Pak. J. Bot., 49(3): 1193-1199, 2017.

EVALUATION OF THE EFFICACY AND MODE OF ACTION OF BIOLOGICAL CONTROL FOR SUPPRESSION OF *GANODERMA BONINENSE* IN OIL PALM

ARNNYITTE ALEXANDER[1], SYAHRIEL ABDULLAH[1], STEPHEN ROSSALL[2] AND KHIM PHIN CHONG[1,3*]

[1]Biotechnology Programme, Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia
[2]Division of Plant and Crop Sciences, School of Bioscience, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, LE12 5RD, United Kingdom
[3]FGV Chair on Sustainable Oil Palm Management, Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, Locked Bag No.3, 90509, Sandakan, Sabah, Malaysia
**Corresponding author's email: chongkp@ums.edu.my; Tel: +6088 320000 (ext 5565)*

Abstract

The ability of potential antagonists, a commercial product containing combinations of microorganisms (TR1) to control *Ganoderma boninense* growth was investigated in this research. TR1 contained multiple strains of *Bacillus* spp. and *Trichoderma* spp. The results from field experiments showed that TR1 was all able to reduce the colonization of *G. boninense*, based on re-isolation of the pathogen onto a selective medium and the reduction of ergosterol content compared to untreated controls. Effectiveness of TR1 was therefore further investigated for mode of action studies. Scanning Electron Microscopy (SEM) observations of *Ganoderma* mycelium, recovered from bioassay plates on which TR1 had inhibited fungal growth, showed that the mycelium was highly disrupted and lysed after exposure to the treatment. The production of potentially antifungal components produced by TR1 microbes in broth cultures was further investigated using Liquid Chromatography Mass Spectrometry (LCMS). Several antimicrobial compounds, which could inhibit *G. boninense* were detected, including pyrene-1,6-dione, 12-deoxyaklanonic acid, N-methyl-a-aminoisobutyric acid, 4-O-8',5"-5'-dehydrotriferulic acid, halstoctacosanolide A, N-acetyl-leu-leu-tyr-amide, 12-oxo-10Z-dodecenoic acid, Gly-Met-OH and lovastatin. These metabolites probably contribute to the antagonistic effect against *G. boninense*. The use of TR1 could offer an alternative to the use of fungicides and is worthy of further investigation for the control of *Ganoderma* infection of oil palm.

Key words: Biological control, Suppression, *Ganoderma boninense* , Palm.

Introduction

Ganoderma boninense is a fungus responsible for Basal Stem Rot (BSR) disease of oil palm. The disease is currently the most destructive disease in oil palm cultivation, especially in Malaysia and other countries in South-East Asia. Basal Stem Rot has been found to infect oil palms as early as one to two years after planting, with increased incidence in 4 to 5 year-old palms, particularly in replanted areas (Henson & Tayed, 2003), or in areas planted with coconut palm trees (Ambak & Melling, 1999). This disease can kill up to 80% of the stand by the time the palms are halfway through their economic life span (Bivi *et al*., 2010). The losses reported due to BSR were estimated to be $70 to 470 million a year in Malaysia (Arif *et al*., 2011). To date, numerous control strategies have been attempted for BSR, but none are completely effective.

Recently, an astute observation by Bivi *et al*. (2010) showed low incidence of BSR, in some natural stands, in areas where pathogenic *G. boninense* was present. This gave rise to assumption that the disease was most likely being kept under control by biological means. Therefore, some recent control programmes to overcome this disease have now been focused on the use of biological control agents (BCAs), and some studies have shown some early promises. Several potential antagonistic BCAs, including *Trichoderma* spp. (Sariah *et al*., 2005; Susanto *et al*., 2005), *Penicillium* spp. (Dharmaputra *et al.*, 1989), *Burkholderia* spp. (Sapak *et al*., 2008), *Bacillus* spp. (Suryanto *et al*., 2012), and *Pseudomonas* spp. (Bivi *et al*., 2010), have shown some efficacy in controlling *Ganoderma boninense* colonization and infection in both nursery and field conditions. The use of biological control agents offers an attractive alternative for the management of BSR disease in oil palms, without any negative impact on the environment. This is because biological agents colonize the rhizosphere and leave no toxic residues, when compared to fungicides (Dubey *et al*., 2007). Several mechanisms have been suggested as being responsible for the effects of BCAs. The effects are either directly or indirecty toward the plant pathogen. Direct effects include competition for nutrients or space, production of antibiotic and lytic enzymes (Chen *et al*., 2000; Ongena & Jacques, 2008), inactivation of the pathogen's enzymes and parasitism (El-Tarabily *et al*., 1997). Indirect effects include biochemical production and morphological alteration in the host plant. This can be observed in plant tolerance to stress via root enhancement and plant development, solubilisation or segregation of inorganic nutrients, and resistant induction (Viterbo *et al*., 2002).

Most of the studies on biological control of plant pathogens deal with single BCAs applied as an antagonist to a single pathogen. However, application of solitary BCAs may not be effective in all soils, as environmental requirements of differing microorganisms will vary. In addition, most cases of naturally occurring biological control probably results from mixed antagonist populations, rather than from high numbers of single antagonist. Mixtures of antagonists are also more stable and has wider spectrum of activity, enhancing the efficacy and reliability of biological control (Mishra *et al*., 2011). Therefore, the present study was conducted to study the effectiveness of combined microbes against *G. boninense*, in field and also *In vitro* experiments.

