EFFECT OF *Trichoderma asperellum* AND *Bacillus cereus* CONSORTIUM ON PLANT GROWTH AND RESISTANCE OF INFECTED *Ganoderma boninense* OIL PALM SEEDLINGS

By

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Despite the enormous economic contribution to Malaysia, oil palm industry is facing devastating threats from serious diseases primarily basal stem rot (BSR) disease. Excessive application of chemicals to control BSR may render negative impacts on the biodiversity and subsequently to the human population. Therefore, biological control would be an ideal alternative as a preventive or curative method of BSR. Thus, an insight of biological control effects *in vitro* and *in vivo* would be crucial in selecting potential biological control agents (BCAs) to counter BSR disease in a sustainable manner. Plant growth-promoting traits of *Bacillus cereus* and *Trichoderma asperellum* such as indole acetic acid (IAA), siderophore production and phosphate solubilization ability were tested. Sealed plate method was conducted to determine the antifungal effects of microbial volatile organic compounds (MVOCs) produced by the BCAs on the growth of *Ganoderma boninense.* An array of treatments was designed to evaluate the potential of BCAs as single and mixture application in *in vivo* nursery trial for six months. Plant height and chlorophyll content were recorded on monthly basis to analyze the vegetative growth of the inoculated seedlings. Data on the vegetative growth were subjected to ANOVA for mean comparison by using SAS software. Induction of oil palm enzymes, phenolic content and metabolites in BSR diseased seedlings in response to the designed treatments were assessed. The ability to solubilize precipitated phosphate and produce siderophore were positively exhibited by *T. asperellum*. Nevertheless, both *T. asperellum* and *B. cereus* were able to produce IAA. Sealed plate method demonstrated MVOCs emitted by *B. cereus,* effectively inhibited *G. boninense* mycelial growth with the mean of 51.3 mm compared to the control plates (85.0 mm). In the *in vivo* trial, *T. asperellum* contributed significantly on the growth of aerial parts while *B. cereus* on the growth of oil palm roots. In addition, the seedlings treated with *T. asperellum* demonstrated the highest disease reduction (DR) (57.2%). Different BCA treatments gave different results on oil palm enzymes, phenolic content and metabolites

produced by seedlings. However, one similarity could be observed in peroxidase assay, lignin assay and phenolic content where *Ganoderma*-infected seedlings treated with *B. cereus* (treatment BG) gave high value in all assays conducted. Gas Chromatography-Mass Spectrometry (GC-MS) analysis showed that phenol, 4-2-aminoethyl- was the most abundant metabolite detected in the mixture treatment of *B. cereus* and *T. asperellum* (treatment BT), followed by acetic acid in the treatment BG. Nevertheless, both microbes demonstrated huge potential as BCAs in BSR disease reduction.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

CAMPURAN *Trichoderma asperellum* DAN *Bacillus cereus* MENINGKATKAN PERTUMBUHAN SERTA KERENTANAN ANAK SAWIT YANG TELAH DIJANGKITI DENGAN *Ganoderma boninense*

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Walaupun memberi sumbangan yang sangat besar kepada ekonomi Malaysia, industri sawit sedang berhadapan dengan ancaman dahsyat dari penyakit-penyakit yang serius terutama sekali penyakit reput pangkal batang (BSR). Penggunaan bahan kimia yang berlebihan untuk mengawal BSR mungkin akan memberi kesan negatif terhadap biodiversiti dan seterusnya kepada populasi manusia. Oleh sebab itu, kawalan biologi boleh menjadi alternatif yang ideal sebagai kaedah pencegahan atau sebagai kaedah penyembuhan penyakit BSR. Oleh itu, pemahaman yang mendalam terhadap kesan kawalan biologi secara *in vitro* dan *in vivo* amat penting dalam pemilihan agen kawalan biologi (BCAs) bagi mengawal penyakit BSR menggunakan kaedah yang mampan. Ciri-ciri pada *B. cereus* dan *T. asperellum* yang akan menyumbang pada pertumbuhan pokok dengan penghasilan indole asid asetik (IAA), pengeluaran siderophore dan kebolehan melarutkan fosfat telah diuji. Kaedah *sealed plate* dijalankan untuk menentukan kesan-kesan antikulat oleh kompaun-kompaun organik yang meruap (MVOCs) yang dihasilkan oleh BCAs terhadap pertumbuhan *G. boninese*. Satu kompilasi rawatan telah direka untuk menilai potensi BCAs yang merangkumi aplikasi tunggal dan campuran BCA dalam percubaan di nurseri secara *in vivo* selama enam bulan. Ketinggian pokok dan kandungan klorofil pada daun telah direkodkan pada setiap bulan untuk mengkaji pertumbuhan vegetatif anak sawit yang telah diinokulasi. Analisis ANOVA telah dijalankan pada data-data pertumbuhan vegetatif yang diperolehi untuk perbandingan purata dengan menggunakan perisian SAS. Induksi enzim-enzim sawit, TPC, dan metabolit dalam anak sawit yang telah dijangkiti penyakit BSR yang bertindak balas dengan rawatan-rawatan yang telah dirangka, dikaji. Kebolehan dalam melarutkan fosfat terendap dan menghasilkan siderophore telah dipamerkan oleh *T. asperellum.* Walaubagaimanapun, kedua-dua *T. asperellum* dan *B. cereus* mampu menghasilkan IAA. Kaedah *sealed plate* telah menunjukkan bahawa MVOCs yang hasilkan oleh *B. cereus* telah berjaya menghalang pertumbuhan miselium

G. boninense secara berkesan dengan purata 51.3 mm berbanding dengan rawatan kawalan (85.0 mm). Dalam ujian *in vivo*, *T. asperellum* telah menyumbang secara signifikan terhadap pertumbuhan bahagian atas anak sawit manakala *B. cereus* pula menyumbang terhadap pertumbuhan bahagian akar sawit. Selain itu, anak sawit yang telah dirawat dengan *T. asperellum* telah berjaya menunjukkan pengurangan penyakit (DR) yang tertinggi (57.2%). Manakala, rawatan BCA yang berlainan telah memberikan keputusan yang pelbagai terhadap enzim-enzim sawit, TPC dan metabolit yang terhasil dari anak sawit. Namun begitu, satu persamaan boleh dilihat dalam ujian peroxidase (PO), ujian lignin dan ujian kandungan fenolik (TPC) dimana rawatan BG telah mempamerkan nilai yang tinggi dalam setiap ujian yang dilaksanakan berbanding rawatan yang lain. Analisis GC-MS telah berjaya mengesan phenol, 4-2-aminoethyl- sebagai metabolit yang paling banyak, diikuti oleh asid asetik dalam rawatan BG. Walaubagaimanapun, kedua-dua mikrob telah mempamerkan potensi yang besar sebagai BCA dalam pengurangan penyakit BSR.

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LIST OF ABBREVIATIONS

^{o}C	Degree celcius
µg/ml	Microgram per milliliter
µl	Microliter
µm	Micrometer
5HM2F	5-hydroxymethyl-2-furaldehyde
ANOVA	Analysis of variance
AUDPC	Area under the disease progress curve
BCA	Biological control agent
bp	Base pair
BSR	Basal stem rot
$CaCI_2$	Calcium chloride
$Ca_3(PO_4)_2$	Calcium phosphate
CAS	Chrome azurol sulphonate
cfu	colony forming unit
cm	Centimeter
cm^3	Cubic centimeter
CO_2	Carbon dioxide
$CuSO_4.5H_2O$	Copper sulfate pentahydrate
DHB	Dihydrobenzofuran
DI	Disease incidence
DNA	Deoxyribonucleic acid
DPD	Dip, Place and Drench technique
DR	Disease reduction
DS	Disease severity
el al.	And others
$FeCl_3$	Iron(III) chloride

$FeCl_3.6H_2O$	Ferric chloride hexahydrate
GAE	Gallic Acid Equivalent
GC-MS	Gas Chromatography-Mass Spectrometry
GSM	*Ganoderma* selective media
H_2O_2	Hydrogen peroxide
H_3BO_3	Boric acid
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
HDTMA	hexadecyl-trimethylammonium bromide
IAA	Indole acetic acid
IBS	Institute of Bioscience
K_2HPO_4	Dipotassium phosphate
KCl	Potassium chloride
KOH	Potassium hydroxide
kPa	Kilopascal
LSD	Least significant difference
M	Molar
MAI	Month after inoculation
$MgCl_2.6H_2O$	Magnesium chloride, hexahydrate
mg l^{-1}	Milligram per liter
mg ml^{-1}	Milligram per milliliter
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
ml	Milliliter
mm	Millimeter
mM	Millimolar
$MnSO_4.2H_2O$	Manganese sulphate dihydrate
MVOC	Microbial volatile organic compound

NA	Nutrient agar
NaCl	Sodium chloride
$NaMoO_4.2H_2O$	Molybdic acid sodium salt dihydrate
NaOH	Sodium hydroxide
NB	Nutrient broth
NBRIP	National Botanical Research Institute's phosphate
$(NH_4)_2SO_4$	Ammonium sulfate
NH_4Cl	Ammonium chloride
nm	Nanometer
OD	Optical Density
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
pH	Potential hydrogen
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
PO	Peroxidase
PVP	polyvinyle pyrrolidone
RCBD	Randomized Complete Block Design
rpm	Rotation per minute
SAS	Statistical analysis software
S.E.	Standard error
SEM	Scanning electron microscope
TPC	Total phenolic content
UV	Ultraviolet
VOC	Volatile organic compound
w/v	Weight per volume
$ZnSO_4.7H_2O$	Zinc sulfate heptahydrate

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Ganoderma boninense is commonly referred to as the major causal pathogen of basal stem rot (BSR) disease on oil palm (Khairuddin, 1990; Rao, 1990; Rees et al., 2009). *Ganoderma* spp. belong to the Family Ganodermataceae (Richter et al., 2015). For about three decades, researchers assumed that *G. boninense* could only infect the older age palms planted more than 10 years (Singh, 1991). However, in the mid-1950s younger palms were detected to have infections in East Asia and this changed the scientist's perspective on this disease (Turner, 1981). In the plantation industry practice, oil palms aged more than 20-25 years will be chopped down and replanted. Basal stem rot disease initially infects the root system and followed by the lower part of the stem. The impact is devastating as the disease often causes the decaying of the palm tissues (Meor et al., 2009; Sundram et al., 2011). Idris et al. (2002) stated that external symptoms of BSR often appear at later stages of infection when the treatment on palms hardly effective anymore. Due to this factor, it is vital to closely monitor via detection and control *G. boninense* infection at an early stage.

Chemical usage has been actively applied in agriculture for decades. Although it is effective in most cases, the excessive use of chemicals could cause negative impacts on the hydrological systems and soil (Salman et al., 2011; Wuana et al., 2011). This remains as a concern and it is crucial to find alternative methods to minimize or even to prevent chemical application. Therefore, studies on biological control agents (BCAs) for BSR disease as preventive or curative measurers are obligatory to suggest as an environmentally safe method. A number of studies have found that *Trichoderma* spp. can significantly help plant growth and boost plant resistance towards biotic and abiotic stresses (Yedidia et al., 2003; Harman et al., 2004; Shoresh et al., 2005; Vinale et al., 2008; Musa et al., 2018). In a study which set out to determine the antagonistic of BCAs, Nusaibah et al. (2017) found that *Bacillus cereus* could help control *Ganoderma* disease when tested *in vivo* in nursery trial.

This study tested on BCAs as a single and consortium treatment, and found that, both treatments were able to reduce BSR in oil palm at nursery stage (Nusaibah et al., 2017). Furthermore, this study also reported that BCAs could also contribute to the vegetative growth of the palms. This view is supported by Zhao et al. (2011) and Naher et al. (2012) that suggested that *Trichoderma* sp. and *Bacillus* sp. could act as plant growth promoters. Therefore, the present study tested on the ability of *Trichoderma asperellum* and *B. cereus* consortium

in enhancing plant growth and inducing resistance in *Elaeis guineensis* against *G. boninense* disease infestation via nursery trial.

1.2 Objectives

I. To assess the plant growth promotion (PGP) potential of *Trichoderma asperellum* and *Bacillus cereus* endophytes.

II. To analyze the vegetative growth parameters and disease suppression in the presence of both endophytes applied as pre-treatment prior to fungal pathogen inoculation.

III. To study the biochemical response that may be involved in the plant growth and disease resistance as a result of biological control treatments.

CHAPTER 2

LITERATURE REVIEW

2.1 Development of oil palm industry

2.1.1 World-wide development of the oil palm industry

According to Oil World (2000), palm oil global yield had increased significantly. Twenty-one million tonnes of palm oil and 6 million tonnes of kernels were produced in the year 2000 and it was significantly higher compared to the yield produced in1972 where the industry only managed to produce 2.2 million tonnes of palm oil and 1.2 million tonnes of kernels. This increase was greatly contributed by Malaysia and Indonesia's oil palm industry. In 2007, the yield of palm oil has surpassed the yield of other vegetable oils except for soybean oil (Corley and Tinker, 2008).

The changing shift worldwide could be observed clearly based on the export figures and it was shown that the African countries' role in the global trade of palm oil has almost come to an end in many countries (Corley and Tinker, 2008). Concurrently, Malaysia and Indonesia have developed as superior players in the business and have gained extensive experience in the manufacturing sector of oil palm products (Foreign Agricultural Service, 2005). The path taken by these two countries were steadily followed by other Southeast Asian countries (Corley and Tinker, 2008). Fast forward to seven years later, with annual world production approximately 50 million tonnes, palm oil has become the highest yield of oil per unit compared to other vegetables oils available in the market (Koushki et al., 2015).

2.1.2 Malaysian oil palm industry

During 1870s, British colonial introduced the oil palm as an ornamanetal plant in Malaya. However, the first planting in huge scale happened in 1917 in Tennamaran Estate, Selangor. This initial plantation marked the start of oil palm industry in Malaysia. 1960s saw the launch of government agricultural transformation program that intended to abstain citizen from too relying only on rubber and tin. As a result, the industry grew rapidly (MPOC, n.d.).

According to the Malaysian Palm Oil Council (MPOC) (n.d.), in order to empower farmers and smallholders, the government's settlement schemes for oil palm plantation were started during late 1960s. Nevertheless, 1980's witnessed another vital achievement in the oil palm industry where oleochemicals industry started to develop rapidly due to sufficient supply of palm oil and palm kernel oil (Wood and Beattie, 1981). In 2016, 17.32 million tonnes of palm oil and 4.19 tonnes of palm kernel oil were produced by 5.74

million hectares of Malaysian oil palm plantation (Kushairi, 2017). This year also saw Malaysia produces 30% of the world's oils and fats and involve in 37% of the export trade of oils and fats, which is the second highest, behind Indonesia (Kushairi, 2017). It is estimated that the Malaysian oil palm industry has created jobs to more than half a million people (MPOC, n.d.).

The oil palm trees cultivated in Malaysia are primarily the Tenera variety (a hybrid between *Dura* and *Pisifera*). About four to five tonnes of crude palm oil (CPO) per hectare per year and one tonne of palm kernels could be produced by this Tenera variety. The oil palm is known as the most efficient oil-bearing crop in the world, compared to soybean, sunflower and rapeseed that require 2.22, 2 and 1.52 hectares of land respectively, to supply a tonne of oil. However, oil palm only requires 0.26 hectares of land to produce the same volume (MPOC, n.d.).

2.1.3 Basal stem rot (BSR) disease and *Ganoderma* spp. in oil palm

Ganoderma is distinguished by large basidiocarps, perennial, woody brackets which are lignicolous and leathery. Typically, their fan or hoof-like forms of fruiting bodies grow on the trunks of the palm trees. Miller (1995) reported that *Ganoderma boninense* distributes through the soil and air. Compatibility test has been conducted and the result showed that the fungi collected from the same site may have distinct origins (Miller, 1995). This study probably proved that *Ganoderma* could transmit not only through the mycelial vegetative growth in soil but also airborne.

Ganoderma is a genus that belongs to the Ganodermataceae family, Aphyllophorales order and class of Basidiomycetes (Karsten, 1881). *Ganoderma* spp. have cosmopolitan distribution because it grows across the globe in suitable environments. They were also referred as 'white-rot' fungi because enzymes that they produce are able to degrade lignin and the degradation of lignin resulting into white rot of the wood substrate. *Ganoderma* was introduced by Peter Adolf Karsten, a French mycologist (Seo & Kirk, 2000).To date; it is safe to say that *Ganoderma* plays a vital role economically as plant pathogen and in the pharmaceutical field.

Both BSR and upper stem rot (USR) were caused by *Ganoderma* spp. (Hasan et al., 2005; Pilotti, 2005; Rees et al., 2012; Wong et al., 2012). Basal stem rot is caused by *G. boninense* (Khairudin, 1990; Rees et al., 2009; Najmie et al., 2011) however USR was mainly caused by *Ganoderma zonatum* (Rakib et al., 2015). However, BSR is a critical and widespread disease of oil palm while USR is considered as minor disease in the oil palm plantations (Rakib et al., 2014). Main contrast between USR and BSR is the initial spot of the disease and their ramification. Basal stem rot decays the palm's bole and cause the fall of the palm (Lisnawati et al., 2016). Upper stem rot decays the upper part of the palm's stem, forms basidiocarp two meters above the ground level and in critical cases able to cause stem fracture (Hasan et al., 2005; Pilotti, 2005;

Rees et al., 2007). Basal stem rot contributes in direct losses of the stands, lowering the yield (Singh, 1991) and results in early replanting (Flood et al, 2002). *Ganoderma boninense* has been identified as the most disastrous fungal pathogen of oil palm in South East Asia region.

Therefore, an action plan for initial detection and disease management would be crucial while requiring improvement for preventive and curative measures.

2.2 Infection and transmission of the causal pathogen

According to numerous studies conducted, root infection is the main method of BSR infection (Pilotti et al., 2002; Rees et al., 2007, 2009, 2012). Root infection may be caused via physical contact of palm roots with the existing *Ganoderma* spp. inoculum from the soil or other adjacent infected roots as suggested by Miller et al. (1999).

However, basidiospores have also been suggested as one of the modes of BSR disease transmission particularly in the case of BSR outbreaks that takes place in new plantations sites where *G. boninense* inoculum was absent in the environment (Pilotti et al., 2003; Rees et al., 2012). Sanderson (2005) found that basidiospores are available in large amount under single a basidiocarp and these basidiospores could be transported in a long distance by air and subsequently would land on ideally wounded palm tissues. In spite that, Hasan et al. (2005) found that multiple trials to replicate BSR infection using basidiospores have been unsuccessful. In addition, Rees et al. (2007) have suggested that the basidiospores of *G. boninense* have weak saprotrophic ability in soil and accumulated organic debris.

Last but not least, not all researchers have the same opinion on the method of BSR infection and how it is transmitted. Nevertheless, they generally agreed that *Ganoderma* spp. is the fungi that cause both BSR and USR (Navaratnam and Chee 1965; Lim et al. 1992; Sariah et al. 1994; Hasan and Turner 1998; Lim and Fong 2005; Breton et al. 2006).

2.3 Symptoms of basal stem rot (BSR) disease on oil palm

The initial diagnosis of BSR disease may be complicated because *Ganoderma* spp. does not show any symptoms at early stages of infection (Najmie et al., 2011). Matured oil palm trees could endure the disease for up to ten years after the appearance of the first symptom while young oil palms could only last for six to 24 months (Corley & Tinker, 2003).

According to Rees et al. (2012), the most important symptom of oil palm BSR disease is the decaying of the roots followed by the bole and the stem. Basidiocarps of *Ganoderma* develop at the bottom of the stem. The limitation of

water and nutrient uptake to the fronds occur due to the stem and root rotting that disrupted the xylem and phloem which finally leads to the chlorosis of the fronds (Riley, 2003; Idris et al., 2006; Chung 2011). Besides that, foliar symptoms such as the presence of necrotic leaves, flattening of the crown and spear leaves remain unopened could be observed at later stages (Liaghat et al., 2014). In worst cases, ruptured stem, production of fruiting bodies or even falling palms could be seen (Nurnadiah et al., 2014).

