ANTAGONISTIC POTENTIAL OF *Trichoderma* SPECIES AGAINST *Ganoderma* DISEASE OF OIL PALM

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ABSTRACT

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Oil palm (Elaies guineensis Jacq.) is an economically important cash crop, grown primarily for its oil and has become one of the main oil crops in the world. To date, oil palm is the world's highest yielding oil producing crop. Unfortunately, a one of the major constraints faced by oil palm industries is a fungal disease, basal stem rot caused by Ganoderma boninense. However, research on sustainable remedy to Ganoderma disease is in an upsurge. Therefore, this study aimed at evaluating the potential of Trichoderma species as biological control agents against Ganoderma disease. Trichoderma species used were T. harzianum, T. virens, T. brevicompactum and T. asperellum. Molecular identification was performed using Qiagen DNA kit and amplified with TvPF and TvPR primer set, which sequenced blast into NCBI and also verified the fungi identity. Dual culture and culture filtrate assay was conducted against Ganoderma boninense. Enzyme assay of chitinase, cellulose, protease and β-1, 3-glucanases activities as well as Siderophore and Phosphate solubilization ability of Trichoderma were also evaluated. The experiments were laid out in a completely randomized design (CRD) with ten replications. The data were subjected to analysis of variance and significant means separated with the Duncan Multiple range test at 5% probability level. The results indicated that Trichoderma species can inhibit Ganoderma growth in vitro with different potentials by producing enzymes that degraded Ganoderma fungal cell-walls. They also possessed the potentials of producing certain chemical compounds which may boost the immune system of oil palm against G. boninense. The use of Trichoderma species could be an excellent solution to basal stem rot disease in younger palms.

Keywords*: Oil palm, Ganoderma boninense, Ganoderma disease, Trichoderma spp*

INTRODUCTION

Oil palm (*Elaies guineensis* Jacq.) is an economically important cash crop, grown primarily for its oil and has become one of the main oil crops in the world. To date, oil palm is the world's highest yielding oil producing crop, producing approximately 5 metric tonnes per hectare per year (Chong, 2011). Unfortunately, one of the major constraints faced by oil palm industry in Malaysia (Chong *et al*., 2011) and Indonesia is the devastating disease known as *Ganoderma* disease caused by *Ganoderma* spp. The fungus was previously reported on older palms; but has been detected on younger palms aged 1-2 years old (Naher, 2013). In Malaysia, the disease incidence was higher in oil palm plantations located on the coastal soils (Khairudin and Chong, 2008). Chong (2011) reported that *Ganoderma* disease not only causes reduction of total oil yield but also resulted in direct loss of stands due to palm death and the economic losses in Malaysia alone have been estimated between $68 to $455 million USD annually. Generally, the losses due to *Ganoderma* disease on oil palm could be either direct or indirect; whereby the direct loss refers to the death of the oil palm, whereas indirect loss may be the decline in fresh fruit bunch (FFB), weight and quality (Susanto *et al*., 2005).

Despite the effectiveness of synthetic chemicals in plant disease management, their continuous utilization has resulted in the development of streams of fungi with multiple resistances, which further complicates the management of plant diseases (Boubaker *et al*., 2009). As a result, many fungicides are now becoming ineffective against most important plant pathogenic fungi. Furthermore, the use of these chemicals in plant protection is also being restricted owing to stringent regulations, health hazard, residual toxicity, long degradation period, environmental contamination and growing public concern about chemical residues in food materials (Palou *et al*., 2008). Therefore, there is an increasing concern and effort to develop a better plant disease management approach that is not only safe, land ecological friendly but economically feasible to the farmers. The most recent studies have shown that biological control approaches could be more effective against many plant pathogenic fungi (Kotasthane *et al*., 2015; Contreras-Cornejo *et al*., 2016).

