Induced Biochemical Changes in *Ganoderma boninense* Infected *Elaeis guineensis* Seedlings in Response to Biocontrol Treatments

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ABSTRACT

Despite massive economic contributions to Malaysia, the oil palm industry faces devastating threats from basal stem rot (BSR) disease. An array of treatments was designed to evaluate the potential of biological control agents (BCAs) as a single and combination of applications in a greenhouse study of six months. Oil palm enzymes, phenolic content, and metabolite induction in BSR-diseased seedlings were also assessed in response to the designed treatments. In the study, seedlings treated with *Trichoderma asperellum* (UPM16) demonstrated the highest disease reduction (DR) (57.2%). Peroxidase (PO), lignin, and total phenolic content (TPC) were evaluated. Treatments on *Ganoderma*-infected seedlings treated with *Bacillus cereus* (UPM15) exhibited the highest reading in all assays. Gas chromatography-mass spectrometry (GC-MS) analysis profiled phenol, 4-2-aminoethyl-as the most abundant metabolite detected in combination treatments with *B. cereus* and *T. asperellum* (BT). Both BCAs complimented and demonstrated huge potential in mitigating BSR diseases in oil palm. However, excessive chemical application to control BSRs negatively impacts biodiversity and the human population. In view of this, studies on biological control are crucial in selecting potential BCAs to counter BSR sustainably. Biological control would be an ideal alternative as a sustainable method for controlling oil palm BSR disease.

Keywords: Basal stem rot, biological control agents, lignin, metabolites, peroxidase, total phenolic content

INTRODUCTION

A disease of oil palm, such as one caused by a genus of polypore fungi, *Ganoderma* spp., continues to cause significant yield reduction and losses through the collapse of standing palms. The most destructive and vital pathogen that causes basal stem rot (BSR) is *Ganoderma boninense*. This pathogen causes damage to up to 80% of palm stands when they are just about halfway through their economic lifespan (Bivi et al., 2010). Synthetic chemical control using hexaconazole is widely used when dealing with this soil-borne pathogen in the plantations. However, synthetic chemical control leads to groundwater pollution, evolving fungicidal resistance variants, and loss of non-target beneficial flora.

Against this background arising from excessive usage of chemicals, the oil palm industry must transform its current practices to more environment-friendly methods. Biological control agents (BCAs) could be the potential alternatives to chemical pesticides. Several potential BCAs, including *Trichoderma* spp. (Nusaibah et al., 2017; Sariah et al., 2005), and *Bacillus* spp. (Nusaibah et al., 2017) have shown their efficacy in inhibiting *G. boninense*'s growth and subsequently reducing infection. In justification, BCA generally occupies the rhizosphere of the soil profile and produces almost no toxic residues as opposed to chemicals (Ashbolt et al., 2013).

The present study was conducted to assess and evaluate BSR disease suppression in the presence of both BCAs applied as pre-treatments prior to *G. boninense* inoculation. Literature has it that a pathogen attack or the presence of an elicitor triggered various plant protective mechanisms specially developed to counteract the invasion of a pathogen causing an infection (Małolepsza & Różalska, 2005). Furthermore, several studies suggested that plant polymers, such as lignin and suberin, played a direct role in the breakdown of the pathogen cell wall (Treutter, 2006; Usall et al., 2000). Furthermore, Surekha et al. (2013) published those phenolic compounds act as antimicrobials, growth interceptors of pathogens, trigger plant defence genes, and structural barriers. Therefore, it is empirical to explore the effects of BCAs, such as *B. cereus* and *T. asperellum*, on their role in the heightening of oil palm defence mechanisms against *G. boninense* infection via enzymes, phenolic content, and metabolites.

MATERIALS AND METHODS

Plant Materials

The present study used three months old commercial oil palm seedlings (*dura × pisifera*). Before transplanting, a 3 : 2 : 1 soil mixture of topsoil, peat moss, and sand was made and sterilised in an autoclave for 30 minutes at 121°C and 100 kPa pressure at Laboratory of Biological Control , Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia (UPM).

Ganoderma boninense Artificial Inoculation

Artificial inoculation of *G. boninense* (UPM13) was carried out following the

dip, place, and drench (DPD) technique as described by Nusaibah et al. (2017). The seedlings were irrigated twice daily at 9 a.m. and 6 p.m. throughout the treatment. Commercial Nitrogen : Phosphorus : Potassium (N : P : K) (15 : 15 : 15) fertiliser (10 g per polybag) was applied at monthly intervals.

Inoculum of Microbes

Trichoderma asperellum isolate and *Bacillus cereus* bacterium was isolated from BSR-infested oil palm plantation soil. These microbes were identified using a universal internal transcribed spacer (ITS) primer set and 16S barcoding, respectively, and morphological identification in a previous study (Nusaibah et al., 2017; Syafiq et al., 2021). *Bacillus cereus* inoculum suspension was prepared using 48-hour grown culture on nutrient agar (NA). The concentration prepared was 10^8 colony-forming units (cfu) mL^{-1} (Zaiton et al., 2008). In addition, a 150 mL *B. cereus* suspension was administered to the seedlings 14 days before artificial inoculation with *G. boninense* inoculum by drenching the soil following a pre-designed treatment, as presented in Table 1.

Three days after the artificial inoculation of the seedlings with *G. boninense*, the application of *B. cereus* was made as a booster dose. Minor adjustments were made to Izzati and Abdullah's (2008) instructions while preparing the conidial suspension of *T. asperellum*.

Whatman® Grade 1 filter paper was used in place of muslin fabric. *Trichoderma asperellum* conidia were obtained from an inoculum cultured on potato dextrose agar (PDA) for seven days. After pipetting an aliquot of 10 mL sterile distilled water onto a PDA plate, the conidia were gently pushed with an L-shaped glass rod. The mixture was then passed via filter paper to eliminate the mycelial debris. The filtrate was diluted to a volume of 1 L using distilled water. The range of 10^7 conidia mL^{-1} was specified for conidia counts. Fourteen (14) days before *G. boninense* artificial inoculation, 250 mL of freshly prepared *T. asperellum* conidial suspension was applied to the seedlings via the drenching technique. Following five days of artificially inoculating the oil palm seedlings with *G. boninense*, a booster dose of *T. asperellum* was applied with a concentration similar to that of the initial treatment.