2.4 Economic importance of the oil palm industry

Enormous net profit of export earnings and one of the major factors that helps to minimize deprivation in certain areas in Malaysia made oil palm as the 'golden crop of Malaysia' (Basiron, 2007). Even with relatively low amount of fertilizer application, it remains as the most productive oil-producing crops, yielding around 3.6 tonnes per hectare, which is six to 10 times the oil yield of rapeseed, sunflower and soybean (Nelson *et al*., 2012). Sayer et al. (2012) suggested that oil palm plantation reduces poverty and with proper governmental policies and guidelines could improve the livelihood of the nation. Oil palm plantation areas have expanded from 54,000 hectares in 1960 (Basiron, 2007) to 5.85 million hectares in 2018 (MPOB, 2019), demonstrating a compound annual growth of 8.29%. Subsequently, oil palm productions also ascend greatly. Within only 58 years, palm oil yield escalated from 94,000 tons in 1960 (Basiron, 2007) to 19 million tons in 2018 (MPOB, 2019), demonstrating a compound annual growth of 9.44%.

Studies by Malaysian Palm Oil Council (MPOC) (2014) and Badan Pusat Statistik (BPS) Jakarta (2015) recorded that in 2014, Indonesia and Malaysia jointly planted 17 million hectares of palm oil. In Malaysia, oil palm could be considered as the almost disease-free crop. However, it is deteriorating from one serious disease which is basal stem rot (BSR). Basal stem rot is caused by *Ganoderma* species and was first detected in Malaysia in 1928 (Sharples, 1928). Abdul Razak et al. (2004) stated that oil palm has an economic life span of 25-30 years and BSR may damage more than 80% of the palms by the time they are halfway through their economic life span. Therefore, without ultimate solutions, BSR could bring down this economically important crop of Malaysia and Indonesia.

2.5 Biological control

Plant disease is a common issue in the agricultural industry. Standard solutions have always been derived based on the application of chemicals even though it is known to cause harmful effects humans and the biodiversity (Schäfer et al., 2012; Woo et al., 2014). Therefore, it is vital to develop alternative methods to control diseases in the oil palm plantations. One of the safest disease control is by using biological control agents (BCAs) (Susanto et al., 2005).

The unique mechanisms of biological control are competition for nutrients orecological niches (Vinale et al., 2006; Sempere et al., 2009; Hermosa et al., 2013; Trabelsi et al., 2013), direct parasitism or mycoparasitism (Benitez et al., 2004; Lorito et al., 2010; Druzhinina et al., 2011; Hermosa et al., 2012; Mukherjee et al., 2013) from physical penetration, antibiosis (Benitez et al., 2004; Mukherjee et al., 2006; Vinale et al., 2006; Sempere et al., 2009; Hanhong, 2011; Mukherjee et al., 2012) and secretion of numerous lytic enzymes (Donzelli et al., 2001; Benitez et al., 2004; Brunner et al., 2005; Woo et al., 2006; Atanasova et al., 2013; Hermosa et al., 2013). However, some studies showed that a combination of these mechanisms gave a better result in reducing the impact of pathogens and improving the health of the plant (Vinale et al., 2008; Berg, 2009; Nusaibah et al., 2017).

2.6 Potential of *bacteria* as a biological control agent (BCA)

According to Droby et al. (2009), the use of natural antagonistic microorganisms to combat pests or suppress plant diseases is called biological control. In accordance with this, some spore-forming bacteria such as *Bacillus* and *Pseudomonas* spp. can be used as biological control agents against plant pathogens due to its beneficial properties, (Perez-garcia et al., 2011).

Bacillus cereus is a Gram-positive, aerobic-to-facultative, spore-forming rod widely distributed environmentally and bearing a close phenotypic and genetic (16S rRNA) relationship to several other *Bacillus* species, especially *Bacillus anthracis* (Ash et al., 1991). *Bacillus cereus* has a saprophytic life cycle in which spores germinated in the soil, with the production of a vegetative cells that could sporulate, maintaining the life cycle (Arnesen et al., 2008). *Bacillus cereus* is the most common aerobic spore bearer in many types of soil and in sediments, dust, and plants (Schoeni & Wong, 2005; Ribeiro et al., 2010).

Endophytic bacteria such as *Bacillus* spp. and *Pseudomonas* spp. have the ability to increase plant's immune system against pathogenic fungi attack (Chen et al., 1995; Alstrom, 2001; Ramli et al., 2016). Some of the methods to strengthen this immune system are by promoting plant growth, solubilizing mineral nutrient and inducing systemic resistance (Van Peer et al., 1991; Francis, 2010). Most plant growth-promoting (PGP) bacteria reside in the plant rhizosphere, and it is the best site to isolate beneficial endophytic bacteria (Kishore et al., 2005). A data obtained by Nusaibah et al. (2017) proved that *Bacillus cereus* is an endophytic bacterium that can significantly improve vegetative growth of oil palm seedlings. Those findings were in line with previous study by Amir et al. (2005) where *Bacillus* spp. exhibits the ability to enhance roots and tops development of the oil palm seedlings.

Some essential mineral nutrients such as nitrogen, phosphorus and iron are fixed in insoluble forms, they are mostly not accessible in the soil to plants (Kim and Rees, 1994; Stevenson and Cole, 1999; Johri et al., 2003; Verma et al., 2010). However, microorganisms may increase the availability of essential

compounds by secreting certain enzymes or chelators (Francis et al., 2010). For example, in the soil, organic phosphorus is stored mainly as insoluble phytate. Many rhizosphere bacteria can solubilize phosphorus from phytate by secreting active phytases (Francis et al., 2010). The most efficient Gram-positive strains that promote plant growth in this manner belong to the genus *Bacillus* (Jorquera et al., 2008). According to Zakry et al. (2012), *Bacillus* sp. could provide nitrogen and reduce nitrogen fertilizer dependence for young palms by encouraging biological nitrogen fixation. In addition, Amir et al. (2005) also found out that *Bacillus* spp. may help to increase the availability of essential nutrients especially nitrogen, phosphorous and potassium for oil palm seedlings.

2.7 Potential of *Trichoderma* fungi as biological control agent

Fungal species belonging to the genus *Trichoderma* are largely spread in the soil and rhizosphere. According to Harman et al. (2004), genus *Trichoderma* has been known as 'an opportunistic avirulent plant symbionts' because they can benefit from and provide benefit in direct interactions with plants. Some of the benefits are improved nutrient uptake, increased growth and yields, increased percentage and rate of seed germination and strengthen plant defenses against various diseases (Harman et al., 2004). Many studies have demonstrated *Trichoderma* spp. as effective biological control agents against various crop diseases (Hermosa et al., 2000; Harman et al., 2004; Dubey et al., 2007; Saber et al., 2009; Zhang et al., 2013; Kim and Knudsen, 2013; Abo-Elyousr et al., 2014). *Trichoderma asperellum*, a less well-studied species could potentially suppress several plant diseases including *Fusarium* wilt pathogen of tomato (Cotxarrera et al., 2002); fungal pathogens attack on cacao (Bailey et al., 2008), and BSR disease of oil palm (Musa et al., 2018).

The capability of *Trichoderma* spp. to fight plant diseases are mostly is due to their direct antagonistic effects on the pathogenic fungi, and specifically due to their ability to excrete lytic enzymes such as chitinases and β-1,3-glucanases (Viterbo et al., 2002; Benítez et al., 2004). These enzymes hydrolyze the pathogen's cell wall thus controlling the growth of fungal pathogens. In addition, *Trichoderma* spp. are effective soil inhabitants and root colonizers especially if the soil is rich with inorganic soil substrates (Yang et al., 2011). *Trichoderma* isolates employ several mechanisms in providing biocontrol of disease, including antibiosis, mycoparasitism, and induced resistance (Gajera et al., 2012; Chaverri & Samuels, 2013; Shoresh et al., 2010). *Trichoderma* spp. parasitic mechanism is clearly demonstrated by the direct penetration and parasitism of the pathogens (Chet et al., 1998; Harman et al., 2004).

Many studies have been conducted to prove the ability of *Trichoderma* spp. as a BCA for oil palm against BSR disease caused by *Ganoderma* spp. Research conducted by Alizadeh et al. (2011) demonstrated induced plant defense response of oil palm seedlings by *Trichoderma* sp. contributed significantly towards the resistance of the seedlings against *G. boninense* infection. Besides that, Alizadeh et al. (2011) also found that *Trichoderma* sp. is a good

plant growth promoter of oil palm seedlings. Another study suggested that the oil palm treated with *Trichoderma* sp. could produce high level of chitinase that may act as defense mechanism against *Ganoderma* sp. infection (Naher et al., 2012). Musa et al. (2018) showed that the production of hydrolytic enzymes by *T. asperellum*, *Trichoderma brevicompatum, Trichoderma harzianum* and *Trichoderma virens* could minimize *G. boninense* spread. Results obtained by Musa et al. (2018) also suggested that *Trichoderma* spp. have potential as good BCAs and these findings were supported by previous studies to control BSR disease on oil palm (Sariah et al., 2005; Sundram et al., 2008; Izzati and Abdullah, 2008).

CHAPTER 3

ABILITY OF *Bacillus cereus* and *Trichoderma asperellum* TO SECRETE BIOCHEMICAL COMPOUNDS CONTIBUTING TO PLANT GROWTH PROMOTION (PGP)

3.1 Introduction

Plant disease is a classic problem in the plantation industry, crop production and storage. Chemical control is one of the most widely used methods by growers and planters to counter or minimize this problem (Agrios, 1988; Cook, 1993). However, excessive application of chemicals such as pesticides and fungicides could lead to various environmental contaminations, and indirectly affect growers' and consumers health (Heydari, 2007; Heydari et al., 2007). As a result, consumers and government called for reduction of chemical application and this situation has encouraged the application of biological control as a potential alternative (Cook, 1993; Heydari and Pessarakli, 2010). Generally, biological control has exhibited many advantages particularly in terms of less toxicity and sustainability compared to chemical control.

Specific objective of this study is:

To determine the plant growth promotion activities of *B. cereus* and *T. asperellum* isolates.

3.2 Materials and methods

3.2.1 Maintenance of microbes

Three microbes used in this study were obtained from the culture collection of the Microbe Control Laboratory, Department of Plant Protection, Faculty of Agriculture, UPM, Selangor, Malaysia. These microbes have been identified via morphological and molecular tools in previous studies; *Ganoderma boninense* (UPM13) and *Bacillus cereus* (UPM15) (Nusaibah et al., 2017) and *Trichoderma* asperellum (UPM16) (Musa et al., 2018). The fungi cultures were sub-cultured onto potato dextrose agar (PDA) (Difco™) plates and maintained at room temperature (27±1ºC) for eight to ten days. *Bacillus cereus* culture was sub-cultured onto nutrient agar (NA) (Difco™) and maintained at 4±1ºC for short term storage. (See Appendix A1, A2, A3, and A4 for more information on isolates)

3.2.2 Determination of plant growth promoting (PGP) activities

3.2.2.1 Indole Acetic Acid (IAA) production

Indole acetic acid (IAA) synthesized by isolate *B. cereus* and *T. asperellum* was detected calorimetrically using Salkowski's reagent (ferric chloride-perchloric acid reagent) by adapting the procedure used by Gorden and Weber (1951) and Glickmann and Dessaux (1995) respectively. This method measures the amount of IAA produced in aqueous solution containing the precursor L-tryptophan. A standard curve using a series of IAA dilutions were generated at 0, 50, 100, 150, 200, 250, 300, 350 and 400 µg/ml to quantify the amount of IAA produced by *B. cereus* and *T. asperellum*.

Bacillus cereus was grown in nutrient broth (NB) (Difco™) at room temperature (28 ± 2°C) on a shaker at 150rpm. After 24 hours, one milliliter of bacterial culture from previous NB was pipetted into 100 ml of modified NB (Difco™) (added with five milliliters of 100 mg l^{-1} of L-tryptophan solution). The bacterium was allowed to grow in the amended NB (Difco™) for 48 hours. Bacterial culture, measured to 1.5 ml, was centrifuged for five minutes at 12000 rpm. Once the centrifugation completed, one milliliter of supernatant was pipetted out and added to two milliliter of Salkowski's reagent. Thermo Scientific Multiskan Go (ThermoFisher Scientific, Finland) spectrophotometer was used to record the color densities of the mixture at 530 nm after incubating for 25 minutes at room temperature (28 ± 2°C).

Trichoderma asperellum was grown in 200 ml of modified potato dextrose broth (PDB) (Difco™) by adding five milliliters of 100 mg l^{-1} L-tryptophan solution to the broth. Conidial suspension of *T. asperellum* (1×10^8 cfu) was used to inoculate in the modified (added with L-tryptophan solution) PDB (Difco™) and incubated for eight days at room temperature. Following this, conidial suspension were centrifuged for ten minutes at 3000 rpm and filtered through a 0.22 µm syringe filter. One milliliter of obtained supernatant was pipetted out and mixed with two drops of orthophosphoric acid (Sigma-Aldrich, USA) and two milliliter of Salkowski's reagent (2% of 0.5 M $FeCl_3$ in 35% $HClO_4$ solution). Thermo Scientific Multiskan Go spectrophotometer (ThermoFisher Scientific, Finland) was used to record the color densities of the mixture after incubating for 20 minutes in the dark at room temperature. The spectrometer was set at 530 nm absorbance. Six replications were prepared for each treatment and the experiment was repeated two times.

3.2.2.2 Siderophore production assay

Siderophore production was tested by adapting the procedure used by Alexander and Zuberer (1991). Four solutions were prepared, sterilized separately and mixed to produce chrome azurol sulphonate (CAS) agar. Solution 1 (Fe-CAS indicator solution) was prepared by mixing ten milliliter ml

of 1mM $FeCl_3.6H_2O$ (in 10mM HCl), 50 ml of CAS solution (1.21mg ml^{-1}) and 40 ml of aqueous solution of hexadecyl-trimethylammonium bromide (HDTMA) (1.82 mg ml^{-1}). Piperazine-N,N-bis [2-ethanesulfonic acid] (PIPES)(Sigma-Aldrich, USA), measured to 30.24 g, and 750 ml of salt solution (mixture of 0.3 g K_2HPO_4, 0.5 g NaCl, and 1.0 g NH_4Cl) were dissolved to prepare solution 2. Distilled water was added to bring the volume to 800 ml, the pH was fixed to 6.8 with 50% KOH. Agar, measured to 15 g, was added prior to autoclaving the solution.

Solution 3 contained two grams glucose, two grams mannitol and trace elements (493 mg $MgSO_4.7H_2O$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4.2H_2O$, 1.4 mg H_3BO_3, 0.04 mg $CuSO_4.5H_2O$, 1.2 mg $ZnSO_4.7H_2O$ and 1.0 mg $NaMoO_4.2H_2O$) in 70 ml distilled water. Solution 3 was autoclaved and cooled to 50ºC. 30 ml of 10% (w/v) casamino acid (R & M Chemicals) were filter-sterilized and mixed with solution 3 to produce solution 4. Chrome azurol sulphonate (CAS) agar was prepared by mixing solution 2 and solution 4, then solution 1 (indicator solution) was added last with adequate stirring to ensure the ingredients were mixed well together. This mixture demonstrated blue to dark green in color. The CAS agar was poured into Petri plates and was allowed to solidify. A cork borer (5 mm in size) was used to punch four holes on each of the CAS agar plate. Bacterial inoculums of 100 µl and conidia suspension were pipetted into the holes of the agar in separate plates respectively. Petri plates were incubated for seven days at 28±2ºC. Lastly, control plate was prepared in a similar method except bacterial inoculums or conidia suspension was replaced by sterilized distilled water. The ability of the isolates to produce siderophore was determined by measuring the diameter of the orange halos exhibited after the stated duration of incubation. Fourteen replications were prepared for each treatment and the experiment was repeated three times.

3.2.2.3 Phosphate solubilization test

Phosphate solubilization test was carried out according to the procedure by Mehta and Nautiyal (1999). National Botanical Research Institute agar (NBRIP) media containing 10 g of glucose, 5 g of $Ca_3(PO_4)_2$, 5 g of $MgCl_2.6H_2O$, 0.25g of $MgSO_4.7H_2O$, 0.2 g of KCl and 0.1 g of $(NH_4)_2SO_4$ (per liter) was freshly prepared. *Bacillus cereus* (UPM 15) and *T. asperellum* (UPM 16) isolates were inoculated into NB (Difco™) and Potato Dextrose Broth (PDB) (Difco™) respectively. A cork borer (5 mm in size) was used to punch four holes on each of the NBRIP agar plate. After 48 hours of inoculation, 100 µl of bacterial inoculum and conidial suspension were pipetted into the holes of the agar. The Petri plates were incubated for seven days at 28±2ºC. The presence of clear zones around the bacterial and fungal colonies was used as an indicator for positive phosphate solubilization. The ability of isolates to solubilize phosphate was determined by measuring the diameter of the clear zone. Fourteen replications were prepared for each treatment and the experiment was repeated three times.

3.2.2.4 Sealed plate method

Over a decade, studies have suggested that volatile organic compounds (VOCs) have the ability to stimulate plant growth and actively restrain fungal growth (Weisskopf, 2013). Double Petri dish assay was conducted based on the procedure described by Gotor-Vila et al. (2017) with some modifications to determine the antifungal effects of VOCs produced by *B. cereus* and *T. asperellum* on the growth o*f G. boninense*. Instead of spraying cell suspension with specific concentration on petri dishes containing growth medai, the study used fully grown B. cereus culture on NA.

Three days old of *B cereus* (fully grown culture) culture grown on NA was stacked facing a PDA plate inoculated with a 5 mm *G. boninense* mycelium plug placed at the center of the plate (5 mm diameter plug) of *G. boninense* without a cover. Both plates were sealed with parafilm tape and incubated at room temperature for ten days. Two types of condition were used in this test; 1) *Bacillus cereus* culture at the base plate, *G. boninense* at the top plate, 2) *Ganoderma boninense* at the base plate, *B. cereus* culture at the top plate. Seven biological replicates were prepared for both conditions mentioned and the experiment were repeat two times. Plates without *B. cereus* culture served as the control. The diameter of *G. boninense* mycelium growth was recorded in millimeter after 12 days of incubation period. Data obtained was converted into the percentage inhibition of radial growth (PIRG) (Skidmore and Dickinson, 1976). The similar protocol as mentioned above was repeated by using a seven day's old *T. asperellum* (fully grown culture) culture grown on PDA. Six replications were prepared for each treatment and the experiment was repeated two times.

3.2.3 Statistical analysis

All data obtained were transformed by arcsine transformed (Gomez & Gomez 1984) and were subjected to ANOVA with the means compared to the least significant difference (LSD) at $p \leq 0.05$ using SAS® software (SAS Institute Inc. 1995).

3.3 Results

3.3.1 Indole acetic acid (IAA) production, siderophore production assay and phosphate solubilization test

IAA production of the isolates was tested according to Gorden and Weber (1951). Table 3.1 and Figure 3.1 showed the amount of IAA production and color changes by both *B. cereus* and *T. asperellum*. *Bacillus cereus* (B) produced more IAA (9.99 ± 0.03 ug/ml) compared to *T. asperellum* (A) (5.83 ± 0.02 ug/ml) based on the data recorded in Table 3.1. Nonetheless, both

isolates were able to produce IAA which is one of the most vital aspects in selecting BCAs in terms of plant growth promotion traits.

