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BIOLOGICAL CONTROL OF BASAL STEM ROT DISEASE IN MATURE OIL PALM (*ELAEIS GUINEENSIS*) IN INLAND AND PEAT SOIL

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ABSTRACT

Oil palm is the main commodity crop cultivated in Malaysia and has made significant contributions to the economies of Malaysia and Indonesia. However, there is a major problem in oil palm, the basal stem rot (BSR) disease caused by *Ganoderma boninense*. This is a serious threat to the oil palm industry as it reduces oil palm yield and shortens the economic lifespan of the crop. In order to control this disease, this present study evaluated the efficacy of *Trichoderma* spp. as a biological control agent (BCA) through the application of different types of bioorganic fertilizers at selected oil palm estates in Palong, Pahang and Tanjung Karang, Selangor with different palm ages and soil types.

Trichoderma spp. was produced on large scale through the submerged fermentation method. Conidial seed culture resulted in higher *Trichoderma* spp. count from the bioreactor (6.32 log cfu/ml) in comparison to mycelial seed culture (4.95 log cfu/ml) and this was statistically significant ($P<0.001$). *Trichoderma* spp. was then mixed with biochar as the carrier at different ratios; 1:0.5 (B50%), 1:1 (B100%) and 1:2 (B200%) for ease of handling and long term storage. For oil palm field applications, *Trichoderma*-biochar (T-biochar) formulations were added into organic fertilizer, Living Organic Fertilizer (LOF) as the final products at concentrations of 0% (T1/T6), 5% (T2/T7), 10% (T3/T8) and 20% (T4/T9). The viability of *Trichoderma* spp. in the carrier and biofertilizer was monitored for up to 24 weeks. It was found that B100% was the best formulation for biochar with *Trichoderma* populations between 5.00-6.00 log cfu/g while there was no significant difference for the *Trichoderma* spp. count in biofertilizers at 10% (T3/T8) and 20% (T4/T9) formulations (around 5.00 log cfu/g).

In the oil palm plantations, the treatments were divided into two bioorganic fertilizer treatments, 1) Fortified Living Organic Fertilizer (FLOF) with customised N, P and K formulations; Treatments T1 to T5 and 2) LOF; Treatments T6 to T10 with different percentages of *Trichoderma* spp. (0, 5, 10 and 20%). Populations of

microorganisms including bacterial, fungal and *Trichoderma* spp. were monitored for up to 24 months. Even though *Trichoderma* spp. count fluctuated over time and between treatments (between 4.00-5.00 log cfu/g), there was a significant difference in treatment T9 (20% *Trichoderma* in LOF) as it had the highest *Trichoderma* spp. populations at both Palong and Tg Karang plantations. Total bacterial and fungal populations were not significantly different ($P \geq 0.05$) at both locations. The soil pH at both locations was acidic whereas the electrical conductivity (EC) in Palong was low, below 0.10 dS/m. Nutrient analyses on soil and leaf samples revealed that the nitrogen, phosphorus and potassium (N, P and K) levels were correlated with the fertilization regime at both estates. Generally, the disease incidence (DI) for BSR disease at Palong was low (below 13.0%) and can only be noticed in few treatments (T1 to T6) with T1 (FLOF control) showed an increment in DI at 20 and 24 months (12.5%). In comparison, at Tg Karang, high DI was recorded for all the treatments especially T8 (10% *Trichoderma* in LOF) and T10 (Grower's standard practice; GSP) with DI of 29.2% and 25.0% respectively at 24 months. Palong plantation also recorded a low percentage of palms with the disease at 5.2% and 3.5% for control and *Trichoderma* treatments respectively whereas the percentage increased between 3-6 folds at Tg Karang. Additionally, while no recovery was recorded for control palms, seven palms from the present *Trichoderma* treatments were found to have completely recovered from BSR disease (two and five palms at Palong and Tg Karang respectively).

Subsequently, *Trichoderma* spp. and other fungal communities in the rhizospheres of healthy, asymptomatic and symptomatic palms were identified through Sanger sequencing method. This is the first study that reported and evaluated the structure of fungal communities in oil palm rhizospheres over the course of BSR disease. *Trichoderma* spp. were present in rhizosphere soils at Palong and Tg Karang but with different distributions across the different symptoms. However, only healthy palms retained unique *Trichoderma* spp. *T. asperellum* was

detected between all the symptoms which indicates that this species might be indigenous to the soil or from residual bioorganic fertilizer applications. Analysis of the fungal-ITS (internal transcribed spacer) DNA region revealed that healthy palms at Palong were similar to each other with 8 common genera. Asymptomatic and symptomatic palms had four and one common genera respectively. In contrast, the fungal communities were distinct across all samples at Tg Karang and ectomycorrhizal fungi (ECM) was absent in asymptomatic and symptomatic palms. Regardless of soil types, it was observed that Ascomycota was the dominant phyla in healthy and asymptomatic palmss. Conversely, symptomatic palms were dominated by Basidiomycetes which suggested a shift in the fungal community. Furthermore, ITS DNA of *Trichoderma* spp. and *Ganoderma* spp. in the palm rhizospheres was quantified via quantitative real-time Polymerase Chain Reaction (qPCR). The quantity of *Trichoderma* and *Ganoderma* DNA was not significant between the different symptoms, but *Ganoderma* DNA was detected ten-fold higher in symptomatic palms. Comparison between the Palong and Tg Karang plots showed that the quantity of *Ganoderma* at Tg Karang was also ten times higher than at Palong. For *Trichoderma,* the quantity was similar at Palong and Tg Karang which was between 0.020-0.035 ng/µl.

Overall, this research revealed that *Trichoderma* spp. had biocontrol activity in mature oil palm estates infected with *Ganoderma* spp., but the effects were influenced by biotic and abiotic factors. Such impacts on *Trichoderma* populations should be studied further. This study also provides evidence on the changes of the fungal communities in oil palm rhizosphere soils with regards to BSR disease. Further evaluation needs to be conducted to elucidate the role of other fungal communities that could co-exist with *Trichoderma* as a biological control agent for the control of BSR disease in oil palm.

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LIST OF ABBREVIATIONS

AAR	Applied Agricultural Resources
ABS	Artificial breeding site
ANOVA	Analysis of variance
AS	Asymptomatic
ATP	Adenosine Triphosphate
AVROS	Algemene Vereniging van Rubberplanters ter Oostkust van Sumatra
BBRC	Bioprocessing and Biomanufacturing Research Centre
BCA	Biological control agent
BLAST	Basal Local Alignment Search Tool
Bp	Base pair
BSR	Basal stem rot
CAT	Catalase
CBD	Complete block design
CFU	Colony forming unit
CHS	Chalcone synthase
COD	Chemical oxygen demand
CPKO	Crude palm kernel oil
CPO	Crude palm oil
Cq	Quantitation cycle
Cr	Chromium
CSR	Common spear rot
CTAB	Cetyltrimethylammonium bromide
CWDE	Cell wall degrading enzyme
DAPG	2, 4-diacetylphloroglucinol
DGGE	Denaturing gradient gel electrophoresis
DI	Disease incidence
DIBA	Dot immunobinding assay
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DSI	Disease severity incidence
E	Efficiency
EC	Electrical conductivity
ECM	Ectomycorrhizal
EDTA	Ethylenediaminetetracetic acid
ELISA	Enzyme-linked immunosorbent assay
e-nose	Electronic nose
ER	Electrical resistance
ESEM	Environmental scanning electron
FFB	Fresh fruit bunches
FLOF	Fortified living organic fertilizer
Foc	*Fusarium oxysporum* f. sp. *cucumerinum*
Foe	*Fusarium oxysporum* f. sp. *elaeidis*
GIS	Geographical Information System
GPS	Global positioning system

GSM	*Ganoderma*-selective medium
GSP	Grower's standard practice
H	Healthy
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HR	Hypersensitive response
HRS	Hyperspectral remote sensing
IAA	Indole-3-acetic acid
IBRC	Industrial Biotechnology Research Centre
ISR	Induced systemic resistance
ISS	in situ sequencing
ITS	Internal transcribed spacer
JA	Jasmonic acid
Lip	Lignin peroxidase
LOF	Living organic fertilizer
MAbs	Monoclonal antibodies
MAMP	Microbe-associated molecular patterns
MC	Moisture content
MEA	Malt extract agar
MEB	Malt extract broth
$MgCl_2$	Magnesium chloride
MnP	Manganese peroxidase
MOP	Muriate of potash
MPOB	Malaysian Palm Oil Board
MRD	Maximum recovery diluent
MSDS	Material safety data sheets
N	Nitrogen
NA	Nutrient agar
NaAc	Sodium acetate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
NMR	Nuclear magnetic resonance
NTC	No template control
OM	Organic matter
P	Phosphorus
PAbs	Polyclonal antibodies
PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PGPR	Plant growth promoting rhizobacteria
PKS	Palm kernel shell
PLFA	Phospholipid fatty acid
PO/POD	Peroxidase
POME	Palm oil mill effluent

PPO	Polyphenol oxidase
PSM	Phosphorus solubilizing microorganisms
PVP	Polyvinylpyrrolidone
qPCR	Real-time Polymerase Chain Reaction
RAFLP	Amplification length polymorphisms
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
rpb2	Ribosomal subunit
Rt-qPCR	Quantitative reverse transcription PCR
S	Symptomatic
SAR	Systemic acquired resistance
SE	Standard error
spp.	Species
TBE	Tris-Borate-EDTA
tef1	*Translation elongation factor*-1α
TGGE	Temperature gradient gel electrophoresis
TPC	Total phenolic content
T-RFLP	Terminal restriction fragment length polymorphism
TSM	*Trichoderma* selective media
UPM	Universiti Putra Malaysia
UV	Ultraviolet
WSNs	Wireless sensor networks

1 CHAPTER 1: INTRODUCTION

1.1 RESEARCH BACKGROUND

The oil palm (*Elaeis guineensis* Jacq.) is an erect monoecious plant, which belongs to the family Arecaceae and is the most important species in the genus *Elaeis* (Syed *et al*., 1982). It has an economic life span of around 25-30 years and it gives the highest yields per hectare of all oil crops (Corley and Tinker, 2003; Uwumarongie-Ilori *et al*., 2012). This has led to a rapidly expanding industry in Asia, Africa and America. Malaysia and Indonesia are the major producers that provide around 90% of the oil entering international trade (Corley and Tinker, 2003).

However, the threat of basal stem rot disease (BSR) that is caused by *Ganoderma* spp. has significantly reduced the yields and this resulted in requirement for early replanting (Flood *et al*., 2002). Young palms usually die within 1 or 2 years once they show symptoms of the disease whereas mature palms can survive for about 3 years or so (Corley and Tinker, 2003). Current control practices involved soil mounding, clean clearing, sanitation, removal of diseased material and digging trenches (Hushiarian *et al*., 2013). Besides, fungicides remain crucial in the field for controlling pathogenic fungal diseases. However, these measures are time-consuming and require high cost of maintenance for the estates.

