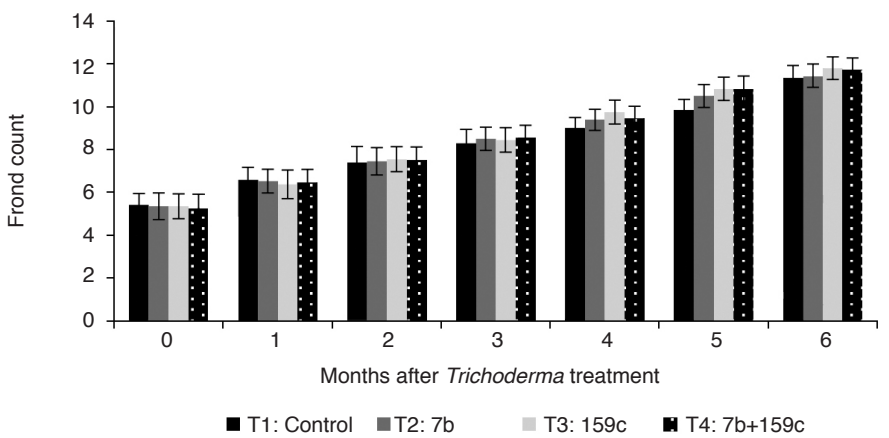

Figure 3. Effect of Trichoderma virens *on frond counts of oil palm seedlings at different growth stages. Value are means of four replicates; vertical bars indicate standard error; 7b* - T. virens; *159c* - T. virens.

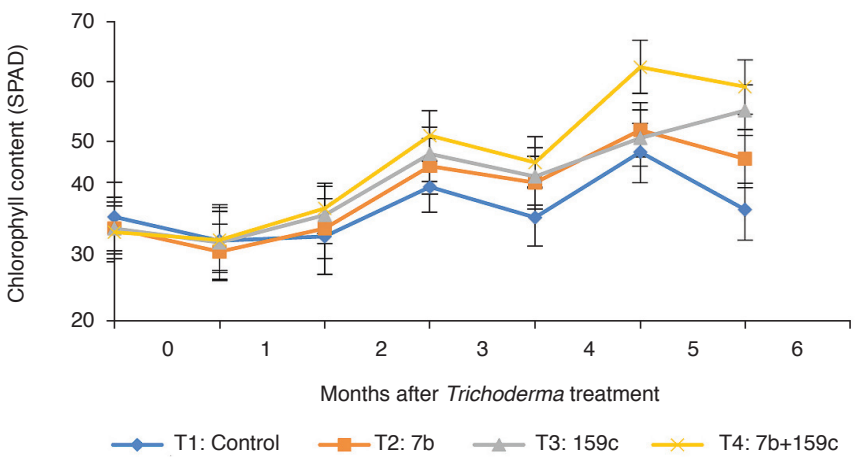

Figure 4. Effect of Trichoderma virens *on chlorophyll content of oil palm seedlings at different growth stages. Values are means of four replicates; vertical bars indicate standard error; 7b* - T. virens; *159c* - T. virens.

to be inhibited and disturbed the synthesis of chlorophyll, even destroy the original chlorophyll in plants (Wolken *et al*., 1955). Precipitation also might affect the synthesis of chlorophyll. Lack of water in leaves also affects synthesis of chlorophyll, and promotes decomposition of chlorophyll and accelerate leaf yellowing (Zhou, 2003). Nevertheless, the current result in this study showed that the chlorophyll content of oil palm seedlings treated with *Trichoderma* was overall increasing throughout the month of observation. Chlorophyll content is an indicator to biotic or abiotic stress in the host plant. Although no stresses were subjected to the seedlings, the treated seedlings had higher chlorophyll content as compared to the control seedlings. In this study, elevated chlorophyll content by inoculating *T. virens* resulted to a beneficial effect in increasing photosynthesis rate in oil palm seedlings thus enhancing the seedlings growth and development.

Assessment of Lignin Content in Oil Palm Roots Treated with *T. virens*

A quantitative assay for investigating the lignifications in root of oil palm seedlings in response to the application of biocontrol agent, *T. virens* isolates 7b and 159c was quantified using thioglycolic acid. Formation of lignin thioglycolic acid in this assay suggested the presence of lignin (Doster, 1988). This study demonstrated more lignin was detected in *T. virens* treated oil palm seedlings and the control seedlings recorded the lowest lignin concentration. *Figure 5* illustrates the lignin concentration in roots of experimental oil palm seedlings. Lignification was significantly increased with the application of *T. virens* isolates 7b and 159c compared to control seedlings (T1). This suggests colonised oil palm root may play a role in strengthening its cell through lignin deposition as defence response. Lignin was found to be the highest at 1.08 min^{-1} g^{-1} with significant difference in seedling treated with *T. virens* isolate 7b (T2), while mixed application of *T. virens* recorded the lowest lignifications among the treated seedlings. Mixed application has a slight increase in lignification as compared to seedlings in control seedlings; however, it was not as effective as compared to T2 and T3. Interference competition between two isolates might have limited one isolate for effective lignification in plant membrane (Wicklow, 1992). The increase of lignification in two other single treatments could be due to more deposition of lignin in newly formed root tissues. This result is an important finding as lignification is known as a mechanism for disease resistance in plants (Bhuiyan *et al*., 2009). This shows that by application of *T. virens*, it increases resistance through lignification of seedlings by preventing further growth and development of plant pathogen which includes *G. boninense*.