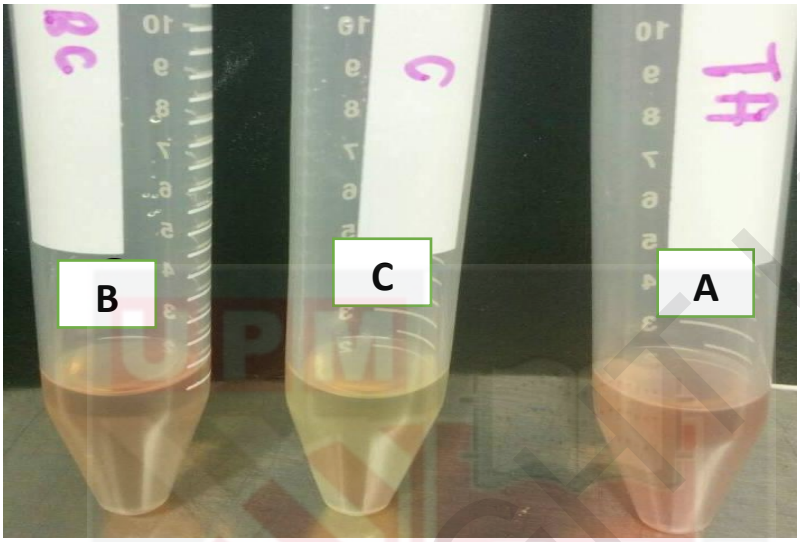


Figure 3.1: IAA produced by A (*Trichoderma asperellum*) and B (*Bacillus cereus*) can be clearly observed and compared to C that act as control treatment

Table 3.1: The result for the plant growth promotion (PGP) potential of the microbes

	IAA PRODUCTION [The amount of IAA production (ug/ml)]	SIDEROPHORE PRODUCTION ASSAY [Average orange halos (mm)]	PHOSPHATE SOLUBILIZATION TEST [Average clear zone (mm)]
Trichoderma asperellum (A)	5.83 ± 0.02^{b}	29.0 ± 0.4^{a}	10.0 ± 0.3^{a}
Bacillus cereus (B)	9.99 ± 0.03^{a}	4.0 ± 0.1^{b}	6.0 ± 0.1^{b}
Control (C)	0^{c}	0^{c}	0^{c}

* Means with the same letters between treatments are not significantly different according to the least significant different (LSD) test at p=0.05. Values are the means ± S.E

Presence of orange halos indicated siderophore production by the isolates (Figure 3.3). After seven days of incubation in the culture chamber, *T. asperellum* (A) showed significantly higher amount of siderophore production with 29.0 ± 0.4 mm orange halos compared to *B. cereus* (B) that only exhibited 4.0 ± 0.1 mm oranges halos (Table 3.1). However, both were found out to have the ability to produce siderophore which is one of the most important criteria that is needed to select a suitable BCA.

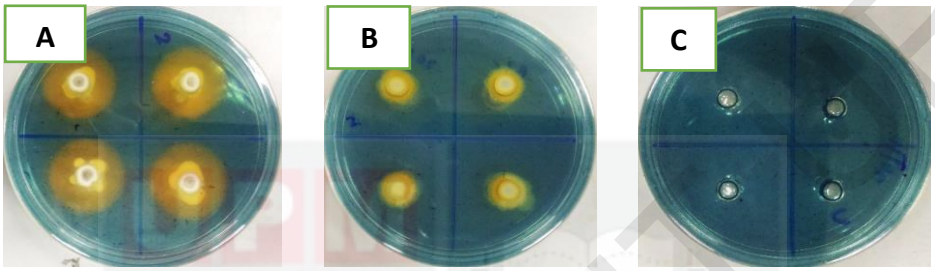


Figure 3.2: A and B displays orange halos indicating positive siderophore production by *Trichoderma asperellum* and *Bacillus cereus*, C showing negative siderophore production by control treatment

After seven days of incubation at room temperature (28 ± 2ºC), *T. asperellum* (A) gave significantly higher average clear zones which are 10.0 ± 0.3 mm compared to *B. cereus* (B) that only gave 6.0 ± 0.1 mm average clear zone (Table 3.1). The higher the measurement of average clear zones, the greater the ability of the BCA to solubilize phosphate.

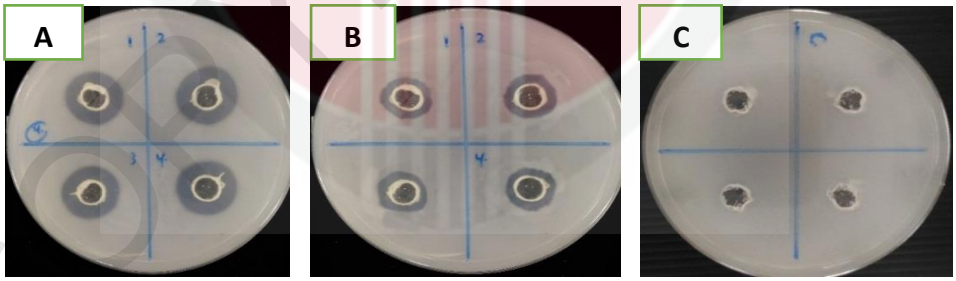


Figure 3.3: A and B demonstrated clear zones indicating positive phosphate solubilization by *Trichoderma asperellum* and *Bacillus cereus*, while C which served as the control treatment indicated negative phosphate solubilization

3.3.2 The effect of MVOC produced by BCA isolate on the growth of *Ganoderma boninense*

Mycelial growth inhibition rate of the pathogenic *G. boninense* indicates the effectiveness of MVOCs produced by the BCA isolates. Table 3.2 and Figure 3.5 demonstrates the mean mycelial growth rate of *G. boninense* when treated with *B. cereus* and *T. asperellum* isolates, recorded after 12 days of incubation. The results of this study showed that *B. cereus* has the ability to produce MVOCs that effectively inhibited *G. boninense* mycelial growth with the PIRG of 39.6% compared to the control plates.

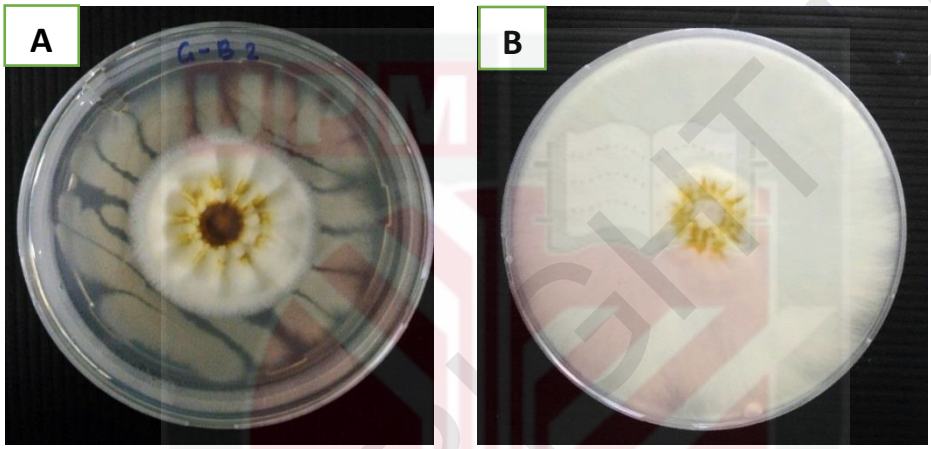


Figure 3.4: Growth inhibition of *Ganoderma boninense* by volatile compound emitted by Bacillus cereus (A) compared to the control plate (B)

Table 3.2: Percentage inhibition of radial growth (PIRG) recorded after treated with *Trichoderma asperellum* and *Bacillus cereus* for 12 days

Isolates	Percentage inhibition of radial growth (PIRG) (%)
Trichoderma asperellum	**
Bacillus cereus	39.6[a]

Means with the same letters between treatments are not significantly different according to the least significant different (LSD) test at p=0.05. Values are the means ± S.E; n=7

** The result for *Trichoderma asperellum* treatment failed to be recorded due to the overgrowth of it on both plates.

3.4 Discussion

Indole acetic acid production, phosphate solubilization, siderophore production and sealed plate method were conducted to assess the ability of both endophytes to secrete biochemical compounds contributing to plant growth promotion (PGP). Work by Simon et al. (2013) showed that one of the most active auxins is IAA. In this study, both *B. cereus* and *T. asperellum* demonstrated the ability to produce IAA. The results showed that both isolates manage to change the yellow color of the broth to pink in color when tested with Salkowski's reagent. The present findings seem to be consistent with other research which also demonstrated that *Bacillus* spp. and *T. asperellum* were able to produce IAA hormone that may promote plant growth and play roles in the plant defense response (Husen, 2003; Hermosa et al., 2012).

Phosphorus is one of the most inadequate elements available in the soil compared to other macronutrients (Bünemann et al., 2011). However, there are some microbes that have the ability to solubilize precipitated phosphate into a suitable form for plant uptake (Kang et al., 2002; Pradnan and Sukla, 2006). Thus, to choose excellent BCAs, the ability of microbes to solubilize phosphate should to be determined. The current study that was conducted using NBRIP media agar demonstrated that *B. cereus* and *T. asperellum* have the ability to solubilize phosphate. These findings are in agreement with Zhao and Zhang (2015)'s findings which showed that *T. asperellum* could solubilize inorganic or organic phosphate. Other than that, the present finding seems to be consistent with Maheswar and Sathiyavani (2012) whom reported that *B. cereus* and *Bacillus subtilis* are active in solubilization of tricalcium phosphate under *in vitro* condition. Jones and Oburger (2010) also stated that several species of soil bacteria such as *Pseudomonas*, *Azotobacter*, *Burkholderia*, *Bacillus* and *Rhizobium* are able to solubilize precipitated phosphate.

Various bacteria, fungi, plants, and yeast have been reported to produce and release siderophore to up take ferric ion from the environment (Chu, 2010). Iron is required in vital processes such as chlorophyll production (Encarnação et al., 2012) and enzyme functions (Grotz and Guerinot, 2006; Ghasemian and Ghalavand, 2010) in maintaining plant health. Therefore, one of the most essential characteristics of potential BCA is the ability to produce siderophore. The results of this study clearly indicated that both *B. cereus* and *T. asperellum* gave positive siderophore production. Orange halos of CAS agar revealed that *T. asperellum* exhibited higher production of siderophore compared to *B. cereus*. This finding is consistent with Qi and Zhao (2013) that displayed *T. asperellum* has the capability to produce siderophores by demonstrating orange halos around the colonies on blue half of the modified CAS agar plate and the production was up to 96.6% siderophore units after two days. Triveni et al. (2013) also demonstrated that *Trichoderma–Bacillus* and *Trichoderma–Pseudomonas* biofilms exhibited higher siderophore production.

To further evaluate the *in vitro* characteristics of the BCAs, sealed plate method was used to determine the antifungal effects of the MVOCs produced by BCAs. Microbial volatile organic compounds are commonly consisting of various lower molecular weight lipophilic compounds that were naturally mixed together. These compounds are the by-products produced by microorganisms as part of their metabolism (Di Francesco et al., 2016; Mari et al., 2016). The results of the present study showed that *B. cereus* has the ability to produce MVOCs that could effectively inhibit *G. boninense* mycelial growth. This result was in line with the studies carried out by Suryanto et al. (2012) and Alexander et al. (2015) where they observed *Bacillus* spp. have the ability to suppress *G. boninense* growth *in vitro*. However, the result of sealed plate test for *T. asperellum* failed to be recorded due to the overgrowth of its mycelium on both surface of the PDA agar.

3.5 Conclusion

Therefore, it was proven that the *in vitro* assessment conducted on isolate *B. cereus* and *T. asperellum* demonstrated their ability in contributing to the plant growth promotion in controlled environment. These results provided further support that these two isolates could be considered as potential BCAs and should be tested via nursery trial (*in vivo*) for evaluating their effectiveness in promoting plant growth and health.

CHAPTER 4

PROLIFERATION OF ENDOPHYTIC BIOLOGICAL CONTROL AGENTS IN THE ROOTS OF OIL PALM SEEDLINGS INOCULATED WITH *Ganoderma boninense*

4.1 Introduction

Disease in oil palm particularly by *Ganoderma* spp. continues to cause significant yield losses and collapse of standing palms. *Ganoderma boninense* is the fungus responsible for BSR and this disease can damage up to 80% of the stands by the time they are just half-way through their economic lifespan (Bivi et al., 2010). Typically, chemical control is recommended when dealing with this soil borne pathogen. However, studies demonstrated that chemical control could lead to groundwater pollution, evolving fungicidal resistance variants and loss of non-target beneficial flora (Sen, 2000).

Due to the problems that arise from excessive usage of chemicals, agriculture industry needs to use an environmentally friendly method. Therefore, BCAs could be the best alternative for chemical usage. Several potential BCAs, including *Trichoderma* spp. (Sariah et al., 2005; Susanto et al., 2005), and *Bacillus* spp. (Suryanto et al., 2012) have shown some efficacy in inhibiting *G. boninense's* growth and subsequently reducing the infection. A BCA occupies the rhizosphere, and produces almost no toxic residues as opposed to chemicals (Saxena and Stotzky, 2001; Kim et al., 2010; Ashbolt et al., 2013).

The previous chapter demonstrated on the *in vitro* potential of UPM 15 (*B. cereus*) and UPM 16 (*T. asperellum*) as biological control agents against *G. boninense.* Results obtained via *in vitro* studies may differ to the *in vivo* assessments conducted (Nusaibah et al., 2017). Thus, a comprehensive conclusion could only be made after analyzing the *in vivo* assessment. This study was conducted to analyze the vegetative growth parameters and disease suppression in the presence of both BCAs applied as pre-treatment prior to *G. boninense* inoculation.

4.2 Materials and methods

4.2.1 Plant material and soil preparation

Ninety-six oil palm seedlings of six months old was purchased from Sime Darby Seeds & Agricultural Services Sdn Bhd., Banting, Selangor. The seedlings were from commercial GH500 variant (Dura×Pisifera) and has been certified as *Ganoderma*-free seedling. The seedlings were placed in a nursery located at Ladang 15, Faculty of Agriculture, UPM, Serdang (shaded with two layers of polynet 30/70). Watering was carried out twice daily, before 11.00 am

and after 4.00 pm (Anon, 2009). The trays consist of six months old seedlings were allowed to adapt to the new nursery environment for two weeks prior to the seedling transplantation from the trays into polythene bag (Figure 4.1). Polythene bags (polybags) that was used has a measurement of 30 cm × 38 cm with a thickness of 500 gauge (0.125 mm) (Halimah et al., 2010) containing 3 kg of soil mixture (3:2:1 v/v/v topsoil: peat: sand) prior to artificial inoculation with UPM13 (*G. boninense*). Prior to the transplanting process, soil mixture was prepared and sterilized with autoclave at 121ºC with 100 kPa pressure for 30 minutes at Microbe Control Laboratory, Faculty of Agriculture, UPM.

Figure 4.1: Trays containing three months old seedlings were left at Ladang 15 Nursery, Faculty of Agriculture, UPM for two weeks before transferring it to polythene bags

4.2.2 Inoculum of biological control agents

Bacillus cereus inoculum suspension was prepared using 48 hours-old growing culture on NA. The inoculum suspensions were prepared using sterile distilled water adjusted to 10^8 colony forming unit (cfu) mL^{-1} (Zaiton *et al.,* 2008). Two weeks prior to artificial inoculation, the seedlings were inoculated with *B.cereus* inoculum suspension by drenching the soil with 150 mL inoculum suspension according to the designed treatments (Table 4.1).

Table 4.1: Treatments for *in vivo* nursery trial

Treatment	Description
T1 (BT)	Unchallenged seedlings treated with the combination of *T. asperellum* and *B. cereus*
T2 (T)	Unchallenged seedlings treated with *T. asperellum*
T3 (B)	Unchallenged seedlings treated with *B. cereus*
T4 (G)	Untreated seedlings challenged by *G. boninense*
T5 (BTG)	Seedlings challenged by *G. boninense,* treated with the

Table 4.1: Continued

	combination of *B. cereus* and *T. asperellum*
T6 (TG)	Seedlings challenged by *G. boninense, treated with T. asperellum*
T7 (BG)	Seedlings challenged by *G. boninense treated* with *B. cereus*
T8 (NC)	Untreated seedlings act as negative control

In addition to that, a booster application with (*B. cereus* was drenched onto the seedling soil in the polybags after three days of artificial inoculation of the oil palm seedlings with *G. boninense*.

Preparation of conidial suspension of *T. asperellum* was carried based on Nur Ain Izzati and Abdullah, (2008) with some modifications where the muslin cloth was replaced with the filter paper. Conidia of *T. asperellum* were harvested from seven days-old culture on PDA. Ten milliliters of sterile distilled water was be pipetted onto the PDA plate and the conidia were gently dislodged with an L-shaped glass rod. Subsequently, the mixture was filtered through filter paper (Whatman® Grade 1) to water remove mycelial debris and made up to 1L by adding distilled water. The conidia counts were fixed in the range of 10^7 conidia/ml using hemocytometer. Two weeks prior to artificial inoculation with *G. boninense* inoculum, the seedlings were inoculated with 250 mL of fresh conidial suspension of *T. asperellum* by applying it onto the soil surrounding the stem of the seedling of each treatment according to the designed treatments (Table 4.1). In addition to that, a booster application of *T. asperellum* with similar concentration as the first treatment was applied after five days of artificial inoculation of the oil palm seedlings with *G. boninense*.

4.2.3 *Ganoderma boninense* artificial inoculation

Ganoderma boninense artificial inoculation with Dip, Place and Drench (DPD) technique was conducted based on Nusaibah et al. (2017). Electric blender (Waring Commercial®, model HGBTWTG4) was used to blend 10 days old *G. boninense* mycelia that were grown in 250 ml of potato dextrose broth (PDB). The recipe mentioned will cater for a suspension size prepared for one seedling. Then, the suspension was transported to the nursery.

The oil palm seedling in the polybag was then carefully uprooted and dipped into the suspension of *G. boninense* fragments (prepared according to the recipe above) with approximately 250 ml of the inoculum per seedling. Subsequent to that, two plates of fully-grown 10 days old *G. boninense* on PDA free from contamination were placed on the soil in the polybag. Following that, the inoculum dipped oil palm seedling roots were then placed onto to the *G. boninense* cultures on PDA which was placed earlier in the polybag with half of the bag filled with soil mixture (3:2:1 v/v/v topsoil: peat: sand) and the remaining suspension for the dip step will be drenched on the roots before

covering with the soil mixture until three-quarters of the polybag. The seedlings were watered daily twice a day before 11.00 am and after 4.00 pm

4.2.4 Experimental design and statistical analysis

The treatments designed for this study are shown in Table 4.1. The experimental design used in the nursery trial are RCBD with eight treatments and 12 biological replications. Disease reduction (DR) was calculated based on the value of AUDPC. All percentage data (disease incidence (DI), severity of foliar symptoms (SFS), and disease severity (DS)) were transformed by arcsine transformed (Gomez & Gomez 1984) and vegetative growth data were subjected to ANOVA with the means compared to the least significant difference (LSD) at $p \leq 0.05$ using SAS® software (SAS Institute Inc. 1995).

4.2.5 Assessment on the oil palm vegetative growth

To evaluate the effect of treatments on oil palm seedling growth in relation to disease development, a number of parameters namely plant height, total root weight (dry and fresh), total top weight (dry and fresh), bole girth size, chlorophyll content or "greenness" via Spectrum SPAD 502 Plus meter (Konica Minolta, Japan) were recorded throughout the nursery trial on monthly basis and followed by destructive sampling at the end of the 24 weeks treatment duration. Plant height was measured starting from ground level to the tallest leaf of the seedling by using a measuring tape. For fresh top and root weight, the harvested seedling parts were measured and recorded immediately using balance available in the lab. Then, the seedling parts were dried in an oven (Memmert, Leading Modell 100-800, Schwabach, Germany) at 70°C for three days to obtain constant weight (Caruso et al., 2013) before dry top and root weight were measured.

4.2.6 Disease progress assessment at nursery trial

The DI percentage represents the number of seedlings assessed as diseased visually (chlorosis and necrosis of leaves, with or without the production of fruiting body) (Idris *et al.,* 2006).

