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Antagonistic Efficacy of *Trichoderma harzianum* and *Bacillus cereus* against *Ganoderma* Disease of Oil Palm via Dip, Place and Drench (DPD) Artificial Inoculation Technique

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Abstract

Sustainability of the oil palm industry is crucial to ensure Malaysia's gross domestic product (GDP) by the agricultural sector. It is crucial to discover a sustainable and eco-friendly remedy for the most devastating *Ganoderma* disease of oil palm. The effects of pre-inoculation of oil palm seedlings with either *Trichoderma harzianum* and/or *Bacillus cereus* on their vegetative growth and the suppression of *Ganoderma boninense* were investigated. The dip, place and drench (DPD) artificial inoculation method was used to assure disease development. Disease severity was assessed based on the root symptoms (DS), disease incidence (DI) and disease reduction (DR). Application of a mixture of *T. harzianum* and *B. cereus* had the highest contribution to the vegetative growth of oil palm seedlings. However, single application of *B. cereus* was found to be the most effective treatment in suppressing *Ganoderma* disease of oil palm with a disease reduction of 94.75% followed by single applications of *T. harzianum* (78.98%) and mixture of both *T. harzianum* and *B. cereus* (68.49%). © 2017 Friends Science Publishers

Keywords: Dip, place and drench (DPD); *Bacillus cereus; Trichoderma harzianum*; Biocontrol

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is a tropical perennial crop, purposely for oil. The oil extracted from this crop is used world-wide for different industrial applications including cosmetics, oleo-chemicals, food and biofuel (Murphy, 2009; Paterson *et al*., 2009). This golden crop has been contributing generously to Malaysia's economy. However a major disease caused by *Ganoderma boninense* has been a treat to Malaysia's oil palm industry for many years and the estimated lost per annum was recorded as earnings of US$20 billion in terms of export earnings (MPOB, 2011) Therefore, there is an obvious need for understanding and developing an early control that will contribute to a sustainable environment along with catering this catastrophic disease (Paterson *et al*., 2009; Flood *et al*., 2010).

Government legislature for reducing the consumption of chemical fungicides had triggered an increasing public awareness on the hazards and the importance in attempting the search for alternatives of synthetic chemical fungicides. Hence, efforts to find an alternative way to control *Ganoderma* disease via biological control agents (BCAs) and utilization of resistant oil palms (Susanto *et al*., 2005) have been a better approach. *In vitro* studies have shown that *Trichoderma* spp., *Aspergillus* spp., and *Penicillum* spp. are antagonistic agents towards *Ganoderma* spp. (Bruce and Highley, 1991; Badalyan *et al*., 2004). These antagonists, particularly *Trichoderma* spp., are good BCAs of *Ganoderma* spp. (Bruce and Highley, 1991; Susanto *et al*., 2005). A part from fungus, endophytic bacteria was reported with the ability to enhance plant's immune system against pathogen attack. This is done by winning competition, antibiosis, induced resistance and promoting plant growth. Endophytic bacteria such as *Pseudomonas* and *Bacillus* spp. were found to be potent BCAs against fungal pathogens on crops such as cotton, oilseed rape, tomato, cucumber and peas (Chen *et al*., 1995; Alstrom, 2001). In addition, some members of the genus *Bacillus* such as *B. cereus* are often considered as microbial factories of biologically active molecules that are potential inhibitors of fungal growth (Pérez-García *et al*., 2011).

Besides that, recent control measures to overcome *Ganoderma* infection in palms are now focused on the use of BCAs. Subsequently, research on the use of BCAs for *Ganoderma* disease control has gained momentum.

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Consequently, in the present study, mixture of different BCAs was studied in an effort to add more potency to biological control solutions for *Ganoderma* disease.

In addition to that, an attempt to design a new artificial inoculation method to establish *Ganoderma* infection in *in vivo* trials has been taken in this study. To date the existing method using Rubber Wood Blocks (RWBs) (Khairudin, 1990; Nur Ain Izzati and Abdullah, 2008) is costly and it has been challenging to obtain the rubber wood supply due to the decreasing production. Apart from that, preparation of RWB inoculums are time consuming (approximately two months based on the RWB sizes) and the possibility of contamination with other fungi are high. Therefore, an alternative *Ganoderma* artificial inoculation method such as DPD would be desirable.