Trichoderma spp. are successful colonizers of their habitats, due to their efficient utilization of the substrates available in the habitat and production of antibiotic metabolites and enzymes (Schmoll *et al*., 2010). Their capacity to produce antibiotics, and parasitize on other fungi and compete with deleterious plant microorganisms has been widely studied. These fungi have been reported of producing a numerous secondary metabolites such as non-ribosomal peptides, terpenoids, pyrones and indolic-derived compounds (Contreras-Cornejo *et al*., 2016). Some strains of *Trichoderma* are antagonistic to other fungi and thereby showing promising potentials as biological control agents of soil-borne diseases (Kacprzak *et al*., 2014). The biological control potential of *Trichoderma* spp. has been demonstrated on a wide spectrum of plant diseases both *in vitro* and *in vivo* (Kotze *et*

al., 2011; Woo *et al*., 2014).Therefore this study aimed at evaluating the potential of *Trichoderma* spp. as biological control agents against *Ganoderma* disease of oil palm.

MATERIALS AND METHODS

Fungal isolates

Four isolates of *Trichoderma* were obtained from the culture collection of the Department of Plant Protection, Faculty of Agriculture, University Putra Malaysia; Selangor, Malaysia. All the *Trichoderma* cultures were stored in 20% glycerol at -80 °C and the isolates sub-cultured on potato dextrose agar (PDA) and maintained at 27 °C ± 2 °C for further studies(Difco™, Becton, USA).

Molecular identification of *Trichoderma* species

Selected *Trichoderma* spp were subjected for molecular identification based on sequence homology of specific genes. Genomic DNA of *Trichoderma* was extracted using Qiagen DNA extraction kit following the manufacturer's instructions. The ITS r DNA regions were amplified using *Trichoderma* spp primer sets of oligonucleotide primers TvPF (5'CCGCCGGAGGACCAACCAA3') and TvPR (5'-GACAGGCATGCCCGCCAGAA-3') for forward and reverse, respectively. Amplification was performed according to the protocols of Qiagen TopTaq Master Mix. Eppendorf Mastercycler® Gradient Thermal Cycler (Hamburg, Germany) was used to run the polymerase chain reaction (PCR).The PCR started with denaturation for 2 minutes at 95 °C, followed by 35 cycles of denaturation for 1 minute at 94 °C, annealing for 30 seconds at 59.9 °C and extension for 2 minutes at 72 °C. The final step of extension was carried out for 10 minutes at 72 °C, before it was maintained at 4 °C. The PCR product obtained was sequenced by an automated sequencer. The same primers as above were used for this purpose. The sequence was compared with the reference species of *Trichoderma* contained in genomic database using the NCBI-BLAST tool.

About 4 µl of PCR product mixed with 2 µl of loading dye (Promega®) was loaded into each well. 100 bp marker (Promega®) was loaded to the second and the final well. Electrophoresis was set using power source from Lightning Volt™ Power Supply Model OSP-300. The power supply was set at 65 V voltage, 500 mA electric and 80 minutes duration. After the completing electrophoresis process, gel was stained in Etidium bromide (0.5 µg per ml) for 20-30 minutes before visualizing under a UV transilluminator (BioRad®).

Dual culture assay

Dual culture assay was conducted on four isolates of *Trichoderma* against *G. boninense*, following the procedure described by Rahman *et al.* (2009). An agar disc (6 mm) was taken from four day old PDA plate cultures of each *Trichoderma* spp. and placed on the periphery of the PDA plates (9 cm). Another agar disc of the same size of *G. boninense* was placed at the other periphery but on the opposing end of the same plate, while another plate inoculated with just a *G. boninense* plug served as a positive control. Each treatment was replicated five times and incubated at 27±2 °C. Antagonistic activity was determined four days after incubation by measuring the radius of the *G.boninense* colony growth in the direction of the antagonist (*Trichoderma*) colony (R2) and the radius of the *G. boninense* colony in the control plate (R1). The experiment was repeated twice. The percentage (%) inhibition of *G. boninense* radial growth was measured at 24 hours intervals by measuring the radial growth (mm) of the developing colony toward the antagonist until the plant pathogen (*G. boninense*) colony was completely surrounded by the antagonist (*Trichoderma* spp.). The percentage growth rate inhibition of pathogen was calculated.

