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Evaluation on the effectiveness of combination of biocontrol agents in managing *Ganoderma boninense* of oil palm

Feodora Grace Japanis[1], Yow San Chan[1] and Khim Phin Chong[1,2]*

[1]Faculty of Sciences and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu Sabah, Malaysia.
[2]FGV Chair of Sustainable Oil Palm Management, Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, Mile 10, Sg Batang, 90000, Sandakan, Sabah, Malaysia.
Email: chongkp@ums.edu.my

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ABSTRACT

Aims: The development of an effective biocontrol formulation for inhibition of *Ganoderma boninense,* a well-known destructive pathogen in oil palm plantation is important to prolong the palm's lifespan and reduce the losses due to this disease. In this paper, we present some new bioformulations with combination of different types of biocontrol agents in managing basal stem rot (BSR) disease.
Methodology: The effectiveness of the treatments designed as T1 (*Trichoderma harzianum* + *Lecanicillium* spp. + *Streptomyces sundarbansensis* + *Pseudomonas aeruginosa*), T2 (*Penicillium simplicissimum* + *Lecanicillium* sp. + *S. sundarbansensis* + *P. aeruginosa*), T3 (*P. simplicissimum* + *P. aeruginosa*) and T4 (LEStani®) was evaluated through treatment on the oil palm seedlings artificial infected by *G. boninense* and the results were expressed in disease severity index (DSI), bole severity index (BSI) and ergosterol content.
Conclusion, significance and impact of study: All tested treatments (T1-T4) managed to control the severity of the *Ganoderma* infection from continuously increasing when the treatments were applied either one month before or after artificial inoculation. The disease severity of infected seedlings without treatments had increased for almost 2-fold at the end of the trial. Moreover, T1 had the greatest inhibition of *G. boninense* with the lowest ergosterol content (a bioindicator of *Ganoderma* colonization) detected (676.67 μg/mL), which is about 1.9-fold lower than control (1273.33 μg/mL) without treatments and a BSI score of 1. Based on the effectiveness among the four tested biocontrol formulations, T1 is the most promising formulation to be further evaluated in the field for control of BSR disease. However, more research is needed in the future to estimate the effective amount for application in open environment.

Keywords: Ganoderma boninense, basal stem rot, *Trichoderma harzianum*, *Penicillium simplicissimum*, *Lecanicillium* spp., *Streptomyces sundarbansensis*

INTRODUCTION

Palm oil is a valuable primary agricultural product which is originated from the African oil palm, *Elaeis guineensis*. It is one of the world's most produced edible oil, followed by soya and rapeseed oil (Ghazani and Marangoni, 2016). Initially, oil palm was introduced as an ornamental plant in Malaysia. The oil palm sector started its expansion into a multibillion dollar industry from 1917 onwards (Alam *et al*., 2015). According to Malaysian Palm Oil Council (2019), Malaysia produces approximately 41% of world palm oil and accounts for about 46% of world exports. Malaysia and Indonesia produce approximately 85% of total palm oil in the world altogether. Based on current statistics, Malaysia produces about 19,519,141 tonnes of palm oil in 2018 (MPOB, 2019). Besides being used in food, palm oil is also widely used in cosmetics, pharmaceuticals and also for bio-diesel production. Many developing countries considered oil palm as an important crop for multipurpose usages and significant tool in eradication of poverty (Feintrenie *et al*., 2010). Thus, Malaysia plays a vital role in fulfilling the expanding global demand for oils and fats.

However, the oil palm industry is facing a huge challenge from a disease threat which is known as basal stem rot (BSR). This disease is caused by the white rot fungus, *Ganoderma boninense* (Hushianian *et al*., 2013). The pathogen has been reported to cause economic losses up to RM 1.5 billion a year. *Ganoderma boninense* was reported to infect mature palms and seedlings as young as 12 months old. Once infected, young seedlings usually die within two months after the first symptoms emerged, meanwhile mature oil palm trees can survive for

***Corresponding author**

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about three years (Arif *et al*., 2011). The symptoms of BSR include the decaying of the bottom of the stem or root, or more severe cases include the fracture of stem. Besides, BSR infection can be observed by chlorosis, fronds wilt and hang down forming a skirt around the trunk, flattening of the crown and spear leaves on the shoot (Rees *et al*., 2012).

For years, none of the strategies for the early detection and management of *G. boninense* have shown promising results (Alexander and Chong, 2014; Tay and Chong, 2016). Thus, this paper presents an evaluation on the effectiveness of four new bioformulations in managing *G. boninense* in oil palm using seedlings as a model. The effectiveness of the formulations in controlling *G. boninense* was evaluated by using several parameters including isolation of the plant pathogen on *Ganoderma* selective media (GSM), evaluation of the disease incidence (DI), disease severity index (DSI), bole severity index (BSI) and ergosterol content.

MATERIALS AND METHODS

Source of pathogen

Pure culture of *G. boninense* was obtained from Genetic Laboratory of Faculty of Science and Natural Resources, Universiti Malaysia Sabah. The identity of *Ganoderma boninense* had been confirmed earlier using DNA sequence analysis after PCR amplification (Chong *et al*., 2011). *G. boninense* culture was subcultured on potato dextrose agar (PDA) and maintained at 27 ± 2 °C for further study.

Source of potential biocontrol agents

Penicillium simplicissimum, *Lecanicillium* spp., *Trichoderma harzianum*, *Streptomyces sundarbansensis* and *Pseudomonas aeruginosa* were obtained from Genetic Laboratory of Faculty of Science and Natural Resources, Universiti Malaysia Sabah. The microbes were originally isolated from Crocker Range, Sabah and had been identified (Chong *et al*., 2019; Lim *et al*., 2015; 2018; 2019a and b). The microbes were subcultured in every two to four weeks in different agar media (PDA for fungi and nutrient agar (NA) for bacteria and actinomycetes) and maintained at 27 ± 2 °C for further study.