Hence, the objectives of this study were (i) to establish *Ganoderma* infection via a newly developed *Ganoderma* artificial inoculation method and (ii) to determine the efficiency of *in vivo* interaction of *Trichoderma harzianum* and/or *B. cereus* mixture for suppression of *G. boninense* infection in oil palm seedlings.

Materials and Methods

Maintenance of Microbes

All the microbes used in this study were isolated from oil palm roots in a preliminary study. These isolates were then sequenced, identified and sustained in the Department of Plant Protection, Universiti Putra Malaysia. Isolate UPM13 (*G. boninense*) and UPM29 (*T. harzianum*) cultures were maintained on Potato Dextrose Agar (PDA) (Difco™*)* and incubated at room temperature (27 ± 1ºC) for eight to ten days prior to usage. While pure freshly cultured bacterium UPM15 (*B. cereus*) cultures were maintained on Nutrient Agar (NA) (Difco™*)* at 4± 1ºC for short term storage.

Plant Material

Oil palm seedlings of three months old, commercial GH500 variant (Dura×Pisifera) placed in trays following normal nursery practices and certified as *Ganoderma*-free were purchased from Sime Darby Seeds and Agricultural Services Sdn Bhd., Banting, Selangor. The Seedlings were placed in a nursery, shaded with two layers of polynet 30/70 at Ladang 15 Nursery, Faculty of Agriculture, UPM, Serdang. Watering was done twice daily, before 11.00 am and after 4.00 pm. The trays containing 3 months old seedlings were left in the nursery for two weeks to stabilize and adapt to the nursery environment before transferring them to polythene bags (polybags) 30 cm × 38 cm with a thickness of 500 gauge (0.125 mm) (Halimah *et al*., 2010) containing 3 kg of sterile soil mixture (3:2:1 v/v/v topsoil: peat: sand) prior to pre-inoculation with the BCAs and artificial inoculation with UPM13 (*G. boninense*). *In vivo* nursery trial was conducted with eight treatments (Table 1), replicated twice with six seedlings per replicate.

Table 1: Percentage of disease incidence (DI) in oil palm seedlings after inoculation with UPM13 (G*anoderma boninense*)

Treatment	Disease Incidence (%)[#]				
	2 MAI[##]	3 MAI	4 MAI	5 MAI	6 MAI
Ganoderma boninense (G)	0	16.6[a]	33.3[b]	66.7[c]	83.3[d]
Bacillus cereus+Trichoderma harzianum+Ganoderma boninense (GTB)	0	0	16.6[a]	16.6[a]	33.3[b]
Trichoderma harzianum+Ganoderma boninense (GT)	0	0	0	16.6[a]	33.3[b]
Bacillus cereus+Ganoderma boninense (GB)	0	0	0	0	16.6[a]

[#]Means with the same letter in the same column are not significantly different with LSD at $P \leq 0.05$, (n=6)
[##]MAI = Months after artificially infected with *Ganoderma boninense*

Table S1: Treatments for experiment

Treatment	Description
T1 (TB)	plant + *T. harzianum* + *B. cereus*
T2 (T)	plant +*T. harzianum*
T3 (B)	plant + *B. cereus*
T4 (G)	plant + *G. boninense*
T5 (GTB)	plant + *G. boninense* + *B. cereus* + *T. harzianum*
T6 (GT)	plant + *G. boninense* +*T. harzianum*
T7 (GB)	plant + *G. boninense* + *B. cereus*
T8 (C)	+plant (Untreated negative control)

Inoculum of Biological Control Agents

Preparation and application of bacterium inoculum: UPM15 (*B. cereus*) inoculum suspension was prepared using 24 h old growing culture on NA. One mL of the 24 h old suspension was dislodged from the NA culture and pipetted into a test tube containing 9 mL of sterilized distilled water labeled as 10^{-1}. A series of serial dilution up to 10^{-7} was carried out and then 0.1 mL bacterial suspension of each dilution factor were spread using sterile L-shaped glass rod on individual NA plates and incubated for 24 h at 27±2°C. After 24 h, the presence of bacterial colonies on the NA plates were counted and expressed as colony forming unit (CFU mL^{-1}). The suspensions were adjusted to a concentration of 1×10^{-8} CFU mL^{-1} for seedling pre-inoculation purpose. Two weeks prior to artificial inoculation, the seedlings were pre-inoculated with UPM15 by drenching the soil with 150 mL of the suspension according to the designed treatments (Table S1). In addition to that, a booster application of the same concentration at pre-inoculation, UPM15 was applied again onto the seedling soil in the polybags after 25 days of artificial inoculation with UPM13.