Culture filtrate assay

The modified Dennis and Webster (1971) method was used. The *Trichoderma* isolates were inoculated in 100 ml sterilized PDB in 250 ml conical flasks and incubated at 25 ± 2 °C on an orbital rotatory shaker at 150 rpm for 15 days. The control flasks were not inoculated with *Trichoderma*. Fungal mats of *Trichoderma* spp. were harvested by centrifugation (Multispeed Centrifuge, PK121: MI Italy) of the culture broth at 4000x g for 30 min at 20 °C in 50 ml sterile plastic tubes (Labcon, SuperClear™: USA) The culture was filtered through Whatmann filter paper to remove the mycelial mats and then sterilized by passing through 0.22 µm pore biological membrane filter. Firstly, the filtrate was observed under a light microscope or by spreading 0.1 ml on PDA plates to confirm if it was a fungal free filtrate. The filtrate was added to prepared molten PDA medium (at 40±3 °C) to obtain a final concentration of 10% (v/v). The PDA amended with culture filtrate was inoculated with six millimeter mycelial plug of the *G. boninense* at the centre and the plates were incubated at 25 ± 2 °C for seven days, while a plate containing no amended PDA was inoculated with *G. boninense* as control. The percentage inhibition was determined.

Cellulase enzyme hydrolysis

Cellulose hydrolysis by *Trichoderma* isolates was determined by using phosphoric acid swollen cellulose (PASC) as a substrate in Mandel and Weber's medium (Mandels and Weber, 1974) and supplemented with Congo red. The plates were inoculated with five millimeter agar plugs from the edge of actively growing colonies of *Trichoderma* spp and incubated at 28±2 °C for 3 days followed by 18 h incubation at 50 °C.

Chitinase enzymes activity

Preparation of colloidal chitin

Five grams of chitin was added to 100 ml of concentrated HCl by constant stirring using a magnetic stirrer and kept in a refrigerator (4 °C) overnight. The resulting slurry was then added to 200 ml of ice-cold 95% ethanol and kept at 25 °C overnight (ethanol neutralization). Then it was centrifuged at 3,000 rpm for 20 minutes at 4 °C. The pellet was repeatedly washed with sterile distilled water by centrifugation at 3,000 rpm for 5 minutes at 4 °C until the smell of alcohol was totally gone. The final colloidal chitin was stored at 4 °C until further use. Chitinase activity was determined using the growing medium and a method developed by Agrawal and Kotasthane (2012). Five day old culture plugs of the *Trichoderma* spp for test of chitinase activity were inoculated into the medium petri plates and incubated at 25±2 °C for four days. Chitinase activity was determined from the formation of purple coloured zone in the inoculated medium. Observations were made of the diameter of purple coloured zone at fourth days after inoculation.

β-1, 3-glucanases enzymes activity

For plate screening method of β-1, 3-glucanases activity, carboxy methyl cellulose agar (CMC agar) medium amended with laminarin was used according to the modified method given by El-Katatny *et al*. (2001). A millimeter culture disc was placed at the centre of the plate and incubated at 25 ±2 °C for three days. The β-1,3-glucanases activity on the plates was visualized by dipping in 0.1% congo red dye for 15–20 min followed by distaining with 1 N NaCl and then with 1 N NaOH for 15 min. The distaining was repeated twice. The β-1, 3-glucanase activity was recorded of the clearance zone formation.

Protease enzymes activity

Protease enzyme activity in *Trichoderma* isolates was determined using Skim milk agar medium (51.5 g per litre) (Berg *et al.*, 2002). Culture disc from five day old isolates was inoculated on the skim milk agar medium and incubated at 25±2 °C for three days. *Trichoderma* with protease activity gave a clear zone around the colony indicating the production of protease enzymes.