Table 1
Treatment design for greenhouse study

Treatment	Description
T1 (BT)	Plant + *Trichoderma asperellum* + *Bacillus cereus*
T2 (T)	Plant + *Trichoderma asperellum*
T3 (B)	Plant + *Bacillus cereus*
T4 (G)	Plant + *Ganoderma boninense*
T5 (BTG)	Plant + *Ganoderma boninense* + *Bacillus cereus* + *Trichoderma asperellum*
T6 (TG)	Plant + *Ganoderma boninense* + *Trichoderma asperellum*
T7 (BG)	Plant + *Ganoderma boninense* + *Bacillus cereus*
T8 (NC)	Plant (Untreated negative control)

Double-Sealed Plate Assay

Various studies have indicated that microbial volatile organic compounds (MVOCs) can stimulate plant growth and actively restrain fungal growth (Weisskopf, 2013). With a few minor adjustments, Gotor-Vila et al. (2017) technique was used to conduct the double-plate assay. The assay was to determine the antifungal effects of MVOCs emitted by *B. cereus* and *T. asperellum* on the growth of *G. boninense*. A 3-day-old *B. cereus* culture previously cultivated on NA was placed facing a PDA plate with a 5 mm *G. boninense* mycelium plug incubated into the plate's centre without a cover. The plates were parafilm-sealed and maintained at room temperature for 12 days. The conditions used in the test were: *B. cereus* culture as the base plate and *G. boninense* culture as the top plate. Seven replication was prepared for this assay. NA plates without *B. cereus* culture served as the control treatment. The diameter of *G. boninense* mycelium growth was recorded in millimetres after 12 days of incubation. A similar procedure was repeated using 7-day-old *T. asperellum* culture grown on PDA.

Experimental Design and Statistical Analysis

Table 1 displays the treatment design for the current trial. The greenhouse experiment was conducted using a randomised complete block design (RCBD) with eight treatments and twelve replications. All oil palm seedlings in the polythene bags were arranged on eight benches in a randomised manner. Every bench would be a block. Block factors, such as light, temperature, and moisture conditions that could affect the response variable were under contemplation. The area under the disease progress curve (AUDPC) was used to calculate disease reduction (DR). All the disease incidences and severity were arcsine transformed (Gomez & Gomez, 1984).

Disease Progress Assessment

The disease incidence (DI) is the proportion of seedlings with leaves that are chlorotic and necrotic, whether they have basidiocarps (Idris et al., 2006).

Equation 1:

$$\text{Disease incidence} = \frac{\text{Number of seedlings infected}}{\text{Total number of seedlings assessed}} \times 100$$

A decrease in the DI compared to the control measures how well a treatment suppresses a disease. In addition, a disease progression curve was created using the data to assess the treatments' effectiveness.

The following formula from Shaner and Finney (1977) was used to determine the AUDPC:

Equation 2:

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

whereby:

n = Number of assessment time
y = Disease incidence (DI)
t = Time (months) after inoculation

The efficacy of treatments in disease reduction (DR) was calculated using the following formula:

Equation 3:

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

The following Tarig et al. (1998) formula was used to determine the percentage of disease severity (DS) in the root tissues, and the severity scale was assessed by the severity index of Breton et al. (2006) (Table 2):

Equation 4:

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

Table 2
The scale used for scoring disease (G. boninense) *severity index based on rotten root tissues of UPM13 seedlings*

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

Source: Breton et al. (2006)

Data on disease incidence and disease severity were analysed by one-way Analysis of Variance (ANOVA). Mean values were compared by least significant difference (LSD) test ($P \leq 0.05$).

Establishment of Basal Stem Rot Disease and Biological Control Treatments

Scanning electron microscope (SEM) was used to examine the disease establishment via colonisation of the *G. boninense* pathogen and the BCAs on the roots of seedlings after one month of inoculation. SEM sample preparation was done following *in-house* procedures of the Microscopy Unit, Institute of Bioscience (IBS), UPM.

Enzyme Assay

Total Peroxidase (PO) Assay. Enzyme extract was created using, with some modifications, Samatha et al. (2012)'s methods. Approximately 1 g of the root was harvested during destructive sampling and subsequently immersed in liquid nitrogen. As soon as possible, the sample was homogenised in 2 mL of cold, 0.05 mol L^{-1} sodium phosphate buffer (pH 5) that had been adjusted with 5 mL of polyvinyl pyrrolidone (PVP) (Sigma-Aldrich, USA). The semi-solid mixture underwent a 20-minute, $18,922 \times g$ centrifugation at 4°C. An aliquot of 200 µL of the supernatant was extracted. Three millilitres of a reaction mixture containing 0.1 mmol L^{-1} sodium acetate buffer (pH 6), 1 mmol L^{-1} H_2O_2 (3%, v/v), and 0.1 mmol L^{-1} *o*-methoxyphenol (guiacol) (Acros

Organics, USA) was prepared. The reaction mixture was mixed well with the previously removed supernatant and then left to sit at room temperature for 2 min. The mixture's absorbance was read at 470 nm with a spectrophotometer (Thermo Scientific Multiskan Go, Thermo Fisher Scientific, Finland). The reaction mixture was used to prepare blanks instead of the supernatant. Change in absorbance $min^{-1}g^{-1}$ protein was used to express PO activity (Kokkinakis & Brooks, 1979).

Data on enzyme concentration and activity were analysed by one-way ANOVA. Mean values were compared by Tukey's Studentized Range (HSD) Test at 5% level of significance.

Lignin Assay. One gram of frozen root from a damaging sample was dissolved in 5 mL of absolute methanol (Merck Schuchardt OHG, Hohenbrunn, Germany) for 48 hours (in four changes of methanol) (Doster & Bostock, 1988). After the root tissue samples were pulverised, 50 mL of them were put into an Eppendorf tube with 0.1 mL of thioglycolic acid and 0.9 millilitres of 2 N hydrochloric acid (HCl) (J.T. Baker®, USA) The samples were then heated for 4 hours in a water bath based on the method proposed by Bruce and West (1989) with some modification where the temperature of the water was reduced from 100°C to 95°C. Following cooling to room temperature, the heated sample test tubes underwent a 5-minute, 13902 × *g* centrifugation. Distilled water was used to clean the residue that had been collected. Finally, the samples were re-centrifuged to obtain the pellet. About 1 mL of 0.5

N sodium hydroxide (NaOH) (Systerm Chemicals, Malaysia) was added to the pellet tubes. The samples were incubated overnight at 28ºC and were later centrifuged. A 1 mL of concentrated HCl (37%) (Avantor Performance Materials, USA) was added to the resulting supernatant, centrifuged, and washed with distilled water. A solution of 1 N NaOH (Systerm Chemicals, Malaysia) in 1 mL was used to dissolve the pellet. After mixing 25 mL of the aliquot with 1 mL of 0.5 N NaOH (Systerm Chemicals, Malaysia), the results were read at 280 nm using Thermo Scientific Multiskan Go (ThermoFisher Scientific, Finland) (Dean & Kuc, 1987).

Total Phenolic Content (TPC) Analysis

The Folin-Ciocalteu colourimetric method was employed to determine the total phenolic content (TPC) (Singleton et al., 1999). The absorbance was measured at 725 nm using methanol as the blank and gallic acid as the reference standard. Gallic acid equivalents (GAE) were used to express the results.

Extraction of Metabolites for GC-MS (Gas Chromatography-Mass Spectrometry) Analysis. For metabolite profiling, the sample extraction method followed the procedure of Nusaibah et al. (2016) with some modifications. An amount of 1 g of oil palm leaf sample from the selected treatment was harvested, washed with sterile distilled water, ground in liquid nitrogen, and then transferred into a Falcon tube. A 5 mL methanol liquid chromatography grade (Merck, Germany) was added, and the

tube was vortexed to fully mix the sample. Subsequently, the mixture was stored for 48 hours at 4°C. The mixture was filtered through a 0.4 μm nylon syringe filter following an incubation period of roughly 40 hours. The compounds collected were evaporated at 38°C on a rotary evaporator until dry. An aliquot of 1 mL of methanol was added to the dried sample, syringe-filtered again, and transferred into a glass insert placed in an amber vial before subjecting it to GC-MS analysis.