Material and Methods

Fungal isolate and growth: A pure culture of *G. boninense* was obtained from a stock culture at the Genetics Laboratory of Universiti Malaysia Sabah. The identity of *G. boninense* had been confirmed earlier using molecular technique as described by Chong *et al*. (2011). The isolate was sub-cultured and maintained at 25ºC on Potato Dextrose Agar (PDA).

Preparation of microbial treatment: The microbial product (TR1) tested in this study was commercially known as, Living Soil Microbes® manufactured by One Good Earth (M) Sdn Bhd. This product is commercially-produced in Malaysia as BCAs and is suited to local environmental conditions. Living Soil Microbes® is a commercial biofungicide formulation with broad spectrum of biocontrol activity. This was considered to be a more-viable approach than that which might be achieved by another study attempting to isolate the biological control equivalent of the 'silver bullet'. The product was applied following the standard operation procedure (SOP) as described by the manufacturer.

TR1 was prepared by mixing 800 mL of product with clean tap water to give a final volume of 40 L. The mixture was sprayed around the palms, with spraying radius of 2-3 m from the base of a palm using a high pressure (350 kPa) motorized knapsack sprayer with a walking speed of 50 m/min, and a nozzle output of 1.5 L/min. Each palm was sprayed approximately with 800 mL of TR1. The process was repeated until all 50 palms were treated.

Assessment on the effectiveness of microbial treatments in the field: Before the application of microbial treatments, a total of 400 mature palms (16 years of age), from areas with a high incidence of BSR disease, located at Genting Plantation, Sapi Nangoh, Sandakan, were screened for ergosterol content. This provides an indication of the initial level of colonization by *Gamoderma* (Chong *et al.*, 2014). A homogeneity test was carried out, using Statistical Programming for Social Science (SPSS), and from the original 400 palms, 100 palms were selected with very similar initial levels of ergosterol content. Fifty palms were treated with TR1, or left untreated as controls. Treated palms received monthly treatments for six months consecutively. Control palms were left untreated. The tree populations of 50 palms were distributed randomly within the plantation. Healthy palms (without ergosterol detected) were also selected for monitoring purpose throughout the project. The experimental strategy for the field trial was a completely randomized design (CRD), with fifty replicates for each treatment, randomly distributed over the plot.

Collection of trunk tissues: Tissues were collected from oil palm trunk at the height of 50 cm from the ground using a drill. The sampling procedure was as described by Chong (2012). The drill bit for collecting the tissues was sterilized using 70% ethanol for 30 s before drilling. The palms were first drilled to a depth of 2 cm to remove any unwanted, saprophytic fungi on the surface. The drill bit was then re-sterilized before further collection of tissues (approximately 10 cm into the trunk) at the same point. Collections of trunk tissues were done at 4 points on the same tree. Approximately 15-20 g of tissue samples were collected and placed into clean zip-locked plastic bags, which were transported to the laboratory in an ice-chest and stored at 0ºC before further use.

***Ganoderma* selective medium (GSM):** Tissues sample were cultured on *Ganoderma* Selective Medium (GSM) to detect the presence of *Ganoderma*. The media provides a useful tool for isolating *Ganoderma*, free from other contaminants. The content of fungicide and antibiotics in GSM eliminates growth of bacteria and other contaminating fungi, while allowing *Ganoderma* to thrive (Ariffin & Idris *et al*., 1992).

Evaluation of *G. boninense* fungal colonization using ergosterol analysis and quantification: Ergosterol is fungal sterol which is absent from vascular plants and other microbial cells. It degrades rapidly after fungal death and thus provides a good quantitative assessment of viable fungi. Ergosterol can therefore be used as an indicator to detect the presence of *Ganoderma* in oil palm tissues. Extraction of oil palm trunk tissue was carried out as described by Chong *et al*. (2011). Aliquots (500 g) of trunk tissue were harvested and homogenized using a commercial blender. Tissue sub-samples (100 mg) were extracted in methanol using a glass rod to disrupt the samples. Extracts were centrifuged at 25200 g for 5 min and the supernatant was made up to 1.5 mL, before being filtered through a 0.45 µm acetate syringe tip filter.

An Agilent Series 1200 Chromatography System, comprising a degasser G1313B, Quat Pump G131A, autosampler ALS G1329A with ChemStation for data manipulation software was used with an Agilent G1313B HPLC VWD detector for the analysis and quantification. A reverse phase Zorbax Eclipse XDB-C_{18} 4.6 mm x 250 mm (with 5 µm particle size) column was used for the separation. The UV detector was set to 282 nm wavelength, and the isolated peak, which eluted with a retention time of 7.0-8.0 min, was identified as ergosterol, based on its co-chromatography and identical absorption spectrum as a pure standard. The system was run isocratically with 100% methanol at flow rate of 1.5 mL/min. A serial dilution of the ergosterol standard, with a concentration range of 5-500 µg/ mL, was injected into the HPLC system to produce a calibration curve for ergosterol quantification.

***In vitro* antagonistic activity of TR1 product against *G. boninense*:** Antagonism bioassays between *Ganoderma* and TR1 were carried out using agar well diffusion assays. A mycelia plug was taken from the edge of a seven day old culture *G. boninense* and placed on the centre of the PDA Petri. Using a 6 mm cork borer, four wells were dug on the same PDA plate, 2 cm away from the *G. boninense* plug. Approximately 0.2 mL of the TR1 treatment mixture was then inoculated in each well. The plates were replicated five times and left at 20ºC for 2 h to allow diffusion from the wells to occur. They were then

incubated face upwards at this temperature for 12 d. The diameters of zones of growth were measured daily. Evaluation of the microbial antagonism against *G. boninense* was assessed based on the pathogen's growth.