Preparation and Application of Conidia Suspension

Conidia of UPM29 (*T. harzanium*) were harvested from seven day-old cultures on PDA. Ten milliliters of sterile distilled water was pipetted onto the PDA plate and the conidia were gently dislodged with a L-shaped glass rod.

Subsequently, the mixture was filtered through filter paper (Whatman® Grade 1, diameter: 9 cm, Pore Size: 11 µm) to remove mycelial debris. Distilled water was added to make 1 L. Two weeks prior to artificial inoculation with *G. boninense* inoculum, seedlings were pre-inoculated with 250 mL of fresh conidia suspension of UPM29 by drenching it onto the soil surrounding the stem of the seedling of each treatment according to the designed treatments (Table S1). In addition to that, a booster application in the same amount applied at pre-inoculation of UPM29 was applied after 30 days of artificial inoculation of the oil palm seedlings with UPM13.

Ganoderma Artificial Inoculation with Dip, Place and Drench (DPD) Technique

Mycelia of *G. boninense* was grown in 250 mL of potato dextrose broth (PDB) for ten days without shaking, and subsequently blender using a kitchen electric grinder (MX-800S, Panasonic Malaysia). The recipe mentioned is for a suspension size for artificial inoculation of one seedling. Once the inoculum suspension was ready, it was transported to the nursery. The oil palm seedling in the polybag was then carefully uprooted by taking out half of the soil from the polybag. The uprooted roots were then immersed or dipped into the suspension of *G. boninense* fragments with approximately 250 mL of the inoculum per seedling. Subsequent to that, two plates of fully-grown *G. boninense* on PDA was placed on the remaining soil in the polybag. The inoculum dipped oil palm seedling roots were then placed in the polybag on top of the *G. boninense* cultures and the remaining suspension for the dip step were drenched on the roots before covering with the same soil mixture taken out previously. The seedlings were watered twice daily throughout the experiment. The treatments designed for this study have been given in Table S1. Infection in the roots was confirmed by re-isolating *G. boninense* from the inoculated seedling roots after six months of inoculation on *Ganoderma* Selective Media (GSM) and via DNA detection using *Ganoderma* specific primer (Utomo and Niepold, 2000). This new DPD technique was validated thrice in a preliminary trial prior to the present study.

Effect of BCAs on *Ganoderma* Disease Affecting Oil Palm Seedlings

Assessment on the oil palm vegetative growth: Plant height, total root and top weight and bole girth were recorded onset, and 6 months after the treatment duration.

Ganoderma Disease Assessment at Nursery Trial

Disease development was monitored by measuring Disease Incidence (DI) percentage at monthly intervals. DI of seedlings was assessed as diseased visually (chlorosis and necrosis of leaves, with or without the production of fruiting body) (Idris *et al.*, 2006). DI referred to the number of seedlings showing symptoms mentioned above in relation to the total number of seedlings assessed by the formula by Campbell and Madden (1990):

$$\%\text{Disease incidence (DI)} = \left[\frac{\text{Number of seedlings infected}}{\text{Total number of seedlings assessed} \times 100}\right]$$

A reduction in the disease incidence compared with the control would be a measure of the treatment effectiveness in suppressing the disease. Disease progress curve (AUDPC) was calculated using the formula by Campbell and Madden (1990):

$$\text{AUDPC} = \sum_{i}^{n-1} (y_i + y_{i+1})/2(t_{i+1} + t_i)$$

Whereby: n = the number of assessment time; y = Disease incidence (DI); t = Observation time (months).
The efficacy of treatments in *Ganoderma* disease reduction was calculated with the following formula:

$$\text{Disease reduction (DR) (\%)} = \frac{\text{(AUDPC in positive control – AUDPC in treatment)} \times 100\%}{\text{AUDPC in positive control}}$$

In addition, the disease development in the seedlings was also rated as Disease Severity (DS). DS referred to the total area of plant tissues that exhibits disease symptoms. The percentage of DS of the oil palm root tissues were scored based on the disease rating scale by Breton *et al.* (2006) (Table 3) and the disease percentage was calculated according to the following formula:

$$\text{DS (\%)} = \frac{\Sigma\,(\text{Number of seedlings in the scale} \times \text{Severity scale}) \times 100}{\text{Total number of seedlings assessed} \times \text{Highest scale}}$$

Experimental Design and Statistical Analysis

The treatments designed for this study are shown in Table S1. The nursery trial experimental design used was Randomized Complete Block Design (RCBD) with eight treatments and six biological replicates. The percentage data were transformed by arcsine square root and analyzed by ANOVA with means compared by the Least Significant Difference (LSD) at $P \leq 0.05$. The vegetative growth data recorded were subjected to ANOVA and the significant data was determined using Tukeys's Studentized Range (HSD) Test at 5% probability level. All statistical analysis was done using SAS® software (v 8.1), Institute Inc. 1995.