On the contrary, application of biological control agents (BCAs) such as fungi to reduce or control phytopathogens and pests and promote the growth of plants can be implemented to overcome BSR problem. Antagonistic microorganisms are often found in nature and can be isolated using selective screening techniques (typically based on particular enzyme activity). The use of BCAs provides an environmental friendly alternative compared to chemical applications. From the early studies of control of wood-rotting fungi, *Trichoderma* spp. has gained attention due to its ability and efficiency in controlling other fungi (Hushiarian et

al., 2013). It has been commercially marketed as biofertilizer, biopesticides and soil amendments (Harman, 2000).

Trichoderma species are saprophytic fungi that can be found in most soils, making them one of the most important groups of antagonistic fungi (Błaszczyk *et al*., 2014). *Trichoderma* also has long been recognised to be effective biological control agent, and they are the most studied genus of fungal antagonists. In Malaysia, many researchers on the development of biocontrol agents for BSR disease concentrate on species such as *T. harzianum, T. asperellum* and *T. virens,* all of which exhibit good biological control potential *in vitro* and in nurseries (Sundram *et al*., 2008; Naher *et al*., 2012; Angel *et al*., 2016; Ho *et al*., 2018; Musa *et al*., 2018).

In the field, however, the survival, establishment, and antagonistic activity of introduced *Trichoderma* species are still inconsistent (Papavizas, 1981), and the influence of farming techniques on indigenous *Trichoderm*a populations is largely unclear. It is critical to understand the ecology and dynamics of *Trichoderma* populations in the field if commercial products are to be successfully incorporated into mainstream agriculture, and if indigenous *Trichoderma* populations are to be enhanced to achieve biological control (Paulitz, 2000). Biocontrol application of *G. boninense* has been the subject of a lot of research, but it has yet to be validated in the field (Siddiqui *et al*., 2021). When compared to the results obtained on an agar plate for the same BCA, the degree of inhibition by BCA towards plant pathogens seems to be lower in some situations when tested in the field. This might be due to the diversity of the environment (O'Brien, 2017) and the intricacy of soil microbial interactions. As a result, it is critical to assess the suppression of BCA towards the pathogen not just *in vitro* but also *in vivo*.

Hence, formulation of *Trichoderma* spp. to obtain a stable product for field application, biocontrol activity of *Trichoderma* spp. under field conditions and the

fungal populations in the oil palm plantation was the main focus of this study since the microorganisms can serve as the first line of a plant's defence against BSR disease. Additionally, field assessments are essential if biocontrol approaches are to become more applicable to be included in the overall management of BSR in oil palm plantations.

1.2 AIMS AND OBJECTIVES OF THE STUDY

The main aim of this study was to evaluate the ability of *Trichoderma* spp. applied as bioorganic fertilizer in different formulations to suppress basal stem rot (BSR) disease caused by *Ganoderma boninense* under field conditions. Even though there were many reports on the use of *Trichoderma* spp. for the control of BSR disease, studies were limited to either controlled (nursery) or *in vitro* conditions and there were very few similar research being conducted under the field conditions. In addition, despite previous researches that have described the soil bacterial and fungal communities in the oil palm plantation, the structure of fungal communities with regards to BSR disease has not been reported.

As such, the novel aspects of this study are;

1. Bio-formulation and application of *Trichoderma* bioorganic fertilizer under field condition in oil palm estates.
2. Identification of fungal communities in oil palm rhizospheres and to understand their roles in the development or suppression of BSR disease.

It was hypothesized that *Trichoderma* spp. can be produced in large quantity using the stirred-tank batch fermentation method and stabilized using inert carrier material for a shelf life of approximately six months. *Trichoderma*-carrier amended with Living Organic Fertilizer (LOF) and Fortified Living Organic Fertilizer (FLOF) will be applied as bioorganic fertilizer in oil palm estates, this will help to improve palms'

health and resistance towards *Ganoderma* spp. This study also set objectives to sequence and identify *Trichoderma* spp. and other fungal species in the rhizospheres of healthy and diseased palms, apart from quantification of the biological control agent and the pathogen via real-time PCR analysis.

Hence, several objectives as followed also act to support the main aim of this study:

1. To develop *Trichoderma* formulations in combination with an inert carrier (biochar), LOF and FLOF for oil palm field applications.
2. To determine the populations of *Trichoderma* spp., other soil microorganisms and soil physicochemical properties in selected oil palm estates in Malaysia.
3. To evaluate the incidences and impacts of BSR disease in oil palm during field trials in selected oil palm estates in Malaysia.
4. To identify *Trichoderma* populations and other fungal communities in the rhizospheres of healthy and diseased palms.
5. To quantify and correlate the relationship between *Trichoderma* spp. and *Ganoderma* spp. in the rhizospheres of healthy and diseased palms.

2 CHAPTER 2: LITERATURE REVIEW

2.1 OIL PALM

2.1.1 The oil palm

The oil palm (*Elaeis guineensis*) came from Western Africa's Gulf of Guinea. It is known as the African oil palm and is grown as an agricultural crop. In the 15th century, the Portuguese introduced this palm to Brazil and other tropical countries (Corley, 1976) whereas the Dutch brought the seeds from Africa to Indonesia in the 18th century (William and Hsu, 1970). The British introduced the African oil palm to Malaya as an ornamental plant in the early 1870s, and the first commercial oil palm plantation took place at the Tennamaram Estate, Selangor, in 1917. (William and Hsu, 1970; Tate, 1996).

Oil palm is a monoecious plant that can grow up to 20-30 metres. It produces approximately eight to fifteen compact bunches per year known as fresh fruit bunches (FFB), weighing between 10-25 kg each. The production of fruit starts 24 to 30 months after planting and the palms will remain productive for the next 20 to 30 years. (Henson, 1990). Oil palm in Malaysia are typically of the tenera variety, which is a cross between the varieties of dura and pisifera (DxP). Higher oil content of about 4 to 5 tonnes of crude palm oil and 1 tonne of palm kernel per hectare per year is produced by the tenera variety. At present, Malaysia has 4.49 million hectares of oil palm land under cultivation. This produces 17 million tonnes of palm oil and 2 tonnes of palm kernel oil, respectively (mpoc.org.my).

2.1.2 Palm oil and its uses

Palm oil consists of a balanced mixture of saturated (40% saturated palmitic acid and 5% saturated stearic acid) and unsaturated fatty acid (44% monounsaturated oleic acid and 10% polyunsaturated linoleic acid). It is cholesterol-free and has no fatty acids that are trans-unsaturated. Palm oil has high antioxidants such as vitamin E and can be found in crude or unrefined oil with

around 60-100 mg of vitamin E and half of it will remain in the cooking oil after refining. Vitamin A is also high in palm oil, where about 50-70 mg of carotenoids are found in 100 g of crude oil (Nagendran *et al*., 2000; Wahid *et al*., 2005).

They are two types of oil derived from the oil palm which are crude palm oil (CPO) and crude palm kernel oil (CPKO). These are produced from the fibrous mesocarp and the palm kernel respectively. The chemical compositions and nutritional contents of these two types of oil vary (Teoh, 2002). Palm oil is high in carotenoids and vitamin E especially tocotrienols whereas saturated fatty acids are mainly found in palm kernel oil. Palm oil fractionation results in the production of palm olein and palm stearin, while palm kernel olein and palm kernel stearin are derived from palm kernel oil fractionation. (Koushki *et al*., 2015).

As the oil has a strong resistance to oxidation at frying temperature, refined palm oil and palm olein are predominantly used for cooking and frying. Palm oil and the products of its fractionation are used as the main ingredients in the production of margarine and shortenings. Furthermore, in the making of confectionaries, coffee whiteners, biscuit cream and many more, palm kernel oil, palm kernel olein and palm kernel stearin are used (Teoh, 2002). Palm oil products are not restricted to food industry alone as it is also used mainly in the oleochemical and biofuel production. Palm oil products play a role in the production of cosmetics, soaps, detergents, candles, lubricants and antifreeze (mpoc.org.my).

2.1.3 Common diseases in oil palm

Before World War II, the oil palm was generally free from devastating diseases and pests. However, with increasing plantation areas of oil palms, there has been a general increase in the outbreak of oil palm diseases being recorded in several parts of the world (Corley and Tinker, 2003). Like other crops, a variety of pathogens such as bacteria, viruses, nematodes and phytoplasma are responsible for diseases in the oil palm. Fungi, which hinder growth and decrease yield, are the

main pathogens (Turner, 1981; Tengoua and Bakoume, 2005). There are about 60 diseases and disorders in oil palm that have been documented from around the world (Turner, 1981). The diseases in oil palm were categorised by Limsriwilai *et al*. (1984) at various growth stages: germination, nursery stage and field planting stages.

In a survey conducted by Pornsuriya *et al*. (2013), the three main diseases found in Thailand were *Ganoderma* basal stem rot, *Curvularia* leaf blight and brown germ. The causal agent for the leaf blight disease is *Curvularia oryzae* and it was prevalent at the seedling stage in the nursery. While in the field, *G. boninense* was particularly damaging at the fruiting stage resulting in the decline and death of the palms (Pornsuriya *et al*., 2013). Moreover, *Fusarium oxysporum* f. sp. *elaedis* that causes vascular wilt disease had been reported to be endemic to Africa, but it is not a major problem in Malaysia and other palm oil-producing countries (Cooper and Rusli, 2014).

Some of the major diseases in oil palm are listed with the causal agents in bracket: vascular wilt (*Fusarium oxysporum)*, leaf freckle (*Cercospora elaeidis)*, *Corticium* leaf rot (*Corticium solani*), *Marasmius* bunch rot (*Marasmius palmivora*), *Ganoderma* trunk rot or basal stem rot (*Ganoderma* spp.), blast (*Pythium splendens*) and *Armillaria* trunk rot (*Armillariella mellea*) (Aderungboye, 1977; Corley and Tinker, 2003). Among all these, the most disruptive disease in Southeast Asia particularly Indonesia and Malaysia is the basal stem rot (BSR) disease caused by *Ganoderma boninense*. In the last few decades, it has spread exponentially and is a major threat to the oil palm industry (Hasan and Turner, 1998). BSR reduced the yield of the palms, caused a direct loss of the stand and this resulted in earlier replanting requirements (Flood *et al*., 2002).

In Malaysia, the BSR disease has decreased the annual production drastically and resulted in palm oil losses of up to RM1.5 billion (USD 500 million) annually (Ommelna *et al*., 2012). As diseased palms produced less oil per bunch,

only 4 tons of crude palm oil/ha/year were obtained instead of the estimated yield of 19 tons of crude palm oil/ha/year. At 31-67% of BSR disease index, fresh fruit bunch (FFB) yields are reduced by 26-46%. Apart from that, through a survey, the BSR incidence was reported to be 3.71% with 59,148 ha of the total area impacted and the worst affected states were Johor, Perak and Sabah (Idris *et al*., 2011). In Sumatra, if the climate change persists, it is projected that after 2050, the oil palm will be less resistant to the disease as the climate is not ideal while *G. boninense* will be more virulent (Paterson, 2019).

2.2 BASAL STEM ROT DISEASE

2.2.1 *Ganoderma* spp.