Sample preparation for scanning electron microscopy (SEM): The zones of hyphal interaction between *Ganoderma* and potential BCAs used in the agar well diffusion assays were prepared for observation with SEM following the procedure described by Chaiyasut *et al*. (2010). Agar plates with fungal mycelia were first excised using scalpel and trimmed to approximately 10 mm x 10 mm in size, and as thin as possible, to reduce the moisture. The samples were dried at 60ºC for 3 h. Observations were performed by mounting the mycelia on carbon tape coated with gold-palladium in an Emitech K550x carbon coater for 1 min and viewed under a Zeiss EVO® 15LS SEM.

Broth bioassay: Approximately 10 mL of sterile Potato Dextrose Broth (PDB) was added to 15 mL Falcon tubes. The tubes were divided into three different groups for subsequent treatment; i) *Ganoderma* control (PDB containing *G. boninense* mycelia plug only (6 mm in diameter), ii) TR1 treatment (PDB containing 1 mL mixture of TR1 microbes only), iii) *Ganoderma* and TR1 treatment (PDB containing *G. boninense* mycelia plug (6 mm in diameter) and TR1 treatment (1 mL mixture of TR1 microbes). Each treatment was replicated three times and incubated for seven days in at 20ºC.

Extraction of potential metabolites from bioassay broths: Bioassay broths prepared as described above were collected and filtered through eight layers of sterile muslin to separate the mycelium from the mixture. The filtrates were then added to 5 mL of HPLC grade methanol to enhance solubility of some metabolites released from microbes during their growth. The mixture was vortexed, dried using a stream of purified nitrogen gas and weighed. The residue was re-dissolved in methanol and the final concentration adjusted to 10 mg/mL. Aliquots (30 µL) of the extracts were diluted with methanol to give a final volume of 1.5 mL. The diluted extracts were filtered with a 0.45 µm PTFE filter into clean HPLC vials, before LCMS profiling.

Liquid chromatography mass spectrometry (LCMS) profiling: LCMS analysis was carried out using an Agilent LC system coupled with Agilent Q-ToF Mass Spectrometry Electron Source Ionization (ESI) detector. The metabolites were identified using an online database (metlin.scripps.edu/metabolies_list). The condition of LC and MS were as described below;

LC conditions: Column: Nacalai Tesque, Cosnosil 5 C18-MS-11 Packed column; 1Pkg (2.1 x 50 mm, 1.8 µm), injection volume; 5 µL, Flow rate: 0.2 mL/min, mobile phase: HPLC water (A) and Acetonitrile (B); A/B= 20: 80 (0 min); 100:0 (10 min); 20: 80 (20 min).

MS/Q-ToF conditions: Accurate mass analysis was conducted with Agilent LC system coupled with Q-ToF system equipped with electrospray ionization positive ionization mode (ESI) probe. Highly accurate mass acquisition of the ion of interest was performed using chromatography injection. Mass spectrometric ionization conditions were as follows: Ion source: Dual ESI; Gas temperature: 300ºC; vacuum capillary; 3500 V; Capillary, 0.027 µA; Chamber, 3.92 µA. For samples analyzed in scan mode, the scan range was 100-1700 m/z with reference mass; 121.05 and 922.009 m/z.

Statistical analysis: Data were statistically analysed using one-way analysis of variance (ANOVA) and significant differences between treatments were detected using Duncan's Multiple-Range Test (DMRT), meanwhile, non-parametric data was analyzed using Kruskall-Wallis test followed by Mann-Whitney test at 5% significant level respectively. Analyses used the Statistical Package for Social Sciences (SPSS) version 19.

Results

***Ganoderma* colonization of oil palm in a field experiment:** Out of the pool of 100 palms infected by *Ganoderma* (with similar level of ergosterol content), 50 palms each were either untreated or treated with TR1. The disease incidence (determined using isolation on GSM) and ergosterol content at the start of the experiment as shown in Figure 1.

Selected palms were treated with TR1 for six months consecutively. After this treatment period the palms were assessed for infection (Fig. 1). In untreated control palms, the Disease Incidence (DI) was maintained at 100% for the duration of the experiment and the ergosterol content of the tissues more than double, which was from 3.478 µg/g to 8.554 µg/g . However, TR1 treated palms showed a significantly recovery, from 100% infection, down to only 12% found infected at the end of observation. This treatment also effectively reduced the amount of ergosterol by 81%, from 3.425 µg/g to 0.663 µg/g of that found before treatment. The concentration of ergosterol in infected, non-treated palms more than double.

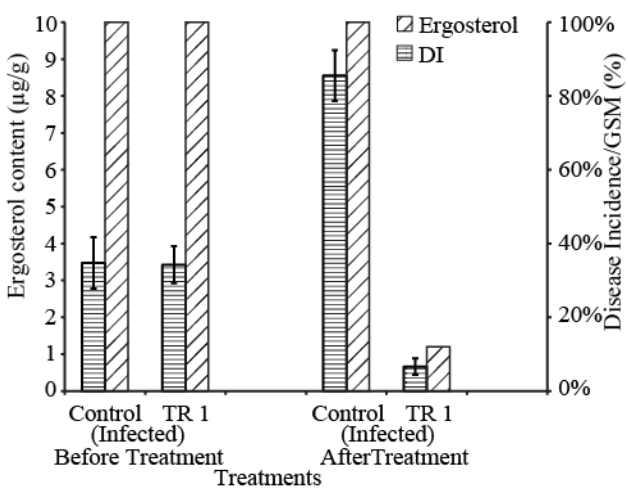

Fig. 1. Quantification of ergosterol content and percentage of Disease Incidence (DI) of palms in field trial before and after BCA treatments. Each treatment has 50 replicates. Each interval was analyzed using one-way ANOVA independently. No ergosterol detected in healthy (Control) seedlings. Bars show S.E of the means.