Untargeted Metabolite Profiling of Treated Oil Palm Seedling Leaves. Based on the modified and improved approach of Fiehn (2002) and Nusaibah et al. (2016), GC-MS analysis was carried out at the Halal Product Research Institute, UPM. A DB5 capillary column (30 m long, 0.25 mm I.D. 155, and a 0.25 μm 5 % phenyl methylpolysiloxane column with an additional 10 m integrated guard column were used for chromatography). Agilent (USA) autosampler for GC (7890A) and MS (5975C) was equipped with a standard 10 μL injection needle. In a splitless mode, 2 μL of each sample was injected.

The column was heated to 180°C for 5 min, 180-260°C for 3 min, 260-280°C for 2 min, and lastly, 280°C for 5 min, with the injector temperature at 280°C and the detector temperature at 290°C. The carrier gas was helium, flowing at a rate of 0.7 mL min^{-1}. Following are the MS operational parameters: Ionisation potential of 70 eV, quadrupole temperature of 100°C, ion source temperature of 290°C, solvent delay of 7 min, the scan rate of 2000 amu sec^{-1}, the scan range of 30-600 amu, and EV voltage of 3000 V. Six biological replicates were run for each treatment and subjected to multivariate analysis. GC-MS results were run through the MetaboAnalyst 4.0 software (Canada, 2019) and based on the multivariate analysis method, specifically Partial Least Squares – Discriminant Analysis (PLS-DA). Metabolites detected from all treatments were discriminated in importance features analysis (Figures 5 and 6). Ten metabolites with the highest concentration were selected to help narrow down the result. The Variable Importance in Projection (VIP) scores estimate the importance of each variable in the projection used in a PLS model. A variable with a VIP Score close to or greater than one can be considered important in given model.

RESULTS

Double-Sealed Plate Test

The mycelial growth inhibition rate of pathogenic *G. boninense* in a double-plate test indicated the effectiveness of MVOCs produced by the BCA isolates. Figure 1 shows the mycelial growth of *G. boninense* when treated with *B. cereus* isolate, recorded after 12 days of incubation. This study showed that *B. cereus* could produce MVOCs that effectively inhibited mycelial growth with a mean of 51.3 mm compared to the control plates (85.0 mm). However, results for the *T. asperellum* assay failed to be recorded due to the overgrowth of *T. asperellum* mycelium on the surface of *G. boninense* culture plates.
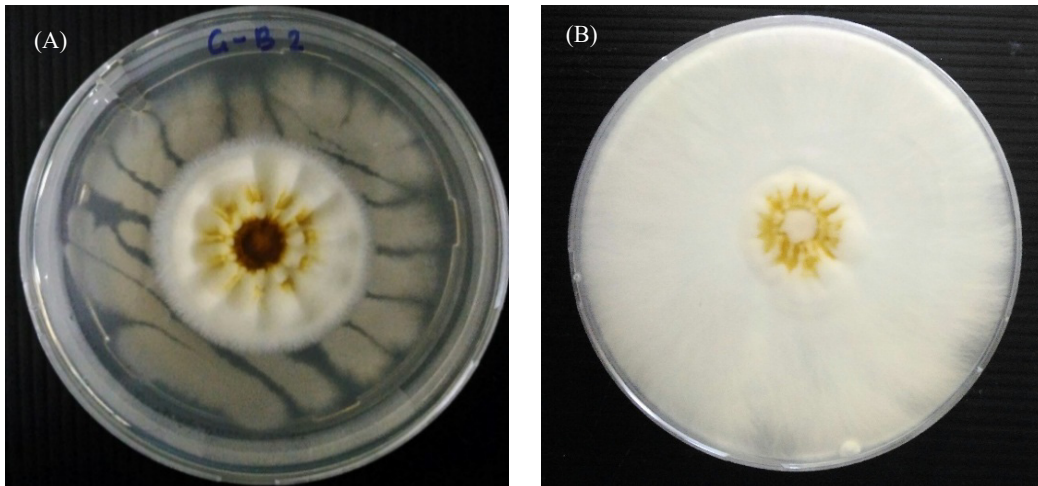
Figure 1. Growth inhibition of *Ganoderma boninense* (UPM13) by volatile compound emitted by *Bacillus cereus* (A) compared to the control plate (B)

Basal Stem Rot (BSR) Disease Assessment

The impact of treatments on disease suppression in oil palm seedlings was investigated during a 6-month greenhouse trial. Figure 2 presents visible proof of disease establishment in terms of root (A and B) and aerial parts (C and D). The percentage of DI was recorded and shown in Table 3. Data on DI recorded those
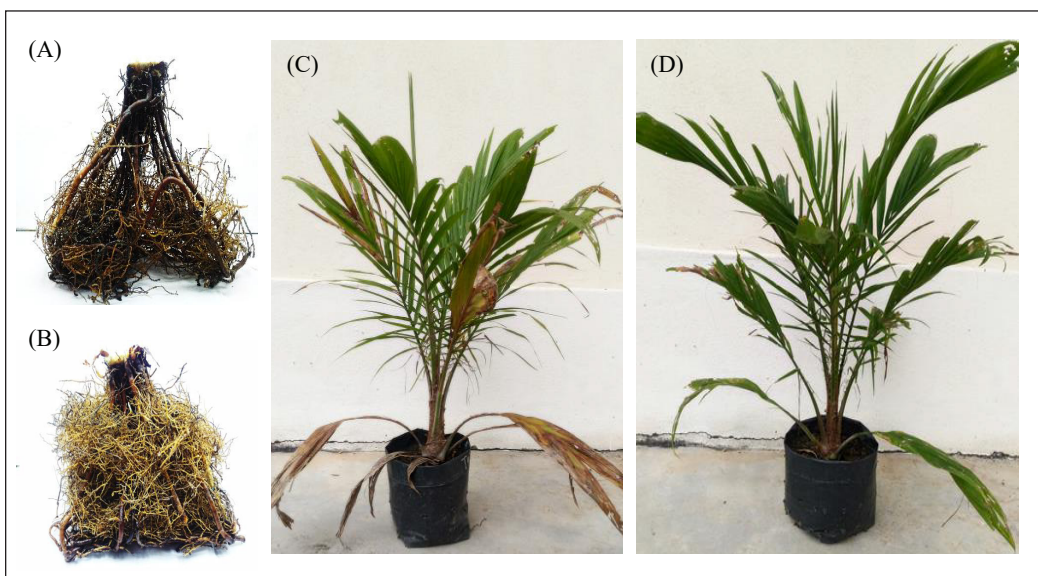

Figure 2. The visual appearance of basal stem rot establishment via artificial inoculation by dip, place, and drench (DPD) technique. (A) *G. boninense* inoculated roots from treatment G; (B) *G. boninense* uninoculated roots served as control; (C) showing external disease symptoms resulting from *G. boninense* inoculation; (D) showing no external disease symptoms observed on control palm

seedlings treated with a consortium or single treatment gave a lower percentage of DI after six months. At 3 months after inoculation (MAI), untreated Ganoderma-infected seedlings demonstrated the first DI at 25% infection, while the remaining treatments displayed DI at 4 to 5 MAI. At 6 MAI, the lowest DI was recorded in combination treatment (BTG) with 81.3%, followed by single treatments of B. cereus (87.5%) and T. asperellum (93.8%).