Detection of *G. boninense* Genomic DNA on Treated Oil Palm Roots at 24 Weeks to Confirm Disease Establishment

DNA extraction, quantification, amplification and sequencing: Extraction method for total DNA was done according to the manual provided in Qiagen DNeasy Plant Mini Kit with a slight optimization according to Nusaibah *et al.* (2011). Seedlings were uprooted and the roots were harvested using a cutter and brought back to the lab that was located just 5 min' walk from the nursery.

Table 2: The effect of biological control agents (BCAs) on the development of *Ganoderma* disease in oil palm seedlings after artificially infected with *Ganoderma boninense* for six months

Treatment	AUDPC[1]	DR[2] (%)
Ganoderma boninense (G)	158.25	-
Bacillus cereus + *Trichoderma harzianum* + *Ganoderma boninense* (GTB)	49.85	68.49
Trichoderma harzianum+*Ganoderma boninense* (GT)	33.25	78.98
Bacillus cereus+*Ganoderma boninense* (GB)	8.3	94.75

[1]Area under disease progress curve (AUDPC) calculated with "t" as time in months and "Y" as the percentage of affected foliage at each reading and "n" as the number of readings
[2]Disease reduction (DR)

Table 3: Scale used to score disease severity index based on rotten root tissues of oil palm seedlings by UPM13 (*Ganoderma boninense*) (Breton *et al.*, 2006)

Scale	Symptoms
0	healthy no internal rot
1	20% rotting of tissue
2	20% to 50% rotting of tissues
3	>50% rotting of tissues
4	> 90% rotting of tissues

Subsequently the roots washed under running tap water until no soil was visible, dipped in 95% ethanol for 3 min and 5 min in sterile distilled water prior to the extraction process. Only rotten and necrotic secondary roots were subjected to the genomic DNA extraction. The extracted genomic DNA was checked for its concentration and purity using Nanodrop in MultiSkanGo instrument.

Polymerase chain reaction (PCR) amplification of *G. boninense* treated oil palm root genomic DNA was performed using the *Ganoderma* specific primers; Gan1: 5′ - TTG ACT GGG TTG TAG CTG - 3′ and Gan2: 5′ - GCG TTA CAT CGC AAT ACA - 3′ (Utomo and Niepold, 2000). Amplification was performed according to the protocols of Qiagen TopTaq Master Mix. Eppendorf Mastercycler® ep Gradient S Thermal Cycler (Hamburg, Germany) was used to run the polymerase chain reaction (PCR).The PCR started with denaturation for 2 min at 95°C. This was followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 30 sec at 59.9°C and extension for 2 min at 72°C. The final step of extension was carried out for 10 minutes at 72°C, before it was maintained at 4°C.

The Amplified PCR Products were Run on 1.7% Agarose Gel, Stained with Ethidium Bromide (EtBr) and Visualized under a UV Transiluminator

Next, DNA sequencing was done to confirm reliability of amplified products. DNA sequencing for forward and reverse primer amplified products were done by purifying the amplified PCR products using QIAquick Gel Extraction Kit (QIAGEN, Germany). And then sequencing service was outsourced to a commercial service provider (First BASE Laboratories Sdn. Bhd. Malaysia). The sequence similarity was matched via BLASTN tool in National Center for Biotechnology Information (NCBI) against the non-redundant nucleotide database in the GenBank for sequence identification purpose.

Results

Effect of BCAs on Vegetative Oil Palm Growth

In the present study, application of BCAs without *G. boninense* inoculation as single or mixture significantly increased plant height compared to the positive control (Fig. 1). From the data obtained, mixture application of TB treatment contributed the most in the progression of oil palm seedlings plant height (50.4 cm) followed by the single application of UPM16 (*T. harzianum*) with 49.3 cm. The lowest plant height was recorded in treatment GTB with 38.6 cm, which is lower than the positive control (43.4 cm). Nevertheless, significant growth of plant height was observed in between onset and after 24 weeks regardless of treatment.