Inhibition of *Ganoderma* by TR1 product in a well diffusion agar assay: Based on the results from the field experiments, the mechanism of action against *Ganoderma* of TR1 was investigated. The results show that TR1 microbes exhibited a strong inhibitory effect against *G. boninense* on PDA, with the fungal final growth of 2.4 ± 0.173 cm, compared to control growth of 8 ± 0 cm, after twelve days incubation at 28±2ºC. The morphology of *G. boninense* growth is illustrated in Figure 2, where suppression of growth and mycelia formation on the portion of the plate antagonized by TR1 clearly observed after six and twelve days of incubation.

Morphology changes in *G. boninense* after exposure to TR1: SEM images of *G. boninense* were prepared from samples of mycelia excised from the interaction zone in the agar well diffusion assay. Severe morphological abnormalities in hyphal structure of the *Ganoderma* were observed in mycelia challenged with TR1, when compared to the control. Healthy, dense and branched mycelium of *G. boninense* is shown in Figure 3 (a). However, the hyphae, when challenged with TR1, were entirely covered and colonized by spores of *Trichoderma* spp. (Fig. 3 (b)). TR1 caused the hyphal structure of the pathogen to become highly disrupted, disaggregated, flattened and shrivelled to a looser mass (Fig. 3 (c) and (d)). The damage to the mycelium structure may eventually inhibit the growth of *G. boninense*.

Identity of metabolites associated with *Ganoderma*-TR1 interaction: To separate and identify metabolites involved in *Ganoderma*-TR1 interaction, LCMS studies were carried out. LCMS identified metabolites from the three combinations (*Ganoderma*, TR1, *Ganoderma* plus TR1). These were further selected based on published reports of antimicrobial activity.

Based on the selected metabolites from the three combinations (*Ganoderma*, TR1 and *Ganoderma* plus TR1), several metabolites were found to be present in all combinations and some were present only in particular treatments. A summary of the major antimicrobial compounds detected in the three culture filtrates is given in Table 1. Some compounds were produced by *Ganoderma* and may have a role in allowing this fungus to compete in the infection court. However, some metabolites are produced by TR1 or by the interaction between TR1 and *Ganoderma* and may have a role to play in biocontrol. Specifically, these compounds are: N-methyl-a-aminoisobutyric acid, pyrene-1,6-dione, 12-deoxyaklanonic acid, 4-O-8',5"-5'-dehydrotriferulic acid, halstoctacosanolide A, N-acetyl-leu-leu-tyr-amide, 12-oxo-10Z-dodecenoic acid, Gly-Met-OH and lovastatin. The active compounds are chemically-diverse in nature.

Discussion

In recent years, research on biological control has gained worldwide interest for controlling plant pathogens. In the current study, a commercial product based on combinations of several microbes (designated TR1), was found to be effective against *G. boninense.* This observation was based on the reduction of ergosterol accumulation in infected palm roots and absence of *G. boninense* growth on GSM. Biocontrol proved effective in field experiments and provided curative effects. Plantation crops, especially those comprising large trees, are not readily amenable to field evaluation, but in the large-scale study reported here the effects of a commercial product tested was very encouraging. Notwithstanding the technical issues of working in commercial plantations, it is recognised that it would be interesting to repeat the field work in different locations of SE Asia where BSR is a problem. Applications of combinations of microorganisms are likely to provide more stable and effective biological control in diverse environments, compared to the use of single organism. In this study the treatment designated TR1 (a combination of *Bacillus* spp. and *Trichoderma* spp.) was effective in controlling the *G. boninense* growth, in field trials. This may reflect possible synergistic effects between the two components in the TR1 treatment. The biological control attributes of both *Bacillus* spp. and *Trichoderma* spp. have been widely documented. Control of fungal pathogens can be brought about by many mechanisms, including production of antibiotic metabolites, competition for space and nutrients, mycoparasitism and production of lytic enzymes (El-Tarabily *et al*., 1997; Shahraki *et al*., 2009; Heydari & Pessarakli, 2010). Thus control of *G. boninense* by TR1 is likely to involve several antifungal processes and be more stable than suppression afforded by a single organism.

The antagonistic activity of TR1 on *G. boninense* was clearly demonstrated in the agar well diffusion assay which showed inhibition of *G. boninense* mycelial growth from day 2 of incubation until the end of the experiment. SEM observation on the morphology of *G. boninense* hyphae in this assay showed spores of *Trichoderma* adhered to fungi hyphae. This can be followed by germination of the spores of the mycoparasite and establishment of a successful parasitic interaction (Kubicek & Harman, 1998). When spores germinated, they utilized the contents of the host hyphae as a nutrient source and parasitized the host. Lectins present in the cell wall of the host are suggested to play a major role in the recognition of hyphae by *Trichoderma* spp. (Motlagh & Samimi, 2013).

The inhibition of *G. boninense* observed may have resulted from the production of non-volatile inhibitory metabolites by components of TR1, which diffused into the agar medium, thus causing inhibition of *Ganoderma* growth. The fatty acid (12-oxo-10z-dodecenoic acid), with possible antifungal properties, was detected in the interaction. The ability of free fatty acids (FFA) to kill or inhibit fungal growth is by interfering with cellular energy production, inhibition of enzyme activity and direct lysis of fungal cells (Desbois & Smith, 2010). Such properties of FFAs are likely to cause the lysis of *Ganoderma* mycelium, as observed in this study. Based on LCMS profiles, two polyketide compounds were also detected, Lovastatin and Halstoctacosanolide A. Lovastatin, also named as Monacolin K, is usually produced by *Trichoderma* spp. and the metabolic pathway targeted by this compound is sterol synthesis, which prevents cellular membrane formation, stops fungal growth and may suppress sporogenesis in established infection agents (Dubey *et al*., 2011). Halstoctacosanolide A is a secondary metabolite usually produced by *Streptomyces* spp. However, detection of this metabolite in both TR1 and combined treatments suggests this antifungal molecule can also be produced by components of the TR1 inoculum.

