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Combination of Biological Agents in Suppressing Colonization of *Ganoderma boninense* of Basal Stem rot

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ABSTRACT

Basal Stem Rot (BSR) is the most destructive disease of oil palm caused by *Ganoderma boninense*. With no remedy to date, a study on the potential of microbes in suppressing colonization of *G. boninense* was designated. Three products contain combinations of Biological Control Agents (BCAs), (designated TR1, TR2 and TR3) to suppress the growth of *Ganoderma boninense* was investigated in this research. To understand the ability of the treatments in suppressing the BSR disease incidence, assessments in nursery and field trial were conducted. The results from both trials showed that TR 1, TR 2 and TR 3 were able to reduce the colonization of *G. boninense* based on the reduction of ergosterol content and Disease Incidence (DI) compared to untreated control. However, in nursery trial, treated seedlings showed an increment in DI after four months with lesser colonization based on the ergosterol quantities. Meanwhile, assessment in the field trial showed that TR 1 and TR 3 had significantly reduced the DI down to 12% and 24% and the amount of ergosterol to 0.663 µg g^{-1} and 1.817 µg g^{-1} of trunk tissues respectively. The use of BCAs could offer an alternative to control the *Ganoderma* infection in oil palm.

Keywords: oil palm, *Ganoderma boninense*, Biological Control Agents, Ergosterol

INTRODUCTION

One of the major diseases contributes to huge losses in oil palm industry is the Basal Stem Rot (BSR) which caused by *Ganoderma boninense*. In earlier years, this disease was reported to affect only mature palms aged 30 years and above, however, infected palms as young as 12-24 months after planting was also detected later [20]. A number of different strategies has been employed to manage the disease but none has shown satisfactory effect. Some recent control measures to overcome this disease are now focused on the use of biological control agents (BCA). Several promising antagonist BCAs such as *Trichoderma* spp. [19,22], *Penicillium* spp. [8], *Burkholderia* spp. [18], *Bacillus* spp. [21] and *Pseudomonas* spp. [4] have shown high efficacy in controlling the growth and infection of *Ganoderma boninense* both in nursery and field. Several mechanisms have been suggested to be responsible for the effects of these BCAs, these include competition for space and nutrients, mycoparasitism, metabolite production, antibiosis and inducing plant systemic resistance [26].

However, effective suppression of plant disease by BCAs is largely affected by environment conditions [10]. Application of single bioagent can have limitation with regard to its consistency and efficacy in different environments. Therefore, more emphasis is laid on the combined use of biocontrol agents, for improved disease control and also to overcome the inconsistent performance of the BCA. Introduction of one or more BCAs to the soil, assuming that each has different ecological requirements, may facilitate disease control without affecting the efficacy of a single organism under diverse conditions, and may result in increased control consistency [10]. Cocktails of biocontrol agents may have advantages of broad spectrum activity, enhancing the efficacy and reliability of the biological control and they communicate with each other to maximize biocontrol efficacy. Therefore, in the present study, combination of several microbes in three microbial based products was evaluated for

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the control of the *G. boninense* colonization in oil palm under nursery and field environment.

Materials and Methods

2.1. Cultivation of oil palm seedlings:

A hundred certified disease free pre-germinated oil palm seeds (AVROS D x P) were purchased from Sawit Kinabalu Sdn. Bhd. Seeds were grown in 13' x 15' cm black polythene bags in Peat Vriezenveen substrate, Product of Holland. The seedlings were maintained in field house with 50% shade at Universiti Malaysia Sabah and watered daily. Granular NPK fertilizer 15:15:6:4 was applied every two weeks with approximately three grams per each seedling. Seedlings were arranged randomly among treatments.

2.2. Artificial inoculation of G. boninense on oil palm seedlings:

Artificial inoculation was conducted as described by Chong *et al*., 2012. A fully grown 14-days old *G. boninense* culture was used to inoculate the oil palm seedlings. Mycelium of *G. boninense* which covered the entire surface of 9 cm Petri dish was scrapped off and suspended in 20 mL of Potato Dextrose Broth (PDB). Tween 20 (50 µL) was added into the mycelia suspension and blended in a commercial blender to get a homogenous mixture. Six months old oil palm seedling planted in peat were carefully uprooted and sprayed with the mycelia suspension with approximately 18 mL in volume and replanted back to the peat.