Ganodermataceae are basidiomycetes that cause white rot of hardwood by decomposing lignin, cellulose and related polysaccharide (Adaskaveg *et al*., 1993). There are more than 250 species in this genus and *Ganoderma* grows on various coniferous, palmaceous and perennial hosts and has a worldwide distribution. It has a role in the breakdown and delignification of woody plants in the forest ecosystem whereas some species of *Ganoderma* being pathogenic and detrimental to economically important trees and perennial crops. Besides oil palm, *Ganoderma* spp. cause root and stem rot disease in many other crops such as coconut, rubber, tea and cocoa (Ariffin *et al*., 2000).

Different species of *Ganoderma* will have a different morphological shape of the sporophores in which the fruiting body consists of stalk or stipe and typically the brackets formed from the stalk (Turner, 1981). Depending on the species, the upper side of the sporophores can be medium to dark brown colour while the lower side is whitish. Most species of *Ganoderma* are poorly described, and there is little detail on their biology. There are two major species of *Ganoderma* noted in the literature; 1) *G. lucidum,* the most important species of mushroom and is the model

medicinal mushroom and 2) *G. boninense*, the primary cause of BSR disease in oil palm and is Southeast Asia's most destructive disease (Mercière *et al*., 2017).

In west Africa, the causal agent of BSR was originally identified as *Ganoderma lucidum* Karst while 4 species of *Ganoderma* in Nigeria comprising *G. zonatum* Muril, *G. encidum*, *G. colassus* and *G. applantum* were said to be the causal agents. In Malaysia, it was also originally identified as *G. lucidum* by Thomson (1931) (Ariffin *et al*., 2000). Turner (1981) mentioned that 15 species of *Ganoderma* from different regions of the world as the likely pathogen to cause BSR disease. Out of these, there were about 7 species (*G. applanatum*, *G. chalceum*, *G. boninense*, *G. lucidum*, *G. tornatum*, *G. miniatocinctum* and *G. psedoferreum*) that had been reported from peninsular Malaysia. It was concluded that the causative agents reported from separate locations in Peninsular Malaysia were of the same species which was *G. boninense* (Ho and Nawawi, 1985) (Figure 2.1) and it is considered as the most aggressive pathogen (Turner, 1981).



Figure 2.1 *G. boninense* at the stem of an infected oil palm. Photo was taken on 16 October 2017.

Ganoderma is a soil-borne fungus and spreads through roots in the soil as well as through the air via the spores. Like other Basidiomycetes, *Ganoderma* reproduces through mycelia and spores. There is a possibility that the dispersal of the basidiospores contributed to the diversity of *G. boninense*. Through sexual recombination, more than one genotype was found in the oil palm plantations (Pilotti *et al*., 2002). Nonetheless, the release of large quantities of *Ganoderma* spores in field experiments did not infect many palms. Hence, instead of the airborne spores, tissue infected in the soil seems to be more likely to transmit the disease to healthy roots (Ho and Zawawi, 1986).

2.2.2 Symptoms and development of the disease

Infection by *Ganoderma* starts when the fungal mycelia penetrate the oil palm roots before spreading to the stem bole area. To degrade the lignin polymer (a component of the plant cell wall), *Ganoderma* secretes ligninolytic enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Xu *et al*., 2017). Cellulose and hemicellulose are hydrolyzed by cellulase and hemicellulase to soluble monomeric sugars after penetration, which is then utilized for fungal growth (Paterson, 2007).

The infection progresses slowly without obvious early symptoms and disease can occur at all stages of the oil palm, from seedlings to old palms. Only when the progression of the infection is between 60-70% that the symptoms start to appear. The earliest symptoms can be seen on the frond, where at least half of the cross-sectional region of the stem has been affected. The deterioration of the inner stem area will lead to a decrease in the supply of water and nutrients to the aerial parts, causing symptoms similar to water stress and malnutrition (Turner and Gillbanks, 1974).

In young palms, the external signs of BSR can be seen as a one-sided yellowing or mottling of the lower frond followed by necrosis (Singh *et al*., 1991).

The unfolded young leaves are necrotic and may be shorter in length. With numerous unopened spears, pale leaf canopy, flattening of the crown and the appearance of basidiocarps, mature palms will also exhibit similar symptoms (Turner, 1981). The pathogen has already destroyed half of the tissues of the plant by the time the foliar symptoms are evident (Ariffin *et al*., 2000). Other than that, the palm roots will be friable and the internal tissues become very dry and powdery (Singh *et al*., 1991).

At the infected root or the stem base, the *Ganoderma* fruiting body (basidiomata) may appear. These appearances provide a guide on the incidence of the disease within the palms (Paterson, 2007). In severe cases, this disease can lead to a fracturing of the palm base and ultimately a collapse of the palm. There is no fixed sequence or symptoms progression and the palms may show only one symptom or combinations of all of the above.

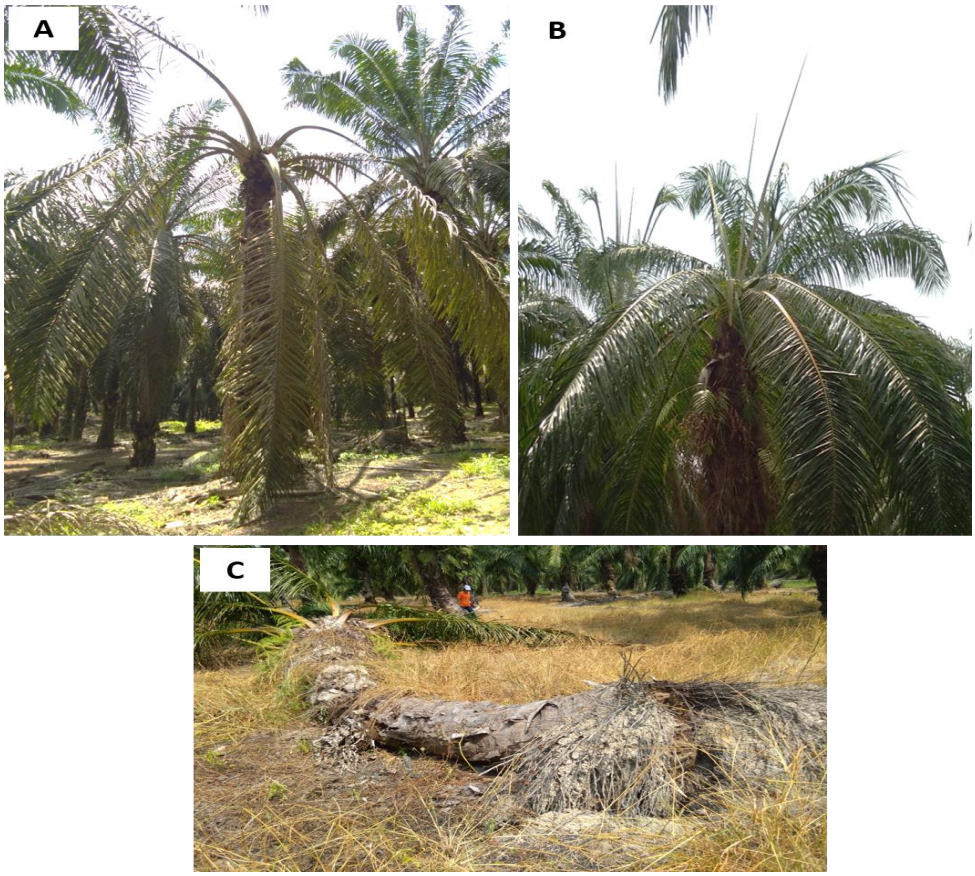


Figure 2.2 (A) *Ganoderma* infected palm, the leaves formed a skirt-like crown, (B) Unopened spears of an infected palm, (C) Fallen palm due to BSR disease. Photos were taken between March to September 2018.

2.2.3 Detection of *Ganoderma* BSR disease

In the early days, the method of diagnosing the disease was through observation of visual signs such as wilting and falling of mature leaves or the appearance of the fruiting body on the stem/bole (Lelong *et al*., 2010). Another conventional diagnostic method was by drilling into the diseased part of the palm and the samples were analyzed by either colourimetric method using ethylenediaminetetraacetic acid (EDTA) (Natarajan *et al*., 1986) or isolation of *Ganoderma* on agar plates by using semi-selective media (Darus *et al*., 1993). Both techniques were time-consuming and has low degrees of accuracy. Meanwhile, it has been recognized that the disease is already well-developed by the time the basidiomata appeared. *Ganoderma* selective medium (GSM) has also been used to detect *Ganoderma* in infected oil palm (Ariffin *et al*., 2000).

Immunological methods such as the use of polyclonal antibodies (PAbs) to serologically detect the fungus through enzyme-linked immunosorbent assay (ELISA) against the mycelial and basidiocarp protein of *Ganoderma* (Utomo and Niepold, 2000; Kandan *et al*., 2010) and dot immunobinding assay (DIBA) (Kandan *et al*., 2010) were developed. Nonetheless, the antibodies showed cross-reactivity against other fungal species and therefore was not-species specific (Utomo and Niepold, 2000). Shamala *et al*. (2006) had raised monoclonal antibodies (MAbs) by using crude mycelium extract of *G. boninense.* The MAbs gave better results as compared to polyclonal antisera but they were still not specific as positive signals were obtained in the cross-reactivity test.

Polymerase chain reaction (PCR) has been used in molecular diagnostics for pathogen amplification and identification. Primers that targeted the internal transcribed (ITS) region 1 of *G. boninense* ribosomal DNA (rDNA) proved to be more accurate than ELISA and is a useful tool for early detection of the disease (Utomo and Niepold, 2000). Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) which are PCR-based approaches have

been utilized to analyse different isolates of *Ganoderma* spp. from different plant hosts (Zakaria *et al.*, 2009) and also for the identification of pathogenic *Ganoderma* in oil palm (Utomo *et al*., 2005). Additionally, an electrochemical DNA biosensor was designed and modified by using a conducting polymer film to specifically detect *G. boninense* (Dutse *et al*., 2012, 2013). The utilization of molecular technique remains a challenge since it involves complex processes, time-consuming and difficulty in the preparation of raw sample materials and DNA extractions (Hushiarian *et al*., 2013).

In a study conducted by Mohd As'wad *et al*. (2011), ergosterol, which is a membrane component of fungi was analysed by using high-performance liquid chromatography (HPLC). This method has made it possible to quantify *G. boninense* in infected palms as it was found that the concentration of ergosterol was correlated with the biomass of the pathogen. Another interesting approach is by measuring the electrical resistance (ER) around the trunk of the palm. It was established that infected palms gave ER reading below 25 Ω whereas the reading for healthy palms was between 25 Ω to 150 Ω, thus detection of an unhealthy palm is possible by using this method (Nurnadiah *et al*., 2014).

The use of diverse sensors and geospatial technologies for the detection of the disease in oil palm plantations was also being explored. Examples include hyperspectral and multispectral remote sensing (Shafri and Anuar, 2008; Santoso et al., 2011), terrestrial laser scanning (Khairunniza-Bejo and Vong, 2014), tomography (Mohd Su'ud *et a*l., 2007), intelligent electronic nose (e-nose) (Markom *et al*., 2009;) and spatial maps (Azahar *et al*., 2011). Apart from rapid and reliable data, these methods provide observation in real-time for the control and management of the estate.