Based on methods developed by Breton et al. (2006), the DS on oil palm seedling root tissues was recorded after 6 MAI. Single treatment of T. asperellum (TG) recorded the lowest root DS at 50%, followed by consortium treatment (BTG) at 63%. Untreated Ganoderma-infected plants (G) gave the highest root DS at 84%.

Table 4 shows AUDPC and percentages of DR. Disease reduction was calculated using AUDPC values and the DR formula as described in Equation 3. Single treatment of T. asperellum gave the highest DR with 57.2%, followed by the consortium and single treatments of B. cereus, with both giving 50% DR.

Pathogen and BCA Colonization in Inoculated Oil Palm Roots

SEM image of root samples showed that BCAs and *G. boninense* had successfully colonised the root tissues, as depicted in Figures 3 and 4. Figure 3A demonstrates activities in the *Ganoderma*-infected seedling treated with a single application of *B. cereus* where *G. boninense* colonised the roots and *B. cereus* cells colonising *G. boninense* hypha.

In *Ganoderma*-inoculated seedlings treated with a single application of *T.*

Table 4

The effects of biological control agents on the progression of basal stem rot disease in oil palm seedlings based on the severity of the root infection following a 6-month artificial infection with G. boninense

Treatment	AUDPC[1]	DR[2]
Plant + *Ganoderma boninense*	263	-
Plant + *Trichoderma asperellum + Bacillus cereus*	131	50
Plant + *Trichoderma asperellum*	112	57.2
Plant + *Bacillus cereus*	131	50

Note. [1]AUDPC = Area under disease progress curve; [2]DR = Disease reduction

Table 3

Percentages of disease incidence in oil palm seedlings following inoculation with UPM13 (G. boninense)

Treatment	Disease incidence (%)*			
	3 MAI**	4 MAI	5 MAI	6 MAI
Ganoderma boninense (G)	25[a]	50[b]	93.8[d]	100[d]
Bacillus cereus + Trichoderma asperellum + Ganoderma boninense (BTG)	0	31.3[a]	56.3[b]	81.3[d]
Bacillus cereus + Ganoderma boninense (BG)	0	37.5[a]	68.8[c]	87.5[d]
Trichoderma asperellum + Ganoderma boninense (TG)	0	43.8[b]	81.3[d]	93.8[d]

* Means with the same letter in the same column are not significantly different by least significant difference (LSD) at $P \leq 0.05$, (n = 6)
** MAI = Months after inoculation with *G. boninense*

Pertanika J. Trop. Agric. Sci. 46 (1): 129 - 151 (2023)

137

asperellum, the hyphae (of *T. asperellum*) were observed colonising *G. boninense* hyphae and the primary roots (Figure 3B). In Figure 4C, both BCAs were observed colonising the oil palm roots in a manner observed in the combination treatment. *Bacillus cereus* cells were also observed on *G. boninense* hyphae (Figure 4D).
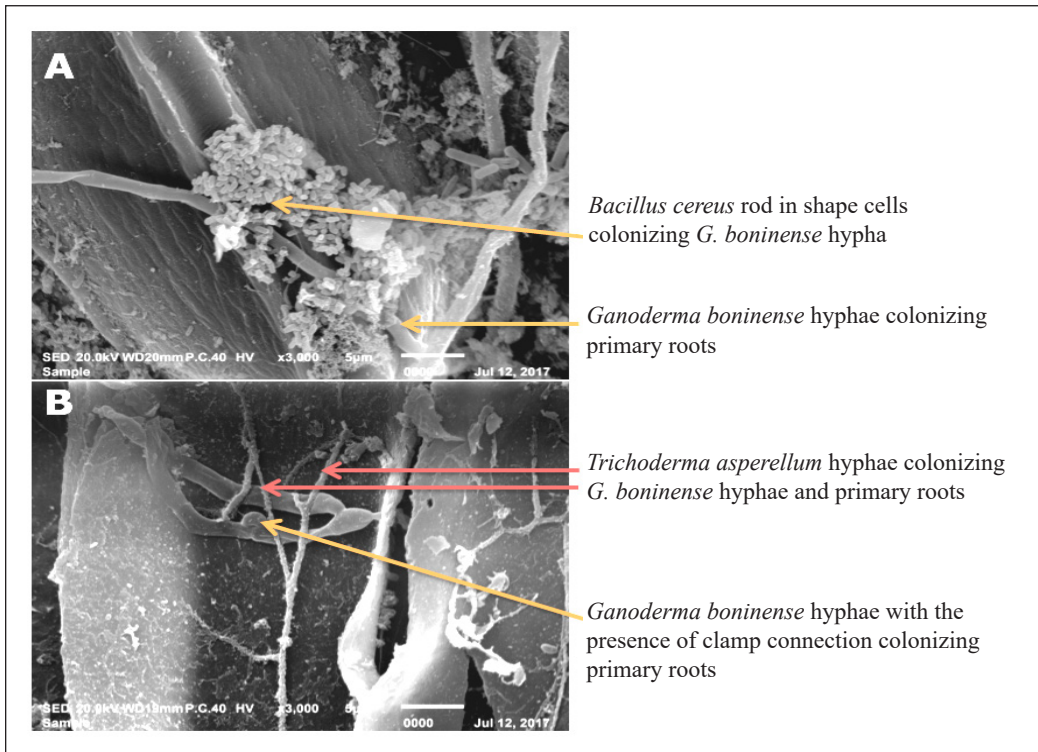

Figure 3. Oil palm seedling roots pre-inoculated with (A) *Bacillus cereus* (B) *Trichoderma asperellum* that were infected with *G. boninense*, and harvested after eight weeks of incubation
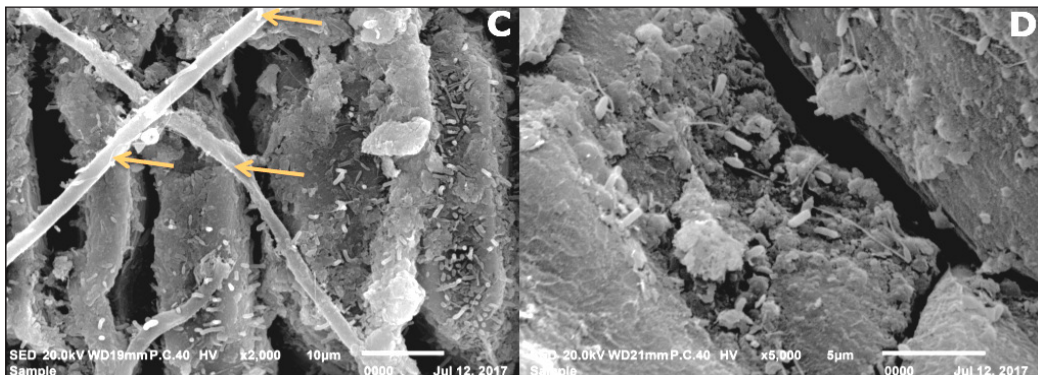

Figure 4. Oil palm seedling roots pre-inoculated with a mixture of *Bacillus cereus* and *Trichoderma asperellum* (C) infected with *G. boninense* and (D) non-infected with *G. boninense*. Roots were harvested after eight weeks of incubation

Enzyme Assay

Peroxidase Assay. Treatment with BG recorded the highest peroxidase (PO) activity with a value of 0.2601 unit^{-1}min^{-1}g, followed by treatment BTG with 0.2278 unit^{-1}min^{-1}g (Figure 5). Generally, all treatments treated with *B. cereus* (BT, B, BTG, and BG) yielded higher PO activities than untreated treatments (T, G, TG, and NC).