Dual culture assays

Mycelial plug (6 mm) was taken from the edge of 7-day-old *G. boninense* pure culture and placed approximately 30 mm from one side of the PDA plate. After two days, a loopful of potential antagonist bacteria were streaked at approximately 30 mm from the *G. boninense* plug. The same procedure was also carried out using actinomycetes. For fungal isolates, a 6 mm mycelial plug of the potential antagonist fungal isolate of 7-day old was placed at 30 mm of the edge of another side of the PDA plate instead of being streaked. *Ganoderma boninense* cultured alone on PDA was served as control. The radial growth of the pathogen in the dual culture and control plates was measured after seven days of incubation at room temperature (27 °C ± 2 °C). The percentage inhibition of radial growth (PIRG) was calculated as described by Bivi *et al*. (2016) as followed:

$$\text{PIRG}\ (\%) = \frac{R1 - R2}{R1}\ x\ 100$$

where, the R1 is the radial growth of *G. boninense* in the control plate and R2 is the radius of *G. boninense* colony in the direction towards the antagonist colony. Antagonists that caused more than 70% PIRG to *G. boninense* were rendered as effective. All assays were done in triplicate.

Table 1: Different combination of the designed bioformulations.

Treatments	Compositions
T1	*Trichoderma harzianum*, *Lecanicillium* spp., *Streptomyces sundarbansensis*, *Pseudomonas aeruginosa*
T2	*Penicillium simplicissimum*, *Lecanicillium* spp., *Streptomyces sundarbansensis*, *Pseudomonas aeruginosa*
T3	*Penicillium simplicissimum*, *Pseudomonas aeruginosa*
T4	LEStani®

Compatibility test of potential biocontrol agents

Potential biocontrol agents that showed PIRG of more than 70% were subjected to compatibility test prior to the preparation of bioformulation to ensure that they will not inhibit each other's growth. Two potential biocontrol agents were placed at approximately 30 mm from opposite sides of the PDA plate. Their growth was observed after 7 days of incubation at room temperature (27 °C ± 2 °C). Normal growth between the two microbes will indicate compatibility. Meanwhile, abnormal or no growth or dominance of one particular microbe over the other microbe will indicate incompatibility.

Preparation of new bioformulations

Potential biocontrol agents that showed good compatibility were combined into three different bioformulations (T1, T2, T3). Another treatment labelled as LEStani® (T4) was obtained from Genetics Laboratory of Universiti Malaysia Sabah. It was a formulation of biofungicide, a registered trademark of UMS product which comprised of combinations of phenolic acids. The effectiveness of the combination of phenolic acids in T4

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was presented earlier by Chong *et al.* (2012). The preparation of T1, T2 and T3 are detailed as follows: T1, T2 and T3 were prepared by suspending 1 mL of sterile distilled water to respective microbial cultures as in Table 1 (except T3 with 2 mL for each culture). The suspension from each culture was transferred into a potato dextrose broth (PDB) with a volume of 996 mL to make up a final volume of 1 L.

Preparation of *Ganoderma* rubber wood block inocula

Method for preparation of *Ganoderma* inocula on 3 cm × 3 cm × 3 cm rubber wood blocks (RWBs) was adopted and slightly modified from Breton *et al.* (2005). RWBs were cleaned with running tap water and then autoclaved for two hours at 121 °C; 15 psi in individual autoclavable polypropylene plastic bags. After that, 100 mL of sterile molten malt extract agar was added into each plastic bag and left to solidify. The RWBs were inoculated with mycelial plugs (6 mm) from a 14-day-old *G. boninense* culture. The inoculated RWBs were sealed and then incubated for 8 weeks in dark and at room temperature (27 ± 2 °C) for the fungal mycelia to fully colonise the block.

Plants materials

Thirty 6-month-old disease-free oil palm seedlings (*DxP tenera*) of in polyethylene bags were purchased from Sawit Kinabalu Sdn. Bhd., Kota Marudu, Sabah, Malaysia. Three oil palm seedlings were used as infected control (without treatments) and another three oil palm seedlings were left uninfected (healthy control). The rest of the seedlings were infected by *G. boninense* and treated with four different bioformulations. The seedlings were watered daily.

Artificial inoculation of oil palm seedlings

The inoculation of oil palm seedlings was done in two different set of experiments. For the first set of experiment, the application of treatments was performed one month before the artificial inoculation of *G. boninense* (an evaluation of treatments applied before infection), while the second set of experiment, the application of treatments was performed a month after the artificial inoculation of *G. boninense* (an evaluation of treatments applied after infection). The RWBs were applied upon transplanting of the oil palm seedlings to bigger polyethylene planting bags (38 × 45 cm) in medium soil and sand mixture (2:1). The seedlings were seated onto the inoculum and the roots were placed in direct contact with the RWBs. Aliquots (100 mL) of each treatment was applied by pouring onto the soil surface surrounding the seedlings. Healthy and infected control oil palm seedlings were left untreated. The progress of *Ganoderma* colonization was monitored. The description and images of macromorphological symptoms (Foliar symptoms) were recorded for two months.

Root harvesting and assessment for disease development

Root samples were cleaned with tap water and air dried for three days. The roots were then mechanically homogenized by using a mechanical blender.