Based on the data obtained on root dry weight of the treated seedlings, it was shown clearly that *B. cereus* contributed enormously on the growth enhancement of oil palm seedling root mass regardless of challenged or not challenged with pathogenic *G. boninense* (Fig. 2). Comparison on the single application of both BCAs pointed out that *B. cereus* (13.3 g) was the BCA accountable in contributing to the root weight significantly unlike *T. harzianum* (3.65 g), where the contribution was about 72% higher. However, seedlings treated with the mixture of BCAs gave the highest significant dry root weight of 17.4 g compared to all other treatments. Surprisingly, the *Ganoderma* control treatment had a significant higher root dry weight (3.32 g) than the negative positive control (1.99 g). Conversely, the data obtained on the aerial or top dry weight of the oil palm seedlings, single application of *T. harzianum* displayed a higher top dry weight of 14.1 g compared to *B. cereus* (8.27 g). On the other hand, the positive control with *G. boninense* exhibited the lowest top dry weight (4.66 g). However, again the highest top dry mass was yielded by mixture treatment of both BCAs (14.6 g) (Fig. 2).

Bole girth or circumference of treated oil palm seedlings only exhibited real significant differences in the treatments with unchallenged seedlings treated with the mixture of *B. cereus* and *T. harzianum* (4.53 cm) compared to the positive and negative controls (1.83 cm and 3.50 cm respectively) (Fig. 3). Unexpectedly, *Ganoderma* treated seedlings applied with single application of *B. cereus* (GB) demonstrated a bole size of 4.03 cm, which is 80.6% growth increase even though it is a diseased treatment compared to onset bole girth size. In general, the best vegetative growth of the bole by the TB treatment showed a growth increase of 82.6% within 24 weeks of inoculation period compared to onset bole girth size.

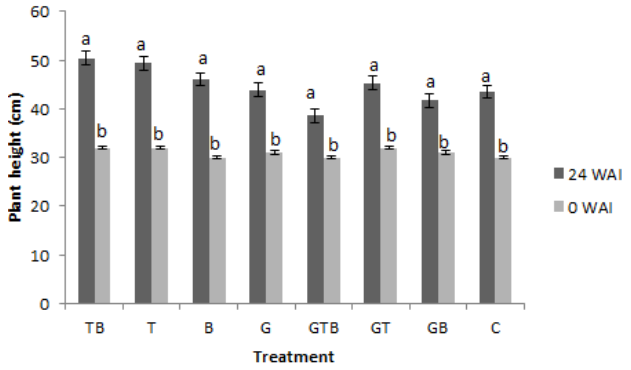

Fig. 1: Effect of *T. harzianum* and *Bacillus cereus* on plant height of oil palm seedlings onset and six months after challenged or not challenged with *G. boninense*. Values are the means ± S.E. (n =6). Means with different letters indicate statistically significant differences between two factors at $P \leq 0.05$

Treatments: TB= (*T. harzianum* + *B. cereus*); T = *T. harzianum;* B = *B. cereus*; G = *G. boninense*; GTB = (*T. harzianum* + *B. cereus* + *G. boninense*); GT (*T. harzanium* + *G. boninense*); GB= (B. *cereus* + *G. boninense*); C=Negative control

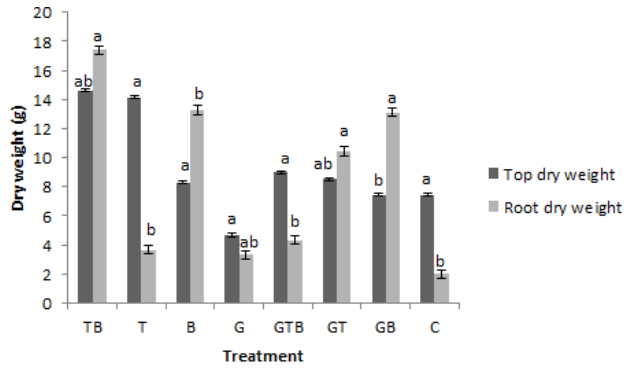

Fig. 2: Effect of *Trichoderm harzianum* and *Bacillus cereus* on the top dry weight and root dry weight of oil palm seedlings six months after challenged or not challenged with *Ganoderma boninense* pathogen. Values are the means ± S.E. (n =6). Means with different letters indicate statistically significant differences between two factors at $p \leq 0.05$