Equation 1:

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

Reduction in the disease incidence compared with the control would be a measure of the treatment effectiveness in suppressing the disease. A disease progress curve was plotted with the data to assess the effectiveness of the

treatments designed. The area under the disease progress curve (AUDPC) was calculated using the formula (Shaner and Finney 1977):

Equation 2:

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

Whereby:

n = the number of assessment time
y = Disease incidence (DI)
t = the time (months) after inoculation

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

Equation 3:

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

The percentage of disease severity (DS) of the foliar symptoms (Table 4.2) and root tissues (Table 4.3) were calculated based on the following formula by Tarig *et al.* (1998):

Equation 4:

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

Table 4.2: Disease severity of foliar symptoms (SFS) due to *Ganoderma* disease was scored according to the scale below

Scale	Symptoms
0	Healthy plants with green leaves
1	Appearance of chlorotic leaves
2	Appearance of chlorotic leaves (>3 leaves)
3	Appearance of dry leaves brown in color
4	Appearance of dry leaves brown in color (>2 leaves)

(Sariah and Zakaria, 2000)

Table 4.3: Scale used to score disease severity index based on rotten root tissues of oil palm seedlings by UPM13 (*Ganoderma boninense*)

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

(Breton *et al.*, 2006)

4.2.7 Detection of basal stem rot disease establishment

The detection of *G. boninense* in the root of the inoculated seedlings was conducted via isolation on PDA, visual observation, scanning electron microscope (SEM) and DNA-based detection using polymerase chain reaction (PCR).

4.2.7.1 Visual observation

After six months of *G. boninense* inoculation, *Ganoderma* infected seedlings and negative control seedlings were observed externally and uprooted to compare the root conditions in order to confirm the disease establishment.

4.2.7.2 Plating out infected root tissues

Necrotic roots from *Ganoderma*-infected seedlings were harvested, cut into small pieces (0.5 cm length), surface sterilized and plated out on the PDA to strengthen the proof of disease establishment.

4.2.7.3 Root sample viewing via Scanning Electron Microscope (SEM)

Scanning electron microscope was used to prove the colonization of *G. boninense* and the applied BCAs on the inoculated roots of oil palm seedlings. Sample preparation was done following the *in-house* method of Microscopy Unit, Institute of Bioscience (IBS), UPM. Samples were cleaned carefully using running tap water and cut into thin-sections (approximately 1 cm^3 slices). Primary fixation was used to fix the structure of the sample. Samples were then put into separate vials and fix in fixative (4% glutaraldehyde) for 48 hours at 4°C. Samples need to be fixed immediately to avoid structural changes in its tissue.

The samples were then washed with 0.1M sodium cacodylate buffer for three changes of 30 minutes each. The samples were fixed with 1% osmium tetroxide for two hours at 4°C to fix lipid structure and give better contrast on sample. The samples were washed again using 0.1M sodium cacodylate buffer and followed by dehydration process for 30 minutes in a series of acetone with different concentration of 35%, 50%, 75% and 95%. At the final step of dehydration, the samples were soaked in 100% acetone for three changes of 20 minutes. The samples were critical-point dried with liquid CO_2 for 30 minutes and coated with gold-palladium. Lastly, the samples were viewed using SEM (JSM-IT100).

4.2.7.4 Detection based on DNA using polymerase chain reaction (PCR)

To further confirm the establishments of basal stem rot disease in the artificially inoculated oil palm seedling, polymerase chain reaction (PCR) was carried out. A primary root of each treatment that was infected with *G. boninense* was harvested, surface sterilized and placed on PDA plates. Fully grown mycelium of *G. boninense* was scrapped and grinded with liquid nitrogen using a mortar and pestle. The fine powder of *G. boninese* was transferred into cooled 1.5 milliliters tube. DNA of the sample was then extracted using DNeasy Plant Mini Kit (Qiagen, Netherlands) and guided by protocol from DNeasy® Plant Handbook (2017). The concentration and purity of extracted DNA was checked using Nanodrop in MultiSkanGo (ThermoFisher Scientific, Finland) spectrophotometer. To determine the Optical Density (OD), 260 nm and 280 nm wavelengths were used. In order to determine the quantity of DNA, the ratio of the absorption wavelength reading of 260 nm and 280 nm was measured. Formula for DNA quantification was calculated based on Equation 5. If the ratio obtained for the samples ranged between 1.7-1.9, the percentage or amount of DNA in the sample can be consider as high, ranging from 85% to 95%.

Equation 5:

$$\text{Ratio } OD_{260}/OD_{280} = \frac{Absorption\ reading\ at\ 260\ nm}{Absorption\ reading\ at\ 280\ nm}$$

Polymerase chain reaction was conducted by amplifying the DNA of these isolates using *Ganoderma* specific primer sets, *Gan*1(5'-TTG ACT GGG TTG TAG CTG-3') and *Gan*2 (5'-GCG TTA CAT CGC AAT ACA-3') (Utomo & Niepold, 2000). DNA amplification was performed according to the protocols of Qiagen TopTaq Master Mix (See Appendix B for the reaction setup using Qiagen TopTaq Master Mix). Eppendorf Mastercycler ep Gradient S Thermal Cycler (Eppendorf, Germany) was used to run PCR. The PCR began with denaturation for two minutes at 95°C and followed by 35 cycles of denaturation for one minute at 94°C, annealing for 30 seconds at 60°C and extension for two minutes at 72°C. Lastly, extension step was carried out for ten minutes at 72°C, before it was maintained at 4°C.

Using 1 × Tris-acetate-EDTA (TAE) buffer (Thermo Fisher Scientific, USA), 1% agarose gel was prepared. A comb with size 0.5 -1.0 millimeter was fixed onto the casting tray to form a well and the gel was poured into the casting tray. The comb was removed once the gel solidified and the gel was sunk with TAE buffer. Next, one microliter of 100 bp ladder (Axil Scientific Pte Ltd, Singapore) was mixed with one microliter of loading dye (Axil Scientific Pte Ltd, Singapore) and then pipetted into the first well as a marker. One microliter of amplified PCR products were mixed with one microliter of loading dye before they were pipetted into the remaining wells. Electrophoresis was set at 85 volt using power source from Lightning Volt™ Power Supply Model OSP-300 (Owl Separation Systems, Inc., UK) and was run for 45 minutes. After that, the gel was placed in the Bio-rad Gel Doc XR Imaging System (Bio-rad, United State) and visualized using ultraviolet (UV) light.

4.3 Results

4.3.1 Effect of BCAs on oil palm seedling vegetative growth

The data obtained on plant height showed that the treatments of BCAs not challenged with *G. boninense* inoculation gave higher reading compared to the treatments of BCAs challenged with *G. boninense* (Figure 4.3)(See Appendix C for the measurement of the vegetative growth parameters). Single application of T (T2) treatment recorded the highest plant height (86.9 ± 3.1 cm), followed by the single application of B (T3) with 85.3 ± 2.7 cm. The lowest plant height was recorded in treatment G (T4) with 74.3 ± 2.6 cm. Nevertheless, the plant height of all treatments has significantly increased between onset and six months after inoculations. (See Appendix D1 for the summary of ANOVA table on the seedling heights). Figure 4.2 demonstrates visible differences on the seedling heights for all the treatments at six MAI.

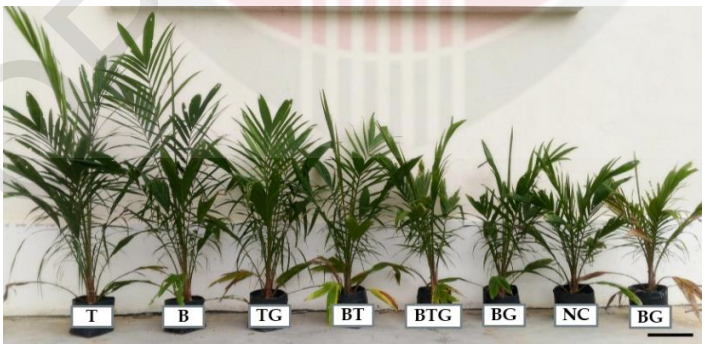


Figure 4.2: Visible differences on the seedling heights for all the treatments at six month after inoculation (MAI). Scale bar represent 10 cm
Treatments: G=*G.boninense*; NC=Negative control; T=*T.asperellum*; B=*B.cereus*;
BT=(*B.cereus*+*T.asperellum*);TG(*T.asperellum*+*G.boninense*);BG=(*B.cereus*+*G.boninense*); BTG=(*T.asperellum*+*B.cereus*+*G.boninense*)

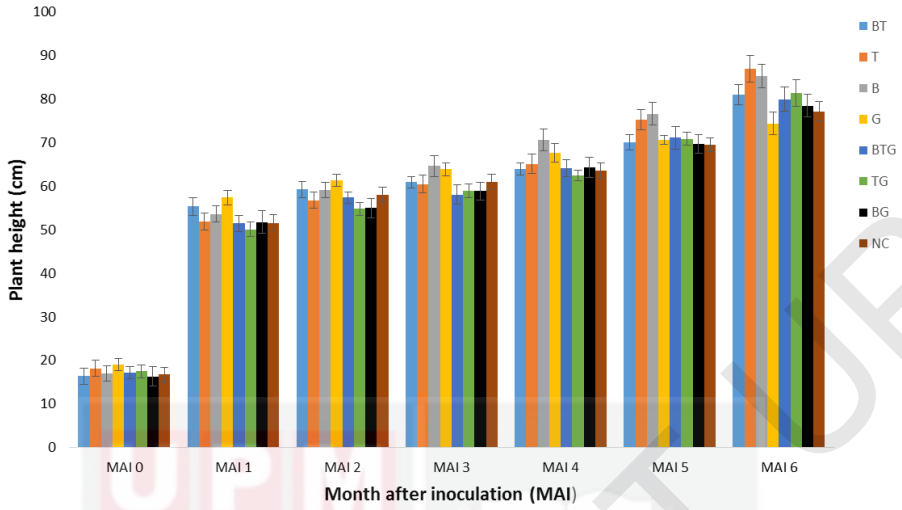


Figure 4.3: Effect of *Trichoderma asperellum* and *Bacillus cereus* on plant height of oil palm seedlings six months after challenged or not challenged with *Ganoderma boninense*. Values are the means ± S.E. (n=12)

Treatments: G=*G.boninense*; NC=Negative control; T=*T.asperellum;* B=*B.cereus*; BT=(*B.cereus*+*T.asperellum*);TG(*T.asperellum*+*G.boninense*);BG=*(B.cereus*+*G.boninense*);BTG=(*T.asperellum*+*B.cereus*+*G.boninense*)

Based on the data obtained on the bole size (Figure 4.4), almost all treatments showed no significant differences from each other except for treatment BT (T1), treatment G (T4) and treatment NC (T8). Mixture application of BT contributed the most in the bole growth with 3.8 ± 0.08 cm. Surprisingly, *Ganoderma*-infected seedlings without any BCA application (T4) exhibited 3.0 ± 0.04 cm in the size of the bole which was similar to untreated negative control (T8).

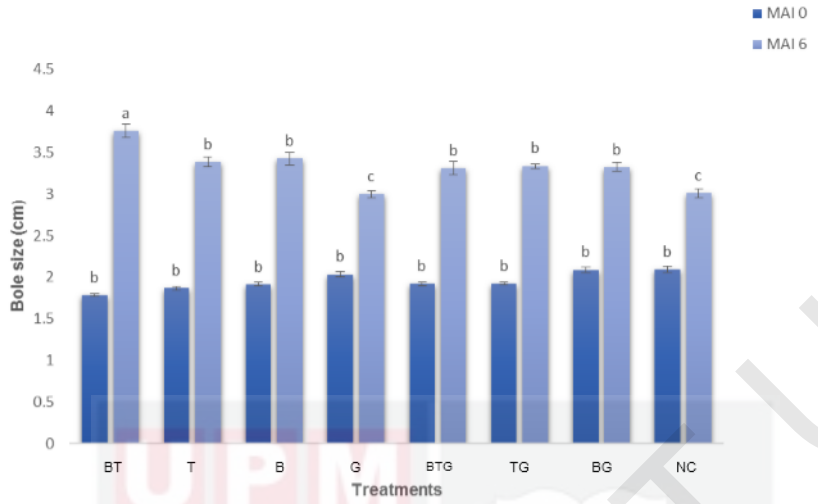


Figure 4.4: Effect of *Trichoderm asperellum* and *Bacillus cereus* on the bole size of oil palm seedlings six months after challenged or not challenged with *Ganoderma boninense*. Means with the same letters between treatments are not significantly different according to the least significant different (LSD) test at *p*=0.05. Values are the means ± S.E. (n=12)

Treatments: G=*G.boninense*; NC=Negative control; T=*T.asperellum;* B=*B.cereus*;
BT=(*B.cereus*+*T.asperellum*);TG(*T.asperellum*+*G.boninense*);BG=*(B.cereus*+*G .boninense*);BTG=(*T.asperellum*+*B.cereus*+*G.boninense*)

Based on the data obtained, it was clear that *B. cereus* contributed better than *T. asperellum* in terms of increasing the bole weight of seedlings according to the data obtained on the effect of BCAs on the fresh and dry bole weight of the seedlings (Figure 4.5). Single application of B treatment recorded higher fresh bole weight with 55.8 ± 1.7 g compared to the single application of T treatment with only 52.8 ± 2.1 g. *Ganoderma*-infected seedlings also gave a similar pattern where BG treatment recorded higher bole weight than TG treatment. Mixture application of BT contributed the most in the fresh bole weight with 61.6 ± 2.3 g followed by the single application of *B. cereus* with 55.8 ± 1.7 g. However, both treatment with TG and BG gave higher dry bole weight of seedling with 38.9 ± 0.5 g and 38.6 ± 0.7 g respectively compared to the mixture application of BT not challenged with *G. boninense* (36 ± 2.5 g). *Ganoderma*-infected seedlings without any BCAs application recorded the lowest fresh bole weight (40.9 ± 2.0 g) and the lowest dry bole weight (28.8 ± 2.0 g) compared to the rest of the treatments. (See Appendix D2 and Appendix D3 for the summary of ANOVA table on the bole weight of the seedlings)

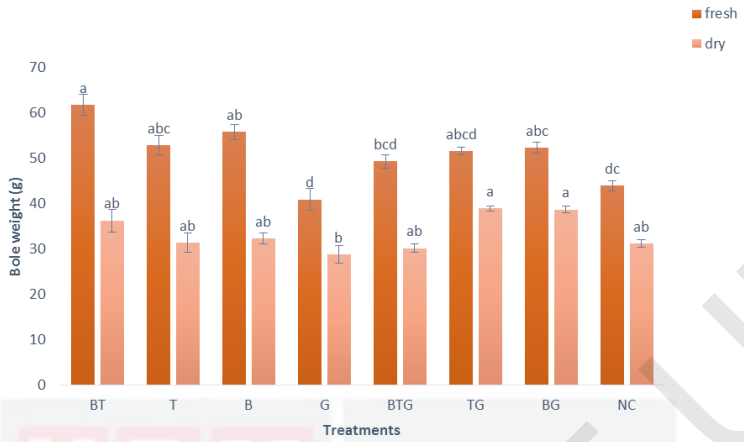


Figure 4.5: Effect of *Trichoderm asperellum* and *Bacillus cereus* on the bole fresh and dry weight of oil palm seedlings six months after challenged or not challenged with *Ganoderma boninense*. Means with the same letters between treatments are not significantly different according to the least significant different (LSD) test at *p*=0.05. Values are the means ± S.E. (n=12)
B=*B.cereus* Treatments: G=*G.boninense*; NC=Negative control; T=*T.asperellum;*; BT= (*B.cereus*+*T.asperellum*);TG(*T.asperellum*+*G.boninense*);BG=(B.*cereus*+*G.boninense*);BTG=(*T.asperellum*+*B.cereus*+*G.boninense*)

Data obtained on the top weight of the seedlings demonstrated that *T. asperellum* helped to promote the growth of the top part of the seedling regardless of challenged or not challenged with *G. boninense* (Figure 4.6). Mixture application of BT and single application of T treatment were significantly better than other treatments with 465 ± 49.3 g and 456 ± 49.6 g of fresh top weight recorded respectively. On the other hand, the lowest fresh top weight was recorded in the treatment negative control with 258 ± 14 g, followed by untreated *Ganoderma*-infected seedlings with 273 ± 20 g. The pattern of the data recorded in the top dry weight was almost similar with the top fresh weight. The only difference was, the top dry weight of seedlings infected with *Ganoderma* and treated with single application of *T. asperellum* (treatment TG) gave significantly higher top weight with 221 ± 9.8 g compared to the *Ganoderma*-infected seedling treated with the mixture application of *B. cereus* and *T. asperellum* (treatment BTG) with 176 ± 10 g of dry top weight. (See Appendix D4 and Appendix D5 for the summary of ANOVA table on the top weight of the seedlings)

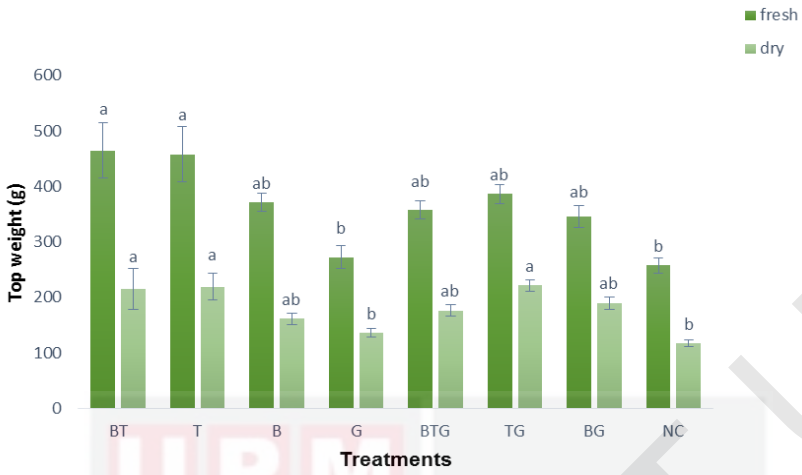


Figure 4.6: Effect of *Trichoderm asperellum* and *Bacillus cereus* on fresh and dry weight of oil palm seedlings top, six months after challenged or not challenged with *Ganoderma boninense*. Means with the same letters between treatments are not significantly different according to the least significant different (LSD) test at *p*=0.05. Values are the means ± S.E. (n=12)

Treatments: G=*G.boninense*; NC=Negative control; T=*T.asperellum;* B=*B.cereus*; BT= (*B.cereus*+T.asperellum);TG(*T.asperellum*+*G.boninense*);BG=(*B.cereus*+*G.boninense*);BTG=(*T.asperellum*+*B.cereus*+*G.boninense*)

Generally, all treatments that were not challenged by *G. boninense* recorded heavier fresh and dry root weight (Figure 4.7). It was found that the mixture application of BT treatment was able to produce the heaviest fresh root weight (213 ± 18 g), followed by the single application of *B. cereus* treatment (198 ± 24.7 g). As expected, untreated *Ganoderma*-infected seedling (T4) gave the lowest fresh and dry root weight. However, all treatments that were challenged by *G. boninense* (T4, T5, T6 and T7) provided no significant differences from each other in terms of the root weight of the seedlings. (See Appendix D6 and Appendix D7 for the summary of ANOVA table on the root weight of the seedlings)

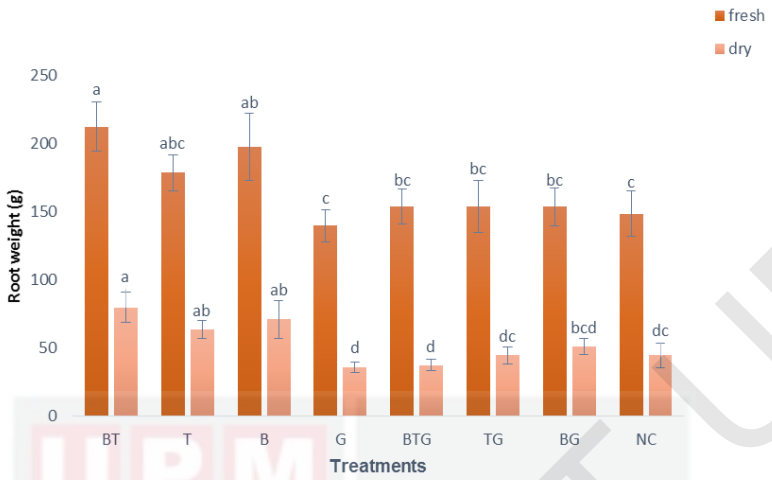


Figure 4.7: Effect of *Trichoderma asperellum* and *Bacillus cereus* on fresh and dry weight of oil palm seedlings roots, six months after challenged or not challenged with *Ganoderma boninense*. Means with the same letters between treatments are not significantly different according to the least significant different (LSD) test at *p*=0.05. Values are the means ± S.E. (n=12)