Isolation of G. boninense

Grinded root samples were cultured on *Ganoderma* selective medium (GSM) as outlined by Ariffin and Seman (1991). The GSM consists of two parts: Part A comprised of Bacto Peptone (Difco) 5.0 g, agar extra pure powder (QRëC) 20.0 g, $MgSO_4 \cdot 7H_2O$ (Merck) 0.25 g, K_2HPO_4 (QRëC) 0.5 g and distilled water pH 5.5 900 mL. Part B consisted of streptomycin sulphate (Sigma) 300 mg, chloramphenicol (Sigma) 100 mg, pentachloronitrobenzene (PCNB) pure (Aldrich) 285 mg, Ridomil (25% WP) 130 mg, Benlate T20 150 mg, ethanol 95% (Sigma) 20 mL, lactic acid (Sigma) 50% 2 mL, tannic acid (R & M Chemicals) 1.25 g and distilled water pH 5.5 80 mL. Part A was stirred on a hot plate at 100 °C until dissolved before being autoclaved for 15 min. Part B was stirred for about 2 h at room temperature. Later part B was added to part A when the autoclaved medium had cooled down to 45-50 °C. The media provides a useful tool in 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.

Disease incidence (DI) assessment

Disease development was assessed based on the percentage of disease incidence (DI) after two months, using the formula as follows:

$$DI = \frac{Number\ of\ infected\ seedlings}{Total\ number\ of\ seedlings\ assessed}\ x\ 100$$

Disease severity index (DSI)

The disease severity of the treated seedlings was assessed based on disease score (0 to 4) as described by Abdullah *et al.* (2003) in Table 2. Then, the DSI was computed according to the formula as described by Abdullah *et al*. (2003). DSI was transformed by arcsine and the mean of each treatment was compared. The DSI was computed for every month to a period of two months.

$$\text{DSI}\ (\%) = \frac{\Sigma abN}{K}$$

where, ∑ab = sum of the product of assessed plants with their corresponding score scale; N = total number of assessed plants; K = highest score scale.

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Table 2: Disease severity of the treated seedlings was assessed based on disease score. The scores (0-4) of basal stem rot disease and their external signs and symptoms of the treated seedlings was based on Abdullah *et al*. (2003).

Disease score	Signs and symptoms
0	Healthy plants with green leaves without appearance of fungal mycelium on any part of plants
1	Appearance of white fungal mass on any part of plants, with or without chlorotic leaves
2	Appearance of basidioma on any part of plants with chlorotic leaves (1 to 3 leaves)
3	Formation of basidioma of any part of plants with chlorotic leaves (more than 3 leaves)
4	Formation of a well-developed basidioma and the plants dried

Bole severity index (BSI)

At the end of the experiment (after two months), the treated seedlings were cut longitudinally to observe the internal symptoms of root and stem decay. Assessment of the disease was based on the scale as described by Breton *et al*. (2005): 0 = healthy, no internal rot; 1 = 1% to 20% rotting of tissues; 2 = 21% to 50% rotting of tissues; 3 = 51% to 90% rotting of tissues; 4 = more than 90% rotting of tissues.

Evaluation of G. boninense colonization by quantifying ergosterol content

Extraction of the ergosterol was done as described by Chong *et al*. (2012). In brief, root tissues (100 mg) were extracted in methanol using bead beating to physically crush the sample. Polyvinylpyrrolidone (PVP) was added (10% w/v) to the methanol to precipitate phenolic compounds. The extract was centrifuged at 15,000 rpm 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. Quantification of ergosterol contents was performed using the Agilent Series 1200 high performance liquid chromatography (HPLC) system with a reverse-phase Eclipse XDB-C18 4.6 mm × 150 mm, 5 µm particle size column. The isolated peak eluted at a retention time from 10 min to 14 min was identified as ergosterol, based on its co-chromatography and identical absorption spectrum with a pure standard at flow rate of 1.5 mL/min. The system was run isocratically with 100% methanol. A serial dilution of the ergosterol standard, with a concentration range of 100-700 µg/mL, was injected into the HPLC system to develop a standard curve, which was then used for ergosterol quantification from oil palm root extracts.

Experimental design and statistical analysis

The experimental design for the nursery trial was a completely randomized design (CRD). All experimental subjects were in triplicates. The ergosterol concentration data obtained were compared by analysing the variance, comparing the averages with ANOVA and a T-test. Analyses were conducted using the Statistical Programming Package for Social Sciences (SPSS) software version 25.

RESULTS AND DISCUSSION

PIRG of potential biocontrol agents against *G. boninense*

Based on the result in Table 3, all antagonists showed PIRG of more than 80% against *G. boninense* and were considered as potential biological control agents (BCA). These antagonists were *T. harzianum*, *P. simplicissimum*, *Lecanicillium* sp., *S. sundarbansensis*, and *P. aeruginosa*. The highest PIRG was achieved by *P. aeruginosa* (97.44%), followed by *P. simplicissimum* with PIRG of 94.87%.

Different antagonist will have different mode of mechanisms to attack the pathogen. The antagonists may affect the pathogen population by direct parasitism and lysis of the pathogen, by competing with the pathogen for space and nutrients, or by antibiotic compounds release that are toxic to the pathogen. The antagonists might also produce mycolytic enzymes such as chitinase and beta-1,3-glucanase that lyse fungal cells. Parasitic mechanism aids parasite entrance into the hyphae of the pathogen (Lim *et al*., 2015). These different mechanisms may be deployed by the BCA in the current experiment to control *G. boninense.*

All the antagonists were found to possess strong competitive effects for space and nutrients against *G. boninense*. The mode of attack by *T. harzianum* against *G. boninense* is by promoting plant's synthesis of biologically active compounds or cause changes in plant morphology and/or physiology.*Trichoderma harzianum* has chitinase and beta-1,3-glucanase in their hyphae, so these enzymes have synergistic effect on each other. *Trichoderma harzianum*also attaches to the hyphae of *G. boninense* and coils it, causing inhibition to the pathogen's growth (Ferreira *et al*., 2007; Shrestha *et al*., 2008). On the other hand, *P. simplicissimum* inhibits the growth of *G. boninense* by producing secondary metabolites such as parahequamides and chromanols that may have antibiotic effects against plant pathogens (Dorothy *et al*., 2001). Differently, *Lecanicillium* spp. is better adapted to various environmental and nutritional conditions and this prevents the spore germination and growth of the plant pathogen. As for *S. sundarbansensis*, it inhibits the growth of *G. boninense* by producing bioactive compounds such as 2-allyloxyphenol that are responsible for many antimicrobial and antioxidant activities (Arumugam *et al*., 2011).