Treatments: TB= (*T. harzianum+B. cereus*); T=*T. harzianum;* B=*B. cereus*; G=*G. boninense*; GTB = (*G. boninense* + *T. harzianum* + *B. cereus* +); GT (*T. harzanium* + *G. boninense*); GB= (*G. boninense* +*B. cereus*); C=Negative control

Effects of BCAs on *Ganoderma* Disease Suppression

The nursery trial was conducted to evaluate the *in vivo* efficacy of BCAs against *Ganoderma* disease of oil palm. After 6 months of treatment duration with *G. boninense* via dip, place and drench (DPD) artificial inoculation technique, seedlings treated with BCAs regardless of single or mixture demonstrated significantly lower percentage of DI (Table 1). The initial DI was observed in *G. boninense* challenged seedlings three months after inoculation (MAI) with 16.6%.

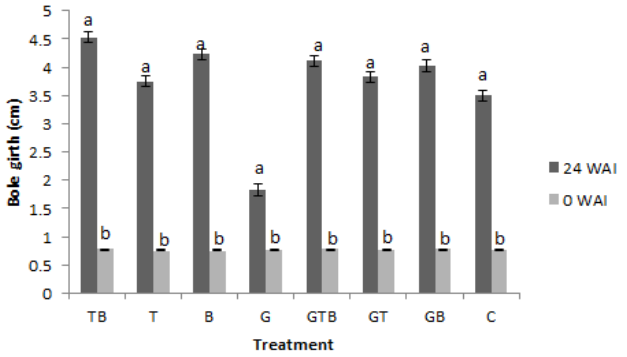

Fig. 3: Effect of *T. harzianum* and *Bacillus cereus* on the bole girth of oil palm seedlings onset and six months after challenged or not challenged with *Ganoderma boninense* pathogen. Values are the means ± S.E. (n =6). Means with different letters indicate statistically significant differences between two factors at $p \leq 0.05$

Treatments: TB= (*T. harzianum+B. cereus*); T=*T. harzianum;* B=*B. cereus*; G=*G. boninense*; GTB = (*T. harzianum* + *B. cereus* + *G. boninense*); GT (*T. harzanium* + *G. boninense*); GB= (B. *cereus* + *G. boninense*); C=Negative control

Nevertheless, a DI (16.6%) was detected in the mixture treatment with BCAs at four MAI. The least DI (16.6%) was exhibited in single treatment with *B. cereus* at six MAI compared with *T. harzianum* (33.3%) on the same MAI.

While the efficacy of the selected BCAs as a mixture or single application to reduce *Ganoderma* disease symptoms was expressed as the percentage of DR derived from the values of AUDPC (Table 2). The AUDPC values suggest the amount of disease developed in each treatment, where the treatment with the lowest AUDPC value indicates the effectiveness of the biocontrol in reducing the disease. The treated seedlings with *B. cereus* gave the highest DR of 94.8%, followed by *T. harzianum* (79.0%) and mixture of BCAs (68.5%).

Root disease severity index was scored after 6 months of inoculation with UPM13 (*G. boninense*) (Fig. 4) based on Table 3 disease scale by Breton *et al*. (2006). *Ganoderma* disease establishment was directly visualized based on the disease severity exhibited by the rotten and necrotic oil palm seedling root tissues. Visual colonization of *G. boninense* mycelium was also observed on both primary and secondary roots at 24 weeks. Based on the score carried out, treatment with *B. cereus* displayed the lowest DS (8.33%) followed by mixture of both BCAs (25.0%), *T. harzianum* (33.3%) and the highest DS observed in *G. boninense* challenged seedlings without any treatment (83.3%).

Detection of *G. boninense* on Inoculated Roots as Proof of Disease Establishment after Six Months of Inoculation Via Dip, Place and Drench Technique

In order to validate the disease establishment of the newly invented artificial inoculation method (DPD), presence of *G. boninense* in the challenged oil palm roots were detected via