Siderophore activity

For siderophore production, an iron free modified Succinate medium (SM) of Meyer and Abdallah (1978) consisting of (g/l): 6.0 K_2HPO_4, 3.0 KH_2PO_4, 0.2 $MgSO_47H_2O$, 1.0 $(NH4)_2SO_4$, 4.0 succinic acid, pH 7.0 with slight modification by adding 15g agar was used. CAS chrome-Azurols agar plates were prepared as CAS (60.5 mg) dissolved in distilled water (50 ml) and blended with 10 ml of iron (III) solution (1 mM $FeCI_3.6H_2O$ in 10 mM HCI) and stirred. The solution was gradually added to 72.9 mg of HDTMA (Hexa decyltrimethyl ammonium bromide) dissolved in 40 ml of water resulting in dark blue liquid, which was autoclaved at 15 lb psi and then mix with the modified SM (Serrate Molide 1-3). Siderophore activity was determined on Chrome-Azurol S (CAS) medium. The isolates of 24-hr old cultures were each spotted on the CAS medium and incubated at 25±2 °C for 48–72 hour Formation of an orange to yellow halo against a dark blue background around the colonies confirmed the siderophore production. Experimental design and statistical analysis

The experimental design used in this study was the CRD with ten replicates for each treatment and carried out in the laboratory. Analysis of variance was conducted using the SAS software [SAS 9.4 Version Institute Inc. (2013) Cary, NC, USA], and statistical means which were significantly different were separated with the Duncan's multiple range test (DMRT) at 5% probability level.

RESULTS

Molecular identification of *Trichoderma* spp

The Dendogram was generated by unweighted pair-group methods with arithmetic mean (UPGMA) method (Kumar *et al*., 2004) using Molecular Evolutionary Genetics Analysis (MEGA) version 6 used to study genetic diversity among the four *Trichoderma* spp. The phylogenetic tree (Figure 2), showed that the isolates studied were separated into four different clades, each represented one *Trichoderma* sp. Clade 1 comprised of 1 isolate (TB003) with accession number (KX0902002.1) and (KC884785.1), identified as *T. brevicompactum*. Clade 2 consisted of one isolate (TH004, KX092003.1), identified as *T. harzianum*, while clade 3 consisted of two isolates (TA003, KX092001.1 and TV005, KX092004.1), identified as *T. asperellum* and clade 4 was an out-grouped isolate with accession number (HB833354.1), identified as *T. viride.* All amplified products with the primer were polymorphic with distinguishable banding patterns which indicated the genetic diversity of *Trichoderma* isolates (Figure 1). A summary of the sequence results presented in Table 1 includes the ITS sequence GenBank accession number that best matched each sample. The result for the polymerase chain reaction amplification products with 245 bp is shown (Fig. 1).

In *vitro* screening of *Trichoderma* spp. against *Ganoderma boninense* (UPM13)

In vitro dual culture, culture filtrate and dual plate assays were performed with four *Trichoderma* spp. against *G. boninense.* The results indicated varying levels of antagonistic responses against the pathogen (Table 2). In the dual culture test, statistical analyses showed that there were significant differences in the percentage inhibition growth rate (PIGR) among the four *Trichoderma* spp. studied. The PIRG values ranged from 76.0 to 84.6%, with the maximum percentage inhibition by *T. asperellum* (84.6%) and the least 76.0% observed with *T. brevicompactum.* In culture filtrate assay, the percentage inhibition of colony diameter growth ranged from 84.4%

(*T. harzianum*) to 71.3% (*T. brevicompactum*) ($P \leq 0.05$). All the *Trichoderma* spp. tested clearly demonstrated certain antifungal activities, and may be used as an antagonist against *G. boninense* on oil palm.