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Table 3: Percentage of radius inhibition (PIRG) growth of potential biocontrol agents against *G. boninense*.

Biological control agent	PIRG (%)
Trichoderma harzianum	$84.62^{d} \pm 1.38$
Penicillium simplicissimum	$94.87^{b} \pm 2.74$
Lecanicillium spp.	$84.62^{d} \pm 1.92$
Streptomyces sundarbansensis	$87.18^{c} \pm 1.37$
Pseudomonas aeruginosa	$97.44^{a} \pm 2.23$

Different alphabets shown significant different (p<0.05) for mean of PIRG by one-way ANOVA analysis.

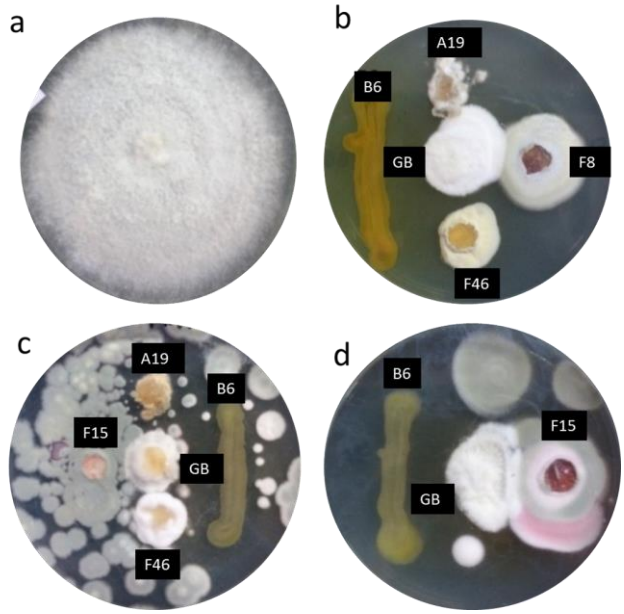

Figure 1: Compatible antagonists were combined into three treatments and were tested on a culture of *G. boninense* for 10 days. (a) *G. boninense* (GB) (control); (b) T1: *T. harzianum* (F46) + *Lecanicillium* spp. (F8) + *S. sundarbansensis* (A19) + *P. aeruginosa* (B6); (c) T2: *P. simplicissimum* (F15) + *Lecanicillium* spp. (F8) + *S. sundarbansensis* (A19) + *P. aeruginosa* (B6); (d) T3: *P. simplicissimum* (F15) + *P. aeruginosa* (B6).

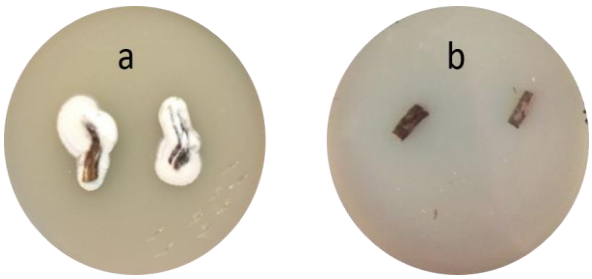

Figure 2: *Ganoderma* mycelium were successfully isolated from all root samples on (a) GSM culture, except for the roots of (b) healthy control.

P. aeruginosa also inhibits *G. boninense* by producing antimicrobial compounds that could cause direct toxic effects on the plant pathogen (Klaenhammer, 1993; Contreras *et al*., 1997). These antimicrobial compounds include pyocins, phenazines, quinolones and phenylpyrroles. The family of *Pseudomonas* are also known to produce iturin which is a lipopeptide that could elicit pores on fungal cell membranes and thus induce apoptosis of pathogen (Chong, 2012; Lim *et al.*, 2015). Since different biological agents possess different mechanisms of action, therefore, a combination of different biological agents to control *G. boninense* may provide a more comprehensive solution for BSR disease.

The compatibility among the tested biocontrol agents

Compatibility test is very important in formulating a new biological treatment. This is to ensure that the antagonistic microorganisms will not kill each other before they could inhibit the growth of *G. boninense*. Microorganisms can be said to be compatible if they are able to grow together. This means that the microorganisms will not be dominant over the other in terms of space and nutrients uptake. The microorganisms will also not grow overlapping each other. As shown in Figure 1, the three different combinations (T1, T2 and T3) of microorganisms were able to grow together without inhibiting each other, but only inhibiting *G. boninense*.

Isolation of *G. boninense* on GSM

All root samples from inoculated seedlings with treatments and without treatments (positive controls) showed the growth of *Ganoderma* mycelia on GSM except for samples from healthy uninoculated seedlings (negative control) (Figure 2). This indicates that the *G. boninense* colonization in the roots was not fully eliminated after treated with the designed bioformulations. This might be because the formulations only suppressed the growth of *G. boninense* but not totally eradicating them. Another reason might be because of the duration of treatments on the infected seedlings (one month) was not long enough.