Lignin Assay. Figure 6 demonstrates that treatments T, BG, and NC recorded the highest lignin concentration with 10, 10.4, and 10.3 mg L^{-1}, respectively. Conversely, treatment G gave the lowest lignin concentration at 5.4 mg L^{-1} compared to other treatments. The lignin concentrations derived from a standard curve of lignin are presented in Figure 7.

Note.
BT = Plant + *Trichoderma asperellum* + *Bacillus cereus*
BTG = Plant + *Ganoderma boninense* + *Bacillus cereus* + *Trichoderma asperellum*
T = Plant + *Trichoderma asperellum*
TG = Plant + *Ganoderma boninense* + *Trichoderma asperellum*
B = Plant + *Bacillus cereus*
BG = Plant + *Ganoderma boninense* + *Bacillus cereus*
G = Plant + *Ganoderma boninense*
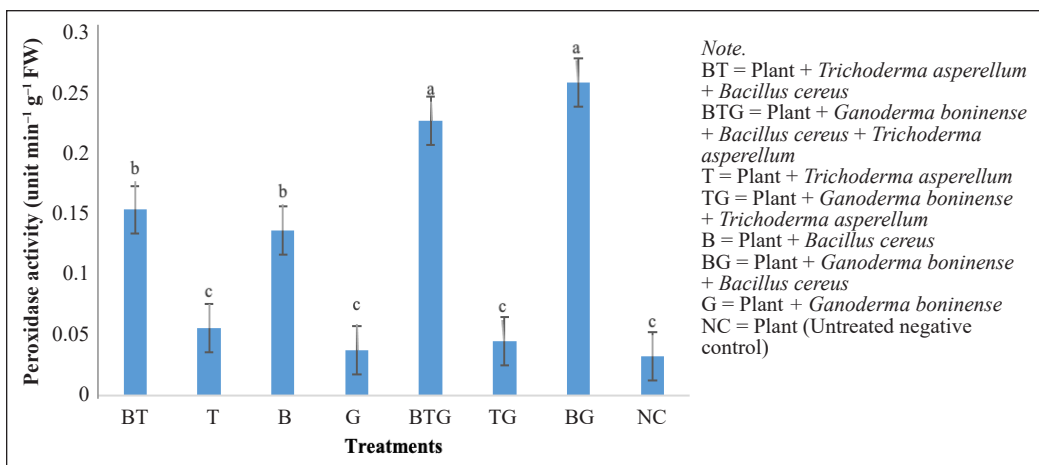NC = Plant (Untreated negative control)

Figure 5. Induction of peroxidase activity in oil palm seedling roots treated with biological control agents. Values are means of five replications, and differences between means are separated by Tukey's studentised range (HSD) test at a 5% level of significance

Note.
BT = Plant + *Trichoderma asperellum* + *Bacillus cereus*
BTG = Plant + *Ganoderma boninense* + *Bacillus cereus* + *Trichoderma asperellum*
T = Plant + *Trichoderma asperellum*
TG = Plant + *Ganoderma boninense* + *Trichoderma asperellum*
B = Plant + *Bacillus cereus*
BG = Plant + *Ganoderma boninense* + *Bacillus cereus*
G = Plant + *Ganoderma boninense*
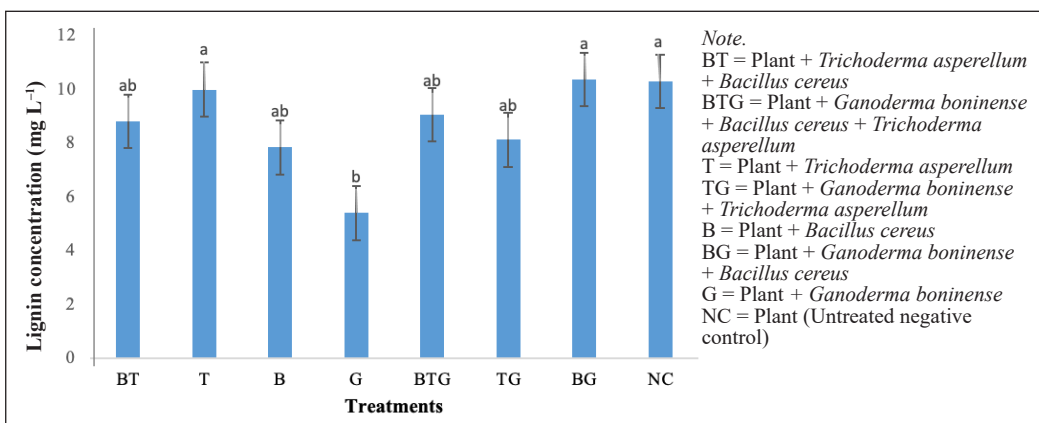NC = Plant (Untreated negative control)

Figure 6. Oil palm lignin concentrations following effects of biological control agents. Values are means of three replications, and differences between means are separated by Tukey's studentised range (HSD) test at a 5% level of significance
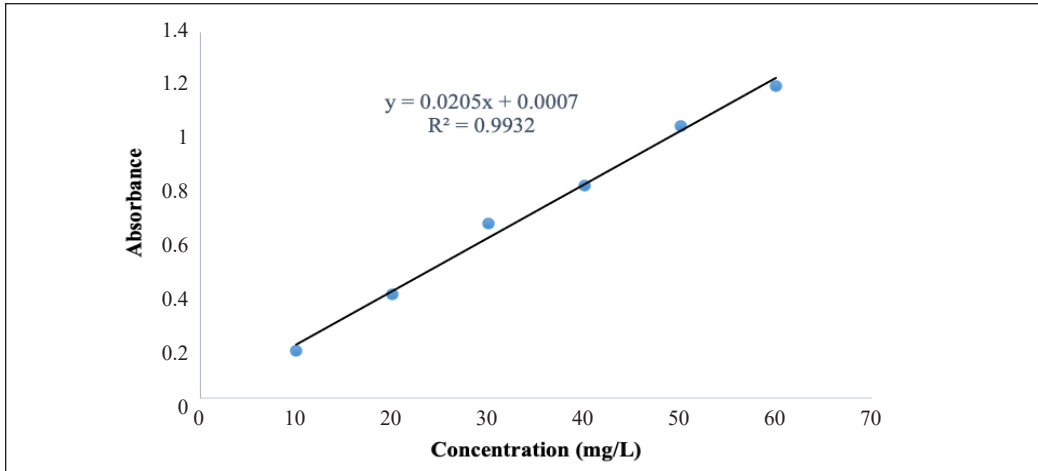
$$y = 0.0205x + 0.0007$$
$$R^2 = 0.9932$$