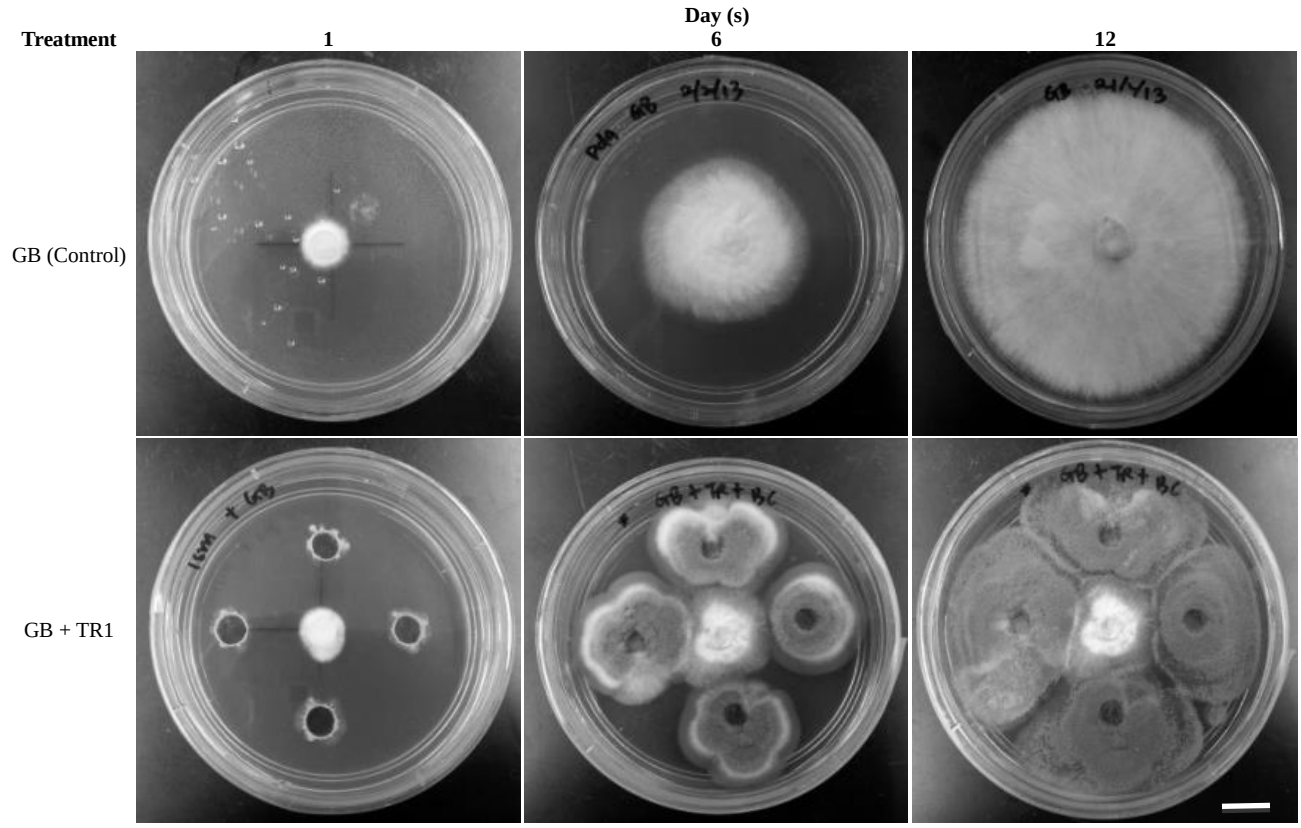

Fig. 2. Antagonistic assay of *G. boninense* on PDA medium with or without the presence of TR1 at 1, 6 and 12 days of incubation. GB denotes for *G. boninense*; GB+TR1 denoted for *G. boninense* cultured with Treatment 1. Bar size: 2 cm