Disease Incidence (DI) of oil palm seedlings

The healthy uninfected seedlings showed 0% of DI (Table 4), as there was no presence of *G. boninense*. The infected seedlings with and without treatments showed 100% of DI, at the end of experiment. This shows that the artificial infection of seedlings were successful. However, none of the treatments have managed to successfully free the seedlings from *G. boninense* infection.

Disease severity index (DSI) of oil palm seedlings

The DSI of infected seedlings without treatments increased from 3% in the first month of inoculation to 6% in the second month of inoculation. Meanwhile, for infected seedlings with treatments, the DSI remained

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Table 4: Disease incidence (DI) of oil palm seedlings inoculated by *G. boninense* and after treated with different types of treatments.

Treatment	DI (%)
Negative control	0
T1	100
T2	100
T3	100
T4	100
Positive control	100

Negative control (Healthy and uninoculated seedlings). T1-T4: Seedlings inoculated by *G. boninense* and treated with various treatments as followed: T1 (*Trichoderma harzianum* + *Lecanicillium* spp. + *Streptomyces sundarbansensis* + *Pseudomonas aeruginosa*), T2 (*Penicillium simplicissimum* + *Lecanicillium* spp. + *S. sundarbansensis* + *P. aeruginosa*), T3 (*P. simplicissimum* + *P. aeruginosa*), T4 (LEStani®) and positive control (inoculated seedlings without any treatment).

constant (3%) throughout the first and second month as shown in Figure 3. This indicates that the formulated biocontrol agents did not eradicate *G. boninense* infection (Figure 2). However, the formulations did reduce the progression and colonization of the pathogen. Whether the treatments were applied before or after the infection, it did not affect the DSI. This might be because the BCAs from T1, T2 and T3 have restricted the growth of *G. boninense* by performing different antagonistic mechanisms against the pathogen as discussed in the previous section. On the other hand, the phenolic acids which are present in T4 have also been reported to have fungitoxicity effects and the ability to limit the growth of *G. boninense* (Chong *et al.*, 2009). Nonetheless, the duration of two months for the observation of DSI may be too short to evaluate further progression of the plant pathogen's colonization.

Bole severity index (BSI) of oil palm seedlings

The bole severity index (BSI) was assessed by visually measuring the browning of the bole tissues which indicates tissue rotting caused by *G. boninense*. The result for BSI is presented in Figures 4 and 5. Based on Figure 5, the healthy uninoculated seedlings had no tissue rots (BSI= 0), whilst the inoculated seedlings without treatment had more than 50% rotten tissues (BSI = 3). The inoculated seedlings treated with T1 showed a BSI of 1, meanwhile T2 and T3 had BSI of 2. Thus, T1 was more effective in limiting the colonization of *G. boninense*. The interaction between the BCAs in a combination can be very unique. Some BCAs interact with plants by inducing resistance or by priming plants without any direct interaction with the targeted pathogen. Other BCAs act via nutrient competition or other mechanisms that modulate the growth conditions for the pathogen (Köhl *et al*., 2019). The combination of BCAs in T1 in this experiment might give the best complementary effects to each other in limiting the progression of *G. boninense* into the bole tissues of the seedlings. *Trichoderma* has the ability to coil the pathogen *G. boninense* (Alexander *et al*., 2017), *Lecanicillium* spp. is able to release hydrolytic enzymes to directly penetrate the cell wall of the fungal plant pathogen (Goettel *et al*., 2008), while *S. sundarbansensis* and *P. aeruginosa* produced different types of antimicrobial compunds which inhibit the pathogen (Lim *et al*., 2018, Lim *et al*., 2019a).

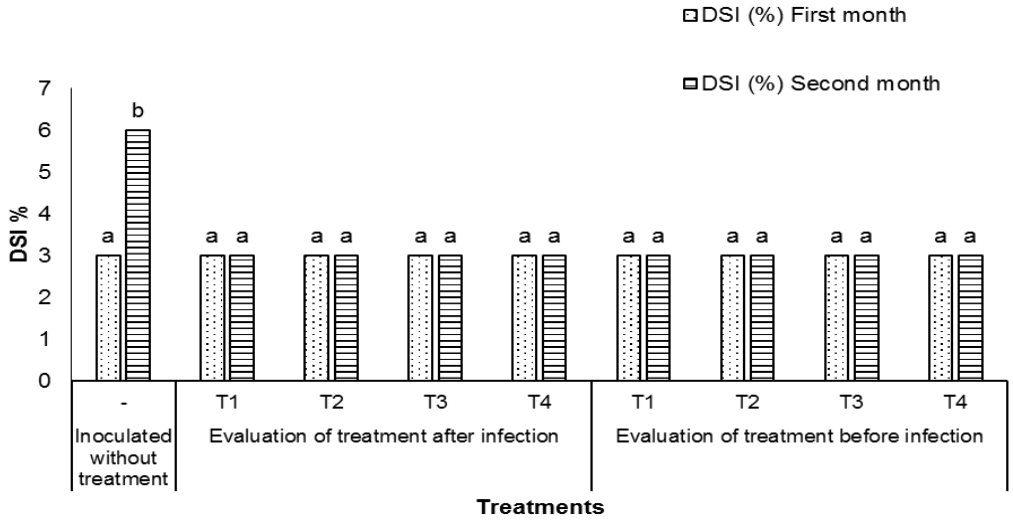

Figure 3: The disease severity index (DSI) of each treatment throughout first and second month of observation. T1: *T. harzianum* + *Lecanicillium* spp. + *S. sundarbansensis* + *P. aeruginosa*; T2: *P. simplicissimum* + *Lecanicillium* spp. + *S. sundarbansensis* + *P. aeruginosa*; T3: *P. simplicissimum* + *P. aeruginosa*; T4: LEStani®. There is no error bar for the data as the triplicates gave the same results for DSI in percentage. Different alphabets showed significant difference (p< 0.05) for the mean of DSI by one-way ANOVA analysis.