Figure 7. Standard curve of lignin with absorbance measured at 280 nm

Total Phenolic Content (TPC). The present study recorded high TPC extracted from oil palm roots in all treatments except for the consortium treatment, which was not infected with *G. boninense* (BT, as well as the untreated *Ganoderma*-infected treatment (G) (Figure 8). *Ganoderma*-infected seedlings treated with *B. cereus* (BG) gave the highest TPC (34.88 mg L^{-1}), followed by seedlings treated with *B. cereus* (B) (34.15 mg L^{-1}). Seedlings treated with consortium treatment (BT) gave the lowest TPC derived from the gallic acid standard curve shown in Figure 9.

Treatments:
1. Plant + *Trichoderma asperellum* + *Bacillus cereus*
2. Plant + *Trichoderma asperellum*
3. Plant + *Bacillus cereus*
4. Plant + *Ganoderma boninense*
5. Plant + *Ganoderma boninense* + *Bacillus cereus* + *Trichoderma asperellum*
6. Plant + *Ganoderma boninense* + *Trichoderma asperellum*
7. Plant + *Ganoderma boninense* + *Bacillus cereus*
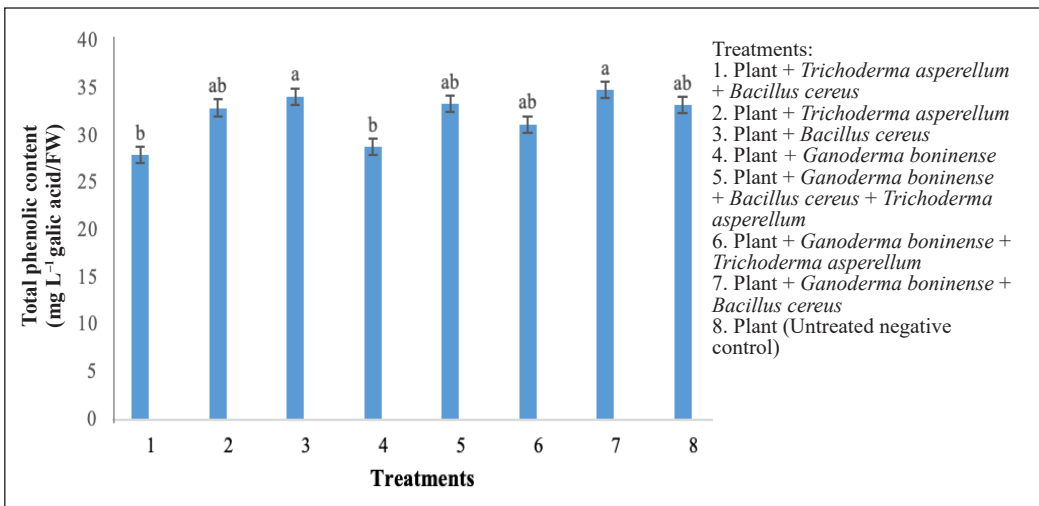8. Plant (Untreated negative control)

Figure 8. Total phenolic content of oil palm seedling roots following effects of treatments. Values are means of three replications, and differences between means are separated by Tukey's Studentized Range (HSD) Test at a 5% level of significance
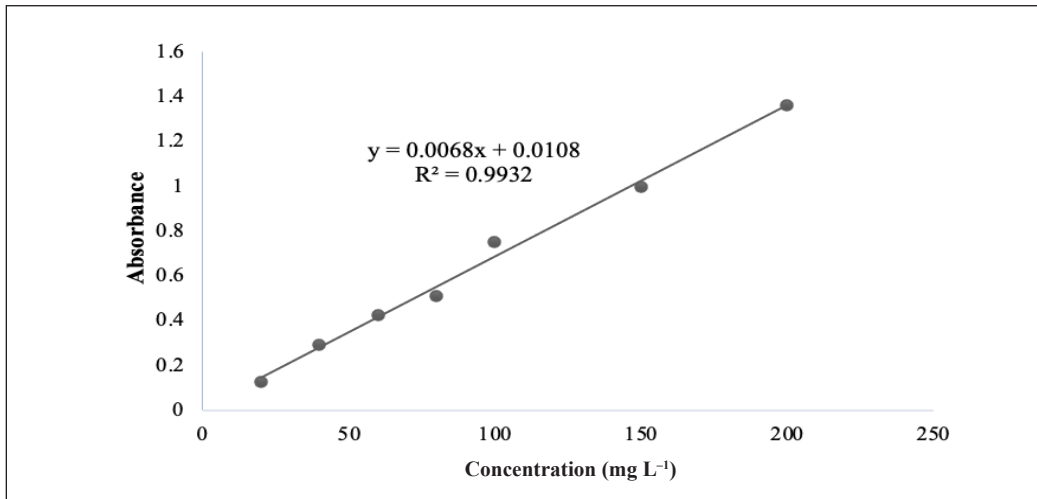
Figure 9. Standard curve of gallic acid with absorbance measured at 725 nm

Metabolite Profiling of Oil Palm Leave Extracts Treated with BCAs

For multivariate analysis, specifically partial least squares-discriminant analysis (PLS-DA), GC-MS findings were profiled and examined using MetaboAnalyst 4.0 software. Metabolites detected from all treatments were discriminated in the importance features analysis (Figure 10). Ten metabolites with the highest concentration were selected to narrow down the findings. Each variable's importance in the projection employed in a PLS model was estimated using the variable importance in projection (VIP) scores. In the presented model, a variable with a VIP score closer to one or higher can be regarded as essential (Gottfried, 2009). Based on VIP scores obtained, treatments BT and T produced three similar metabolites in relatively high concentrations, which were identified as phenol, 4-2-aminoethyl- (2.7-fold change), benzofuran, 2,3-dihydro- (2.4-fold change),

and 9, 12, 15-octadecatrienoic acid (2.3-fold change). Treatments with BG and NC also had noticeable similarities in terms of metabolites profiled. They produced acetic acid, aminooxy-(2.7-fold-change), 2-furancarboxaldehyde, 5-hydroxymethyl-(2.3-fold change), and methyl 11, 14, 17-eicosatrienoate (1.9-fold change). The list of major metabolites detected in the leaves of treated seedlings and their potential bioactivity are presented in Table 5.