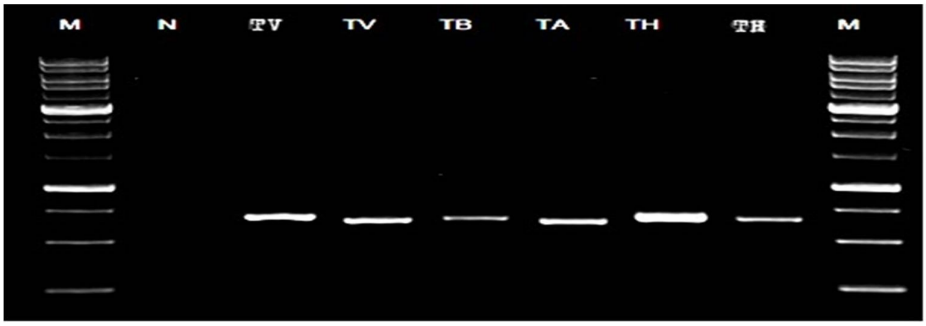

Fig. 1: PCR amplification of ITS regions of *Trichoderma* isolates. Lane 1 and 9 M- 100bp ladder; Lane 2- N (negative control with distilled water); Lane 3 and 4 TV-*Trichoderma virens*; Lane 5-TB (*Trichoderma brevicompatum)*; Lane 6-TA (*Trichoderma asperellum*); Lane 7 and 8-TH (*T. harzianum*).

Table 1: Sequencing results for *Trichoderma* isolates with 100% maximum identity

Isolate no.	*Trichoderma* species	ITS Gene bank accession no.	References
TA002	*T. asperellum*	KX092001.1	Druzhinina *et al.* 2006
TB003	*T. brevicompactum*	KX092002.1	Zhang and Xian, 2010
TH004	*T. harzianum*	KX092003.1	Druzhinina *et al*., 2006
TV005	*T. virens*	KX092004.1	Druzhinina *et al.* 2006

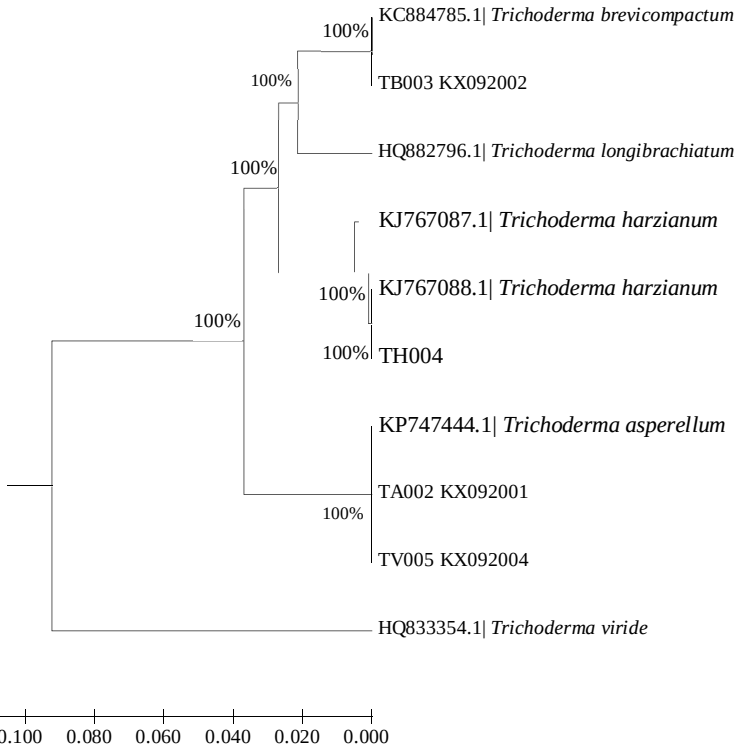

Fig. 2: Phylogenetic tree of *Trichoderma* spp resulting from the sequence results of ITS regions of the genomic rDNA gene and gene bank data using UPGMA analysis of Nei-Li distances. The scale bar indicates fixed nucleotide substations per sequence position.

Determination of different enzymatic activities and siderophore production of *Trichoderma* spp

The production of different types of hydrolytic enzymes by *Trichoderma* spp.was determined. The results were recorded based on the diameter (mm) produced in the enzyme detection media. The cellulose activities produced

by the tested *Trichoderma* spp.ranged from 35.7 to 55.5 mm with the maximum activity recorded by *T. asperellum*, followed by *T. virens* and *T. harzianum*, while the least actvity was recorded in *T. brevicompactum*. All the four species of *Trichoderma* were screened for their ability to hydrolyse chitin in a chitinase medium. The results of chitin hydrolysis showed varied responses. Chitinase activity was significantly higher ($p<0.05$) in *T. asperellum*, while *T. brevicompactum* produced the lowest chitinase activity on chitinase medium. Significant differences ($p<0.05$) was observed between *T. asperellum* and other species for cellulose and chitinase production. *Trichoderma asperellum* also significantly ($p<0.05$) produced the highest protease actvity, while the lowest was recorded in *T.brevicompactum*. Significant difference ($p<0.05$) in the glucanase activity was demonstrated by all the *Trichoderma* spp. tested. The highest glucanase activity was observed in *T. asperellum*, followed by *T. virens* and by *T. harzianum* also while the least glucanase actvity was observed in *T. brevicompactum.*