2.3. Preparation of microbial treatments (for nursery trial):

Three microbial products (TR1, TR2 and TR3) were tested in this study;

TR1; Living Soil Microbes® product of One Good Earth (M) Sdn Bhd

TR2: High Technology Yield® product of Agrinos (M) Sdn Bhd

TR3; Agriorganica® product of Organica Biotech Sdn Bhd

All products are commercially-produced in Malaysia as BCAs and suited to local environmental conditions. Each product was applied to oil palm following the standard operation procedure (SOP) of respective company.

a. Treatment 1 (TR1):

This treatment contained a combination of *Bacillus* spp. and *Trichoderma* spp. TR1 was prepared by mixing 4 mL of the material with 50 mL clean tap water. Aliquots (50 mL) of this suspension were applied to the surface immediately surrounding the seedlings.

b. Treatment 2 (TR2):

This treatment consists a combination of *Bacillus* spp., *Pseudomonas* spp. and *Aspergillus* sp. TR2 was prepared by mixing 2 mL of the combined product, 2 mL of L-amino acid complex, 2 mL of chitin and 2 g of amino acid powder into 200 mL of chlorine-free water. Approximately 200 mL of the mixture was applied to the seedlings.

c. Treatment 3 (TR3):

TR3 consists of three food-associated microorganisms, *Lactobacillus, Nattobacillus* and *Saccharomyces cerevisiae.* TR3 was prepared by mixing 2 mL of the material with 2 g of humic acids and adding to 50 mL of clean tap water. Approximate 20 mL of the mixed suspension was sprayed directly onto the peat surface and the seedlings.

2.4. Application of BCAs to reduce Ganoderma colonization of infected seedlings:

Oil palm seedlings were first inoculated with G. boninense as described by Chong *et al*., [7], incubated for two months in the field house, to allow successful infection to be developed, and then treated with TR1, TR2 and TR3 as described above. Treatments were applied monthly for four months consecutively. Infection was assessed every two months, which was two months after the first inoculation with BCAs, followed by four months after the BCAs.

2.5. Collection of root tissues:

The infected oil palm seedling roots were uprooted carefully from the growing medium. The uprooted roots were washed and cleaned under running tap water to remove any unwanted materials. The cleaned roots were then surface sterilized by immersing them into pure ethanol (99%) for 1 minute and air dried. This step will remove any saprophytic fungi on the roots' surface. The roots were then cut into smaller pieces of about 5 cm long, homogenized in a commercial blender [7] and placed into clean zip-locked plastic for further use.

2.6. Collection of trunk tissues:

Mature oil palm tissues samples were collected from high incidence of Basal Stem Rot disease areas located at Genting Plantation in Sabah. The tissues were collected from oil palm at the height of half metre (approximately 50 cm) from the ground using a power driller. Sampling procedure was done according to Chong [7]. The drill bit for collecting the tissues was first sterilized using 70% ethanol for 30 seconds before proceeding to drill. The palms were first drilled for 2 cm depth to remove any unwanted or possible saprophytic fungi on the surface. The drill bit was later re-sterilized again in 70% ethanol before further collection of tissues (approximate 10 cm into the trunk) at the same point. Collections of trunk tissues were done at 4 points of the same tree with 90º to each other. Approximate

15-20 g of tissues samples were collected and placed into a clean zip-locked plastic, which was then transported to the laboratory in an ice-chest and stored at 0ºC cold room for further use. Tissues were collected before and after the microbial treatments.

2.7. Evaluation of G. boninense fungal colonization by ergosterol analysis and quantification:

Extraction of oil palm seedlings roots were carried out as described by Chong *et al*. [6]. Oil palm roots were harvested and homogenized using a commercial blender. Roots samples (100 mg) were extracted in methanol using glass rod to physically crush the sample. The extract was centrifuged at 15000 rpm for 5 min and the supernatant was made up to 1.5 mL before filtered through a 0.45 µm acetate syringe tip filter. The same procedures were used for extraction of trunk tissues. An Agilent Series 1200 Chromatography System was used 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^{-1}. The presence of *Ganoderma* in the tissues was also re-confirmed with *Ganoderma* Selective Media as described by Ariffin and Idris [1].