Assessment of Induced Systemic Resistance Enzyme Activities upon Application of *T. virens* in Oil Palm Seedlings

Trichoderma spp. can act as elicitor to initiate the ISR in plants as one of biocontrol mechanism of disease (van Loon *et al*., 1998; Yedidia *et al*., 2003; Harman, 2006). Plant defence enzymes activities including POX, PPO, PAL and SOD in leaves were measured at various time intervals. We observed that treatment of *T. virens* raised these enzyme activities in oil palm leaves (*Figure 6*). Increase in enzyme activity in the leaves suggests a systemic response to application of *T. virens* isolates 7b and 159c in the rhizosphere. In comparison with control seedlings, seedlings treated with combination of both *T. virens* isolates recorded the highest enzyme activities. The remaining treatment recorded a good

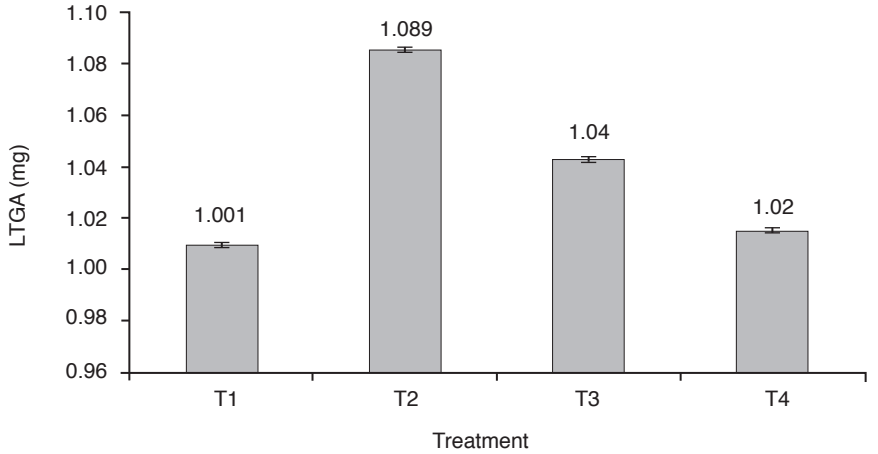

Figure 5. Effect of Trichoderma virens *isolates 7b and 159c on oil palm root lignification. Values are means of four replicates; vertical bars indicate standard error; T1- control; T2* - T. virens *isolate 7b; T3* - T. virens *isolate 159c; T4* - T. virens *isolates 7b and 159c; LTGA - lignin thioglycolic acid.*