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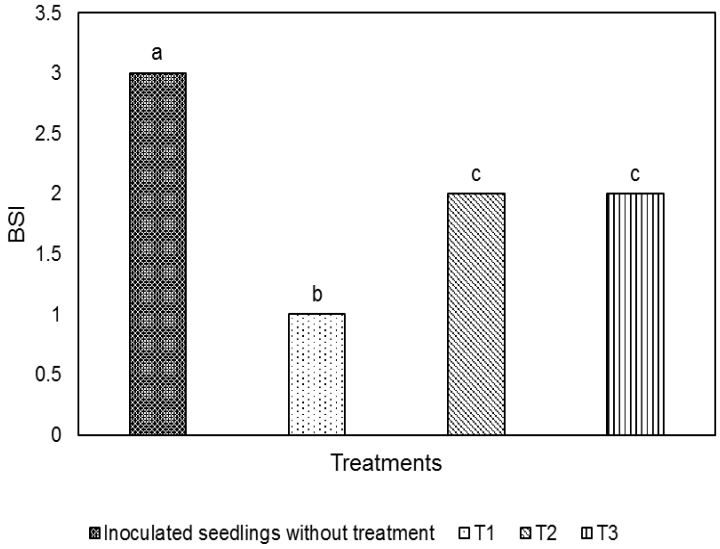

Figure 4: Bole severity index (BSI) of oil palm seedlings treated with different bioformulations. Seedlings were applied with treatments one month after the artificial inoculation of *G. boninense*. T1: *T. harzianum* + *Lecanicillium* spp. + *S. sundarbansensis* + *P. aeruginosa*; T2: *P. simplicissimum* + *Lecanicillium* spp. + *S. sundarbansensis* + *P. aeruginosa*; T3: *P. simplicissimum* + *P. aeruginosa*. Different alphabets showed significant difference (p<0.05) for the mean of BSI by one-way ANOVA analysis.

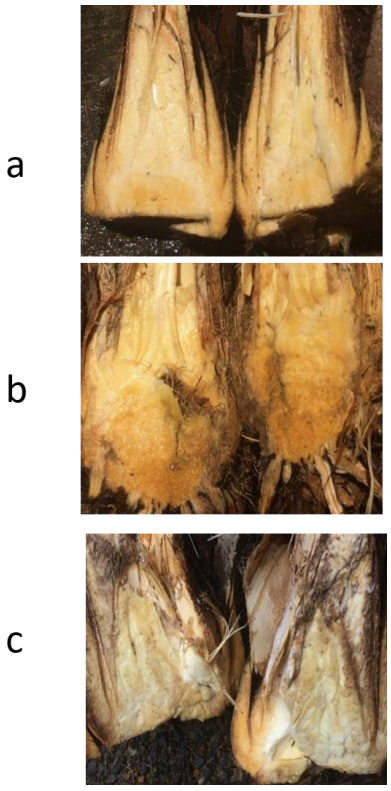

Figure 5: Bole severity index (BSI) correlated with different types of treatments. (a) Healthy uninoculated seedlings, BSI = 0; (b) inoculated seedlings without treatment, BSI = 3; (c) inoculated seedlings with T1, BSI = 2. T1: *T. harzianum* + *L. lecanii* + *S. sundarbansensis* + *P. aeruginosa*.

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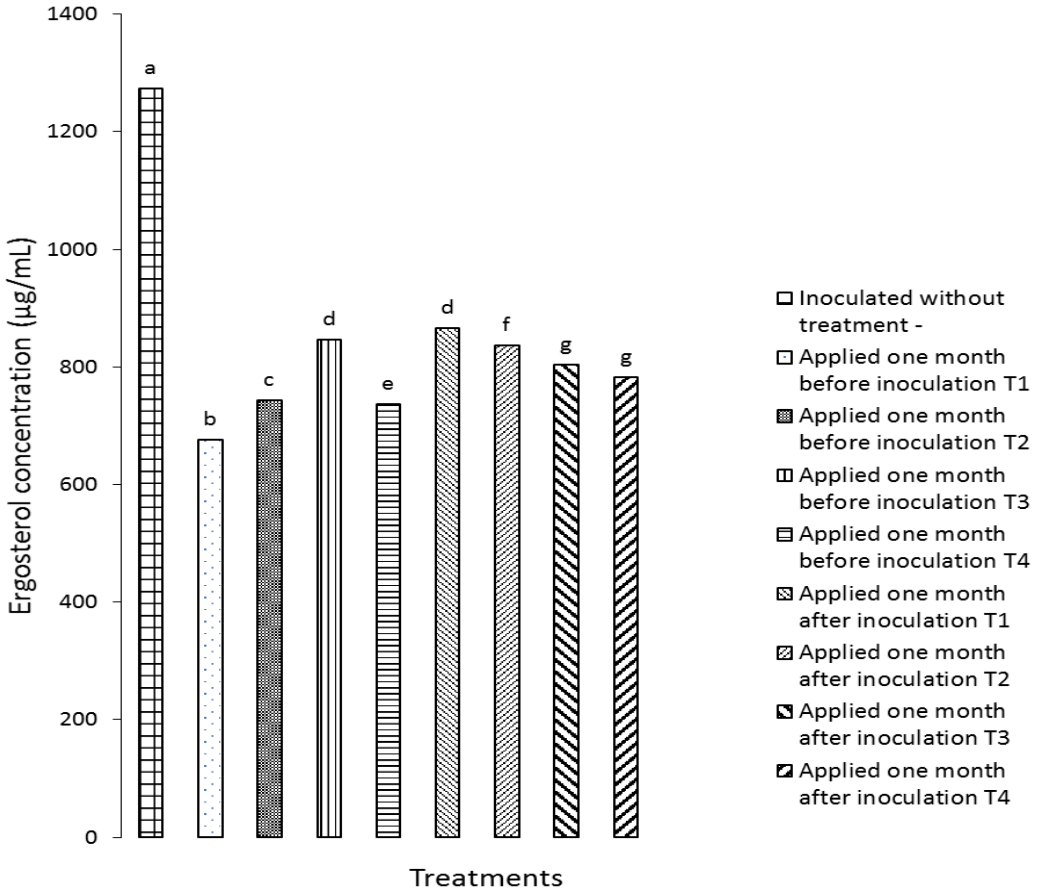