2.8. Preparation of microbial treatment (for field trial):

Only two products (TR1 and TR3) were tested for field. The products containing the same content as described previously were prepared following their SOP respectively.

a. TR 1 :

TR 1 was prepared by mixing 400 mL of *Bacillus* mixture and 400 mL of *Trichoderma* mixture into tap water until the final volume was 40 L. The mixture was sprayed around the palms with spraying radius of 2-3 m from the base using a high pressure (350 kPa) motorized knapsack sprayer at 50 m min^{-1} walking speed. With nozzle output 1.5 L min^{-1}, each palm was sprayed approximately 800 mL of the mixture. The step was repeated until all 50 palms were treated.

At four months (Figure 2), seedlings were harvested for quantification of ergosterol content and root tissues were also plated on GSM. Disease incidence of all treatments ;TR 1, TR 2 and TR 3 showed an increment up to 80%, 90% and 70% respectively after four months of treatments. However, there was a significant difference in amount of ergosterol among the TR 1, TR 2 and TR 3 treatment. TR 1 was the most effective treatment in reducing the ergosterol colonization compared to TR 2 and TR 3, while TR 3 was the least effective. The ergosterol content in Control infected seedlings increased drastically and with significantly higher amount compared to BCAs treated seedlings. Although the disease incidence also increased in all treated seedlings, *Ganoderma* colonization in TR 1, TR 2 and TR 3 was lower compared to untreated seedlings based on the lower ergosterol content detected in their root tissues. The present study confirms all three treatments were potential to reduce the colonization of *Ganoderma* in infected oil palm tissue.

b. TR 3:

TR 3 was prepared by mixing 265 mL of the product with 130 g of humic acids in tap water, to make up a final volume of 40 L. The mixture was sprayed around the palms as described above.

2.9. Assessment on the effectiveness of microbial treatments in field trial:

A total of infected palms were screened for homogenous ergosterol group. Homogeneity test was carried out using SPSS and 150 palms with no significant difference of ergosterol content ($p>0.05$) were selected for further testing. Selected palms were subjected to treatments with TR 1 and TR 3 for six months consecutively following the SOP of each treatment. Healthy palms without ergosterol were selected for control.

2.10. Evaluation of Disease Incidence (DI):

The infection of *G. boninense* was detected based on fungi growth on GSM and the presence of ergosterol. Disease incidence was calculated using the formulas described by(Masood *et al*. (2010) as below;

$$DI = \frac{\text{mlap detcefni fo rebmuN}}{\text{latoT rebmun fo desessa malp}} \times 100$$

2.11. Statistical analysis:

Data was statistically analyzed using one-way ANOVA (Analysis of Variance) by Statistical Package for Social Science (SPSS) 19.0 programme. Significant differences among treatments were analyzed using Duncan's multiple range test (DMRT) at 5% significant level. Non-parametric data was analyzed using Kruskall-Wallis test at 5% significant level followed by Mann-Whitney test.

Results:

3.1. Ganoderma colonization in nursery trial:

There was no significant difference in the amount of ergosterol among the infected seedlings treated with various treatments (TR 1, TR 2 and TR 3). Their amounts of ergosterol which represented the degree of *Ganoderma* colonization were

significant lower than untreated control. All treatments also show greatest reduction of disease incidence with 20% reduction in both TR 1 and TR 2, and 10% reduction in TR 3 treatment.