By using tomography images, the palms were classified as intact stem tissue, infection by *Ganoderma* and non-*Ganoderma* infection. These were established based on the expert's knowledge in the Fuzzy Inference Systems (Mohd

Su'ud *et al*., 2007). For e-nose device, it can imitate the human olfactory sense and this system has been able to select healthy and infected palms with high precision through various odour parameters. This method provides a fast indication of plant stress results in the field, but it is not specific to *G. boninense* as odour profiles will be generated when palms are infected by other pathogens (Markom *et al*., 2009).

Hyperspectral remote sensing (HRS) is an imaging technique that has the capability to identify diseased crops and this is probably the most widely used technique being tested in the field currently (Khosrokhani *et al*., 2018). Depending on the molecular texture and composition, different materials uniquely absorb, reflect and emit electromagnetic radiation and this is recognized by HRS (Khosrokhani *et al*., 2018). Lelong *et al*. (2010) proposed the use of HRS data and calibration of a statistical model to distinguish oil palm canopy spectra into several levels of *Ganoderma* attack. From the derived algorithm, oil palm can be classified into 4 stages of disease severity with 94% accuracy. It seems promising to use this tool to differentiate between healthy and diseased palms, although more analysis needs to be carried out to accurately distinguish the levels of infection involved (Hushiarian *et al*., 2013).

Recently, the use of nuclear magnetic resonance (NMR) and electrochemical sensors has enabled the characterization of the metabolites present in healthy and infected oil palms. It has been reported that some metabolites were more prominent in infected oil palm leaves such as sucrose and fructose sugars which might be the energy source for the pathogen. In addition, alanine, choline, indole-3-acetic acid (IAA) and phenolic compounds play a role in plant defence mechanisms (Isha *et al*., 2019).

Another latest approach is by using DNA fragments linked to *G. boninense* via colourimetric assay. When the complementary DNA of the target fungus/organism is present, the DNA-nanoparticle conjugates will aggregate and

the colour change can be observed visually. This assay has the potential to be applied in the field since it is sensitive, provides fast results and do not require specific equipment. Yet, further improvement is necessary to reduce the occurrence of mismatches and decrease the assay volume for easier field application (Rani *et al*., 2020).

2.2.4 Management of BSR disease

In the oil palm industry, there was a consensus that the roots of the oil palms are the main route of BSR infection. *G. boninense* can colonise any woody materials and this can act as the source of inoculum for the present stand while the remaining debris from the infected stands may become the inoculum for the subsequent planting (Flood *et al*., 2010). Thus, much emphasis has been placed on the control and management of this disease through the physical, chemical and biological methods.

Physical methods such as digging trenches aimed to prevent the spread of *Ganoderma* mycelia to neighbouring healthy palms. However, this involved a high cost of maintenance and the trench depth might not be adequate to prevent roots from slipping underneath (Turner, 1981; Ariffin *et al*., 2000). Besides, sanitation practices such as windrowing and clean clearing during replanting are considered important practices to control BSR. An open burning technique was employed prior to the 1990s, but this was controversial from the environmental perspective and there are now strict regulations in place where open burning in Malaysia is prohibited (Chung, 2011).

Sanitation is one of the commonly tested commercial practices. This is done by chipping and falling diseased palms and deboling the infected roots at the existing planting or during replanting in order to reduce the amount of infected tissues buried or left in the soil. If done properly, this may reduce the infection rate of the disease (Chung, 2011; Hushiarian et al., 2013). Another typical strategy is soil mounding to cover the fruiting body at the stem base. It involves heaping soil

around the trunks of palm trees up to 75 cm height with a radius of 1 m. This strategy has been generally recognised as successful by planters/growers, particularly for supporting healthy root development but it does not not prevent the development of the disease (Turner, 1981).

The chemical approach, which involved the use of fungicide based treatment has also been attempted. To limit BSR incidence in the field, most oil palm plantations utilise chemicals such as carboxin and quintozene. The fungicide is applied using hand-knock pressure to prevent chemical loss during treatment. This is in contrast with fungicide hexaconazole which does not show effective control of the disease (Chung, 2011). Since *G. boninense* is a soil-borne pathogen with various resting stages (mycelium, pseudosclerotia, basidiospores and chlamydospores), the fungicides may already have degraded before reaching the target, making them ineffective (Susanto *et al*., 2005). Fungicides also should be used with caution as they hinder the growth of beneficial microorganisms in the soil (Naher *et al*., 2013). Additionally, this process necessitates the use of labour and costly chemicals, making it uneconomical (Shamala et al., 2006).

Chitosan which is a chitin-derived polysaccharide can be obtained commercially as a by-product of shellfish. It has been shown that chitosan is toxic and hinders fungal growth and production, thus have the potential to control plant diseases (Ommelna *et al*., 2012). Chitosan had been tested in the oil palm nursery and 0.5% (w/v) of chitosan reduced the fungal sterol to a minimum and showed a low level of BSR disease (Ommelna *et al*., 2012). A study by Ganeson (2016) also showed promising results in which the phenolic contents and plant defence enzymes such as peroxidase (PO), phenylalanine ammonia-lyase (PAL) and chitinase were upregulated in the infected roots of oil palm seedlings. Besides, the use of chitosan as agronanofungicides has also been explored. Hexaconazole and dazomet fungicides were encapsulated with chitosan nanoparticles and used as an antifungal

agent against *G. boninense.* The findings revealed a dual mode of action of chitosan, as a carrier for the fungicides and as a biocide itself (Maluin *et al*., 2020).

Other than that, disease resistance in a plant is influenced by its nutritional status. Nutrient supplementation such as copper, calcium and silicon (Sabrina *et al*., 2012; Najihah *et al*., 2015; Rahamah Bivi *et al*., 2016) and also application of fertilizer GanoCare® (Rebitanim *et al*., 2020) was used in an attempt to improve the plant's resistance against pathogen attack. These nutrients were reported to decrease oil palm disease severity by amplifying peroxidase production, reduction in ergosterol activity and enhanced lignification in treated plants (Nur Sabrina *et al*., 2012; Rahamah Bivi *et al*., 2016).

Research and development of oil palm progenies resistant to *Ganoderma* have been earmarked for future control of this disease. An array of planting materials with a broad genetic base were used in a screening test in the nursery and it was found that Deli material was the most susceptible while African material was partially resistant (Idris *et al*., 2004). This was in line with the field observation carried out by Durand-Gasselin *et al*. (2005), although the genes for *Ganoderma* resistance have not been investigated. Incorporation of this resistance into commercial varieties requires a longer period as it is originated from a semi-wild genotype and it involved 5–6 generations (30–40 years) of backcross breeding to advanced breeding parents. Furthermore, since defence-related genes have not been isolated, this makes the development of a fully resistant oil palm difficult (Siddiqui *et al*., 2021).

The oil palm AVROS (*Algemene Vereniging van Rubberplanters ter Oostkust van Sumatra*) is the most often planted variety, and it is claimed to be more resistant to *G. boninense* than other commercial varieties (Chong et al., 2012). Recently, based on data obtained from 25 years of field observation, Tisné *et al*. (2017) have identified four *Ganoderma* resistance loci in oil palm. Two loci control the development of the first *Ganoderma* symptoms while the other two are related

to the death of the palms. Also, from the ongoing breeding programmes, favourable haplotypes have been detected among a major gene pool (Tisné *et al*., 2017). This provided important information for the selection and improvement of resistant varieties of oil palm against *Ganoderma* disease. This transgenic approach appears to be promising, but it is unlikely to produce an economically viable solution immediately.

One alternative to control BSR disease is using biological control agents. Extensive research had been done in recent years to identify effective BCA that includes fungi, bacteria and actinomycetes for managing BSR disease. Dual culture, poison agar and culture filtrate assays are the most used methods for evaluating the effectiveness of BCA. The higher the proportion of inhibition of radial growth (PIRG), the more effective the BCA is in suppressing the pathogen (Lai and Nusaibah, 2021).

Pseudomonas aeruginosa was effective against *Ganoderma* and has been shown to improve plant growth (Lim *et al*., 2019; Muniroh *et al*., 2019) while *Streptomyces* spp. released antimicrobial compounds such as salinomycin, ribostamycin, landomycin B and benzymalic acid that may contribute to the antagonistic effect against the phytopathogen (Lim *et al*., 2018). Ting *et al*. (2014) isolated three isolates of actinomycetes from empty fruit bunch of oil palm. The isolates showed antagonistic effect against *G. boninense* and were identified as *Nocardiopsis* sp., *Streptomyces violaceorubidus* and *Streptomyces* sp., with percentages of inhibition of 91.4%, 86.4% and 69.1%, respectively. Arbuscular mycorrhizal fungi (AMF) have been shown to be associated with the roots of oil palm, and it has been suggested that they may be able to suppress *G. boninense* (Sundram *et al*., 2015).

Besides, studies have shown that the growth of *Ganoderma in vitro* has been inhibited by fungi such as *Penicillium* spp., *Aspergillus* spp. and *Trichoderma* spp. (Badalyan *et al*., 2004). In Malaysia, the most common *Trichoderma* spp. used as

BCA are *T. harzianum*, *T. asperellum* and *T. virens* (Sundram *et al*., 2008; Naher *et al*., 2012; Angel *et al*., 2016; Ho *et al*., 2018; Musa *et al*., 2018). New biocontrol candidates, such as ascomycetous mycoparasitic and mycophilic fungi like Cladobotryum semicirculare (Marzuki *et al*., 2015) and Scytalidium parasiticum (Goh *et al*., 2016) have recently been described as having the ability to inhibit BSR disease. In a mycoparasitism test, *C. semicirculare* has the ability to reduce the regeneration or recovery of Ganoderma mycelia (Marzuki et al., 2015). For *S. parasiticum*, the fungus could also impede regeneration of *G. boninense* fruiting bodies and was proven to be non-pathogenic to oil palm seedlings in the nursery study (Goh *et al*., 2016). Nevertheless, the majority of these studies focused on *in vitro* assessment, with only a few reporting on nursery or greenhouse trials. Field trials are still lacking and it is the most critical step in determining the efficacy of these BCAs in the field since the BCA may act differently in the field than on an agar plate or in a controlled setting (Lim *et al*., 2019; Siddiqui *et al*., 2021).

Mass production of these antagonists, especially *Trichoderma* spp. has been done by many researchers and this preparation could be used to control *Ganoderma* by application around the roots of infected oil palms (Ariffin *et al*., 2000). Though the use of BCA has not completely provided the solutions for BSR, in contrast to the others, the technique has become a notable method. Hence, many products such as biofertilizers and biofungicides are available in the market for the control of BSR (Naher *et al*., 2013).

2.3 Biological control of plant diseases

The aim of biological control is to suppress or control diseases by applying BCAs to the plant or soil, such as fungi or bacteria, or a combination of both. Therefore, the BCA works by preventing infection or the establishment of the pathogen in the plant (O'Brien, 2017). The relationship between the plant (as the host), the pathogen, the BCA (as the antagonist), the microbial population on and around the plant and the physical environmental conditions determines the

effectiveness of the use of BCA. The use of microorganisms as BCA is considered better than chemicals as they are pathogen-specific and are safe to non-target organisms and the environment (Handelsman and Stabb, 1996). In the last decade, as summarised in Table 2.1, several studies have been conducted on the use of BCAs for plant disease control and commercial BCAs production.

Table 2.1 List of biological control agents that have been commercially produced (O'Brien, 2017).