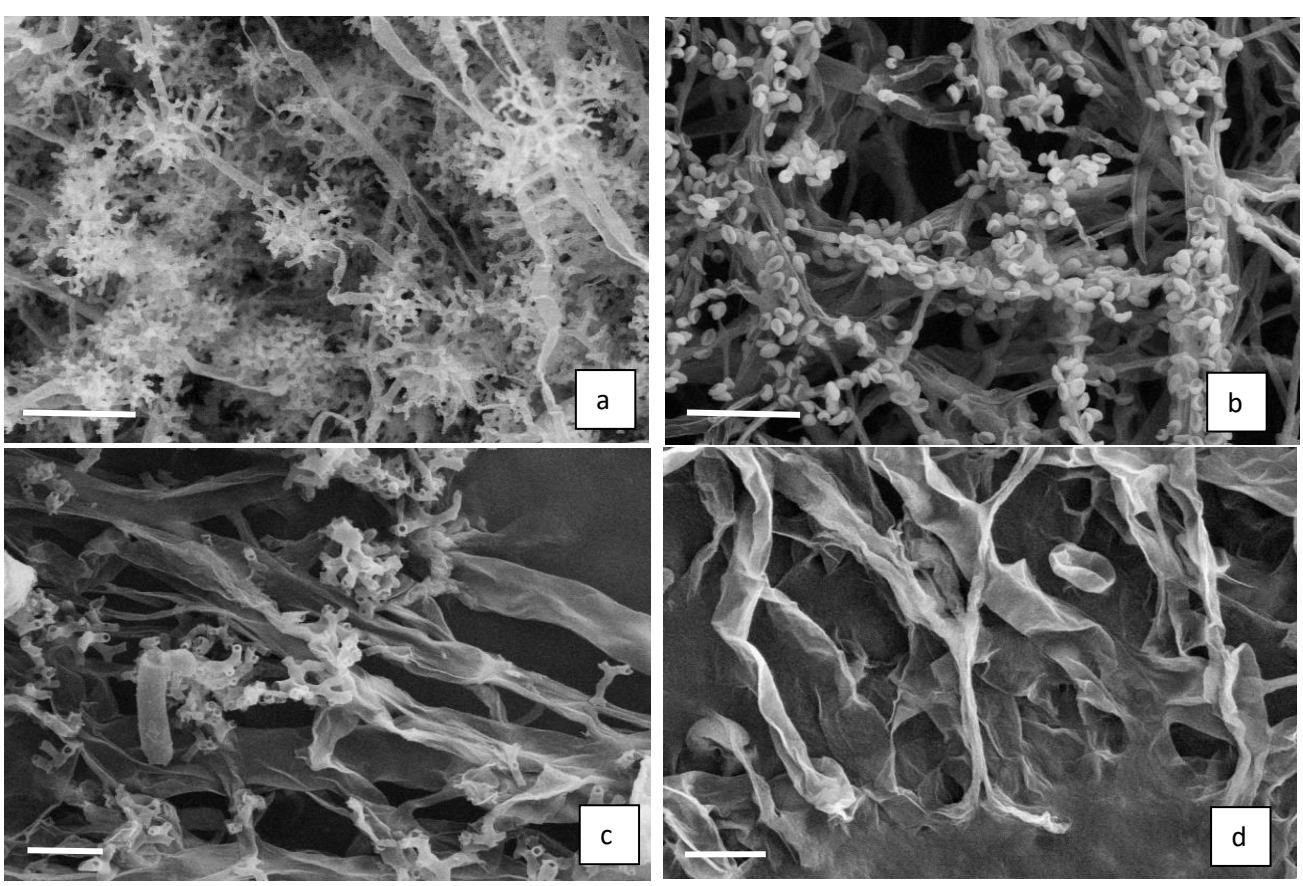

Fig. 3. Antagonistic effects of TR1 on *G. boninense* mycelium in agar well diffusion assay at day 12. (a) The healthy and dense branching mycelium of *G. boninense* from a control plate cultured in the absence of TR1. (b) Spores of *Trichoderma* spp. from TR1 attached to *G. boninense* hyphae. (c) Hyphal branches of *G. boninense* were highly disrupted and disaggregated in the presence of TR1. (d) Highly shrivelled and flattened/lysis mycelium of *G. boninense* in the presence of TR1. Scale bars: (a)-(b): 10 µm. (c)-(d): 2 µm.

Table 1. Comparison of compounds produced by GB, TR1 and GB-TR1 interaction based on LCMS.

Compounds detected based on LCMS		
GB	**TR1**	**GB-TR1**
- 4-chloro-3,5-dimethoxybenzyl alcohol	- 4-chloro,3,5-dimethoxybenzyl alcohol	- 4-chloro-3-5-dimethoxybenzyl alcohol
- alpha, alpha-trehalose 6-palmitate	- alpha, alpha'-trehalose 6-palmitate	- alpha, alpha'-trehalose 6-palmitate
- triterpenoid		- titerpenoid
- tetracenomycin D3		- tetracenomycin D3
- L-valine		
- 16-feruloyloxypalmitate		
	- pyrene-1,6-dione	- pyrene-1,6-dione
	- 12-deoxyaklanonic acid	- 12-deoxyaklanonic acid
	- N-methyl-a-aminoisobutyric acid	- N-methyl-a-aminoisobutyric acid
	- 4-O-8',5"-5'-dehydrotriferulic acid	- 4-O-8',5"-5'-dehydrotriferulic acid
	- halstoctacosanolide A	- halstoctacosanolide A
	- N-Acetyl-leu-leu-tyr-amide	- N-acetyl-leu-leu-tyr-amide
	- Ala-Trp-OH	
	- Lys Tyr Leu	
		- 12-oxo-10Z-dodecenoic acid
		- Gly-Met-OH
		- lovastatin

Note: GB indicate for *G. boninense* and GB-TR1 indicate for *G. boninense* incorporated with TR1

Two compounds of peptide group, gly-met-OH and N-acetyl-leu-leu-tyr-amide were also detected in TR1 and TR1 plus *Ganoderma* treatments, suggesting that those peptides were produced by TR1 microbes. *Bacillus* spp. are known to produce many potent antimicrobial peptides. Study by Kwak *et al.* (2014), demonstrated that peptide compounds extracted from *Lactobacillus* showed remarkable antifungal activity aganist *G. boninense*. Such peptides can spontaneously insert themselves into membranes and thus interact with the pathogen to increase their membrane permeability. This is either by the effect of their positive charge on negatively charged target membrane lipids or by membrane destabilization through lipid rearrangements, due to the enormous changes in the net charge of the composed system (Marshall & Arenas, 2003). It is therefore suggested that the mechanism of action of these metabolites may be attributed to the disruption and disaggregation of *G. boninense* hyphae as seen in Figure 3 (c).