Treatments: G=*G.boninense*; NC=Negative control; T=*T.asperellum;* B=*B.cereus*; BT=(*B.cereus*+T.asperellum);TG(*T.asperellum+G.boninense*);BG=(B.*cereus+G.boninense*);BTG=(*T.asperellum+B.cereus+G.boninense*)

Data obtained on the chlorophyll content demonstrated that chlorophyll content started to decrease at two MAI. However, a slight increase was noted at six MAI (Figure 4.8). At six MAI, treatment BG gave the highest chlorophyll content with 44.6 ± 2.1 SPAD unit, followed by treatment G with 43.6 ± 1.3 SPAD unit. However, there were no significant differences in chlorophyll content between all treatments. (See Appendix D8 for the summary of ANOVA table on the chlorophyll content of the seedlings)

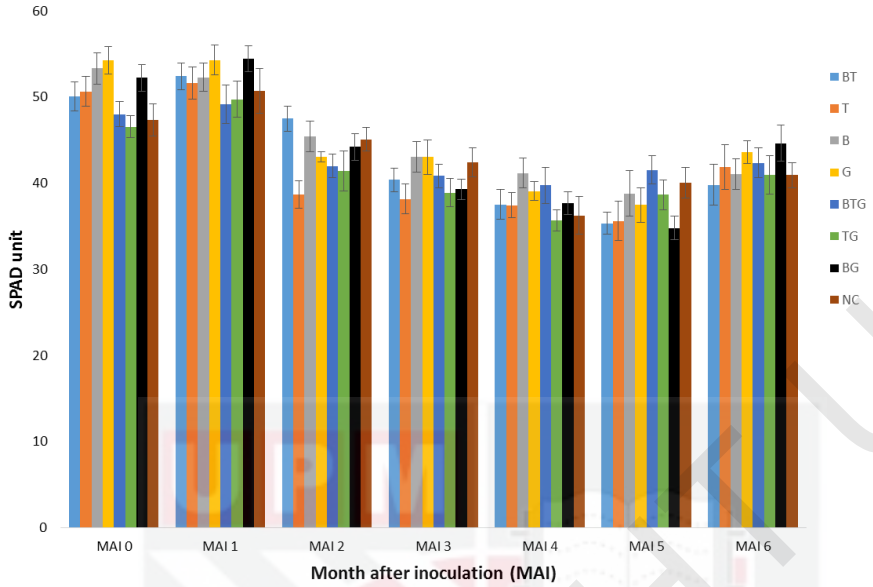


Figure 4.8: Effect of *Trichoderma asperellum* and *Bacillus cereus* on chlorophyll content of oil palm seedlings six months after challenged or not challenged with *Ganoderma boninense*. Values are the means ± S.E. (n=12)

Treatments: G=*G.boninense*; NC=Negative control; T=*T.asperellum;* B=*B.cereus*; BT=(*B.cereus*+*T.asperellum*);TG(*T.asperellum*+*G.boninense*);BG=*(B.cereus*+*G .boninense*);BTG=(*T.asperellum*+*B.cereus*+*G.boninense*)

4.3.2 Basal stem rot disease assessment

The nursery trial was conducted to examine the effect of treatments on the disease suppression in oil palm seedlings. The percentage of disease incidence (DI) was recorded as in Table 4.4. Data recorded showed that seedlings treated with consortium or single treatment gave a lower percentage of DI after six months of treatment duration. Three months after inoculation (MAI), untreated *Ganoderma*-infected seedlings demonstrated the first DI with 25%. However, the rest of the treatments recorded DI at four MAI. At six MAI, the least DI was recorded in consortium treatment (BTG) with 81.3%, followed by single treatment of *B. cereus* (87.5%) and single treatment of *T. asperellum* (93.8%).

Table 4.4: Percentage of disease incidence (DI) in oil palm seedlings after inoculation with UPM13 (G*anoderma boninense*)

Treatment	Disease incidence (%)*			
	3MAI**	4MAI	5MAI	6MAI
G. 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 with LSD at $P \leq 0.05$, (n=6)
**MAI = Months after inoculated with *Ganoderma boninense*

The disease severity on oil palm seedlings root tissues was recorded after six MAI based on Breton et al. (2006) (Figure 4.9). Single treatment of *T. asperellum* (TG) demonstrated the lowest root disease severity with 50%, followed by the consortium treatment (BTG) with 63%. The untreated *Ganoderma*-infected treatment (G) gave the highest root disease severity with 84%.

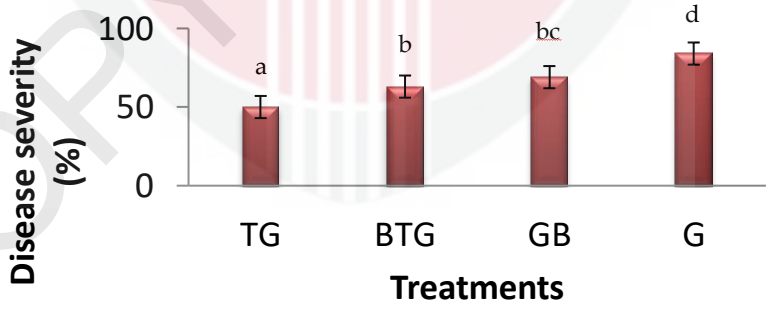


Figure 4.9: Bar graph shows the percentage of disease severity (DS) on oil palm seedlings root tissues, six months after challenged with *Ganoderma boninense*. Means with the same letters between treatments are not significantly different according to the least significant different (LSD) test at p=0.05. Values are the means ± S.E. (n=12)
Treatments:G=*G.boninense*;TG(*T.asperellum+G.boninense*);BG=(*B.cereus+G. boninense*);BTG=(*T.asperellum+B.cereus+G.boninense*)

Table 4.5 shows the area under disease progress curve (AUDPC) and the disease reduction (DR) percentage. Disease reduction was calculated using AUDPC values and DR formula (Equation 3). Single treatment of *T. asperellum* gave the highest DR with 57.2%, followed by both consortium treatment and single treatment of *B. cereus* with 50% DR.

Table 4.5: The effect of BCAs on the development of basal stem rot disease in oil palm seedlings based on the root disease severity after artificially infected with *Ganoderma boninense* for six months

Treatment	AUDPC[1]	DR[2]
plant + *G. boninense*	263	-
plant + *T. asperellum* + *B. cereus*	131	50
plant +*T. asperellum*	112	57.2
plant + *B. cereus*	131	50

[1]Area under disease progress curve (AUDPC)
[2]Disease reduction (DR)

4.3.3 Basal stem rot disease establishment in the inoculated oil palm roots

4.3.3.1 Visual observation

The root of *Ganoderma*-infected seedlings was less dense than the roots of negative control seedling (Figure 4.10). The root of *Ganoderma*-infected seedlings was also darker in colour compared to the root of negative control seedling. The external symptoms of the upper parts could also be observed clearly. The *Ganoderma*-infected seedlings demonstrated three symptoms which are unopened spear leaf, severely necrotic of the lower fronds and tip necrosis of the leaflets. These supplementary data provide the visual proof that *G. boninense* have been established via DPD technique.

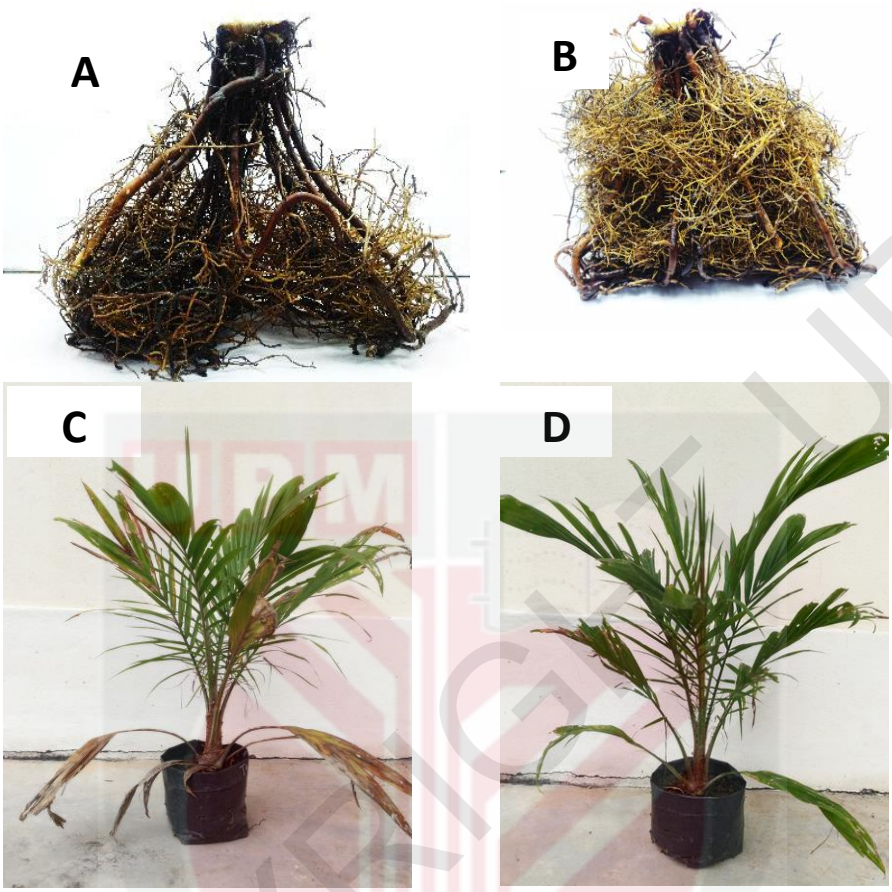


Figure 4.10: Visual proof of basal stem rot establishment via artificial inoculation with Dip, Place and Drench (DPD) technique. A demonstrating *Ganoderma boninense* inoculated roots from treatment G; B displaying uninoculated roots that served as the control; C showing external disease severity symptoms resulted from *Ganoderma boninense* inoculation; D no external disease severity observed on the control palm

4.3.3.2 Plating out infected root tissues

To further support the disease establishments, necrotic roots from *Ganoderma*-inoculated seedlings were harvested, surface sterilized and plated out on the potato dextrose agar (Figure 4.11). After two weeks, the plates of agar were fully covered by the mycelium of the *Ganoderma boninense*.

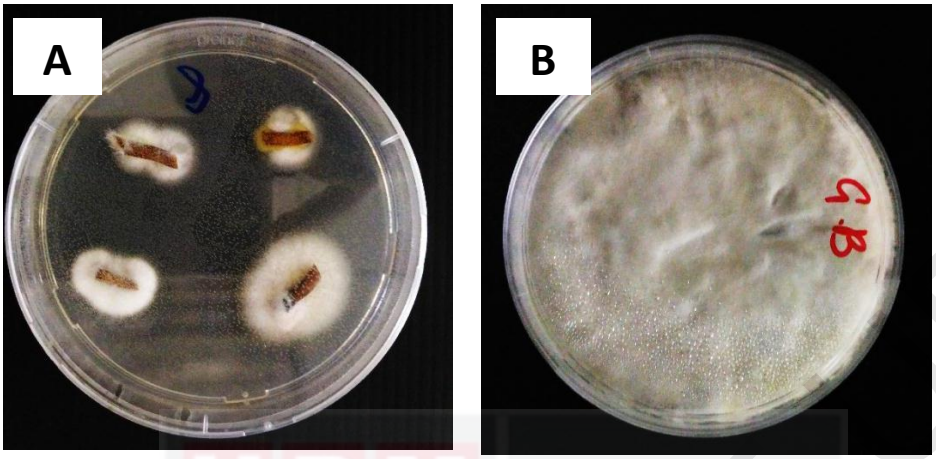
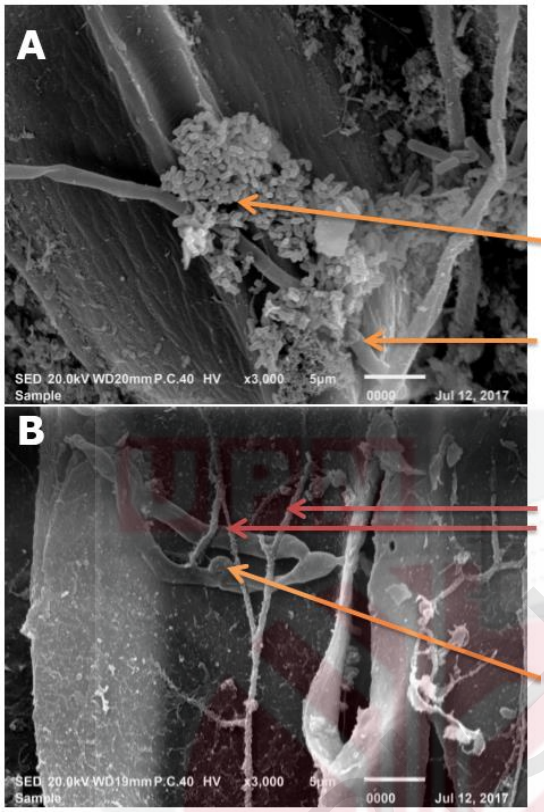


Figure 4.11: Cultural proof of basal stem rot establishment via artificial inoculation with Dip, Place and Drench (DPD) technique. A= the white cottony mycelium of *Ganoderma boninense* started to grow after six days; B= the plate of potato dextrose agar was fully covered by pure culture *Ganoderma boninense* after two weeks

4.3.3.3 Root sample viewing via Scanning Electron Microscope (SEM)

Scanning Electron Microscope viewing of the root samples showed that BCAs and *G. boninense* were successfully established in the root tissues as shown in Figure 4.12 and Figure 4.13. Both *G. boninense* and BCAs were able to colonize the rhizosphere of the roots. Figure 4.12 (A) demonstrated activities in the *Ganoderma*-infected seedling treated with single application of *B. cereus* where *G. boninense* colonizing the roots and *B. cereus* cells colonizing *G. boninense* hypha. While, in the *Ganoderma*-inoculated seedling treated with single application of *T. asperellum, T. asperellum* hyphae were observed colonizing *G. boninense* hyphae and the primary roots (Figure 4.12 (B)).



B. cereus rod in shape cells colonizing *G. boninense* hypha

G. boninense hyphae colonizing primary roots

T. asperellum hyphae colonizing *G. boninense* hyphae and primary roots

G. boninense hyphae with the presence of clamp connection colonizing primary roots

Figure 4.12: Oil palm seedling roots pre-inoculated with (A) *Bacillus cereus* (B) *Trichoderma asperellum* both infected with *Ganoderma boninense*, and harvested after eight weeks of incubation period

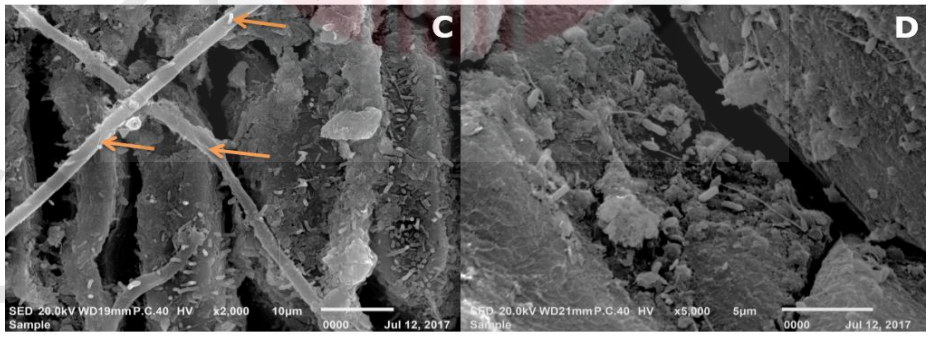


Figure 4.13: Oil palm seedling roots pre-inoculated with mixture of *Bacillus cereus* and *Trichoderma asperellum* (C) infected with *Ganoderma boninense* and (D) non-infected with *Ganoderma boninense.* Roots harvested after eight weeks of incubation period.

4.3.3.4 Polymerase Chain Reaction (PCR) and gel electrophoresis

In order to strengthen the evidence of BSR disease establishment using DPD technique, presence of *G. boninense* in the challenged oil palm roots were detected via PCR and gel electrophoresis. Based on the gel electrophoresis results on amplified PCR products obtained (Figure 4.14), PCR product intact bands were present in the seedlings roots inoculated with *G. boninense*.

Figure 4.14: Amplified DNA utilizing *Ganoderma* specific primer sets (Gan1 and Gan2) showing band size of 167 bp on 1.7% agarose gel for detection of *Ganoderma boninense* (UPM13) fungus on treated seedlings at six months of post-inoculation

M: 100 bp ladder
L1: PCR negative control (non-template)
L2: *Trichoderma asperellum+Ganoderma boninense* (TG)
L3: *Bacillus cereus+Ganoderma boninense* (BG)
L4: *Bacillus cereus+Trichoderma asperellum+Ganoderma boninense* (BTG)
L5: *Ganoderma boninense* (G)
L6: PCR positive control [identified *Ganoderma boninense* (UPM13) pure culture]

4.4 Discussion

This study set out with the aim of assessing the ability of *T. asperellum* and *B. cereus* as potential biological control agents against *G. boninense.* Many studies on BCAs as an alternative method in plant protection have been done in recent years demonstrated positive results regardless a single treatment or as a consortium (Berg et al., 2009; Atanasova et al., 2013; Hermosa et al., 2013; Nusaibah et al., 2017). This time both *B. cereus* and *T. asperellum* potential were assessed in the nursery trial after positive outcomes recorded in *in vitro* test.

To analyze the vegetative growth parameters and disease suppression in the presence of both endophytes against basal stem rot disease, the artificial inoculation with Dip, Place and Drench (DPD) technique was conducted. Due to the fact that this technique is relatively new, several methods were used to detect *G. boninense* on inoculated roots as proof of disease establishment after six months of inoculation. Visual observation, direct isolation from infected roots, polymerase chain reaction and SEM viewing were carried out and the results obtained successfully proved the establishment of basal stem rot disease on the root of the inoculated seedlings. These findings further support a study by Nusaibah et al. (2017), where they conclude that DPD technique could be considered as an efficient technique in the disease establishment since *G. boninense* displays vegetative growth with hyphae as the main mode of vegetative growth (Sundram et al., 2011; Naher et al., 2014).

The effect of BCAs on the oil palm vegetative growth was studied. Generally, higher vegetative growth was demonstrated in all BCA treated seedlings compared to the untreated seedlings. However, mixture of *T. asperellum* and *B. cereus* (T1) showed to be significantly effective based on the data of bole weight, top weight and root weight parameters. This finding is in agreement with Nusaibah et al. (2017)'s findings that reported the mixture of both *Trichoderma harzianum* and *B. cereus* proved to be significantly better compared to other treatments in inducing palm's vegetative growth.

Another important finding was that application of *T. asperellum* (T2) was superior compared to other treatments when it comes to the plant height contribution. The present findings seem to be consistent with Harman et al., (2004) where they found that *Trichoderma* sp. improved nutrient uptake and subsequently enhanced the plant growth of maize.

In the present study, *B. cereus* performed better than *T. asperellum* in terms of increasing the bole weight and root weight of the seedlings. In accordance with the present results, previous study by Zhao et al. (2010) demonstrated that bacterial polysaccharide extracted from *B. cereus* significantly improved the biomass of *Salvia miltiorrhiza* hairy root. Besides that, these results obtained in the current study were also in agreement with Dawwam et al. (2013) whom

recommended *B. cereus* application as a biofertilizer component due to its promising plant growth promoting characteristics.