Table 2: *In vitro* antagonistic activity of *Trichoderma* spp. against *Ganoderma boninense*

Isolates	Percentage inhibition growth rate PIRG (%)	
	dual culture	culture filtrate
T. harzianum	81.1 ± 0.21^{b}	84.4 ± 1.80^{a}
T.asperellum	84.1 ± 1.37^{a}	83.8 ± 0.72^{a}
T. virens	78.7 ± 1.58^{c}	78.9 ± 3.25^{b}
T. brevicompactum	76.0 ± 2.10^{d}	71.3 ± 3.02^{c}

± =Standard deviation (SD), the mean values followed with the same superscript letter within the column were not significantly different ($P\leq 0.05$).

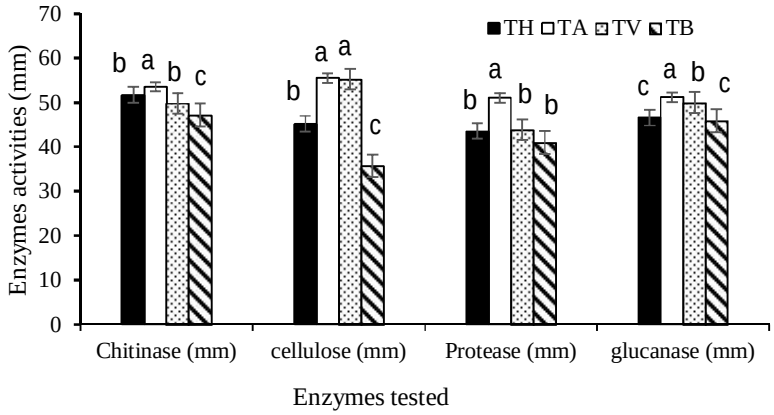

Figure 3: Activity profile of hydrolytic enzymes secreted by the antagonist *Trichoderma* spp. Error bars represent SE of the mean. Column with same letter above vertical bars are not significantly different ($p<0.05$).

In addition, the ability of *Trichoderma* spp to produce siderophore was also determined and the result showed varied levels of siderophore production, There were significant differences ($p<0.05$) in siderophore production among the *Trichoderma* spp tested, with the highest siderophore in *T. harzianum* (57.7 %), followed by *T. asperellum*(45.38%), *T. harzianum* (44.5%), and *T. brevicompactum* (35.88%) (Fig. 4).

DISCUSSION

Trichoderma spp. are mainly known as biological control agents with a wide range of applications in plant protection (Lorito *et al*., 2010). They act on other microbes, notably plant fungal pathogens (Chaverric and Samuels, 2013). In this study, the results demonstrated that *Trichoderma* spp. tested have the potential as biological control agents of *G. boninense* by significantly inhibiting the growth of the pathogen, *G. boninense*. All the species tested inhibited *G. boninense* growth by more than 70%. This effect could be most likely due to the production of antibiotic, secondary metabolite compounds or lytic enzymes which contributed to the direct antagonistic degrading effect on the *G. boninense* cell wall and mycoparasitic activities. Selection of *Trichoderma* spp. as biological control agents depends largely on their effectiveness in the *in vitro* results. The potential of *Trichoderma* in producing antibiotic and lytic enzymes capable of hydrolyzing the fungal pathogens cell wall contents has been reported (Vinale *et al*., 2013; Martinez-Medina *et al*., 2014). In addition, studies have shown that *Trichoderma* strains are capable of improving plant growth and disease resistance (Hermosa *et al*., 2012; Vos *et al*., 2015). In this study, dual culture and culture filtrate tests with *T. asperellum* and *T. harzianum* showed a remarkable PIRG rate of 80%. It could be argued that the *in vitro* antagonistic activity is not a property of a