Biocontrol agent	Crop	Supplier	Country
Ampelomyces quisqualis M-10	Powdery mildews	EcoGen	USA
B. subtilis MB1600	Fungal pathogens of cotton, Large seeded legumes Soybeans	Beker Underwood	USA
B. subtilis MB1600 + Rhizobium	Fungal pathogens of soybeans, peanut	Beker Underwood	USA
Bacillus amyloliquefaciens GB99+ *B. subtilis* GB122	Bedding plants in potting mixes	Gustafson, Inc.	USA
Bacillus lichenformis SB3086	Turf Grass, Sclerotinia	Novozymes Biologicals, USA	USA
Bacillus pumillus GB34	Soybean fungal diseases	Gustafson, Inc.	USA
Bacillus subtilis GB03	Cotton, legume fungal diseases	Gustafson, Inc.	USA
Bacillus subtilis GB03, other *B. subtilis B. lichenformis, B. megaterium*	Fungal pathogens of greenhouse and nursery plants.	Growth Products Ltd	
Bacillus subtilis QST 713	Vegetables, Fruits	AgraQuest	USA
Coniothyrium minitans	Root rot	Prophyta Biologischer	Germany
Coniothyrium minitans	Root rot	Bioved	Hungary
Escherichia coli phage	Bacterial pathogens of fruit and vegetables	Intralytix	USA
Fusarium oxysporum non-pathogenic	Wilt	S.I.A.P.A.	Italy
Fusarium oxysporum non-pathogenic	Wilt	Natural Plant Protection	France
Gliocladium catenulatum	Vegetables, Fruits	ArgaQuest	
Gliocladium catenulatum	Root rot wilt	Verdera	Finland
Listeria monocytogenes phage	Bacterial pathogens of fruit and vegetables	Micreos	The Netherlands
Listeria monocytogenes phage	Bacterial pathogens of fruit and vegetables	Intralytix	USA
P. fluorescens A506, and 1629RS *P syringae* 742RS	Certain fruits, almond, potato, tomato	Frost Technol Corp.	
Pseudomonas aureofaciens Tx-1	Fungal opathogens of turf Grass	Turf Science Laboratories	
Pseudomonas chlororaphis 63–28	Wilt diseases of ornamentals and vegetables in GH	Turf Science Labs	
Pseudomonas syringae	Pome fruit, citrus, cherries, potatoes	JET Harvest Solutions	
Pseudozyma flocculosa	powdery mildew	Plant Products	Canada
Pythium oligandrum	Root rot	Bioreparaty	Czech Republic
Salmonella phage	Bacterial pathogens of fruit and vegetables	Intralytix	USA
Streptomyces griseoviridis	Vegetables, Fruits	ArgaQuest	
Streptomyces griseoviridis K61	Field ornamental, vegetable fungal pathogens	AgBio	
T. harzianum	Grey mold	Makhteshim Chemical Works	Israel
T. harzianum ATCC20476 *Trichoderma polysporum* ATCC20475		Binab	Sweden
T. harzianum T-22	Root rot	Bioworks	USA
Trichoderma harzianum	Vegetables, Fruits	ArgaQuest	
Trichoderma harzianum	Root rot	Efal Agri	Israel
Trichoderma spp	Root rot wilt	Binab	Sweden
Trichoderma spp.	Root rot wilt	Bioplant	Denmark
Trichoderma spp.	Root rot	Agrimm Technologies	New Zealand
Trichoderma virens GL-21	Root rot	Certis	USA
Trichoderma viride	Root rot wilt	Ecosense Laboratories	India
Xanthomonas campestris phage *Pseudomonas syrinage* phage	Bacterial pathogens of fruit and vegetables	Omnilytics	USA

2.3.1 Effectiveness of biological control

The degree of disease control obtained by applying BCAs to a crop has been documented to be equal to or comparable to that achieved by applying fungicides (O'Brien, 2017). The use of *Bacillus amyloliquefaciens* as BCA against *Penicilium digitatum* in mandarin fruit resulted in 77% suppression of the disease in comparison to 96% by the use of fungicide imazalil (Hao *et al*., 2011). By adding a fungicide, the efficacy of the BCAs may be improved, providing that the fungicide does not negatively affect the BCAs. In control of *Botrytis cinerea*, the pathogen of strawberry, application of *T. atroviridae* reduced the disease to a lower level, but the application of fungicide together with the BCAs is able to eliminate the pathogen (Card *et al.*, 2009).

On top of that, by using a mixture of BCAs and fungicide to inhibit powdery scab in potato, Nakayama (2017) reported a similar improvement in disease control. Under greenhouse conditions, the development of three pathogens, *Fusicoccum aesculili*, *Phomopsis macrospora* and *Cytospora chrysosperma* was suppressed by a *Bacilus pumilis* strain isolated from the poplar endosphere (Ren *et al.,* 2013). Although comparative disease suppression experiments are conducted in glasshouse and field trials, it should be noted that the level of disease control appears to be lower in field trials. This can be seen in the study conducted by Fu *et al.* (2010) in which the suppression level of *Pseudocercospora musae,* the pathogen of banana by *Bacillus subtilis* B106 was observed to be 24% lower in the field. This may be attributed to numerous environmental factors.

2.4 *TRICHODERMA* SPP. AS A BIOLOGICAL CONTROL AGENT (BCA)

Trichoderma is a fungal genus that was first described in 1794 and it can be found in all types of soil, manure and decaying plant tissues. The fungi have also been isolated from uncommon sites such as shellfish, termites and marine bivalves (Błaszczyk *et al*., 2014). It is classified as imperfect fungi that belong to the order

Hypocreales of Ascomycota. In 1865, the Tulasne brothers suggested a relation to the sexual state of a *Hypocrea* species. Nevertheless, it was difficult to morphologically differentiate the numerous species belonging to the genus *Trichoderma*/*Hypocrea* (Samuels, 2006). It was not until 1969 that the development of an identification concept was introduced, and several new *Trichoderma*/*Hypocrea* species were subsequently identified. By 2006, there are over 100 phylogenetically described species of the genus (Druzhinina *et al*., 2006). They are characterized by rapid growth, profuse conidial spores and the ability to produce sclerotia (Schmoll and Schuster, 2010). They produce several pigments, from greenish-yellow to reddish tinge but some colourless species are also present while the conidia have various colouration from colourless to different hues of green colour (Błaszczyk *et al*., 2014) (Figure 2.3).

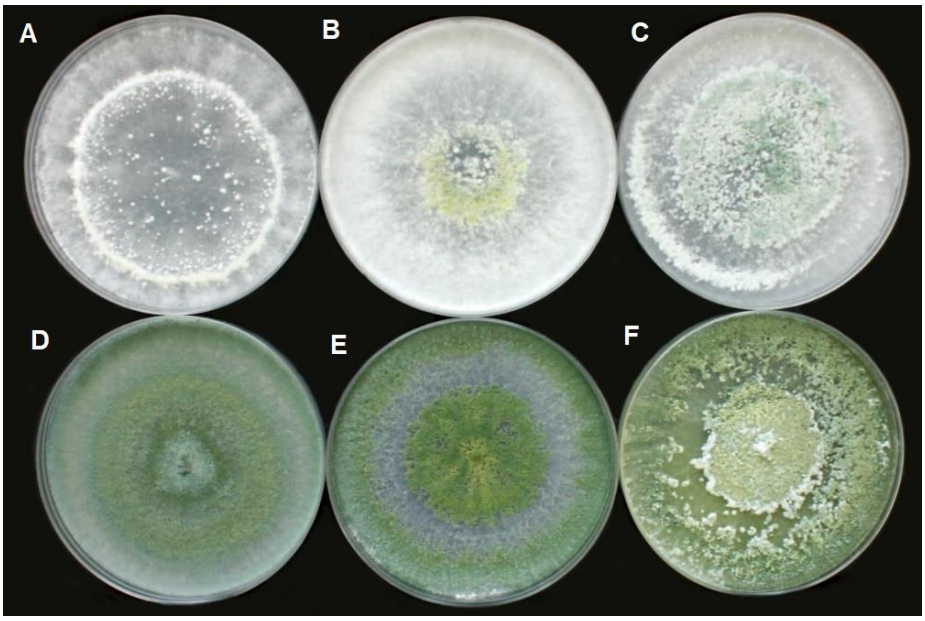


Figure 2.3 The variety in morphology and colours of *Trichoderma* spp. grown on potato dextrose agar (PDA). (A) *T. cremeum*, (B) *T. longipile*, (C) *T. viride*, (D) *T. harzianum*, (E) *T. atroviride* and (F) *T. citroviride* (Błaszczyk *et al*., 2014).

Trichoderma spp. are extremely effective colonisers in their environments, expressed in both their productive use of the substrate on hand and in their ability to secrete enzymes and antibiotic metabolites. By controlling growth, conidiation and enzyme production, they conform to their surroundings and thereby adjust

their lifestyle to the present circumstances that can be used for humanity's advantage (Schmoll and Schuster, 2010). The species in this genus are rapidly growing saprophytes which can be detected by coconut-like odour due to the secretion of volatile compounds like 6-pentyl-α-pyrone (Schmoll and Schuster, 2010; Waghunde *et al*., 2016).

Trichoderma spp. have been extensively researched to enhance plant growth, parasitism of other fungi and competition with plant pathogens in order to be used as biological control agents and biofertilizers (Akladious and Abbas, 2012). Some species of *Trichoderma* can colonize plant root surfaces, thus interact with the plants and exchange compounds that can cause changes in plant metabolism. This interaction increases the availability of nutrients, improves crop yield and improves disease tolerance (Harman *et al*., 2004; Haque *et al*., 2010). Recently, biofertilizers containing microorganisms such as *Trichoderma* have emerged as an integral component of the integrated supply chain of nutrients, as they hold a significant promise to increase the quality and yield of crops. Other than that, the successful use of biofertilizers enriched with *Trichoderma* will minimise the use of nitrogen (N) fertilisers, increase soil quality and reduce soil-borne pathogens (Haque *et al*., 2010).

2.4.1 Mode of action of *Trichoderma*

In the early 1930s, *Trichoderma*'s ability as a BCA was first presented and several studies indicated that many species of *Trichoderma* are capable of controlling and managing various genera of seed, foliar and soil-borne pathogens (Ghazanfar *et al*., 2018). *Trichoderma* expresses cell wall degrading enzymes (CWDEs) like chitinases, cellulases and glucanases as a secondary opportunistic invader. Also, this genus produces a lot of spores as well as antibiotics. It has been reported that many species of *Trichoderma* are 'rhizosphere competent', in which they have the ability to break down hydrocarbons, polysaccharides, chlorophenolic compounds and xenobiotic pesticides that are used in agriculture (Harman *et al*.,

2004). *Trichoderma* spp. are environmentally friendly and can act as an alternative to synthetic toxic chemicals. Apart from that, it formed a symbiotic rather than parasitic relationship with plants (Ghazanfar *et al*., 2018). *Trichoderma* employs various biocontrol mechanisms against phytopathogens and this can be seen in Figure 2.4.