This current study found that the presence of both BCAs employed did not have any significant impact on the chlorophyll content. These findings were consistent with Pereira et al. (2015), where they found out that *Azospirillum brasilense* did not significantly influence the chlorophyll content of maize tested. However, the findings of the current study do not support the previous research conducted by Nulit et al. (2015), where they showed that *Phlebia* sp. isolate increased all vegetative growth parameters measured of oil palm including total chlorophyll content.

Other than examining the effect of BCAs on the vegetative growth of oil palm seedlings, BSR disease suppression were assessed at nursery trial. Both BCAs tested in the current study exhibited its ability in suppressing BSR disease of oil palm according to the data recorded in DS and DR analysis. Surprisingly, single treatment of *T. asperellum,* with 50% DS and 57.2% DR, was more efficient in disease suppression compared to both consortium treatment and single treatment of *B. cereus*. Several studies showed that *T. asperellum* could potentially suppress several plant diseases (Cotxarrera et al., 2002; Bailey et al., 2008; and Musa et al., 2018). In addition, according to Yang et al. (2011), *Trichoderma* spp. are shown to be effective soil inhabitants and root colonizers. However, consortium used in this study still demonstrated better DR and with lower DS to the seedlings roots compared to the single treatment of *B. cereus*.

4.5 Conclusion

In conclusion, the purpose of this study is to investigate the effects of biological control agent application on the oil palm seedlings in the nursery trial. Based on the results obtained, treatments applied did influence the vegetative growth parameters of the seedlings and BSR disease suppression. Generally, it is safe to conclude that *T. asperellum* contributed significantly on the growth of aerial parts and *B. cereus* on the growth of oil palm roots. However, further validation through the study of oil palm enzymes, phenolic content and plant metabolites in response to BCAs may be helpful in understanding the BCAs interaction with the seedlings particularly in the plant disease mechanism.

CHAPTER 5

ENHANCEMENT OF OIL PALM ENZYMES, PHENOLIC CONTENT AND METABOLITES IN BASAL STEM ROT DISEASED SEEDLINGS IN RESPONSE TO *Bacillus cereus* and *Trichoderma asperellum* TREATMENTS

5.1 Introduction

Pathogen attack or the presence of elicitor could trigger various plant protective mechanisms that have been specially developed to counteract pathogen development (Malolepsza and Rozalska, 2005). According to Singh et al. (2009), in order to trigger plant resistance, plant enzymes work collaboratively with each other. Several studies suggested that plant polymers such as lignin and suberin could play direct role in the breakdown of pathogen cell wall. (Usall et al., 2000; Treutter, 2006). Surekha et al. (2013) also suggested that phenolic compounds also act as antimicrobial, growth interceptors of pathogen, triggers plant defense genes and structural barrier. Therefore, it is vital to explore the response of oil palm enzymes, phenolic content and metabolites upon *B. cereus* and *T. asperellum* treatments.

5.2 Materials and methods

5.2.1 Plant materials

Refer to section 4.2.1

5.2.2 Oil palm seedling inoculation

Methods are as described in section 4.2.3

5.2.3 Application of biological control agent

Biological control agents were applied following the method as described in section 4.2.2

5.2.4 Experimental design

Refer to section 4.2.4. The treatments used in this experiment are shown in Table 4.1.

5.2.5 Enzyme assay

5.2.5.1 Total peroxidase (PO) assay

One gram of root that was harvested during destructive sampling was immersed in liquid nitrogen and instantly homogenized in two milliliters of cold 0.05 mol L^{-1} sodium phosphate buffer (pH 5) altered with five milligram polyvinyle pyrrolidone (PVP) (Sigma-Aldrich). At 4°C, the semi-solid mixture was centrifuged for 20 minutes at 14 000 r/min. 200 µL of the supernatant was extracted. Three milliliters of reaction mixture consisting of 0.1 mmol L^{-1} sodium acetate buffer (pH 6), 1 mmol L^{-1} H_2O_2 (30%, v/v) and 0.1 mmol L^{-1} o-methoxyphenol (guiacol) (Acros Organics) was prepared. The previously extracted supernatant was fully mixed with the reaction mixture and incubated at room temperature for two minutes. Then the mixture absorbance was read at 470 nanometers with a spectrophotometer (Thermo Scientific Multiskan Go, Thermo Fisher Scientific, Finland). Blanks were prepared using the reaction mixture without the supernatant. The PO activity was expressed as change in absorbance $min^{-1}g^{-1}$ protein (Kokkinakis and Brooks 1979).

5.2.5.2 Lignin assay

One gram of frozen root that was harvested during destructive sampling was immersed in five milliliter of absolute methanol (Merck Schuchardt OHG) for 48 hours (four changes of methanol). The tissues were grinded and 50 milligram was transferred into an Eppendorf tube (containing 0.1 milliliter thioglycolic acid and 0.9 milliliter 2 N hydrochloric acid (HCl). The sample was heated at 95°C for four hours. The cooled test tube was centrifuged at 12 000 r/min for five minutes and the residue was washed with distilled water. To obtain the pellet, the sample was centrifuged again. One milliliter of 0.5 N sodium hydroxide (NaOH) was added to the tube containing pellet. The sample was incubated overnight and then centrifuged. The supernatant was mixed with one milliliter of concentrated HCl, (37%) (Avantor Performance Materials, Inc.) centrifuged and washed with distilled water. The pellet was dissolved in one milliliter 1 N NaOH. Lastly, 25 µL aliquot was mixed with one milliliter of 0.5 N NaOH and read at 280 nm using Thermo Scientific Multiskan Go, (ThermoFisher Scientific, Finland) (Dean and Kuc, 1987).

5.2.5.3 Determination of total phenolic content (TPC)

The total phenolic content was analyzed by using the Folin-Ciocalteu colorimetric method (Singleton, 1999). One gram of root that was harvested during destructive sampling was grinded in a chilled mortar and pestle. The sample was transferred into an Eppendorf tube and four milliliters of methanol (Merck Schuchardt OHG) was added (two changes in 18 hours). The sample was centrifuged at 5000 rpm for five minutes. One milliliter of supernatant was extracted and added into 0.5 milliliter of Folin-Ciocalteu reagent (diluted with

distilled water, ratio 1:1). After three minutes of sample incubation at 25°C, 0.5 milliliter of 1M sodium carbonate was added and incubated again for one hour. Lastly, the absorbance was measured at 725 nm with gallic acid (R & M Chemicals) as reference standard and methanol (Merck Schuchardt OHG) as blank. Results were expressed as Gallic Acid Equivalent (GAE).

5.2.6 Metabolites extraction for Gas Chromatography-Mass Spectrometr (GC-MS) analysis

For metabolites profiling activity, method by Nusaibah et al. (2016) was applied. One gram of leaf sample from selected treatment was harvested, washed with sterile distilled water, grinded in liquid nitrogen and then transferred into Falcon tube. Five milliliter of HPLC-grade methanol (Merck Schuchardt OHG) was added and the tube was shaken to fully mix the sample. The mixture was stored for 48 hours at 4 °C. The mixture was filtered through a 0.4-µm nylon syringe filter (Nalgene). The compounds collected were evaporated at 38˚C on a rotary evaporator (BUCHI B-491) until dry. One milliliter of methanol (Merck Schuchardt OHG) was added to the dried sample. 200 µl of sample from round-bottom flask was extracted and syringe-filtered. The sample was then transferred into a glass insert placed in an amber vial.

5.2.7 Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The sample extracted from section 5.2.7 was sent to Halal Product Research Institute (Malaysia) and GC-MS analysis was conducted by Halal Product Research Institute's staff based on the modified and optimized method of Fiehn (2002). Chromatography was carried out on a DB5 capillary column (30 m long, 0.25 mm I.D. 155 and a 0.25 µm five percent phenyl methylpolysiloxane column with an additional 10 m integrated guard column). A standard ten microliter injection needle was mounted onto an auto sampler. Each sample (two microliter) was injected in a splitless mode. The following conditions were used: carrier gas, helium, with a flow rate of 0.7 mL/min; column temperature: five minutes at 180 °C, 180-260 °C at 3 °C/min, five minutes at 260 °C, 260-280 °C at 0.2 °C/min, and finally five minutes at 280 °C, with the injector temperature at 280 °C and the detector temperature at 290 °C. The MS operating parameters were as follows: ionization potential, 70 eV; ion source temperature, 290 °C; quadrupole, 100 °C; solvent delay, seven minutes; scan speed, 2000 amu/sec; scan range, 30-600 amu; and EV voltage, 3000 V. Identified metabolites were analyzed using MetaboAnalyst 4.0 software (Canada, 2019). Metabolites information was further confirmed with mVOC 2.0 software.

5.3 Results

5.3.1 Enzyme assay

5.3.1.1 Total peroxidase (PO) assay

Treatment with BG recorded the highest peroxidase (PO) activity with 0.2601 unit/min/g, followed by treatment BTG with 0.2278 unit/min/g (Figure 5.1). Generally, all treatments treated with *B. cereus* (BT, B, BTG and BG) yielded high PO activity compared to the treatments untreated with *B. cereus* (T, G, TG and NC).

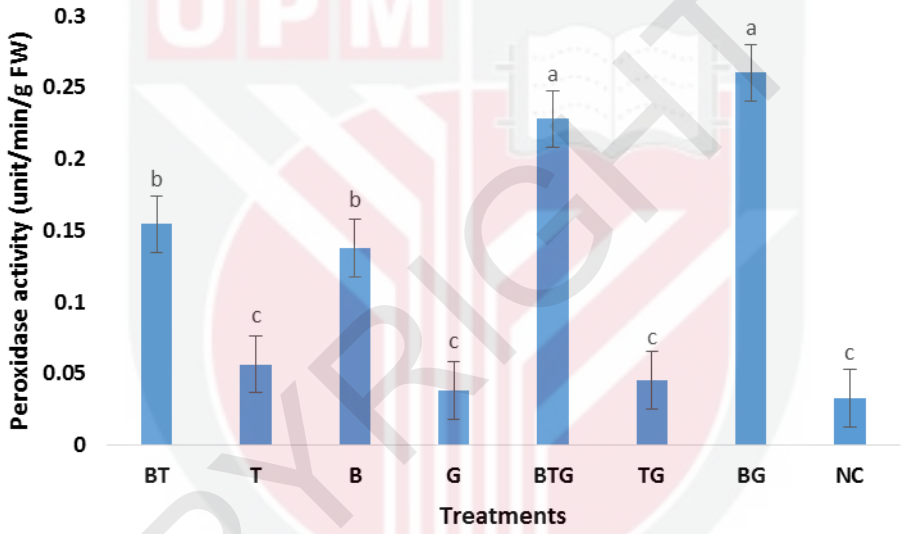


Figure 5.1: Induction of peroxidase (PO) activity in oil palm seedling roots treated with biological control agent/s. Values are means of five replications and difference between the means are separated by Tukey's Studentized Range (HSD) Test at 5% level of significance

5.3.1.2 Lignin assay

Figure 5.3 demonstrated that treatment T, BG and NC recorded the highest lignin concentration with 10, 10.4 and 10.3 mg/L respectively. However, treatment G gave the lowest concentration of lignin with 5.4 mg/L compared to other treatments. The concentration of lignin was derived from the standard curve of lignin shown in Figure 5.2.

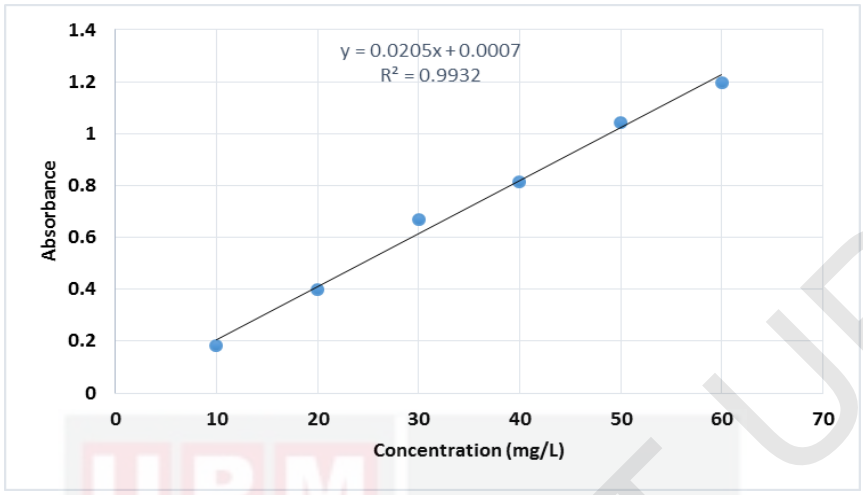


Figure 5.2: Standard curve of lignin. The absorbance was measured at 280 nm

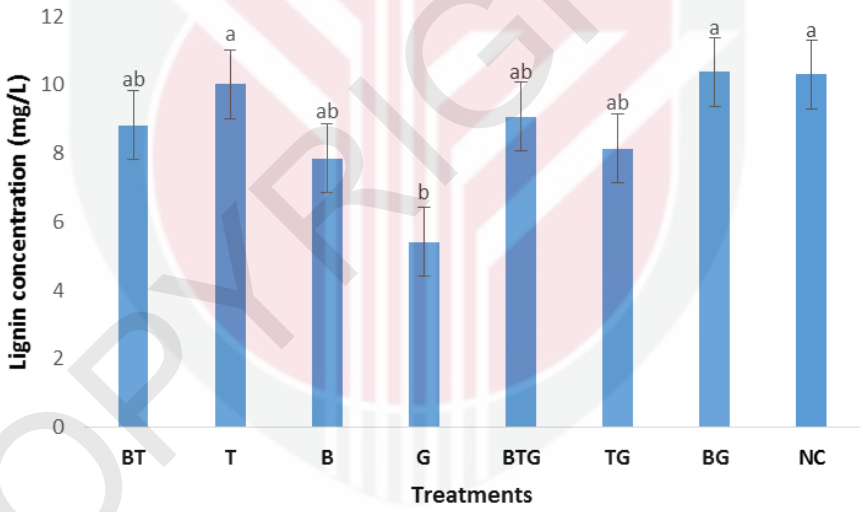


Figure 5.3: Effect of biological control agent/s on oil palm lignin concentration.Values are means of three replications and difference between the means are separated by Tukey's Studentized Range (HSD) Test at 5% level of significance

5.3.1.3 Total phenolic content

The TPC extracted from oil palm roots was high in all treatments except for the consortium treatment not infected with *G. boninense* (BT) and untreated *Ganoderma*-infected treatment (G) (Figure 5.5) *Ganoderma*-infected seedling

treated with *B. cereus* (BG) gave the highest TPC (34.88 mg/L), followed by unchallenged seedling treated with *B. cereus* (B) (34.15 mg/L), unchallenged seedling treated with consortium treatment (BT) gave the lowest TPC reading. The concentration of TPC was derived from the gallic acid standard curve shown in Figure 5.4.

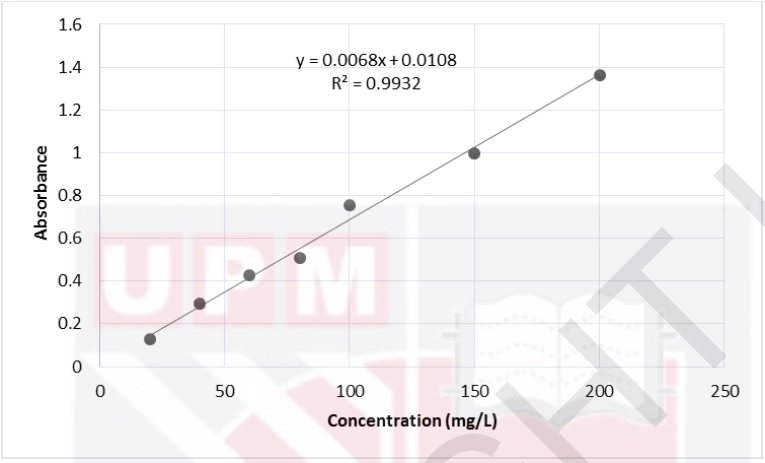


Figure 5.4: Standard curve of Gallic acid. The absorbance was measured at 725 nm

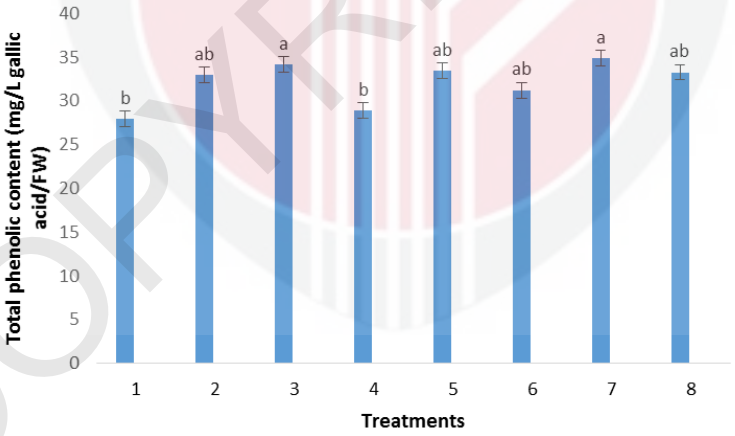


Figure 5.5: Effect of treatments on total phenolic content on oil palm seedling's root. Values are means of three replications and difference between the means are separated by Tukey's Studentized Range (HSD) Test at 5% level of significance

5.3.2 The result of metabolite extraction for Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Gas Chromatography-Mass Spectrometry 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 (Figure 5.6). 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 (Gottfried, 2009). Based on VIP scores, treatment BT and treatment T produced three similar metabolites in relatively high concentration which were 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). Treatment BG and NC also had noticeable similarities. 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 treatments and their potential bioactivity was recorded in Table 5.1.

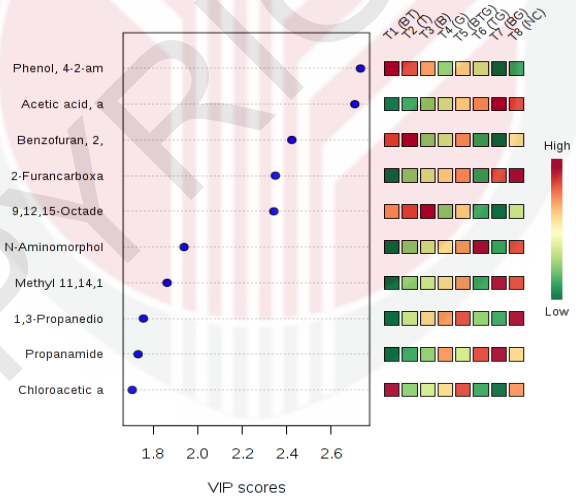


Figure 5.6: The Importance Features analysis of the metabolites detected in the treatments. The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in each group under study.

Table 5.1: Major metabolites detected in the treatments and their potential bioactivity.

Treatments	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	Antimifungal (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	Antimicrobial (Ölgen et al., 2014)
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	

5.4 Discussion

Plant enzymes such as PO and polyphenol oxidase (PPO), helps the formation of lignin and oxidative phenols that involve in plant defense mechanisms against pathogen (Avdiushko et al., 1993). The current study found that the seedlings treated with single treatment of *B. cereus* recorded the highest PO enzyme activity. In fact, all treatments involving *B. cereus*, regardless of single application or consortium gave better PO enzyme activity than the treatments without *B. cereus*. In harmony to the present study, Ramarathnam et al. (2011) found that, the enzyme activity for strain *B. cereus* was higher than the other treatments when inoculated with the pathogen. Other than that, a study by Halfeld-Vieira et al. (2006) reported that the leaf tissues that was exposed to *B. cereus* and inoculated with *Pseudomonas syringae* as pathogen in tomato, exhibited high PO enzyme activities compared to other treatments designed. These studies proved that *B. cereus* could induce plants with more PO enzyme activities.