Figure 6: The ergosterol concentration (µg/mL) in oil palm seedlings treated with different bioformulations. The treatments were applied one month before and after the inoculation of *G. boninense*. T1: *T. harzianum* + *L. lecanii* + *S. sundarbansensis* + *P. aeruginosa*; T2: *P. simplicissimum* + *L. lecanii* + *S. sundarbansensis* + *P. aeruginosa*; T3: *P. simplicissimum* + *P. aeruginosa*; T4: LEStani®. No ergosterol was detected in healthy seedlings. Different alphabets showed significant difference (p<0.05) for the mean of ergosterol concentration by one-way ANOVA analysis.

Ganoderma colonization on oil palm seedlings

Ergosterol is the main fungal sterol and it plays a vital role in the cell membrane and other cellular components (Nielsen and Madsen, 2000). Thus, ergosterol analysis is important in provisionally estimating *G. boninense* colonization in oil palm. Previous report had shown that the ergosterol concentration increased significantly as the degree of root colonization by *G. boninense* increases, meanwhile ergosterol was not detected in healthy oil palm seedlings (Chong and Dayou, 2014). The quantification of ergosterol concentration by different treatments is presented in Figure 6. In present study, the results showed that the infected seedling without treatments was found to have ergosterol concentration of 1273.33 µg/mL. For the treatments applied one month before artificial infection, the lowest ergosterol concentration (676.67 µg/mL) was found in seedlings treated with T1. Whereas for treatments applied one month after artificial infection, the lowest ergosterol concentration (783.33 µg/mL) was found in seedlings treated with T4. Ergosterol was not detected in the roots of healthy oil palm seedlings.

CONCLUSION

The current work had successfully evaluated four different types of new bioformulations *in vitro* and at the nursery level. The established bioformulations with newly designed combination of microbes showed great effect in inhibiting *G. boninense* colonization. The disease severity index (DSI) revealed that the treatments were able to control the colonization from continuously increasing, either before or after infection. In short, T1 (*T. harzianum* + *Lecanicillium* spp. + *S. sundarbansensis* + *P. aeruginosa*) was found to be most effective as it had the lowest Bole Severity Index (BSI) which is 1 and the lowest ergosterol content (676.67 µg/mL) in comparison to other treatments and if applied before infection. These bioformulations have the potential to be further developed and assessed in the field for *G. boninense* management.

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REFERENCES

Abdulllah, F., Ilias, G. N. M., Nelson, M., Nur Ain Izzati, M. Z. and Umi Kalsom, Y. (2003). Disease assessment and the efficacy of *Trichoderma* as a biocontrol agent of basal stem rot of oil palm. *Research Bulletin Science Putra* **11, 31-33.**

Alam, A. F., Er, A. C. and Begum, H. (2015). Malaysian oil palm industry: Prospect and problem. *Journal of Agriculture and Environment* **13(2), 143-148.**

Alexander, A., Abdullah, S. and Chong, K. P. (2017). Evaluation of the efficacy and mode of action of biological control for suppression of *Ganoderma boninense* in oil palm. *Pakistan Journal of Botany* **49 (3), 1193-1199.**

Alexander, A. and Chong, K. P. (2014). Combination of biological agents in suppressing colonization of *Ganoderma boninense* of basal stem rot. *American-Eurasian Journal of Sustainable Agriculture* **8(7) (Special), 1-7.**

Arif, M. A., Roslan, A., Idris, A. S. and Ramle, M. (2011). Economics of oil palm pests and *Ganoderma* diseases and yield loses: *Proceedings of the Third International Seminar Integrated Oil Palm Pests and Management*. Malaysia Pail Oil Board, Bangi, Malaysia. **pp. 83-89.**

Ariffin, D. and Seman, I. A. (1991). A selective medium for isolation of *Ganoderma* from diseased tissues. *In: Proceedings of the 1991 International Palm Oil Conference, Progress, Prospects and Challenges towards the 21st Century*. September 9-14. Malaysian Palm Oil Board, Bangi, Selangor, Malaysia. **pp. 517-519.**

Arumugam, M., Mitra, A., Pramanik, A., Saha, M., Gachhui, R. and Mukherjee, J. (2011). *Streptomyces sundarbansensis* sp. nov., an actinomycete that produces 2-allyloxyphenol. *International Journal of Systematic and Evolutionary Microbiology* **61, 2664-2669.**

Bivi, M. S., Paiko, A. S., Khairulmazmi, A., Akhtar, M. S. and Idris, A. S. (2016). Control of basal stem rot disease in oil palm by supplementation of calcium, copper, and salicylic acid. *The Plant Pathology Journal* **32(5), 396-406.**

Breton, F., Hasan, Y., Lubis, Z. H. and Franqueville, H. (2005). Characterization of parameters for the development of an early screening test for basal stem rot tolerance in oil palm progenies. *Journal of Oil Palm Research* **(Special issue, April), 24-36.**