N-methyl-a-aminoisobutyric (Aib) acid, a peptaibol compound, is also produced mainly by the *Trichoderma* genus. Such compounds are secondary metabolites, which function as antimicrobials against both bacteria and fungi. They forms pores in bilayer membranes, making them leaky and leading to the death of the cells. A phenolic compound, 4-O-8',5"-5"-dehydrotriferulic acid was also detected and was produced by TR1 microbes. *In vitro* studies on the effect of phenolic compounds, such as syringic acid, caffeic acid and 4-hydroxybenzoic acid extracted from oil palm roots also showed that these compounds have significant fungitoxic effect towards *G. boninense* at a concentration of 0.5 mg mL^{-1} (Chong *et al*., 2009). It is suggested that phenolic compounds inhibit *Ganoderma* growth through different mechanisms involving the inhibition of extracellular fungal enzymes, inhibition of fungal oxidative phosphorylation, nutrient deprivation (metal complexation, protein insolubilisation), and antioxidant activity (Scalbert, 1991).

Quinones and anthraquinones are well known antimicrobial compounds. Such molecules were also produced by TR1. Quinones have the ability to enter cell membranes via passive transport, due to their molecular size and the permeability of the hydrophilic side of the bilayer lipid membrane. They are known to form irreversible complex with protein nucleophilic amino acids, leading to protein deactivation and loss of function (Stern *et al*., 1996). Anthraquinones are known to be one of the antifungal metabolites produced by *Trichoderma* spp. and are usually produced in a small quantities (Reino *et al.*, 2008). However, an interesting study by Dervilla & Sheridan (1989) found that *Trichoderma polysporum*, when challenged with *Heterobasidium annosum*, produced higher amount of anthraquinone metabolites, which had antagonistic effect on *H. annosum*. Similarly, the current study also detected greater amounts of 12-deoxyaklanonic acid, an anthraquinone compound, after the interaction between *Ganoderma* and TR1. It is suggested that the mechanism of this anthraquinone compound against *Ganoderma* is similar to that reported in the study by Stern *et al*. (1996).

Thus antimicrobial metabolites produced by the microbes in TR1 have probably contributed to the activity against *Ganoderma*. The production of secondary metabolites from TR1 microbes may act synergistically with the production of cell wall degrading enzymes from both biological components. These include chitinses, and glucanases that breakdown polysaccharides, chitins and ß-glucans, thereby destroying the pathogen cell wall integrity (Devaki *et al*., 1992; Elad, 2000). Production of such enzymes could also explain the observation of severe morphological abnormalities, with shrivelling and distortion of the fungal hyphae observed under SEM. Thus, the well-documented ability of *Trichoderma* and *Bacillus* species to produce a number of inhibitory metabolites, as well as some fungal cell wall degrading enzymes, may be involved in the growth inhibition of *G. boninense* by TR1.

In conclusion, combinations of microbial antagonists could provide a stable, versatile biocontrol agent against *G. boninense*, which could be worthy of commercial exploitation. The use of BCAs also offers an environmentally safe mean of managing oil palm BSR disease, and may provide an alternative to chemical

fungicides, which are often expensive and may cause adverse effects on the environment. It would be interesting to explore the possibility that BCAs could become established in oil palm plantation ecosystems and provide long-term antagonism towards *Ganoderma*.

References

Ambak, K. and L. Melling. 1999. Management practices for sustainable cultivation of crop plants on tropical peatland. In: *Proceedings of the International Symposium on Tropical Peatlands*. Bogor, Indonesia.

Arif, M.S., A. Roslan and A.S. Idris. 2011. Economics of OP pests and *Ganoderma* disease and yield losses. In: *Proceedings of the Third MPOB-IOPRI International Seminar: Integrated OP Pests and Diseases Management*. Kuala Lumpur Convention Centre, Kuala Lumpur, Malaysia.

Ariffin, D. and A.S. Idris. 1992. The *Ganoderma* Selective Medium (GSM). *PORIM Information Series no.8.* Palm Oil Research Institute of Malaysia, Malaysia.

Bivi, M.R., M.S.N. Farhana, A. Khairulmazmi and A. Idris. 2010. Control of *Ganoderma boninense*: A causal agent of basal stem rot disease in oil palm with endophyte bacteria *In vitro*. *Int. J. Agric. Biol.*, 12:833-839.

Chaiyasut, C., C. Krutama and S. Sirilun. 2010. Breaking the spores of *Ganoderma lucidum* by fermentation with *Lactobacillus plantarum*. *Afr. J. Biotechnol.*, 9(43): 7379-7382.

Chen, C., R.R. Belanger, N. Benhamou and T.C. Paulitz. 2000. Defense enzymes induced in cucumber roots by treatment with plant growth promoting rhizobacteria (PGPR) and *Pythium aphanidermatum*.*J. Physiol. Mol. Plant Path.*, 56: 13-23.

Chong, K.P. 2012. An evaluation of the *Ganoderma* fungal colonisation using ergosterol analysis and quantification. *The Planter*, 88(1034): 311-319.

Chong, K.P., C.P. Foong, C.M.V.L. Wong, S. Rossall and M. Atong. 2011. First identification of *Ganoderma boninense* isolated from Sabah based on PCR and sequence homology. *Afr. J. Biotechnol.*, 10(66): 14718-14723.

Chong, K.P., P.A. Eldaa and J. Dayou. 2014. Relation of *Ganoderma* content to basal stem rot disease severity index. *Adv. Environ. Biol.*, 8(14):14-19 .