Nevertheless, variables such as the availability of sunlight and pathogen infection have been shown to influence lignin content in plants (Zhang et al., 2007; Xu et al., 2011). High levels of root lignin could be a means of limiting fungal infection (Bennet et al., 2015). In this study, lignin assay demonstrated that *Ganoderma*-infected seedlings treated with *B. cereus* (BG), unchallenged seedling treated with *T. asperellum* (T) and negative control (NC) produced the highest reading of lignin with no significant difference from each other. This data supported the previous findings obtained on the vegetative growth in the current study where consortium treatment and unchallenged seedling treated with *B. cereus* contributed to higher root weight compared to the other treatments. *Ganoderma*-infected seedling gave the lowest lignin accumulation. This finding is in line with Adaskaveg et al. (1991) that concluded white rot fungi such as *Ganoderma* spp., degrade wood components specially lignin, thus made the plant more susceptible towards pathogen.

According to research by Hu and Kitts (2001), epidemiological data suggested that phenolic acids have strong inhibitory activity on oxidation induced by peroxyl radicals. While Nikraftar et al. (2013) concluded that phenolic compounds in plants and their synthesis in response to infection is associated with plant resistance. The current study showed that *Ganoderma*-infected seedlings treated with *B. cereus* gave the highest TPC reading, followed by unchallenged seedlings treated with *B. cereus*. These results match those observed in earlier studies by Shoko et al. (1999) and Baydar et al. (2004) whom confirmed that phenolics were the most important compounds active against bacteria. This may explain the induction of TPC in seedlings treated with *B. cereus*. Presence of the endophytic *B. cereus* may have triggered the palms to produce TPC higher than the basal level as a defense tool to the proteins synthesized by this bacterium. One unanticipated finding was that, unchallenged seedlings treated with consortium contributed to the lowest reading of TPC. A possible explanation on this observation could be; no virulent factors detected by the palm and endophytic *Trichoderma* spp. have

always played a symbiotic role in plant-pathogen interaction. In addition, *T. asperellum* may have colonized the palm roots over *B. cereus* where this was also proven in the SEM observation conducted earlier in this current study.

Based on the GC-MS analysis of extracted oil palm roots, phenol, 4-2-aminoethyl- (syn: tyramine) was detected in treatment BT. Like any other biogenic amine, tyramine is mainly produced due to the microbial enzymes or tissue bioactivity that caused a certain amino acid to undergo enzymatic decarboxylation process (Halasz et al., 1994). Tyramine is the key enzyme that helps hydroxycinnamic acid amides (HCAA) production (Campos et al., 2014). According to several studies, HCAA contributed in strengthening the cell wall of a plant thus creates a barrier against microbial degradation (Grandmaison et al., 1993; Hagel and Facchini, 2005). Moreover, HCAA can act directly as antimicrobial agents. Newman et al. (2001) found that coumaroyltyramine (CT) and feruloyltyramine (FT) that accumulated in pepper plants infected with the bacterial pathogen *Xanthomonas campestris*, demonstrated antibacterial activity *in vitro*. Besides that, FT extracted from allium roots has been recorded to show antifungal activity (Fattorusso et al., 1999).

The study also detected acetic acid production in treatment BG. Acetic acid (syn: aminooxyacetic acid, AOA) is known for its flavoring and preservative properties against microorganisms in a variety of food products. It also a natural compound found throughout the biosphere (Davidson et al., 2002; Alawlaqi and Alharbi, 2014). *In vitro* study by Giorgio et al. (2015) proved that acetic acid were able to reduce mycelium growth arising from the fungal plug of *Sclerotinia sclerotiorum*. However, Ueno et al. (2011) demonstrated that AOA were able to suppress the resistance induced by IAA and tryptophan. This is in line with previous study by Watanabe et al. (1979) where they found that AOA suppresses disease resistance of plants.

The 2, 3-dihydrobenzofuran (DHB) skeleton is widespread in many natural products and biologically active molecules (Donnelly and Meegan, 1984). Derivatives of benzofurans are sometimes discovered as metabolites of fungi, including endophytes of trees (Findlay et al., 1997; Xia et al., 2011; Richardson et al., 2015). Several DHB products were reported to have antioxidant, cytoprotective properties and insecticidal activity (Chin et al., 2008; Huang et al., 2009). Several benzofuran products were found to have antifungal properties and successfully reduced the growth of *Microbotryum violaceum* when tested *in vitro* in a study carried out by Richardson et al. (2015). Jang et al. (2006) also found that a dihydrobenzofuran derivative, namely Awajanoran, exhibited antimicrobial activities against five strains of bacteria.

5-hydroxymethyl-2-furaldehyde (5HM2F) was studied by Subramenium et al. (2018) and it was found to have the antibiofilm and antivirulence activities against *Candida albicans,* opportunistic pathogenic yeast (Gow and Yadav, 2017). Based on the results of antifungal susceptibility testing, the combination of antifungal and 5HM2F was more effective than the single treatment of

antifungal in reducing *C. albicans* biofilm (Subramenium et al., 2018). Nevertheless, Chen et al. (2014) also discovered that 5HM2F and its derivatives extracted from plant, displayed antioxidant activity when tested in the lab. 5-hydroxymethyl-2-furaldehyde has been revealed to be naturally available in a number of plants (Sharma et al., 2004, Lin et al., 2008), honey (Coco et al., 1996) and heat treated food products (Almeida et al., 2009).

According to Burr et al. (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's essential fatty acids. A-linolenic acid has been found to have antibacterial activities against several oral pathogens such as *C. albicans*, *Streptococcus mutans* and *Porphyromonas gingivalis* when studied *in vitro* (Huang and Ebersole, 2010). In a study by Walters et al. (2004), ALA demonstrated antifungal activities against mycelial growth of plant pathogenic fungi namely, *Crinipellis pernicosa, Pyrenophora avanae, Pythium ultimum* and *Rhizoctonia solani.* This finding suggested that ALA could play an important role in the search for alternative methods to controlling crucial plant pathogens (Walters et al., 2004). This fatty acid could be found naturally in plants. Shafaghat (2011) reported that ALA was a main component in flower, leaf, stem and seed of *Hypericum scabrum* (St. John's wort).

CHAPTER 6

SUMMARY, CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

Malaysia and Indonesia have evolved into major producers of palm oil products. However, the disastrous BSR disease in oil palm plantations in both countries causes the decaying of the roots, followed by the bole and stem. In most critical scenario, ruptured stem, production of fruiting bodies or even collapse of the palms could be seen during advance stage of the disease. This disease contributed significantly to economic loss in the plantation industry. To date, the common BSR management practice employed by established plantations is by applying synthetic fungicides. Even though it can cause harmful effects on humans and the biodiversity, standard solutions have always been derived based on the application of chemicals. Therefore, it is crucial to establish alternative methods to control diseases in the oil palm plantations. One of the safest disease controls is by using biological control agents (BCAs). Thus, the study was conducted to analyze the potential of *B. cereus* and *T. asperellum* in terms of inducing resistance in *G. boninense* infected oil palm seedlings and as potential plant growth promoters.

Indole acetic acid production, phosphate solubilization, siderophore production and sealed plate method were conducted to assess the plant growth promotion activities of the BCAs studied. The results showed that both isolates manage to change the yellow color of the broth to pink in color when tested with Salkowski's reagent and this result suggested that both *B. cereus* and *T. asperellum* have the ability to produce IAA. The present study that was conducted using NBRIP media agar also demonstrated clear zones and indicated both isolates were able to solubilize phosphate. Other than that, the ability of both isolates to produce siderophore was clearly exhibited. This can be observed in orange halos of CAS agar that revealed both isolates tested gave positive siderophore production. To further study the *in vitro* characteristics of the BCAs, sealed plate method was conducted to determine the antifungal effects of the MVOCs against *G. boninense*. The results of the present study showed that *B. cereus* has the ability to produce MVOCs that could effectively inhibit *G. boninense* mycelial growth. However, the result for *T. asperellum* failed to be recorded due to the overgrowth of its mycelium on both surface of the PDA agar.

Both *B. cereus* and *T. asperellum* potential were assessed in the nursery trial after *in vitro* test demonstrated promising results. The effect of single and consortium treatments application on seedling's vegetative growth and disease suppression were recorded. Generally, enhanced vegetative growth was demonstrated in all BCA treated seedlings compared to the untreated seedlings. However, consortium of *T. asperellum* and *B. cereus* found to be significantly effective based on the data of bole weight, top weight and root weight parameters. This study also showed that the application of *T. asperellum* was superior compared to other treatments when it comes to the

plant height contribution. However, in terms of increasing the bole weight and root weight of the seedlings, *B. cereus* single application performed better than *T. asperellum* single application. Based on the results obtained from the vegetative growth parameters data, we could conclude that, *T. asperellum* contributed significantly on the growth of aerial parts while *B. cereus* contributed on the growth of oil palm roots. These results provided further support that these two isolates could be considered as potential BCAs. Similarly, both BCAs tested in the current study also exhibited its ability in suppressing BSR disease of oil palm according to the data recorded based on the DS and DR analysis. Nevertheless, single treatment of *T. asperellum,* with 50% DS and 57.2% DR, was more efficient in disease suppression compared to both consortium treatment and single treatment of *B. cereus*.

Oil palm PO enzyme activity, lignin content, TPC and metabolites in BSR diseased seedlings in response to *B. cereus* and *T. asperellum* treatments were also assessed. All treatments involving *B. cereus*, regardless of single application or consortium recorded better PO activity than the treatments that does not involve *B. cereus*. This result suggested that *B. cereus* could induce plant to produce more PO. Similarly, the current study also showed that infected seedlings treated with *B. cereus* gave the highest TPC reading, followed by unchallenged seedlings treated with *B. cereus*. Lignin assay showed that *Ganoderma*-infected seedling treated with *B. cereus*, unchallenged seedling treated with *T. asperellum* and negative control all produced the highest reading of lignin with no significant difference from each other. The result from GC-MS analysis showed that phenol, 4-2-aminoethyl- was the most abundant metabolite detected in the consortium treatment, followed by acetic acid in the infected seedlings treated with *B. cereus*.

The main goal of the current study was to determine the ability of *T. asperellum* and *B. cereus* as potential BCAs that could enhance plant growth and induce resistance in *G. boninense* infected oil palm seedlings. Based on the data obtained from the bioactivity test, vegetative growth analysis, disease suppression and biochemical test in response of both BCAs, this study has demonstrated that both BCAs have huge potential as BCAs against *G. boninense*.

Assessing disease symptoms visually could be confusing and misleading, especially when conducted by the amateurs. One of the limitations of the visual observation is that various symptoms appear almost indistinguishable. The initial symptoms of BSR such as unopened spears are interchangeable to drought symptom. Other than that, visual observation is also limited by time. Limited time available could affect the result of disease assessment, for instance in some cases, the external symptoms is not visible enough during the early stages of infection. Due to these factors, other detection method namely plating out infected tissues, root sample viewing via SEM and detection based using PCR method were conducted in this study to strengthen the findings obtained from the visual observation method.

In the future research, the data on enzyme assays should be recorded on monthly basis to observe the pattern of enzyme production related to time after inoculation. This may help in understanding more on the production of enzymes in response to the designed treatments. The nursery trial should be setup in a more systematic arrangement to minimize external effects that may influence the growth of the seedlings.

It is also recommended to study the expression of genes that are involved in induced defense mechanism as a result of BCA/s application. Nevertheless, trial on formulations using these potential BCAs would be an extended advantage to add more sustainable and environmentally safe fungicides in the market to control BSR disease.

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APPENDICES

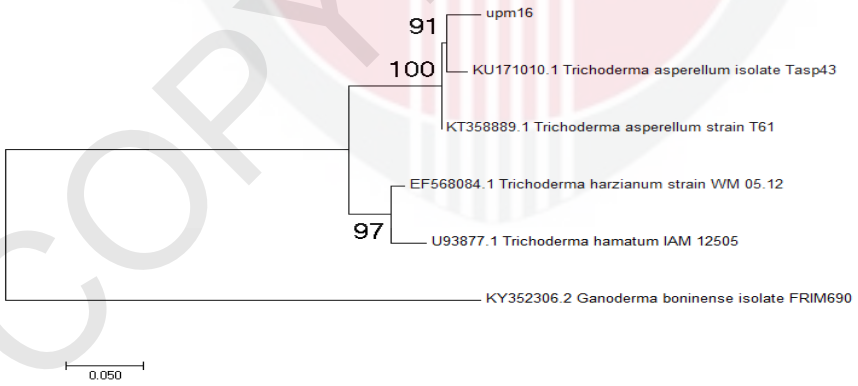
Appendix A1

Sequence of *Trichoderma asperellum* (UPM16)

GGAAATGCAATCAACGTGATCAGAGGTCACTTTCGAGGTGGGGTGTTTTACGGACGTGAACC
CGCCGCGCTCCGGTGCGAGTTGTGCAAACTACTGCGCAGGAAAGGCTGCGGCGAGACCGCC
ACTGTATTTCGGGGCCGGAACCCGTGTGAGGGGTCCCGATCCCCAACGCCGATCCCCCGGA
GGGGTTCAAGGGTTGAAATGACGTTCGAACAGGAATGCCCGCCAAAATACGGGCGGGCGCA
GTGTGCGTTCAAGGATTCAATGATTCACTAAATTCTGCAATTCACATTACTTATCGAATTTCGCT
GCGTTCTTCATCTATGCCGGAACCAAAAGATCCGTTGTTAAAAGTTTTGATTCATTTTAAATTTT
TGCTCAAAGCTGTAAAAAATACCTCCGCGAGGGGACTACAAAAAGAGTTTGGTTGGTTCCTCC
GGCGGGCGCCTGGTTCCGGGGCTGCAACGCACCCGGGGCGTGACCCCGCCAAGGCAACAG
TTTGGTAACTTTCACATTGGTTTTGGGAGTGGTAAACTCGGTAATGATCCCTCCGCAGGTTCAC
CCTACGGAAGAA
GGGGGGNCTCCGGGCTTCACTCCGAACCATGTGACGTTACCAAACTGTTGCCTCGGCGGGG
TCACGCCCCGGGTGCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAACCAAACT
CTTTCTGTAGTCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAAAATTCAAAATGAATCAA
AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGT
AATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATT
CTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGCGTT
GGGGATCGGGACCCCTCACACGGGTGCCGGCCCCGAAATACAGTGGCGGTCTCGCCGCAGC
CTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGCGTCCACGTCCGTAAAA
CACCCAACTTTCTGAAATGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATAT
CAATAAGCGGAGGAAGGAGGAA

Appendix A2

Phylogenetic tree of *Trichoderma asperellum* (UPM16)



Appendix A3

Sequence of *Bacillus cereus* (UPM15)

GGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCCGCGGCATGCTGATCCGCGATTACTAG
CGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTACAATCCGAACTGAGAACGGTTTTATGAGA
TTAGCTCCACCTCGCGGTCTTGCAGCTCTTTGTACCGTCCATTGTAGCACGTGTGTAGCCCAG
GTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCA
CCTTAGAGTGCCCAACTTAATGATGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTA
ACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCTCCCGAA
GGAGAAGCCCTATCTCTAGGGTTTTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGC
TTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGC
CTTGCGGCCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAACTTCAGCACTAAAGGGCGGAA
ACCCTCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTG
CTCCCCACGCTTTCGCGCCTCAGTGTCAGTTACAGACCAGAAAGTCGCCTTCGCCACTGGTG
TTCCTCC

Appendix A4: Phylogenetic tree of *Bacillus cereus* (UPM15)

Appendix B

Reaction setup using Qiagen TopTaq Master Mix

Component	Volume/reaction	Final concentration
TopTaqMaster Mix	12.5 µl	1 unit Qiagen TopTaq DNA Polymerase
Diluted primer mix		
Primer A (GAN 1)	2 µl	2 µM
Primer B (GAN 2)	2 µl	2 µM
RNase-free water	Variable	
Template DNA	Variable	150 ng/reaction
Total reaction volume	25 µl	

Appendix C

Measurement of oil palm seedling vegetative growth

Measurement of plant height and chlorophyll content (SPAD meter) in treatment BT from MAI zero to MAI six

Table 1.1: Measurement of plant height and chlorophyll content (SPAD meter) at MAI zero.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	12	52
2	16	44.6
3	14	53.1
4	18	42.1
5	20	46
6	14	48.7
7	16	55.1
8	11	55.9
9	18	60.2
10	17	53.7
11	18	43
12	23	46.3

Table 1.2: Measurement of plant height and chlorophyll content (SPAD meter) at MAI one.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	47	51.3
2	59	64
3	42	56.4
4	62	50.2
5	53	47
6	59	48.7
7	50	47.2
8	48	53.5
9	60	48.2
10	58	48.9
11	60	59.1
12	66	54.4

Table 1.3: Measurement of plant height and chlorophyll content (SPAD meter) at MAI two.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	49	50.9
2	62	56.4
3	48	43.2

4	64	39.1
5	59	47.2
6	61	53.6
7	58	48.4
8	53	49.3
9	60	41.1
10	62	47
11	67	48.9
12	68	44.8

Table 1.4: Measurement of plant height and chlorophyll content (SPAD meter) at MAI three.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	58	35.9
2	66	40.1
3	52	45.3
4	64	38
5	60	37.5
6	64	41.3
7	58	48.3
8	55	47
9	60	35.6
10	63	39.1
11	65	42.9
12	66	33.6

Table 1.5: Measurement of plant height and chlorophyll content (SPAD meter) at MAI four.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	68	46.4
2	73	29.6
3	56	42.6
4	66	31
5	62	33.6
6	64	40.9
7	59	45.3
8	58	41.4
9	61	29.1
10	65	38.5
11	68	36.6
12	66	35.5

Table 1.6: Measurement of plant height and chlorophyll content (SPAD meter) at MAI five.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	70	39.4

2	75	37.3
3	59	36.9
4	74	39.1
5	67	34
6	64	39.9
7	58	37.2
8	60	37.4
9	65	26.9
10	73	27.2
11	68	33.8
12	68	35

Table 1.7: Measurement of plant height and chlorophyll content (SPAD meter) at MAI six.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	90	39.1
2	80	51.2
3	72	33.4
4	97	44.2
5	67	53.2
6	71	43.6
7	79	28.6
8	78	37
9	72	47.3
10	91	36.3
11	71	34.7
12	68	29.2

Measurement of plant height and chlorophyll content (SPAD meter) in treatment T from MAI zero to MAI six

Table 2.1: Measurement of plant height and chlorophyll content (SPAD meter) at MAI zero.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	14	52.5
2	23	52.2
3	19	38.4
4	10	59.1
5	18	55.8
6	14	55.1
7	16	56.4
8	12	46
9	22	53.1
10	22	47.4
11	23	47
12	15	45

Table 2.2: Measurement of plant height and chlorophyll content (SPAD meter) at MAI one.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	42	56
2	52	47.3
3	54	55.8
4	43	59.2
5	52	59.2
6	46	47.3
7	56	58
8	48	39.7
9	64	52.4
10	60	51.4
11	57	50.7
12	47	42.4

Table 2.3: Measurement of plant height and chlorophyll content (SPAD meter) at MAI two.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	52	44.7
2	64	39.6
3	53	42.1
4	47	25.9
5	61	43.9
6	52	42
7	58	36.1
8	49	37.1
9	64	45.9
10	65	35
11	62	35.1
12	54	36.5

Table 2.4: Measurement of plant height and chlorophyll content (SPAD meter) at MAI three.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	54	44.9
2	72	44.5
3	64	38.2
4	49	25
5	64	40.2
6	52	41.6
7	62	33.9
8	53	37.1
9	66	46.6
10	64	33.9
11	67	37.4

12	58	34.6

Table 2.5: Measurement of plant height and chlorophyll content (SPAD meter) at MAI four.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	60	35.6
2	79	38.9
3	70	37.2
4	52	38.5
5	67	48.8
6	55	44.4
7	62	36
8	58	33
9	65	37.8
10	61	32.7
11	69	35.9
12	58	30.5