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

Chong, K. P., Atong, M. and Rossall, S. (2012). The roles of syringic, caffeic and 4-hydroxybenzoic acids in *Ganoderma*-oil palm interaction. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences* **14 (2), 157-166.**

Chong, K. P. and Dayou, J. (2014). Relation of *Ganoderma* ergosterol contant to basal stem rot disease severity index. *Advances in Environmental Biology* **8(14), 14-19.**

Chong, K. P., Lo, R. K. S. and Gansau, J. A. (2019). Bioactivity of *Alcaligenes faecalis* and *Lecanicillium* sp. isolated from crocker range of Sabah against *Erwinia psidii* of papaya dieback disease. *Malaysian Journal of Microbiology* **15(5), 387-393.**

Chong, K. P., Rossall, S. and Atong, M. (2009). *In vitro* antimicrobial activity and fungitoxicity of syringic acid, caffeic acid and 4-hydroxybenzoic acid against *Ganoderma boninense*. *Journal of Agricultural Science* **1(2), 15-20.**

Contreras, B. G., De. Vuyst, L., Devreese, B., Busanyova, K., Raymaeckers, J., Bosman, F., Sablon, E. and Vandamme, E. J. (1997). Isolation, purification, and amino acid sequence of Lactobin A, one of the two bacteriocins produced by *Lactobacillus amylovorus* LMG P-13139. *Applied and Environmental Microbiology* **63(1), 13-20.**

Dorothy, E. T., Jens, C. F. and Martha, C. (2001). Systematics of *Penicillium simplicissimum* based on rDNA sequences, morphology and secondary metabolites. *The Mycological Society of America* **93(2), 298-308.**

Feintrenie, L., Schwarze, S. and Levang, P. (2010). Are local people conservationists? Analysis of transition dynamics from agro forests to monoculture plantations in Indonesia. *Ecology and Society* **15(4), 37.**

Ferreira, R. B., Monteiro, S., Freitas, R., Santos, C. N., Chen, Z., Batista, L. M., Duarte, J., Borges, A. and Teixeira, A. R. (2007). The role of plant defence proteins in fungal pathogenesis. *Molecular Plant Pathology* **8(5), 677-700.**

Ghazani, S. M. and Marangoni, A. G. (2016). Healthy fats and oils. *In:* Encyclopedia of Food Grains. The World of Food Grains. 2nd edn. Volume 1. Wrigley, C., Corke, H., Seetharaman, K. and Foubion, J. (eds.). Elsevier, Oxford, England. **pp. 257-267.**

Goettel, M. S., Koike, M., Kim, J. J., Aiuchi, D., Shinya, R. and Brodeur, J. (2008). Potential of *Lecanicillium* spp. for management of insects, nematodes and plant diseases. *Journal of Invertebrate Pathology* **98(3), 256-261.**

Hushianian, R., Yusof, N. A. and Duyse, S. W. (2013). Detection and control of *Ganoderma boninense*: Strategies and perspectives. *SpringerPlus* **2, Article number 555.**

Klaenhammer, T. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews* **12, 39-86.**

Köhl, J., Kolnaar, R. and Ravensberg, W. J. (2019). Mode of action of microbial biological control agents

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against plant diseases: Relevance beyond efficacy. *Frontier in Plant Science* **10(845), 1-19.**

Lim, P. H., Gansau, J. A. and Chong, K. P. (2015). Isolation of potential biological control agents of *Ganoderma boninense* from crocker range, Sabah. *Advances in Environmental Biology* **9(25), 55-60.**

Lim, P. H. Gansau, J. A. and Chong, K. P. (2018). *Streptomyces* spp. as a potential biocontrol agent against *Ganoderma boninense* of basal stem rot in oil palm. *Journal of Oil Palm Research* **30(2), 265-275.**

Lim, P. H., Gansau, J. A. and Chong, K. P. (2019a). Biocontrol of basal stem rot pathogen *Ganoderma boninense* by *Pseudomonas aeruginosa*. *Bangladesh Journal of Botany* **48(2), 209-215.**

Lim, P. H., Gansau, J. A. and Chong, K. P. (2019b). Preliminary screening and characterization of fungi isolated from forest soil as biocontrol agent against *Ganoderma boninense. Malaysian Journal of Microbiology* **15(2), 103-110.**

Malaysian Palm Oil Board (MPOB). (2019). Production of Crude Palm Oil For 2018. MPOB: http://bepi.mpob.gov.my/index.php/en/statistics/production/186-production-2018/850-production-of-crude-oil-palm-2018.html. [Retrieved on 26 March 2019].

Nielsen, K. F. and Madsen, J. O. (2000). Determination of ergosterol on mouldy building materials using isotope dilution and gas chromatography-tandem mass spectrometry. *Journal of Chromatography A* **898, 227-234.**

Rees, R. W., Flood, J., Hasan, Y., Wills, M. A. and Copper, R. M. (2012). *Ganoderma boninense* basidiospores in oil palm plantation: Evaluation of their possible role in stem rots of *Elaeis guineensis*. *Plant Pathology* **61(3), 567-578.**

Shrestha, C. L., Oña, I., Muthukrishnan, S. and Mew, T. W. (2008). Chitinase levels in rice cultivars correlate with resistance to the sheath blight pathogen *Rhizoctonia solani*. *European Journal of Plant Pathology* **120(1), 69-77.**

Tay, Z. H. and Chong, K. P. (2016). The potential of papaya leaf extract in controlling *Ganoderma boninense*. *IOP Conference Series: Earth and Environmental Science* **36 (012027), 1-7.**

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