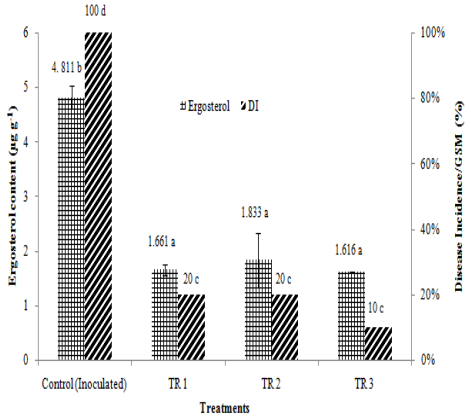

Fig. 1: Quantification of ergosterol content and percentage of Disease Incidence (DI) two months after artificial inoculation. Each treatment had 10 replicates. Means followed by the same letters within each column of ergosterol content are not significantly different at $\alpha = 0.05$, as determined by DMRT. Means within each column of Disease Incidence followed by the same letter are not significantly difference at $\alpha = 0.05$ after Kruskall-Wallis test followed Mann-Whitney test. No ergosterol detected in healthy (Control) palms (Data not shown).

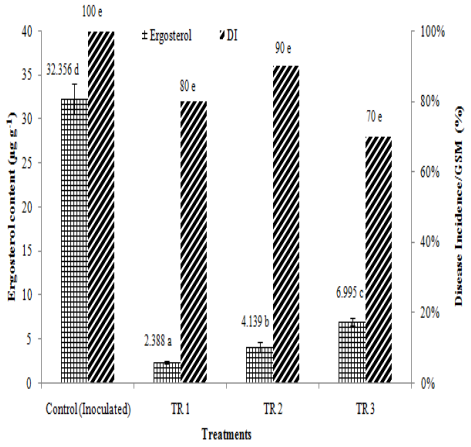

Fig. 2: Quantification of ergosterol content and percentage of Disease Incidence (DI) four months after artificial inoculation. Each treatment had 10 replicates. Means followed by the same letters within each column of ergosterol content are not significantly different at $\alpha = 0.05$, as determined by DMRT. Means within each column of Disease Incidence followed by the same letter are not significantly difference at $\alpha = 0.05$ after Kruskall-Wallis test followed Mann-Whitney test. No ergosterol detected in healthy (Control) palms (Data not shown).

3.2.Ganoderma colonization in field trial:

Disease Incidence (DI) and ergosterol content at the beginning of the experiment is shown in Figure 3. After six months of continuous treatment the palms were re-assessed for the *Ganoderma* colonization. In infected but untreated control palms the DI of *Ganoderma* was remained at 100% with amount of ergosterol double up the treated palms. However, TR 1 palms showed a significantly recovery, to only 12% of DI found at the end of the observation. This treatment also effectively reduced the amount of ergosterol by 81% compared to before treatment. TR 3 palms successfully reduced the percentage of fungal colonization to 24%, with reduction in ergosterol to 47%.

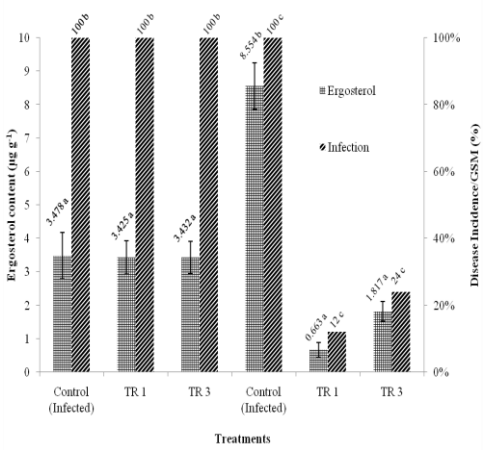

Fig. 3: Quantification of ergosterol content and percentage of Disease Incidence (DI) of palms in field trial before and after BCA treatments. Each treatment has 50 replicates. Each interval was analyzed using one-way ANOVA independently. Means followed by the same letters within each column of ergosterol content are not significantly different at $\alpha = 0.05$, as determined by DMRT. Means within each column of Disease Incidence followed by the same letter are not significantly difference at $\alpha = 0.05$ after Kruskall-Wallis test followed Mann-Whitney test. No ergosterol detected in healthy (Control) palms (Data not shown).