Table 2.6: Measurement of plant height and chlorophyll content (SPAD meter) at MAI five.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	61	36.3
2	81	41.7
3	78	35.5
4	55	33
5	68	37.8
6	57	46.8
7	65	32.7
8	65	21
9	71	48.8
10	65	27.4
11	73	38.7
12	61	27.9

Table 2.7: Measurement of plant height and chlorophyll content (SPAD meter) at MAI six.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	84	49.2
2	100	52.5
3	87	44.5
4	64	51.1
5	83	45
6	69	34.5
7	76	50.3
8	79	31.2

9	106	47.2
10	71	25.7
11	97	33.3
12	78	38.1

Measurement of plant height and chlorophyll content (SPAD meter) in treatment B from MAI zero to MAI six

Table 3.1: Measurement of plant height and chlorophyll content (SPAD meter) at MAI zero.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	18	60
2	15	51.5
3	18	59.1
4	20	53
5	18	43.1
6	24	53.4
7	14	61.3
8	15	48.7
9	20	62.9
10	15	50.4
11	14	49.2
12	13	47.3

Table 3.2: Measurement of plant height and chlorophyll content (SPAD meter) at MAI one.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	56	52.5
2	55	60.8
3	58	47.7
4	56	54.7
5	48	58.7
6	64	47.1
7	59	54
8	40	56.1
9	54	51.1
10	57	39.3
11	49	53.6
12	47	51.8

Table 3.3: Measurement of plant height and chlorophyll content (SPAD meter) at MAI two.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	65	53

2	58	53.8
3	63	44.4
4	59	39.2
5	64	49
6	68	45
7	61	44.8
8	49	35.1
9	58	54.5
10	63	43.7
11	50	42.8
12	51	39.8

Table 3.4: Measurement of plant height and chlorophyll content (SPAD meter) at MAI three.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	77	47.7
2	64	51.3
3	66	34.1
4	66	38.7
5	67	47
6	79	50.5
7	61	40.9
8	60	46.9
9	62	47.8
10	68	34
11	55	37.5
12	50	40.8

Table 3.5: Measurement of plant height and chlorophyll content (SPAD meter) at MAI four.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	80	52.8
2	73	47.5
3	74	39.5
4	70	35.6
5	68	44.4
6	86	38.8
7	62	35.6
8	67	44.4
9	77	45.4
10	74	40
11	61	30.9
12	55	39

Table 3.6: Measurement of plant height and chlorophyll content (SPAD meter) at MAI five.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	62	59.2
2	81	40
3	78	35.4
4	73	46.7
5	80	38.5
6	92	35.5
7	71	36.1
8	71	49.9
9	80	34.8
10	86	30.2
11	67	34.2
12	58	25.3

Table 3.7: Measurement of plant height and chlorophyll content (SPAD meter) at MAI six.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	83	48.3
2	92	45.8
3	87	37.3
4	86	40.7
5	86	36
6	100	40.8
7	84	49.9
8	84	37.2
9	93	37.6
10	95	50.7
11	69	32.8
12	64	35.7

Measurement of plant height and chlorophyll content (SPAD meter) in treatment G from MAI zero to MAI six

Table 4.1: Measurement of plant height and chlorophyll content (SPAD meter) at MAI zero.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	22	52.9
2	16	54.7
3	21	64.7
4	24	54.4
5	20	51.9
6	21	44.7
7	19	56.7

8	14	52
9	18	59
10	18	57.8
11	18	45.3
12	18	56.9

Table 4.2: Measurement of plant height and chlorophyll content (SPAD meter) at MAI one.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	65	52.7
2	55	56.7
3	66	48.3
4	60	54.3
5	59	59.2
6	62	50
7	47	49
8	51	55.7
9	51	52.4
10	57	60.6
11	59	66.9
12	56	45.6

Table 4.3: Measurement of plant height and chlorophyll content (SPAD meter) at MAI two.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	66	42.3
2	63	44.9
3	68	46.5
4	63	39.7
5	61	43.7
6	65	43.4
7	54	40.5
8	55	41.6
9	54	42
10	61	45.9
11	66	42.5
12	59	43.2

Table 4.4: Measurement of plant height and chlorophyll content (SPAD meter) at MAI three.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	68	49.5
2	69	51.5
3	70	45.4
4	68	42.8
5	62	39.9

6	67	46.9
7	53	28.7
8	57	40.9
9	57	40.6
10	66	52.6
11	67	34.7
12	60	42.7

Table 4.5: Measurement of plant height and chlorophyll content (SPAD meter) at MAI four.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	79	41.9
2	80	40.2
3	73	42
4	72	41.9
5	66	35.7
6	67	45.2
7	53	40.5
8	59	32.3
9	64	33.7
10	72	40
11	68	39.2
12	63	36.5

Table 4.6: Measurement of plant height and chlorophyll content (SPAD meter) at MAI five.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	83	46.5
2	87	34.1
3	77	42.6
4	78	43
5	71	35.6
6	67	42.4
7	59	44.3
8	67	23.4
9	80	29.4
10	78	32.2
11	70	36
12	67	40.4

Table 4.7: Measurement of plant height and chlorophyll content (SPAD meter) at MAI six.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	65	35.6
2	67	45.6

3	90	45.9
4	92	50.1
5	82	43.5
6	70	40
7	79	51.5
8	84	39.8
9	88	43.4
10	85	39.3
11	76	44.7
12	72	43.6

Measurement of plant height and chlorophyll content (SPAD meter) in treatment BTG from MAI zero to MAI six

Table 5.1: Measurement of plant height and chlorophyll content (SPAD meter) at MAI zero.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	20	43.5
2	21	55
3	19	53.1
4	17	53.4
5	18	45
6	15	40.6
7	13	46.5
8	16	46.4
9	17	44.8
10	20	48.3
11	21	55.7
12	15	43.8

Table 4.2: Measurement of plant height and chlorophyll content (SPAD meter) at MAI one.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	54	64.5
2	55	54.3
3	53	57.7
4	55	42.6
5	56	43.7
6	53	51
7	35	50.7
8	46	44.2
9	45	38.3
10	55	51.3
11	58	52.3
12	52	39.5

Table 4.3: Measurement of plant height and chlorophyll content (SPAD meter) at MAI two.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	57	38
2	64	43.5
3	59	47.3
4	59	35.1
5	62	38.5
6	56	52.2
7	37	45.9
8	51	43.6
9	50	41.6
10	55	38.5
11	63	42.1
12	52	37.8

Table 4.4: Measurement of plant height and chlorophyll content (SPAD meter) at MAI three.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	60	46.2
2	67	44.8
3	62	38.7
4	64	48.6
5	62	40.6
6	57	36.4
7	41	39
8	50	44.1
9	51	32
10	59	42.2
11	67	42.1
12	56	35.4

Table 4.5: Measurement of plant height and chlorophyll content (SPAD meter) at MAI four.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	71	52.9
2	66	42.6
3	65	41.1
4	72	41.4
5	68	39.5
6	67	31.2
7	49	43.3
8	57	35.1
9	57	38.5
10	68	41.3
11	67	24.3

12	62	46

Table 4.6: Measurement of plant height and chlorophyll content (SPAD meter) at MAI five.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	76	49.2
2	72	46.3
3	75	40.8
4	83	47.6
5	78	40.3
6	78	48.2
7	52	38.5
8	62	40.7
9	61	43.5
10	78	30
11	65	36
12	62	37.5

Table 4.7: Measurement of plant height and chlorophyll content (SPAD meter) at MAI six.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	77	43.9
2	80	42.1
3	87	48.8
4	86	51.9
5	92	48.2
6	101	42.5
7	65	41
8	76	42.6
9	69	44.5
10	85	39
11	73	33.7
12	68	30.2

Measurement of plant height and chlorophyll content (SPAD meter) in treatment TG from MAI zero to MAI six

Table 6.1: Measurement of plant height and chlorophyll content (SPAD meter) at MAI zero.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	15	49
2	15	57.4
3	18	43.7
4	20	44.6
5	24	47.7

6	17	38.8
7	16	47.9
8	18	43
9	19	45.2
10	20	46.7
11	17	46.6
12	12	47.8

Table 6.2: Measurement of plant height and chlorophyll content (SPAD meter) at MAI one.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	47	35.8
2	43	53.9
3	46	47.5
4	59	49.7
5	62	65.3
6	50	47.3
7	43	51.3
8	50	55.1
9	52	44.9
10	54	42.7
11	48	52.3
12	47	51.2

Table 6.3: Measurement of plant height and chlorophyll content (SPAD meter) at MAI two.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	51	28.6
2	48	56.8
3	59	38.1
4	60	43.3
5	64	51.6
6	54	40.8
7	49	45.2
8	57	32.8
9	55	42.2
10	56	47.3
11	55	35.9
12	49	34.3

Table 6.4: Measurement of plant height and chlorophyll content (SPAD meter) at MAI three.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	55	38.9
2	50	48.9

3	63	37
4	65	34.1
5	65	42.3
6	63	36.7
7	54	47.5
8	58	39.9
9	62	40.5
10	60	39.4
11	61	32.6
12	50	28.9

Table 6.5: Measurement of plant height and chlorophyll content (SPAD meter) at MAI four.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	61	34.9
2	59	36.8
3	65	30.8
4	67	33.7
5	66	41.9
6	67	31.4
7	58	36.8
8	66	42.5
9	63	33
10	63	41.5
11	62	31.7
12	49	33

Table 6.6: Measurement of plant height and chlorophyll content (SPAD meter) at MAI five.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	63	41.5
2	68	30.4
3	71	39.4
4	71	34.1
5	71	45.2
6	68	30.8
7	67	41
8	77	50.5
9	68	42
10	69	37.2
11	67	31.6
12	54	40.1

Table 6.7: Measurement of plant height and chlorophyll content (SPAD meter) at MAI six.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	74	43.6
2	68	38.3
3	81	42
4	82	57.8
5	77	50.1
6	88	38
7	88	40.3
8	112	35.1
9	84	46.3
10	76	35.6
11	76	34.6
12	70	29.8

Measurement of plant height and chlorophyll content (SPAD meter) in treatment BG from MAI zero to MAI six

Table 7.1: Measurement of plant height and chlorophyll content (SPAD meter) at MAI zero.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	13	52.8
2	18	55.4
3	20	44.5
4	11	41.9
5	19	57.9
6	20	55.5
7	19	48.2
8	17	53.4
9	18	49.4
10	20	59.3
11	16	50.8
12	17	57.6

Table 7.2: Measurement of plant height and chlorophyll content (SPAD meter) at MAI one.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	45	52.1
2	51	60.3
3	62	53.8
4	33	62
5	54	51.4
6	59	60.2
7	40	54.9

8	50	42
9	51	54.7
10	65	57.2
11	54	51.4
12	57	53.5

Table 7.3: Measurement of plant height and chlorophyll content (SPAD meter) at MAI two.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	53	51.7
2	55	40.9
3	60	48.3
4	38	32.5
5	54	42.1
6	59	45.4
7	48	37.4
8	51	45.7
9	53	46.2
10	68	49.6
11	63	43.6
12	58	47.4

Table 7.4: Measurement of plant height and chlorophyll content (SPAD meter) at MAI three.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	60	45.3
2	62	35.7
3	64	39.1
4	39	36.5
5	55	37.5
6	61	34.9
7	48	34.9
8	54	42.7
9	61	42.6
10	69	41.9
11	68	35.1
12	59	45.3

Table 7.5: Measurement of plant height and chlorophyll content (SPAD meter) at MAI four.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	67	41.8
2	64	37
3	66	31.2
4	43	39.2

5	61	32.6
6	60	32.2
7	51	39.1
8	62	42.9
9	74	37.9
10	79	45
11	70	32.7
12	64	40.8

Table 7.6: Measurement of plant height and chlorophyll content (SPAD meter) at MAI five.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	72	33.2
2	72	28.8
3	71	28.3
4	46	41.1
5	67	31.8
6	66	38.8
7	66	32.1
8	66	33.9
9	78	43.4
10	83	31.3
11	72	38.1
12	70	36.8

Table 7.7: Measurement of plant height and chlorophyll content (SPAD meter) at MAI six.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	77	42.1
2	80	35.6
3	81	36.6
4	59	46.2
5	80	42.2
6	78	59.6
7	70	52.5
8	75	53.9
9	81	44.4
10	87	41
11	80	39.3
12	78	42.3

Measurement of plant height and chlorophyll content (SPAD meter) in treatment NC from MAI zero to MAI six

Table 8.1: Measurement of plant height and chlorophyll content (SPAD meter) at MAI zero.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	15	45.6
2	22	43.5
3	16	52.3
4	14	48.2
5	16	56
6	22	50
7	19	46.6
8	16	53.5
9	13	52.4
10	17	47.2
11	17	33.7
12	15	39.1

Table 8.2: Measurement of plant height and chlorophyll content (SPAD meter) at MAI one.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	46	31.6
2	58	42.3
3	45	46.3
4	54	53.1
5	53	55.8
6	62	54
7	57	43
8	45	51.6
9	47	52.2
10	55	68.1
11	54	53
12	43	57.5

Table 8.3: Measurement of plant height and chlorophyll content (SPAD meter) at MAI two.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	50	39.8
2	65	39.6
3	53	47.5
4	60	41.2
5	59	47.3
6	66	48.5
7	66	45
8	51	36.8
9	57	46.3
10	60	47.3
11	58	53.4
12	51	48.5

Table 8.4: Measurement of plant height and chlorophyll content (SPAD meter) at MAI three.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	53	41.4
2	66	35.9
3	60	45.9
4	64	44.8
5	57	37.5
6	67	39.8
7	68	42.7
8	53	34.7
9	67	47.4
10	65	48
11	59	36.8
12	52	54

Table 8.5: Measurement of plant height and chlorophyll content (SPAD meter) at MAI four.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	54	26.8
2	65	29.3
3	63	40.5
4	64	38.6
5	63	33.3
6	68	27.6
7	69	38.4
8	53	31.7
9	71	34.4
10	72	43.5
11	64	37.6
12	55	53.3

Table 8.6: Measurement of plant height and chlorophyll content (SPAD meter) at MAI five.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	60	41.7
2	66	41.3
3	65	35
4	70	43.4
5	71	36.9
6	74	34.9
7	72	34.6
8	64	35.1
9	75	36.9
10	77	49.4
11	70	38.1

12	62	53.4

Table 8.7: Measurement of plant height and chlorophyll content (SPAD meter) at MAI six.

Seedling replicates	Plant height (cm)	Chlorophyll content (SPAD meter)
1	64	44.7
2	76	39.7
3	78	51.1
4	87	40.6
5	78	41.6
6	80	34.3
7	83	38
8	73	35.9
9	81	41.9
10	85	38
11	72	36.9
12	68	48.4

Appendix D1

Summary of ANOVA table for the effect of *Trichoderma asperellum* and *Bacillus cereus* on plant height of oil palm seedlings at 6 MAI

Means with the same letter are not significantly different.					
t Grouping			**Mean**	**N**	**trt**
	A		86.917	12	T2
	A				
B	A		85.250	12	T3
B	A				
B	A	C	81.333	12	T6
B	A	C			
B	A	C	81.000	12	T1
B	A	C			
B	A	C	79.917	12	T5
B		C			
B		C	78.417	12	T7
		C			
		C	77.083	12	T8
		C			
		C	74.333	12	T4

Appendix D2

Summary of ANOVA table for the effect of *Trichoderma asperellum* and *Bacillus cereus* on the fresh bole weight of oil palm seedlings

Means with the same letter are not significantly different.						
Tukey Grouping				**Mean**	**N**	**trt**
		A		61.625	12	t1
		A				
B		A		55.750	12	t3
B		A				
B		A	C	52.750	12	t2
B		A	C			
B		A	C	52.250	12	t7
B		A	C			
B	D	A	C	51.500	12	t6
B	D		C			
B	D		C	49.250	12	t5
	D		C			
	D		C	43.875	12	t8
	D					
	D			40.875	12	t4

Appendix D3

Summary of ANOVA table for the effect of *Trichoderma asperellum* and *Bacillus cereus* on the dry bole weight of oil palm seedlings

Means with the same letter are not significantly different.				
Tukey Grouping		**Mean**	**N**	**trt**
	A	38.875	12	t6
	A			
	A	38.625	12	t7
	A			
B	A	36.125	12	t1
B	A			
B	A	32.250	12	t3
B	A			
B	A	31.375	12	t2
B	A			
B	A	31.125	12	t8
B	A			
B	A	30.125	12	t5
B				
B		28.750	12	t4

Appendix D4

Summary of ANOVA table for the effect of *Trichoderma asperellum* and *Bacillus cereus* on the fresh oil palm seedlings top

Means with the same letter are not significantly different.				
Tukey Grouping		**Mean**	**N**	**trt**
	A	465.00	12	t1
	A			
	A	457.50	12	t2
	A			
B	A	386.25	12	t6
B	A			
B	A	371.25	12	t3
B	A			
B	A	357.50	12	t5
B	A			
B	A	345.00	12	t7
B				
B		272.50	12	t4
B				
B		257.50	12	t8

Appendix D5

Summary of ANOVA table for the effect of *Trichoderma asperellum* and *Bacillus cereus* on the dry oil palm seedlings top

Means with the same letter are not significantly different.				
Tukey Grouping		**Mean**	**N**	**trt**
	A	221.25	12	t6
	A			
	A	218.75	12	t2
	A			
	A	215.00	12	t1
	A			
B	A	190.00	12	t7
B	A			
B	A	176.25	12	t5
B	A			
B	A	161.25	12	t3
B				
B		136.25	12	t4
B				
B		117.50	12	t8

Appendix D6

Summary of ANOVA table for the effect of *Trichoderma asperellum* and *Bacillus cereus* on the fresh weight of oil palm seedlings roots

Means with the same letter are not significantly different.					
t Grouping			**Mean**	**N**	**trt**
	A		212.50	12	t1
	A				
B	A		197.50	12	t3
B	A				
B	A	C	178.75	12	t2
B		C			
B		C	153.75	12	t6
B		C			
B		C	153.75	12	t5
B		C			
B		C	153.75	12	t7
		C			
		C	148.75	12	t8
		C			
		C	140.00	12	t4

Appendix D7

Summary of ANOVA table for the effect of *Trichoderma asperellum* and *Bacillus cereus* on the dry weight of oil palm seedlings roots

Means with the same letter are not significantly different.					
t Grouping			**Mean**	**N**	**trt**
	A		80.00	12	t1
	A				
B	A		71.25	12	t3
B	A				
B	A	C	63.75	12	t2
B		C			
B	D	C	51.25	12	t7
	D	C			
	D	C	45.00	12	t8
	D	C			
	D	C	45.00	12	t6
	D				
	D		37.50	12	t5
	D				
	D		36.25	12	t4

Appendix D8

Summary of ANOVA table for the effect of *Trichoderma asperellum* and *Bacillus cereus* on the chlorophyll content of oil palm seedlings at 6 MAI

Means with the same letter are not significantly different.			
Tukey Grouping	**Mean**	**N**	**trt**
A	42.813	12	T7
A			
A	42.563	12	T4
A			
A	41.750	12	T5
A			
A	40.375	12	T1
A			
A	40.250	12	T8
A			
A	40.000	12	T2
A			
A	40.000	12	T3
A			
A	39.188	12	T6

BIODATA OF STUDENT

Tuan Muhammad Syafiq Bin Tuan Hassan was born on 18th August 1993 at Kampung Teratak Pulai, Bachok, Kelantan, Malaysia. He obtained his primary education at Sekolah Kebangsaan Demit, Kelantan. He received his secondary education at Sekolah Menengah Kebangsaan Dato' Ahmad Maher, Kelantan. He studied at UiTM Puncak Alam, Selangor for foundation and bachelor degree at Universiti Malaysia Sarawak, Sarawak. He graduated with Bachelor of Science with Honours (Plant Resource Science and Management) in 2015. He went to Universiti Putra Malaysia to further his study in Master of Science with the field of plant pathology. During his study period, he became a lab demonstrator for two years as he was supported by Graduate Research Fellowship (GRF) scheme from Universiti Putra Malaysia.

LIST OF PUBLICATIONS

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