DISCUSSION

In recent years, studies on BCAs as an alternative method in plant protection have indicated positive results regardless of a single treatment or a combination of treatments (Hermosa et al., 2013; Nusaibah et al., 2017). The present study evaluated the ability to reduce BSR disease severity by *B. cereus* and *T. asperellum* in a greenhouse after positive outcomes were recorded *in vitro*.
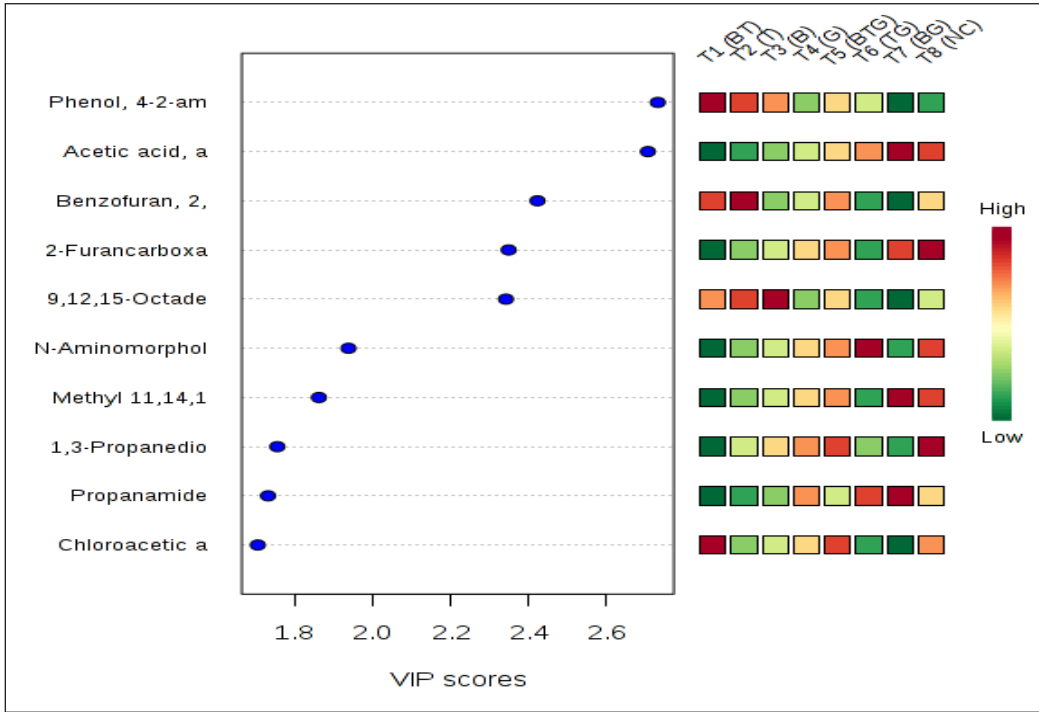
Figure 10. The importance features analysis of metabolites detected in the treatments. Coloured boxes on the right indicate relative concentrations of the corresponding metabolite in each group under study

The double-sealed plate method was used to determine the antifungal effects of MVOCs emitted by BCAs. Generally, MVOCs consist of various lower molecular weight lipophilic compounds that were naturally mixed. These compounds are by-products produced by microorganisms as part of their metabolism (Di Francesco et al., 2016; Mari et al., 2016). The study showed that *B. cereus* could produce MVOCs inhibiting *G. boninense* mycelial growth. The results were in line with Alexander et al. (2015), who observed that *Bacillus* spp. could suppress *G. boninense* growth *in vitro*.

In analysing disease suppression in the presence of both endophytes against BSR disease, the DPD technique (Nusaibah et al., 2017) was adopted. Since this technique is relatively new, SEM was used to determine disease establishment and efficacy of BCA treatment. The images observed through SEM effectively showed BSR disease establishment and successful BCA treatment on the roots of inoculated seedlings. Furthermore, the findings supported a study by Nusaibah et al. (2017), which concluded that the DPD technique could be considered an efficient technique in disease verification since *G. boninense* displayed vegetative growth with hyphae as the main mode of vegetative growth (Naher et al., 2014; Sundram et al., 2011).

BSR disease suppression was also assessed at a nursery trial. Both BCAs tested in the current study demonstrated efficacy in reducing BSR disease severity of oil

Table 5
Major metabolites detected in oil palm leaves and their potential bioactivities

Treatment	Metabolite	Synonyms	Activity / Reference
BT	Phenol, 4-2-aminoethyl-	Tyramin Tyramine Tyrosamine	Antimicrobial (Campos et al., 2014)
	Chloroacetic acid, 2,2-dimethylpropyl ester	Chloro-acetic acid neopentyl ester 2,2-dimethylpropyl 2-chloroacetate neopentyl 2-chloroacetate	
T	Benzofuran, 2,3-dihydro-	Coumaran Dihydrobenzofuran Dihydrocoumarone	Antifungal (Richardson et al., 2015), insecticidal activity (Huang et al., 2009)
B	9,12,15-Octadecatrienoic acid, Z, Z, Z-	α-linolenic acid Industrene 120	Antibacterial (Huang & Ebersole, 2010), antifungal (Walters et al., 2004)
TG	*N*-aminomorpholine	4-aminomorpholine 4-morpholinamine	
BG	Acetic acid, aminooxy-	Aminooxyacetate Aminooxyacetic acid Carboxymethoxyamine	Antifungal (Giorgio et al., 2015)
	Methyl 11,14,17-eicosatrienoate	Methyl 11,14,17-icosatrienoate Methyl icosa-11,14,17-trienoate	
	Propanamide	Propionamide Propylamide Propionic amide Propionimidic acid	Antimicrobial (Ölgen et al., 2008)
NC	2-furancarboxaldehyde, 5-hydroxymethyl-	2-furaldehyde, 5-(hydroxymethyl)- 5-oxymethylfurfurole 2-hydroxymethyl-5-furfural	Antifungal (Subramenium et al., 2018)
	1,3-propanediol, 2-ethyl-2-hydroxymethyl-	Ethriol Ethyltrimethylolmethane Etriol	

Treatments:	BT = Plant + *Trichoderma asperellum* + *Bacillus cereus* B = Plant + *Bacillus cereus* BG = Plant + *Ganoderma boninense* + *Bacillus cereus*	T = Plant + *Trichoderma asperellum* TG = Plant + *Ganoderma boninense* + *Trichoderma asperellum* NC = Plant (Untreated negative control)

palm based on data recorded in DS and DR analysis. Single treatment of *T. asperellum*, with 50% DS and 57.2% DR, was more efficient in disease suppression compared to both single and combination treatments of *B. cereus*. Several studies have also indicated that *T. asperellum* could suppress several plant diseases (Bailey et al., 2008; Musa et al., 2018). According to Yang et al. (2011), *Trichoderma* spp. were shown to be effective soil inhabitants and root colonisers. However, the consortium treatment used in

this study still demonstrated better DR and lower DS to seedling roots compared to a single treatment of *B. cereus*.

Plant enzymes, such as PO and polyphenol oxidase (PPO), assist the formation of lignin and oxidative phenols that involve plant defence mechanisms against the pathogen (Avdiushko et al., 1993). The present study observed that seedlings treated with a single treatment of *B. cereus* recorded the highest PO enzyme activity. All treatments involving *B. cereus*, regardless of single application or combination, gave better PO enzyme activity than those without *B. cereus*. Parallel to the present study, Ramarathnam et al. (2011) observed that enzyme activity for strain *B. cereus* was higher than other treatments when inoculated with the pathogen. A study by Halfeld-Vieira et al. (2006) reported that leaf tissues exposed to *B. cereus* and inoculated with *Pseudomonas syringae* as a pathogen in tomatoes exhibited higher PO enzyme activities compared to other treatments. These studies confirmed that *B. cereus* efficiently induced PO enzyme activities.