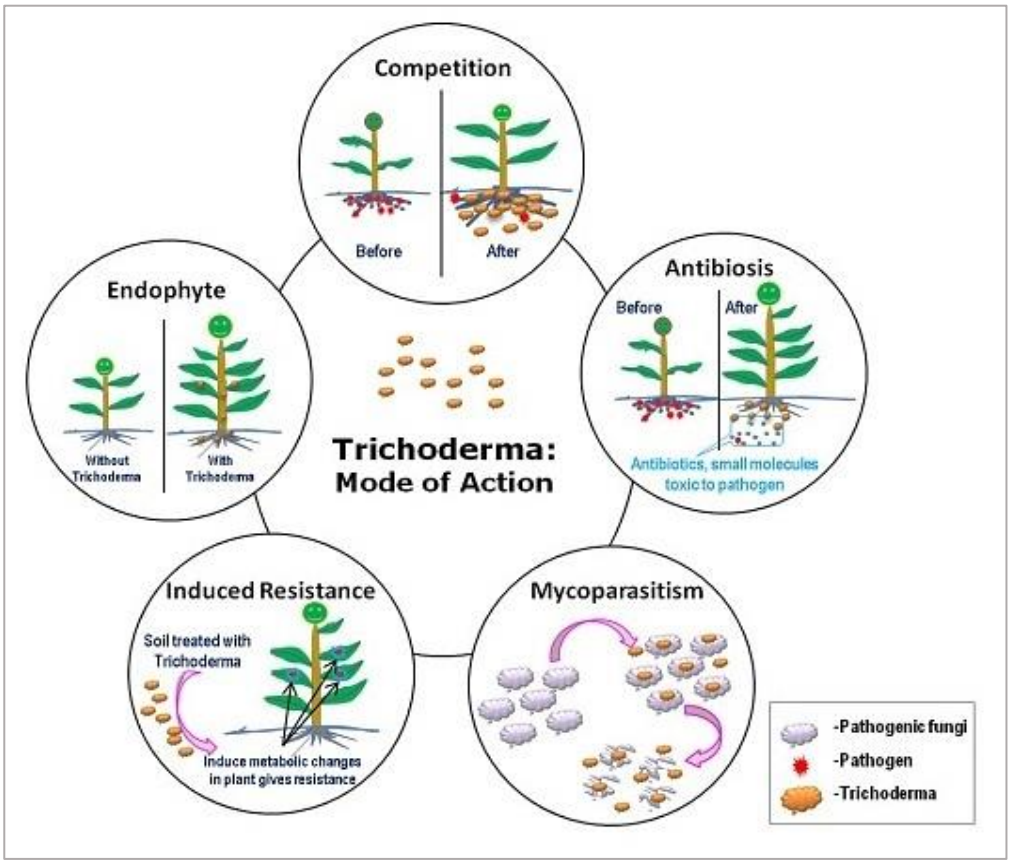


Figure 2.4 Model depicting the interaction of *Trichoderma* spp. with the pathogen and improvement in plant growth (Waghunde *et al*., 2016).

2.4.1.1 Competition

Trichoderma has been well known to compete with the pathogens in plant rhizosphere for nutrients, biological niches or infection sites. In contrast to other rhizospheric microorganisms, *Trichoderma* seems to be more capable of mobilising and absorbing soil nutrients. Competition of *Trichoderma* spp. with other pathogens for nutrients (carbon and iron), which are the two essential elements for the viability of filamentous fungi (Waghunde *et al*., 2016), is known to be one of the

effective strategies for the control and management of pathogens (Sood *et al*., 2020). *Trichoderma* has the capability to utilize different kinds of sugars that are available in the fungal environment such as glucan, chitin and cellulose, converting them into glucose to obtain the ATP (adenosine triphosphate) energy (Chet *et al*., 1997). Even though the role of glucose transport system in *Trichoderma* is yet to be explored, it is undeniable that it plays a crucial role in the survival of *Trichoderma* as it has been reported that *Trichoderma* competes with *Fusarium oxysporum*, *Rhizoctonia* solani and other pathogenic fungi for carbon sources (Sood *et al*., 2020).

In iron (Fe) deficient environment, fungi will release ferric-ion specific chelators to mobilize Fe ions from its surrounding. Certain *Trichoderma* isolates are able to produce highly effective chelators for Fe known as siderophores, depriving other fungi and hindering their growth (Waghunde *et al*., 2016; Sood *et al*., 2020). Siderophores also convert the insoluble Fe^{3+} ions into soluble form (Fe^{2+} ions), making them available for plant's root absorption (Sood *et al*., 2020). Moreover, in order to mobilize the immobile nutrients, *Trichoderma* releases fumaric, gluconic and citric acids which are organic acids that lower the soil pH. This enables the solubilisation of essential micronutrients and mineral cations (phosphates, Fe, magnesium and manganese) for plant metabolism (Harman *et al.*, 2004).

2.4.1.2 Antibiosis

The process involving diffusible compounds of low molecular weight to slows or fully inhibit the growth of other microorganisms is known as antibiosis. There are about 180 secondary metabolites that have been isolated from the species of *Trichoderma* (Reino *et al*., 2008). Among these are several antibiotics against fungal pathogens such as viridin, viridol, gliotoxin, gliovirin, koninginins, peptaibols and pyrones (Howell, 2003; Harman *et al*., 2004). Peptaibols have also shown potent activity against certain viruses and gram-positive bacteria (Błaszczyk *et al*., 2014). Meanwhile, gliotoxin and gliovirin belong to P and Q group strains

respectively of *Trichoderma* (*Gliocladium*) *virens* in which the P group negatively affect *Pythium ultimum* while the Q group was active against *Rhizoctonia solani* (Howell *et al*., 2000).

Several *Trichoderma* species (*T. viride*, *T. virens*, and *T. koningii*) are known to produce viridins that can inhibit spore germination in *F. caeruleum*, *Colletotrichum lini*, *Botrytis allii*, *Aspergillus niger*, *Peniclium expansum* and *Stachybotrys atra* (Singh *et al*., 2005). Similarly, harzianic acid obtained from *T. harzianum* exhibits antibiotic activity against *R. solani*, *Pythium irregular* and *Sclerotinia sclerotiorum* (Manganiello *et al*., 2018). In general, antibiosis is typically combined with lytic enzymes since the dual activity resulted in more effective antagonism against plant pathogens as compared to the individual function of antibiotics or enzymes. This can be seen in the control of *F. oxysporum* and *B. cinerea* by *Trichoderma* where lytic enzymes caused the initial disintegration of the fungal cell walls and enhanced the secretion of antibiotic into the target hypha (Howell, 2003).

2.4.1.3 Mycoparasitism

Hyperparasitism or mycoparasitism is a complex event in which it involves direct contact between the antagonist and the pathogen. Around 75 species of *Hypocrea*/*Trichoderma* have been recorded with mycoparasitic potential and various studies have shown that *Trichoderma* has the potential to attack and disintegrate phytopathogens that include *Alternaria alternata*, *S. sclerotiorum*, *R. solani*, *Ustilago maydis*, *Fusarium* spp. and *Pythium* spp. through mycoparasitism (Harman *et al*., 2004; Druzhinina *et al*., 2011).

This complex interaction involves sequential events in which *Trichoderma* will first need to sense and detect the signals from the host fungus before making contact, triggering morphological changes that involve coiling and appressorium formation that leads to the formation of a hole on the surface of targeted fungi.

This enables *Trichoderma* hyphae to penetrate the host's lumen, and then continues to destroy the target fungi. The host's cell wall contents are digested by *Trichoderma* CWDEs which inhibit the hyphal growth and eventually result in parasitism of the host fungi (Harman *et al*., 2004; Kumar, 2013). A number of chitinolytic enzymes and ß-1,3-glucanases play a significant role in mycoparasitism of *Trichoderma* and the latter has been observed to break down the cell walls of filamentous fungi, yeast and bacteria (Sood *et al*., 2020). Aside from CWDEs production, *Trichoderma* inactivates the enzymes of pathogenic fungi (e.g. pectinases, etc.) that are used for colonization and penetration of plant tissues (Harman *et al*., 2004).

2.4.1.4 Induced resistance

The addition of numerous species of *Trichoderma* to the rhizosphere has enhanced plant defence against many pathogenic organisms by inducing the development of various plant resistance mechanisms, primarily induced systemic resistance (ISR), hypersensitive response (HR) and systemic acquired resistance (SAR) (Harman *et al*., 2004). This response seems to be induced in a variety of crops; for instance, induced resistance was exhibited in plants as diverse as maize and tomato by *T. harzianum* T-22, suggesting the induced resistance in plant by *Trichoderma* spp. has little or non-host specific (Waghunde *et al*., 2016).

Resistance induction in plant can be attributed to the increase in the concentration of defensive metabolites and enzymes including phytoalexins, PAL, chalcone synthase (CHS), chitinase and glucanase (Djonović *et al*., 2006; Seidl *et al*., 2006). When chitinase (Chit42) derived from *T. harzianum* was expressed in potato and tobacco plants, the transgenic lines were totally resistant or became extremely tolerant towards soil-borne and foliar pathogens (*R. solani*, *B. cinerea*, *A. alternata* and *A. solani*) (Howell, 2003). Other than that, the use of elicitor filtrate from *T. harzianum* (PDBCTh10 isolate) against *Phytophtora capsici* that causes root rot in pepper plant was explored. Pepper seedlings that were treated with the

elicitors showed reduced infection up to 23% in comparison to control treatment (93% infection rate) (Sriram *et al*., 2009).

2.4.1.5 Endophytes

Endophytes are organisms that are able to form colonies inside plant tissues without causing any disease or harming the plants. Endophytic organisms can stimulate the expression of numerous plant genes that regulate stress responses. Besides, it was observed that colonization of the roots of crop plants by certain species of *Trichoderma* causes the up-regulation of genes and pigments which resulted in improvement in the photosynthesis of plants. When plants are under stress, their photosynthetic capability is reduced because of the damage to the photosystems caused by reactive oxygen species (ROS). *Trichoderma* helps to alleviate this problem by activating biochemical pathways that convert ROS to less harmful molecules (Harman *et al*., 2019).

Recently, endophytic *T. citrinoviride* was isolated from the ginseng roots that were grown in the mountains. This *Trichoderma* species showed great potential as a biological control agent against the pathogens of ginseng plant, particularly *Cylindrocarpon destructans* and *B. cinerea*. In addition, *T. citrinoviride* induced the synthesis of ginsenocides which are bioactive metabolites with pharmacological properties in ginseng (Park *et al*., 2019). In sugarcane, *Trichoderma* is stated to be the fourth most abundant endophytes while Joshi *et al*., (2019) were the first to report the presence of *T. longibrachiatum* and *T*. *asperellum* as endophytic microorganisms in sugarcane. The biocontrol activities of these two species were also evaluated against *Colletotrichum falcatum*, which is a pathogen for sugarcane. With the exception of *T*. *stromaticum*, *T*. *koninggiopsis* and *T. stilbohypoxyli*, phylogenetic review classifies all recognized endophytic *Trichoderma* as a separate taxon, indicating that endophytism of *Trichoderma* has been recently evolved within this genus (Druzhinina *et al*., 2011).