particular *Trichoderma* spp, as different isolates within same species can exhibit varying potential of biocontrol (Anees *et al*., 2010). However, some researchers were of the view that results from dual culture and filtrate culture are not enough predictors of *Trichoderma* spp potential as biological control agents under field conditions (Martina-Medina *et al*., 2014; Saravankumar *et al*., 2016). Therefore, it is important to combine both *in vitro* and *in vivo* study to screen the biocontrol potential of *Trichoderma* spp. (Martinez-Medina *et al.*, 2014).

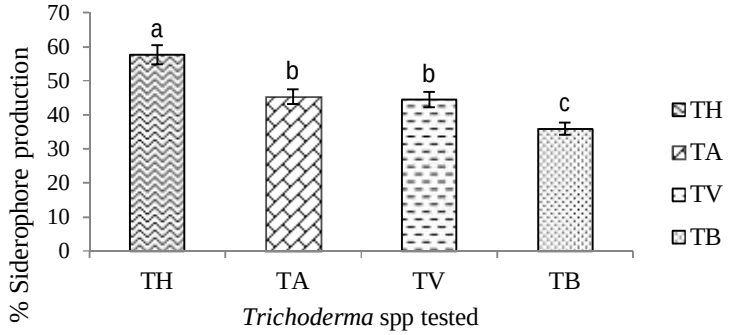

Fig. 4: Percentage of siderophore production by *Trichoderma* spp. Error bars represent SE of the mean. Column with same letter above vertical bars are not significantly different ($P \leq 0.05$). (TH- *Trichoderma harzianum*; TA = *Trichoderma asperellum*; TV = *Trichoderma virens* and TB = *Trichoderma brevicompactum*

The findings of this study demonstrated that *T. asperellum, T. harzianum* and *T. brevicompatum* have strong potential for the production of hydrolytic enzymes such as chitinase, cellulose, 1, 3, β-glucanase and protease. The production of these cell wall degrading enzymes could be responsible for the inhibition of *G. boninense* growth *in vitro*. These findings are in conformity with the previous research works (Naher *et al*., 2014; Yoeh *et al*., 2012). The production of extracellular cell wall degrading enzymes such as β-1,3 glucanase, cellulase, chitinase and protease by *Trichoderma* have an important role in the inhibition of the fungal pathogens (Gajera *et al.*, 2012). In addition, the enzymes could provide many benefits to the plants via interactions and degradation of pesticides, used in crop production (Vinale *et al.*, 2014). The cell wall degrading enzymes could hydrolyse or degrade the cell wall of fungal pathogens and inhibit their growth (Woo *et al.*, 2014). Reports also showed that some released cell wall degrading products are groups of the elicitors that induce Damage-Associated Molecular Pattern Molecules (DAMPs) effect against pathogen infection (Boller and Felix, 2009). The hydrolytic enzymes produced by Trichoderma spp. may be responsible for their antagonistic activities against plant pathogens. These results showed that *Trichoderma* spp. have good potential for enzymatic production. Nevertheless, the result indicated that *T. harzianum* championed in the production of siderophore among all the four species studied leaving *T. brevicompatum* as the least producer of siderophore. However in a study by Qi and Zhao, (2013) *T. asperellum* was identified as the most efficient species of *Trichoderma* in producing siderophore under salt stress condition and alleviating iron deficiency in plants. The ability of *Trichoderma* spp. to produce siderophore and its efficacy in phosphate solubilization does not only contribute to the plant growth-promoting attributes, but also stimulates plant resistance against biotic and abiotic stresses (Qi and Zhao 2013).

CONCLUSION

The four *Trichoderma* spp. identified as *T. asperellum, T. brevicompatum, T. harzianum and T. virens* based on the in molecular characters were found to inhibit *G. boninense in vitro* by producing an array of hydrolytic enzymes. Therefore, *Trichoderma* was identified with large potential effect as biocontrol agent against *G. boninense* causing *Ganoderma* disease of oil palm.

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