Variables, such as the availability of sunlight and pathogen infection, have been reported to influence lignin content in plants (Xu et al., 2011). High levels of root lignin were a means of limiting fungal infection (Bennett et al., 2015). In their studies, lignin assays recorded that *Ganoderma*-infected seedlings treated with *B. cereus* (BG), seedlings treated with *T. asperellum* (T), and negative control (NC) yielded the highest lignin with no significant difference from each other. The data supported findings recorded on vegetative growth in the present study, where combination treatment and seedlings treated with *B. cereus* contributed higher root weight than the other treatments. *Ganoderma*-infected seedlings gave the lowest lignin accumulation. Adaskaveg et al. (1991) concluded that white rot fungi, such as *Ganoderma* spp., degraded wood components, especially lignin, making plants more susceptible to pathogens.

Epidemiological data by Hu and Kitts (2001) suggested that phenolic acid has strong inhibitory activity on oxidation induced by peroxyl radicals. According to Nikraftar et al. (2013), phenolic compounds in plants and the synthesis of those compounds in response to infection were linked to plant resistance. The present study also demonstrated that *Ganoderma*-infected seedlings treated with *B. cereus* yielded the highest TPC, followed by disease-free seedlings treated with *B. cereus*. These results matched those observed in an earlier study by Baydar et al. (2004), who confirmed that phenolics were the most important compound active against bacteria and explained the induction of TPC in seedlings treated with *B. cereus*. Endophytic *B. cereus* could have triggered the palms to produce TPC higher than the basal level as a defence tool against proteins synthesised by the bacterium. Seedlings treated with a combination of treatments contributed the lowest reading in TPC. It could be due to no virulent factors detected by the palm and endophytic *Trichoderma* spp. played a symbiotic role in plant-pathogen interaction.

Trichoderma asperellum had colonised the palm roots over *B. cereus*, as was seen in previous SEM observation.

Based on the GC-MS analysis of extracted oil palm leaves, phenol 4-2-aminoethyl- (syn: tyramine) was detected in treatment BT. Like any other biogenic amine, tyramines are mainly produced due to microbial enzymes or tissue bioactivity that causes certain amino acids to undergo an enzymatic decarboxylation process (Halász et al., 1994). Tyramine is the key enzyme that helps the production of hydroxycinnamic acid amides (HCAA) (Campos et al., 2014). HCAA strengthens a plant's cell walls, thus creating a barrier against microbial degradation (Hagel & Facchini, 2005). HCAA could also act directly as an antimicrobial agent. Newman et al. (2001) published that coumaroyltyramine (CT) and feruloyltyramine (FT) accumulated in pepper plants infected with the bacterial pathogen *Xanthomonas campestris* demonstrated antibacterial activity. FT extracted from *Allium* roots had been recorded to exhibit antifungal activity (Fattorusso et al., 1999). This study also detected acetic acid production in treatment with BG. Acetic acid (syn: aminooxyacetic acid, AOA) is known for its flavouring and preservative properties against microorganisms in various food products. It is also a natural compound throughout the biosphere (Alawlaqi & Alharbi, 2014). An *in vitro* study by Giorgio et al. (2015) observed that acetic acid could reduce mycelium growth arising from fungal plugs of *Sclerotinia sclerotiorum*.

The 2, 3-dihydrobenzofuran (DHB) skeleton is reportedly widespread in many natural products and biologically active molecules (Katritzky & Rees, 1984). Derivatives of benzofurans are sometimes discovered as metabolites of fungi, including endophytes of trees (Richardson et al., 2015). Several DHB products have been reported to have antioxidant, cytoprotective properties, and insecticidal activity (Z. Huang et al., 2009). In a study by Richardson et al. (2015), several benzofuran products were found to have antifungal properties and successfully reduced the growth of *Microbotryum violaceum*. Jang et al. (2006) also found that a dihydrobenzofuran derivative, awajanoran, exhibited antimicrobial activities against 5 strains.

Subramenium et al. (2018) studied 5-hydroxymethyl-2-furaldehyde (5HM2F) and found it to have antibiofilm and antivirulence activities against *Candida albicans*, pathogenic yeast. Based on the antifungal susceptibility testing results, the combination of antifungal and 5HM2F was more effective than a single antifungal treatment in reducing *C. albicans* biofilm. Chen et al. (2014) also discovered that 5HM2F and its derivatives extracted from the plant displayed antioxidant activity when tested in the laboratory. Furthermore, it was revealed that 5HM2F was naturally found in several plants (Lin et al., 2008), honey (Coco et al., 1996), and heat-treated food products (Almeida et al., 2009).

According to Burr and Burr (1930), 9, 12, 15-octadecatrienoic acid, Z, Z, Z- (syn: α-linolenic acid, ALA) is a polyunsaturated

fatty acid and is one of two human essential fatty acids. *In vitro* studies have shown that α-linolenic acid has been reported to have antibacterial activities against several oral pathogens such as *C. albicans*, *Streptococcus mutans*, and *Porphyromonas gingivalis* (C. B. Huang & Ebersole, 2010). In addition, Walters et al. (2004) reported that ALA demonstrated antifungal activities against mycelial growth of plant pathogenic fungi, namely, *Crinipellis pernicosa*, *Pyrenophora avanae*, *Pythium ultimum*, and *Rhizoctonia solani*. These findings suggested that ALA could play an important role in the search for alternative methods for controlling crucial plant pathogens.

CONCLUSION

The primary purpose of this study was to determine the contribution of BCA application on oil palm defence mechanism against *G. boninense* infection via greenhouse trial. Based on the results obtained, treatments positively impacted BSR disease suppression. PO enzyme activity, lignin content, TPC, and metabolites in BSR-diseased seedlings were affected in response to *B. cereus* and *T. asperellum* treatments. Treatments involving *B. cereus*, regardless of single application or combination, recorded enhanced PO activity. The present study showed that *B. cereus* induced the palms to produce more PO and TPC. Lignin assay showed that *Ganoderma*-infected seedlings treated with *B. cereus*, seedlings treated with *T. asperellum*, and negative control all produced the highest reading of lignin

with no significant difference between them. The GC-MS analysis revealed that two metabolites with antifungal properties, phenol, and 4-2-aminoethyl, were the most abundant metabolite detected in the combination treatment, followed by acetic acid in the infected seedlings treated with *B. cereus*. Therefore, it could be concluded that treatments with a single or consortium of *T. asperellum* and *B. cereus* upregulated the oil palm defence mechanism against *G. boninense* infection. To further strengthen and support the outcome of this study, a field trial should be conducted using the currently designed treatments on palms still at the early stages of BSR infection.

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148

Pertanika J. Trop. Agric. Sci. 46 (1): 129 - 151 (2023)

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