Discussion:

In recent years, research on biological control (BCA) has received attention for controlling *Ganoderma boninense*. In current study, investigation was carried out on the efficacy of microbes of TR 1, TR 2 and TR 3 against *G. boninense*. Their ability to control this plant pathogen in nursery and field was found to be effective based on the reduction of ergosterol accumulation in plant's root and the absence of *G. boninense* growth on GSM when root tissues were plated. In this study, the results from using TR 1 revealed the combination of *Bacillus* spp. and *Trichoderma* spp. was effective in controlling the colonization of *Ganoderma* both in nursery and field. A similar outcome was reported by Yobo *et al.* [25] which describe the combination of *Trichoderma* spp. and *Bacillus* spp. had significant effect in decreasing the disease severity of *Rhizoctonia solani* in cucumber in both nursery and field condition. Some possible mechanisms of antagonism employed by *Trichoderma* spp. was include mycoparasitism, competition of nutrients and space or the production of inhibitory compounds from the *Trichoderma*, thus, modifying the rhizospere to a condition where *G. boninense* cannot strive. Meanwhile *Bacillus*, an endophytic bacteria, has the ability to penetrate plant's vascular system [18], which play a significant role in inhibiting the penetration of *G. boninense* into the vascular system of oil palm. Their ability to compete within the vascular system may limit *Ganoderma* for both nutrients and space during its proliferation.

The ability of TR 2 to control *G. boninense* was associated with the presence of *Bacillus* spp., *Pseudomonas* spp. and *Aspergillus* spp. Synergistic effects of *Bacillus* spp. and *Pseudomonas* spp. have been reported in the control of *Fusarium* wilt of chickpea [14]. *Pseudomonas* is known to restrict pathogens by producing metabolites with antimicrobial activity [5]. Several researches have shown *Aspergillus* species has the potential as BCA against wood decay fungi and *Ganoderma* of oil palm [1,24]. Major mechanisms involved in the biocontrol activity of *Aspergillus* spp. are competition for space and potential plant infection sites and production of numerous hydrolytic enzymes [23] which partially degrade *Ganoderma* cell wall and lead to its parasitisation.

The results from current study also proved the potential of yeasts from TR 3 as a biocontrol agent against *G. boninense*. Combination of *Lactobacillus*, *Nattobacillus* and *Saccharomyces cerevisiae* in TR 3 may work synergistically against *G. boninense* and lead to the success of TR 3 in reducing the pathogen's colonization both in nursery and field. This was in agreement with the finding of Moustafa & Mohamed [17], whom reported application of *S. cerevisiae* as biocontrol agents have reduced the infection of soil-borne fungal plant pathogen, *Fusarium oxysporum* causing damping-off symptoms in sugar beet seedlings. *S. cerevisiae* was used as biocontrol agent and inducer of plant systemic resistance mechanisms which is associated with increasing certain hydrolytic enzymes and pathogensis-related protein [9]. Application of *Lactobacillus*, a Lactic acid bacterium (LAB) as a biocontrol agent has been reported against some pathogens such as *Xanthomonas* campestris, *Erwinia carotovora* and *Staphylococcus aureus [3,13]*. The antagonist effects of *Lactobacillus* were due to the production of antimicrobial agents such as organic

acids, hydrogen peroxide and related substances [15]. The addition of *Nattobacillus* in this treatment was to help in activating, strengthening and enhancing *Lactobacillus* in producing metabolic compound such as lactic acid, producing strong protein degradation enzymes and starch degradation enzyme which is against *Ganoderma* growth [12].

The results from this study confirmed the application of BCAs treatments had decreased the amount of ergosterol in treated palms compared to untreated control. It is suggested the treated palms had acquired some form of tolerance to the *Ganoderma* infection from the microbes' treatments. Development and induction of the host defence mechanism, such as formation of structural barriers, like lignified cell walls, to keep out the pathogen and production of antifungal metabolites to slow down the infection progress and improved growth and plant vigour may possibly contribute to disease suppression of *G. boninense* [11,18].

Conclusion:

In conclusion, TR 1, TR 2 and TR 3 were found to be a potential biocontrol agents against BSR disease of oil palm based in nursery and field. Introducing antagonist microbes to the soil in order to control *G. boninense* is to manipulate and activate the antagonistic activity in the soil microbial community, in a matter which can lead to the suppression of BSR incidence by reducing the pathogen colonization. Long term effectiveness of biological control may an ideal solution in oil palm disease management in comparison to the use of hazardous chemicals in controlling *G. boninense.*

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