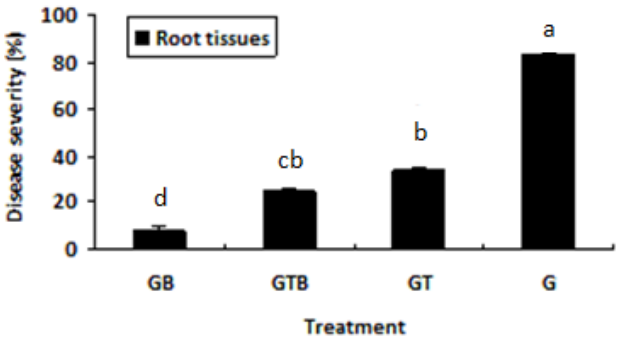

Fig. 4: Bar graph shows the percentage of disease severity (DS) on oil palm seedlings root tissues, six months after inoculation with *Ganoderma boninense* and treated with *Trichoderma harzianum* and *Bacillus cereus*. The means with the same letters between treatments are not significantly different at $P \leq 0.05$. Values are the means ± S.E. (n =6)

Treatments: G=*G. boninense*; GT = (*T. harzanium* + *G. boninense*); GB = (B. *cereus*+*G. boninense*); GBT = (*T. harzianum*+*B. cereus*+*G. boninense*)

Fig. S1: Amplified DNA showing band approximately 170 bp on 1.7% agarose gel for detection of *Ganoderma boninense* (UPM13) on treated seedlings at six months of post-inoculation

Treatments on four months old oil palm seedlings inoculated with; Lane 1: *Ganoderma boninense* (G); Lane 2*: Trichoderma harzianum*+*Ganoderma boninense* (GT)*;* Lane 3*: Bacillus cereus*+*Trichoderma harzianum*+*Ganoderma boninense* (GTB)*;* Lane 4*: Bacillus cereus*+*Ganoderma boninense* (GB); Lane 5: *Bacillus cereus* (B): Lane 6: *Trichoderma harzianum* (T); Lane 7: *Bacillus cereus*+*Trichoderma harzianum* (TB); Lane 8*:* Control (un-inoculated roots); L9: Negative control (non-template); M: 100 bp ladder

Fig. S2: Proof of *Ganoderma* disease establishment via artificial inoculation with Dip, Place and Drench (DPD) technique. Treatments: G=*G. boninense*; C= negative control (un-inoculated seedling)

DNA and sequenced for identification after six months of post-inoculation period in a qualitative analysis. To date, detection based on DNA is the most reliable and accurate method. Based on the gel electrophoresis results on amplified PCR products obtained, high intensity of amplified PCR product band was present in the disease control seedlings (Fig. S1 and L1). However, in seedlings treated with BCAs (L2, L3 and L4), low intensity bands were detected, displaying the efficacy of BCAs in suppression or inhibition of *G. boninense* growth. Nonetheless, no bands or detection of *G. boninense* in L5-L9, indicates the reliability of this analysis. In addition, sequenced PCR products also gave 100% similarity with the UPM 13 isolate that was used for *Ganoderma* disease establishment in the nursery trial conducted. While Fig. S2 (supplementary data) displays visual proof on the reliability of the new artificial inoculation method (DPD) in establishing *Ganoderma* disease in oil palm seedlings. Rotten and necrotic primary and secondary root tissues were demonstrated in seedlings inoculated with UPM13 (*G. boninense*) compared to un-inoculated seedling. To further strengthen the disease establishment results, harvested roots (for treatment G, GTB, GT and GB) were yielded on GSM for UPM13 isolation and identification, and all the results obtained were positive on the GSM with UPM 13.

Discussion

In order to assess control options, in *in vivo* trials, the reliability of an artificial inoculation method for disease establishment is crucial. The current established method for *Ganoderma* artificial inoculation requires rubber wood blocks (RWBs), which is costly and difficult to obtain nowadays. In addition to that, preparations of RWB inoculum are time consuming (approximately two months according to RWB sizes) and the possibility of contamination with other fungi is high. In addition to that, most importantly a fully colonized RWB in standard sizes used (6 cm × 6 cm × 6 cm and 6 cm × 6 cm × 12 cm) often exaggerates the disease severity on the young oil palm seedlings as old as 3 to 5 months that is often used in the nursery trials. This is way far from the actual disease establishment in the real-time environment where abundant inoculums present on-site. Therefore, an alternative potent *Ganoderma* artificial inoculation method such as DPD is essential. The present study presents a new artificial *Ganoderma* disease inoculation method the Dip, Place and Drench (DPD). Based on the trials conducted, this method can be classified as effective on disease establishment, economical, non-laborious and not time consuming.