2.4.2 Interaction between *Trichoderma* spp. and plants

Apart from controlling pathogens, *Trichoderma* helps to improve plant and root growth and enhances plant defence mechanisms. As *Trichoderma* is drawn by chemical signals emitted from plant root, subsequent attachment, penetration and colonisation of *Trichoderma* within plant roots establish symbiosis relationship between *Trichoderma* and host plants. *Trichoderma* produces hydrophobin, which is a cysteine-rich protein that facilitates the root anchoring process and expansin-like proteins help root invasion (Sood *et al*., 2020). *Trichoderma*'s rapid colonisation of root tissues is accomplished by reducing plant defences, such as reducing phytoalexin production. This phenomenon was previously described by Masunaka *et al*. (2009) in the penetration of *Lotus japonicus* roots by *T. koningii*. Furthermore, *Trichoderma* interacts with other beneficial microorganisms in the rhizosphere which also helps to improve plant growth and survival (Lace *et al*., 2015).

2.4.2.1 Influence on the germination of seeds

Trichoderma-seed treatment and biopriming allow plant hormones and enzymes that play a role in seed germination to be released and/or produced. This has also resulted in a quicker seed germination rate and increased vigour of the seedlings, so seed-borne pathogens such as *Pythium* is not able to invade the host (Mastouri *et al*., 2010). *T. harzianum*-producing gliotoxin has been reported to mimic gibberellic acid, the plant growth hormone that plays a role in seed germination (Bezuidenhout *et al*., 2012). In addition, it has been shown that *Trichoderma* has increased the rate of seed germination of various crops such as chickpea, tomato, okra, maize, chilli and mustard (Singh *et al*., 2018).

2.4.2.2 Improvements in plant morphology and physiology

There are reports that mentioned *Trichoderma* application to plant rhizosphere has improved plant morphology such as height, biomass, leaves number, root-shoot ratio, etc. (Halifu *et al*., 2019; Sood *et al*., 2020). For example,

in *Arabidopsis thaliana*, the number of root hairs and the lateral roots increased when the soil was inoculated with *T. atroviride* (Contreras-Cornejo *et al*., 2015) whereas in cabbage, *T. tomentosum* and *T. longipile* have significantly increased the fresh weight and the total leaf area of treated plants in comparison to the untreated ones (Rabeendran *et al*., 2000).

Aside from that, *Trichoderma* positively influences plant physiological processes such as photosynthesis (as mentioned in Section 2.4.1.5), stomatal conductance, water use efficiency, absorption and assimilation of nutrients and many more. *Trichoderma* releases cellulase that degrades cellulose in the rhizosphere and increases organic matter and nutrients contents in the soil (Singh *et al*., 2018). The uptake of magnesium (Mg), the main constituent of chlorophyll involved in catalysing enzymatic activity and regulating genes involved in photosynthesis, was significantly improved by *Trichoderma* treatment (Sood *et al*., 2020). Furthermore, *Trichoderma*-treated rice plants showed a three-fold increment in stomatal conductance and photosynthetic rate and a two-fold increment in water use efficiency relative to treatment with nitrogen, phosphorus and potassium (N, P and K) (Doni *et al*., 2014). In another study of rice plants, plant senescence was delayed while the water holding capacity and drought tolerance was improved in *T. harzianum* treated plants (Shukla *et al*., 2012).

2.4.2.3 Improvements in yield and product quality

The effect of *Trichoderma* on seed germination, plant morphology and physiology accelerates vegetative and reproductive growth of the plant and contributes to improved field stand. Hence, this improves the number of plant leaves, flowers and fruits, as well as the increment in the mean weight of individual fruit (Singh *et al*., 2018). Nevertheless, as *Trichoderma* alters the proteome and transcriptome of plants which in turn benefit the plant itself, the mechanisms involved in *Trichoderma*-plant interactions are still not very clear. It has been documented that wheat treated with *T. harzianum* (Th3) has substantial

improvement in yield under field conditions and this is directly reflected in an increase in farmers' overall income (P. Sharma *et al*., 2012). In addition, there are various studies on the effect of treatments with *Trichoderma* and improvement in the yield of the plants such as corn, sugarcane, tomato, mustard, etc. (Singh *et al*., 2018; Sood *et al*., 2020).

2.4.2.4 Plant tolerance to abiotic and biotic stress

Plants are constantly subjected to diverse abiotic and biotic stresses. Under stressful environments, it has been proven that different *Trichoderma* strains are able to improve plant's growth and reproduction. There are few reports where *T. harzianum*, *T. asperellum* and *T. asperolloides* alleviate the salinity stress on rice, cucumber and *A*. *thaliana* respectively (Rawat *et al*., 2012; Brotman *et al*., 2013; Qi and Zhao, 2013). Besides mitigating chilling stress in tomatoes, *T. longibrachiatum* ameliorates oxidative stress in plants by increasing the gene expression of antioxidants peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) (Zhang *et al*., 2016).

As described in Section 2.4.1.4, *Trichoderma* helps plants to deal with biotic stresses through induced resistance. *Trichoderma* has the ability to release MAMPs (microbe-associated molecular patterns) which are conserved molecular signatures in microbes' population such as flagellin in bacteria and chitin in fungi. The release of molecular recognition MAMPs would result in a signal cascade through the signalling molecules of plants such as jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) (Singh *et al*., 2018). *T. asperellum* produces an induced systemic resistance (ISR) via SA signalling cascade in *Arabidopsis* root, whereas both the SA and JA/ET signalling pathways involved in the signalling of ISR by cell-free culture filtrates of *Trichoderma* (Yoshioka *et al*., 2012).

Even though the plant is pathogen-free, *Trichoderma* is able to induce long-term upregulation of SA gene markers. Therefore, when the plant is challenged by

a pathogen, the SA-dependent gene expression can be modulated by pre-treatment with *Trichoderma* and the expression of defence genes caused by the JA signal transduction pathway occurs soon after infection, allowing ISR to increase over time (Tucci *et al*., 2011). Priming for improved defence is a general trait of mediated resistance responses of beneficial microbes. Defence responses are not triggered immediately in primed plants but are increased by pathogens or other stimuli upon attack, resulting in quicker and stronger resistance to the intruder (Van Wees *et al*., 2008). This induces plant immunity against pathogens and increases the growth of plants in exchange.

2.4.3 *Trichoderma* spp. for the control of BSR disease in oil palm

The potential of *Trichoderma* spp. as a BCA has been demonstrated on a variety of plant diseases including BSR disease in oil palm. Moreover, this biological antagonist is considered to be able to successfully control *Ganoderma* (Soepena et al., 2009). *Trichoderma* spp. such as *T. harzianum*, *T. asperellum*, *T. viride* and *T. virens* had been applied as a single BCA or in combination with other BCAs (*Bacillus* spp., *P. aeruginosa,* etc.) against *G. boninense in vitro* (Musa *et al*., 2018; Haryadi *et al*., 2019; Muniroh *et al*., 2019) and in nurseries (Nur Ain Izzati and Abdullah, 2008; Sundram *et al*., 2008; Nusaibah *et al*., 2017). In Indonesia, the oil palm field treated with *T. harzianum* showed a significant disease reduction as compared to the untreated field (Susanto *et al*., 2005). Moreover, Alexander *et al*. (2017) reported that oil palm field experiments using TR1, a commercial product containing *Trichoderma* spp. and *Bacillus* spp. was able to decrease *G. boninense* colonisation on the basis of reduction of the content of ergosterol and re-isolation of the pathogen using *Ganoderma* selective medium (GSM).

In a study conducted by Naher *et al*. (2011), the root tissues of the oil palm treated with either *G. boninense* or *T. harzianum* or a mixture of both, chitinases that play a role in the inducible plant defence response were upregulated. However, further research on oil palm leaves revealed contrasting results in which plants

exposed to *G. boninense* did not show a significant increase in chitinase expression. Only *Trichoderma*-treated plants showed higher expression of chitinases, which suggested that plants with low expression of chitinases during BSR infection may not be sufficient to ward off pathogen (Naher *et al*., 2012). Furthermore, analysis of the root transcriptome of oil palm seedlings indicated that *Trichoderma* regulates genes linked to phytohormone biosynthesis, antioxidant, unique secondary metabolites as well as phytotoxic compound detoxification. This finding shed light on the biocontrol mechanisms employed by *T. harzianum* against the causal agent of BSR disease (Ho *et al*., 2018).

2.4.4 Production and formulation of *Trichoderma* spp.

The effectiveness of any biological control agent depends not only on its virulence, but also on its efficiency of mass production, maintaining cost and shelf life. Regardless of the choice of organisms, a significant criterion for effective application of BCA is the preparation of microbial biomass with sufficient population counts that is viable and vigour. If the biocontrol agent is identified and proven to be successful over multiple reproducible outcomes against plant pathogens, then the processes of mass development, fungal formulation with carrier and application of BCA products shall be followed. Hyphae, conidia and chlamydospores are the three types of propagules that can be used for the production and formulation of *Trichoderma*, and either solid or liquid fermentation can be considered for the development of sufficient quantities of viable and active *Trichoderma* inocula (Cumagun, 2014). Solid fermentation involves the use of substrates, whereas for submerged fermentation, microorganisms are grown in liquid media (Ramanujam *et al*., 2010).

2.4.4.1 Solid-state fermentation

The market potential of *Trichoderma* is increasingly growing each year. Mass production of inoculum is depending mainly on solid-state fermentation, more

specifically through the use of solid substrates involving cereal grains (Mascarin *et al*., 2015), in which sorghum seems to be very useful and a cheaper option (Waghunde *et al*., 2016). The grains are moistened, sterilized and inoculated with *Trichoderma* and the incubation will take around 10-15 days. This leads to the coating of the grains with dark green spore layers of *Trichoderma* (Kumar *et al*., 2014). Mass production of *Trichoderma's* aerial conidia using cereal grains is a well-known approach generally used for the solid-state fermentation of biological control agents (BCAs) such as *Metarhizium anisopliae*, *Bauveria bassiana* and *Clonostachys rosea* (Mascarin *et al*., 2015; Zhang *et al*., 2015). Through this method, usually the aerial conidia obtained from the mass production are of high viability and effectiveness (de Rezende *et al*., 2020).

Apart from using cereal grains, some other economical media that can be used for the production of these fungi are agricultural wastes (groundnut shells, sugarcane bagasse, ground corn cob, spent malt, tapioca rind, tea waste, etc.) and those materials high in organic matter (farmyard manure, neem cake, cow dung, poultry manure, goat manure, peat, etc.) (Cumagun, 2014; Waghunde *et al*., 2016). This production method requires only minimal costs, particularly in small-scale manufacturing. However, it needs sufficient space for production, inoculation and storage (Cumagun, 2014). Additionally, it is labour-intensive, time-consuming and it is difficult to standardize fungal inoculum through different production batches, which can contribute to uneven consistency and uniformity of the product (de Rezende *et al*., 2020).

2.4.4.2 Liquid fermentation

Liquid fermentation is a more promising choice to overcome the limitations of solid-state fermentation as it is cost-effective and efficient in mass production required for agricultural uses (de Rezende *et al*., 2020). It can be divided into two categories known as 1) submerged liquid fermentation where the fungus is submerged to form microcycle conidia, blastospores or microsclerotia in a

continually agitated and aerated liquid media; and 2) stationary liquid fermentation, where mycelium and aerial conidia are formed by sporulation on a still, liquid surface (Jaronski and Mascarin, 2017). Liquid media such as potato dextrose broth, molasses-yeast medium, V8 juice and wheat bran are generally used for the production of *Trichoderma* (Cumagun, 2014; Kumar *et al*., 2014; Waghunde *et al.*, 2016).