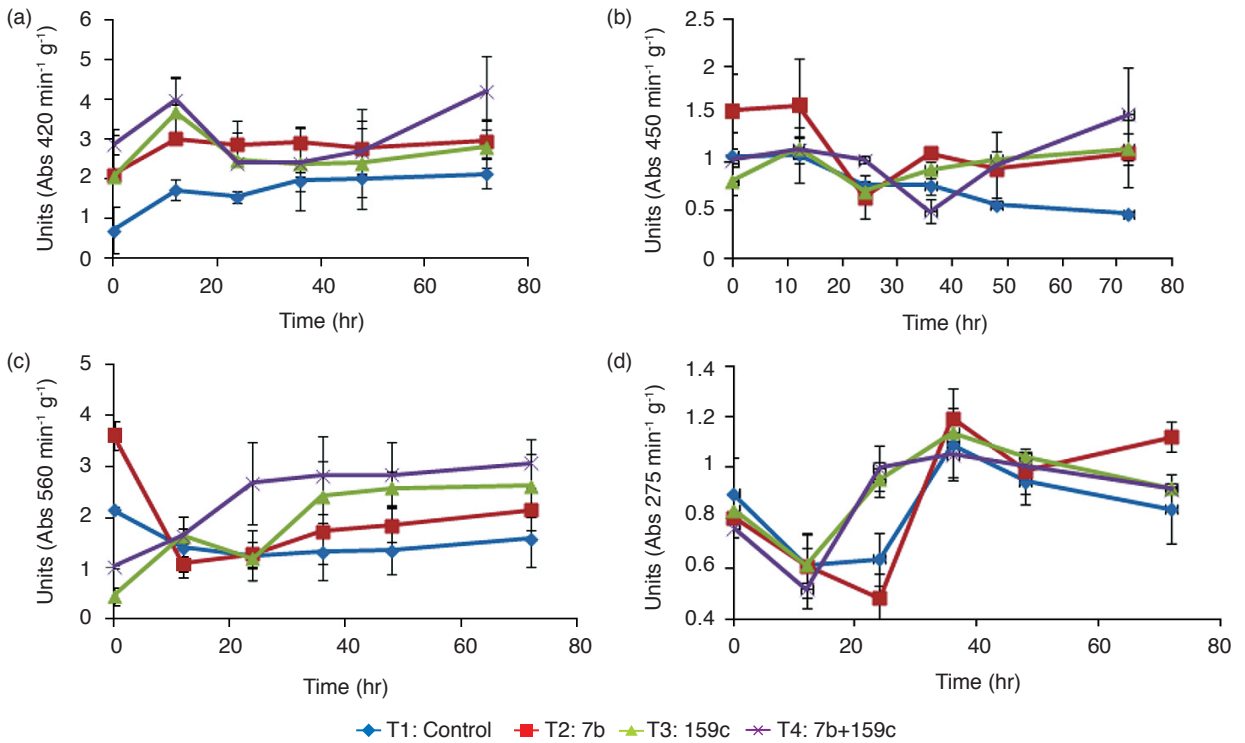

Figure 6. Total peroxidase (a), polyphenol oxidase (b), superoxide dismutase (c) and phenyl alanine lyase (d) activity in leaves of oil palm seedlings treated with endophytic Trichoderma virens *7b and 159c isolates. Values are mean of three replications with vertical bars representing standard error.*

increase in PPO, POX and SOD activity in tested time intervals (*Figure 6*). A similar trend showed by these enzymes where the activities peaked at the initial application of *T. virens* isolates prior to a decrease in their activities and followed by a gradual increase in the activities. This is correlated to colonisation of the oil palm roots by *T. virens* prior to produce substances or molecules to initiate systemic resistance by the plants. *Trichoderma* needs to fully colonise the root of oil palm seedlings in order to effectively induce the systemic defence mechanism and this is demonstrated by the gradual increase of enzyme activity in each inducible enzyme (Benitez *et al*., 2004). Sudden increase of POX, PPO and SOD activities were recorded between 0 to 12 hr post infection (hpi) upon *T. virens* 7b and 159c isolates application to seedlings. This might be due to plant's response to the inoculation which recognised the antagonists as detrimental at initial inoculation. POX, PPO and SOD are defence enzymes related to resistance inducement in plants (Prasannath and De Costa, 2015). POX has a role in defence-related processes, which includes hypersensitive response, lignification, crosslinking of phenolics and glycoproteins, suberization and phyloalexin production (Nicholson and Hammerschmidt, 1992). Whereas for PPO, this enzyme catalyses phenolic compounds to quinones derivatives, which is highly toxic to pathogens (Lamb *et al*., 1989). SOD are uniquitous metalloenzymes that are able to catlyse its dismutation of superoxide radicals to hydrogen peroxide and oxygen (Bowler *et al*., 1994). This enzyme has a major role in defence against toxic-reduced oxygen species and crucial in plant stress tolerance. Therefore, the elevation of these enzymes level after inoculation of *T. virens* further explains that *Trichoderma* has the ability to induce resistance in oil palm seedlings. Similar observation was recorded in PAL activity in the leaves of *Trichoderma* treated oil palm seedlings, where PAL activity elevated significantly. This is due to enough accumulation of phenolic compounds prior to triggering the plant defence mechanism (Dixon and Paiva, 1995). However, combination of both *T. virens* isolates had moderate effects in enhancing PAL compared to single application of *T. virens* isolates 7b which recorded the highest enzyme activity. PAL is the key enzyme that is responsible in linking primary metabolism of aromatic amino acids with secondary metabolic products (MacDonald and Dcunha, 2007). This enzyme has been extensively studied for its role in plant defence system due to synthesis of various phenolic compounds and anthocyanin which are responsible for plant's resistance to pathogen (Dixon and Paiva, 1995). Various defence enzymes act together may further contribute to the development of an effective chemical and mechanical defence barrier in oil palm seedlings. Thus, this may be an effective strategy to induce systemic resistance in oil palm seedlings by introducing this endophytic *T. virens* as prophylactic treatment.

CONCLUSION

Based on the present study, it can be concluded that endophytic *T. virens* significantly improves growth and development of host plants. However, *T. virens* plays less important role in increasing the frond number of seedlings and future studies should extend the study period and continue to the field observation before a conclusion on fronds is made. Vegetative growth and lignification of oil palm seedlings were recorded to be enhanced with the application of *T. virens* isolates 7b and 159c and they act better with individual treatment. In a nursery trial conducted previously by Sundram *et al*. (2016), the results shows that *T. virens* isolates 7b and 159c managed to reduce disease severity of oil palm seedlings. This was further affirmed with the response in plants with elicitors produced by biocontrol agent primed the defence mechanism against the potential pathogens. The activity of PPO, POX, SOD and PAL in oil palm seedlings were recorded to be elevated prior to *T. virens* inoculation and were in an increasing trend up to 72 hpi. This enhancement of enzymatic activity suggests application of *T. virens* may enhance the oil palm's capacity to protect itself against subsequent infection of pathogen through the elicitation of plant defence enzymes.

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