Chong, K.P., S. Rossall and M. Atong. 2009. *In vitro* synergy effect of syringic acid, caffeic acid and 4-hydroxybenzoic acid against *Ganoderma boninense*. *Int. J. Eng. & Technol.*, 1(4): 282-284.

Dervilla, M.X.D. and M. Sheridan. 1989. Anthraquinones from *Trichoderma polysporum*. *J. Agri. Food Chem.*, 25(10): 7288-7292.

Desbois, A.P. and V.J. Smith. 2010. Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. *Appl. Microbiol. Biotechnol.*, 85(6): 1629-1642.

Devaki, N.S., S.S. Bhat, S.G. Bhat and K.R. Manjunatha. 1992. Antagonistic activities of *Trichoderma harzianum* against *Pythium aphanidermatum* and *Pythium myriotylum* on tobacco. *J. Phytopathol.*, 136: 82-87.

Dharmaputra, O.S., H.S. Tjitrosomo and A.L. Abadi. 1989. Antagonistic effect of four fungal isolates to *Ganoderma boninense*, the causal agent of basal stem rot of oil palm. *J. Biotropical*, 3: 41-49.

Dubey, S.C., M. Suresh and B. Singh. 2007. Evalution *Trichoderma* species against *Fusarium oxysporum* f. sp. *ciceris* for integrated management of chickpea wilt. *Biol. Control.*, 40: 118-127.

Dubey, S.C., T. Aradhika, P. Dureja and G. Anita. 2011. Characterization of secondary metabolites and enzymes produced by *Trichoderma* species and their efficacy against plant pathogenic fungi. *Indian J. Agr. Sci.*, 81: 455-461.

Elad, Y. 2000. Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Protection*, 19: 709-714.

El-Tarabily, K.A., G.E.St.J. Hardy, K. Sivasithamparam, A.M. Hussein and D.I. Kurtböke. 1997. The potential for the biological control of cavity-spot disease of carrots, caused by *Phytium coloratum*, by streptomycete and non-streptomycete actinomycete. *New Phytologist*, 137: 495-507.

Henson, I.E. and D. Tayed. 2003. Physiological analysis of an oil palm density trial on a peat soil. *J. Oil Palm Res.*, 15(2): 1-27.

Heydari, A. and M. Pessarakli. 2010. A review on biological control of fungal plant pathogens using microbial antagonists. *J. Biol. Sci.*, 10: 273-290.

Kubicek, C.P. and G.E. Harman. 1998. *Trichoderma and Gliocladium. Vol. 1. Basic Biology, Taxonomy and Genetics*. Taylor & Francis, London.

Kwak, M.K., R. Liu, M.K. Kim, D. Moon, A.H. Kim, S.H. Song and S.O. Kang. 2014. Cyclic dipeptides from lactic acid bacteria inhibit the proliferation of pathogenic fungi. *Microbiol.*, 52(1): 64-70

Marshall, S.H. and G. Arenas. 2003. Antimicrobial peptides: A natural alternative to chemical antibiotics and a potential for applied biotechnology. *Electron. J. Biotech.*, 6(2): 0717-3458.

Mishra, D.S., A.K. Gupta, C.R. Prajapati and U.S. Singh. 2011. Combination of fungal and bacterial antagonists for management of root and stem rot disease of soybean. *Pak. J. Bot.*, 43: 2569-2574.

Motlagh, M.R.S. and Z. Samimi. 2013. Evaluation of *Trichoderma* spp. as biological agents in some of plant pathogens. *Ann. Biol. Res.*, 4(3): 173-179.

Ongena, M. and P. Jacques. 2008. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol.*, 16(3): 115-125.

Reino, J.L., R.F. Guerrero, R. Hernandez-Galan and I.G. Collado. 2008. Secondary metabolites from species of the biocontrol agent *Trichoderma*. *Phytochem. Rev.*, 7: 127-132.

Sapak, Z., S. Meon and Z.A.M. Ahmad. 2008. Effect of Endophytic Bacteria on Growth and Suppression of *Ganoderma* Infection in Oil Palm. *Int. J. Agric. Biol.*, 10: 127-132.

Sariah, M., C.W. Cjoo, H. Zakaria and M.S. Norihan. 2005. Quantification and characterization of *Trichoderma* spp. from different ecosystems. *Mycopathologia*, 159: 113-117.

Scalbert, A. 1991. Antimicrobial properties of tannins. *Phytochemistry*, 30: 3875-3883.

Shahraki, M., A. Heydari and N. Hassanzadeh. 2009. Investigation of antibiotic, siderophore and volatile metabolites production by *Bacillus* and *Pseudomonas* bacteria. *Iran J. Biol.*, 22: 71-85.

Stern, J.L., A.E. Hagerman, P.D. Steinberg and P.K. Mason. 1996. Phlorotannin-protein interactions. *J. Chem. Ecol.*, 22:1887-1899.

Suryanto, D., R.H. Wibowo, E.B. Siregar and E. Munir. 2012. A possibility of chitinolytic bacteria utilization to control basal stem disease caused by *Ganoderma boninense* in oil palm seedling. *Afr. J. Microbiol. Res.*, 6(9): 2053-2059.

Susanto, A., P.S. Sudharto and R.Y. Purba. 2005. Enhancing biological control of basal stem rot disease (*Ganoderma boninense*) in oil palm plantations. *Mycopathology*, 159: 153-157.

Viterbo, A., M. Montero, O. Ramot, D. Friesem, E. Monte, A. Llobell and I. Chet. 2002. Expression regulation of the endochitinase *chit36* from *Trichoderma asperellum* (*T.harzianum* T-203). *Curr. Genetic*, 42: 114-122.

(Received for publication 23 March 2016)