The current study evaluated two different BCAs as single and consortium application on oil palm seedlings. The first was the effect on the vegetative growth of oil palm seedlings, while the second was the ability of BCAs to

suppress *Ganoderma* disease. In general, higher vegetative growth was recorded in all BCA treated seedlings when compared to the untreated seedlings. However, mixture of both *T. harzianum* and *B. cereus* proved to be significantly superior among all the treatments in enhancement of vegetative growth. This was followed by the single application of *T. harzianum* and *B. cereus*, which also significantly improved the vegetative growth of oil palm seedlings studied. Nevertheless, it was noticed that *T. harzianum* (T) application contributed to the most significant higher dry weight for top among all treatments. These show that, *T. harzianum* contributes enormously in growth of oil palm foliar and stem, which classifies it as an excellent plant growth promoter. This was also in line with studies carried out by Harman *et al*. (2004) and Bal and Altintas (2006) where they observed increased growth response induced by *Trichoderma* species in several crops. Apart from that, several work that also yielded positive results on *Trichoderma* sp. as oil palm growth promoter and BCA were reported by Susanto *et al*. (2005), Nur Ain Izzati and Abdullah (2008) and Naher *et al*. (2012). *Trichoderma* spp. increases the uptake and concentration of soil nutrients.

Work by Dawwam *et al*. (2013) recommended *B. cereus* isolated from roots of potato plant as a biofertilizer component due to its excellent plant growth promoting characteristics. In addition, Zhao *et al*. (2011) also classified *B. cereus* isolated from *Sophora alopecuroides* root nodules as a potent plant growth promoter. Thus, these studies were in harmony with the present study.

On the other hand, both BCAs studied in the present work demonstrated its ability in suppressing *Ganoderma* disease of oil palm *in vivo* via DR and DS of roots analyses. *Trichoderma* spp. have been one of the most established BCA used worldwide due to its potent antagonistic effects against numerous plant fungal pathogens, including *Fusarium* spp., *Sclerotinia* spp. and *Rhizoctonia* spp. (Pandey *et al*., 2005; Bernal *et al*., 2009). To date, strains of *Trichoderma* including *T. harzianum* are currently being commercialized because they exert strong competitive effects for space and nutrients; more importantly, they produce toxins against phytopathogenic species, thus making them excellent biocontrol agents (Zimand *et al.*, 1996; Susanto *et al.*, 2005; Sharoni *et al*., 2006). Hence, this BCA would be the most preferred component in an amendment of a biological control compost or biofertilizer. According to Pugliese *et al*. (2011) and Harman *et al*. (2004), *Trichoderma* as a component in compost could lead to a substrate with broader–range suppressive effects. However, this present study demonstrated that consortium of *T. harzianum* and *B. cereus* was less effective in controlling *Ganoderma* disease of oil palm at nursery trial compared to individual application of the BCAs. This may be due to some incompatibility between them, as BCAs were typically selected based on their individual antagonistic behavior towards pathogens *in vitro* rather than for their combined potency (Leeman *et al.*, 1996; Meyer and Roberts, 2002). Even though, this consortium did show some level of *Ganoderma* disease reduction by 68.49%.

Remarkably, it was also found single *B. cereus* treatment was able to display significantly higher percentage of *Ganoderma* disease suppression compared to *T. harzianum* with 94.75% DR rate. In harmony to the present study, Zaiton *et al.* (2008) and Ramli *et al*. (2016) also listed *B. cereus* as one of potential endophytic bacteria against *G. boninense* growth *in vitro*. However, Zaiton *et al.* (2008) and Ramli *et al.* (2016) did not test the ability of *B. cereus in vivo* or in *Ganoderma* disease suppression in oil palm nursery trials. This shows that, contrary results may be obtained in *in vivo* trials compared to *in vitro* experiments, as these are very different environments. Besides that, the reduction in DS suggested that *T. harzanium* and *B. cereus* played an independent role in the inhibition of *Ganoderma* disease symptoms. In line to that, most bacteria used as BCAs are endophytes or common rhizosphere-colonizing bacteria such as *Bacillus* spp., *Enterobacter* spp., *Pseudomonas* spp., *Serratia* spp., and *Burkholderia* spp. (Van Loon and Bakker, 2004).

Conclusion

Promising results were obtained on the vegetative growth enhancement and suppression of *Ganoderma* disease by *B. cereus* and *T. harzianum in* the *in vivo* nursery trial on oil palm seedlings. The association and effect of these endophytic microbes on these abilities need to be assessed further based on the biochemical changes and gene expression at the molecular level.

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