Experiments have been carried out on various biocontrol agents in recent years, primarily entomopathogens, by using liquid substrates for aerobic fermentation in bioreactors (Jaronski and Mascarin, 2017). The number of studies to achieve multiple propagules have also increased for *Trichoderma* (Watanabe *et al*., 2006; Kobori *et al*., 2015; Mascarin *et al*., 2015; de Rezende *et al*., 2020). The benefits of this type of fermentation are that the production procedures can be controlled and optimized with a consistent volume of initial inoculum, which makes it easier to monitor the nutrients, temperature, pH and other factors during liquid fermentation while minimizing contamination (Cumagun, 2014).

2.4.4.3 Formulation of *Trichoderma* spp. with carrier media

Mass production of biological control agents should be followed by the formulation of the products in which the type of application will influence the type of formulation. This involves the mixing of active ingredients (e.g. pure spores, conidia suspensions, liquid culture filtrates, etc.) with inert/carrier materials in order to allow the biocontrol agents to remain viable during a prolonged period of storage (Cumagun, 2014). In general, products derived from solid or semi-solid fermentation may not need specialised formulation with carrier medium before use. Before application, the solid medium where *Trichoderma* is grown in solid-state fermentation may be dried or grounded.

For liquid fermentation, *Trichoderma* biomass can be extracted and concentrated with or without the liquid media. If the *Trichoderma* biomass is

extracted together with media, then it can be integrated into pellets, wettable powders, granules, dust or emulsifiable liquids (Ramanujam *et al*., 2010).

Furthermore, according to Cumagun (2014), the final formulation should be simple to manage and stable over a range of temperatures (-5 to 35 °C). Also, the viability of BCAs should not be compromised by the carrier materials. About 55% of *Trichoderma* formulations are sold in wettable powder, where it contains a certain concentration of dried conidia in fine dust to be combined with water. The next widely used formulation is in granular form at 13.6%, followed by liquid and solid forms at 10.3% and 6.2% respectively (Woo *et al*., 2014). To date, *Trichoderma*-based products are widely commercialized to protect crops from various diseases or to improve plant growth and productivity.

2.4.5 Application methods for *Trichoderma* spp.

For effective disease control, the delivery and establishment of *Trichoderma* spp. to the site of action is very crucial. Depending on the types of formulation, liquid products may be used for soil amendment, seed treatments or foliar spray while solid products are mainly for seed or soil treatments (Berninger *et al*., 2018).

2.4.5.1 Seed treatment

One of the simple and efficient ways of supplying antagonist for soil/seed-borne disease prevention in plant is to coat the seeds with *Trichoderma.* Before sowing, the seeds shall be covered with a layer of dry *Trichoderma* powder or dust (Kumar *et al*., 2014). *Trichoderma* propagules have been reported to germinate on the pigeon pea seed surface and colonised the roots rhizosphere of the germinated seedlings (Kumar *et al*., 2009). Seed treatment in wheat with *T. longibrachiatum* had improved seed germination, shoots and roots weight of wheat seedlings, under both control and salt-stress conditions. In addition, the transcriptional level of ROS was induced in *Trichoderma*-treated seeds, enhancing the wheat seedlings to be tolerant to salt stress (S. Zhang *et al*., 2019). Moreover, aflatoxin contamination

caused by *Aspergillus flavus* and *A. parasiticus* were reduced when the groundnut seeds were treated *T. harzianum*. Mycoparasitism was involved to suppress the pathogens in groundnut seeds as it was revealed through environmental scanning electron (ESEM) that *Trichoderma* coiled around the hyphae of *Aspergillus* (Chiuraise *et al*., 2015).

2.4.5.2 Root treatment

By using spore or cell suspension of the antagonists, the roots of seedlings can be drenched with *Trichoderma* either in nursery beds or before transplanting (Kumar *et al*., 2014). This technique is suitable for rice and vegetable crops where transplanting is carried out and it is another means of *Trichoderma* application that aims to protect the plants from pathogens. This can be seen in tomato, chilli, aubergine, capsicum and rice in which there was a reduction in disease severity and enhancement in seedlings growth (Singh and Zaidi, 2002).

2.4.5.3 Foliar spraying

The liquid suspension of various *Trichoderma* has been successfully applied at the phyllosphere, mainly for the control of foliar pathogens. Foliar spray of *Trichoderma* on wheat and tomato decreased the severity of disease caused by *Fusarium graminearum* (Panwar *et al*., 2014) and *Xanthamonas perforans* (Chien and Huang, 2020) respectively. Besides, the P, K and Mg contents were significantly increased in tomato plants, where P and K can minimise host plant susceptibility while Mg has a direct effect on plant disease resistance (Chien and Huang, 2020). Also, the use of the *Trichoderma* consortium (*T. harzianum* and *T. asperellum*) for foliar treatment of *Passiflora caerulea* (passionflower) has resulted in higher contents of flavonoids and polyphenol, improved plant physiology and increased yield (Şesan *et al*., 2020). Nevertheless, it might not be practically feasible since a large volume and frequent application of *Trichoderma* suspension might be needed for the treatment to be continually effective (Kumar *et al*., 2014).

2.4.5.4 Soil treatment

The distribution of *Trichoderma* in the soil would increase the population dynamics of enhanced fungal antagonists and thus reduces the development of pathogenic microbes in the infection court. Several studies have been published on the use of biocontrol agents in the soil, either before or after planting, to treat a variety of soil-borne fungal diseases (Kumar *et al*., 2014). For example, He *et al*. (2019) used *T. asperellum* granules for the control of stalk rot caused by *F. graminearum* in maize. For the pot trial, 2 g/pot of *Trichoderma* granules were applied while for the field trial, the granules were combined with compound fertilizer and were added to the soil before sowing at a density of 75 kg ha^{-1}. Both pot and field trials showed a high disease control efficiency (~50%) while the plant height, weight and yield were significantly improved. Additionally, the control of *F. oxysporum* and *F. solani* by using three isolates of *Trichoderma* together with the fungicide thiophanatemethyl were also being explored. In greenhouse and field settings, all treatments resulted in a substantial reduction of damping-off and root rot/wilt diseases in dry beans with improved plant survival rates. Further analysis revealed that by activating the defence enzymes, including peroxidase, chitinase and polyphenol oxidase, the applied treatments triggered systemic defence responses in dry bean plants (Abd-El-Khair *et al.*, 2019).

2.5 THE ROLE OF MICROBIAL COMMUNITY IN DISEASE SUPPRESSIVE SOIL

As sessile organisms, plants are contained in one position and are not able to escape from their environment, including their encounters with microorganisms. These encounters or interactions between the plants and microorganisms may take place at various points such as in the rhizosphere or the aerial parts of the plants. The rhizosphere refers to the narrow soil zone adjacent to plant roots which are affected by root exudates and can support up to 10^{11} microbial cells per gram of root (Antoniou *et al*., 2017). The microbes that can be found in the rhizosphere are

known as root microbiome, with many microorganisms having a neutral effect on the plant while others may lead to positive or negative plant fitness outputs (Raaijmakers *et al*., 2009; Berendsen *et al*., 2012). The role of the root microbiome in plant health becomes more obvious in disease suppressive soils, where even in the presence of a pathogen in these soils, a plant is unlikely to become infected by the pathogen (Weller *et al*., 2002).

Disease suppressive soils have been recognised for over 100 years and can be identified as those the progression of the disease is limited, even in the presence of a virulent pathogen and a susceptible plant host. The mechanisms involved in suppressive soils include parasitism, competition and antibiosis (Chandrashekara *et al*., 2001) and this is related to the activity of indigenous microbiota (Weller *et al*., 2002; Haas and Défago, 2005). Most disease suppressive soil will lose its characteristics when sterilized or pasteurized, thus this highlighted the role played by microorganisms as the main contributor in disease suppression (Weller et al., 2002; Haas and Défago, 2005). Also, in disease suppressive soils, greater microbial diversity has been observed than in conducive (non-suppressive) soils (Garbeva *et al*., 2006).

Plants themselves can select the root microbiome by actively secreting compounds that can stimulate or suppress the microorganisms. The microbial population densities in the rhizosphere are far greater than in the adjacent bulk soil, as plants secrete their photosynthates (up to 40%) into the rhizosphere. However, the microbes in the rhizosphere are typically less diverse as compared to those in the bulk soil (Berendsen *et al*., 2012). Also, within species, different plant genotypes are able to develop unique root microbiome (Micallef *et al*., 2009). Besides plant genotype, the type of soil is another important factor that influences the composition of the microbial rhizosphere (Garbeva *et al*., 2008). The root microbiome of various plant species growing on the same soil is often distinct (Berg

et al., 2006; Garbeva *et al*., 2008) and certain plant species have been found to be capable of producing identical populations in various soils (Miethling *et al*., 2000).

2.5.1 Concept of disease suppressive soil

Soil suppressiveness towards diseases can be divided into general suppression and specific suppression. Generally, due to the presence and activity of soil microorganisms, all natural soils have the potential to inhibit phytopathogens' activities. It was observed that the disease progressed quickly and was more severe in pasteurized soil in comparison to the same natural soil after the artificial introduction of the phytopathogens to those soils. This phenomenon is known as general suppression and is assumed to be influenced by the overall microbial activity in the soil (Mazzola, 2002). As the name implies, general suppression is not specific and works against most, if not all pathogens (Chandrashekara *et al*., 2001).

For specific suppression, it only works against certain types of pathogens (Chandrashekara *et al*., 2001) and involves an individual or a certain group of microorganisms that acts to antagonize the pathogens (Mazzola, 2002). Contrasted to general suppressiveness, the suppressive factor could be passed to conducive soil by the addition of around 1-10% (w/w) of the suppressive soil (Mazzola, 2002; Weller *et al*., 2002). Nonetheless, for extended periods, some specific suppressive soil can maintain their characteristics and persist even when the soils are left bare, while some other soil established the specific suppressive characteristics after many years of crop monoculture. This is a remarkable process considering that monoculture practices can also lead to the build-up of a specialised group of plant pathogens in the rhizosphere (Bennett *et al*., 2012).

Soils that are disease suppressive have been reported for numerous diseases. For instance, the wilt disease in several plants species caused by *Fusarium* spp., potato scab disease in which the causal agent is *Streptomyces* species, *Rhizoctonia* damping-off disease in sugar beet and the take-all disease caused by

Gaeumannomyces graminis var. *tritici* in wheat (*Triticum aestivum*) (Weller *et al*., 2002; Raaijmakers *et al*., 2009; Mendes *et al*., 2011). Microorganisms that play a role in suppressiveness have been isolated from these types of soil. Among these, one of the best examples can be seen from take-all disease during monoculture of wheat, where the disease suddenly showed a decrement in incidence and severity after a serious outbreak of the disease. In a phenomenon known as 'take-all decline', it was contributed by the build-up of fluorescent *Pseudomonas* spp. in the soil as the disease suppressing agent. The pseudomonads produce an antibiotic known as 2, 4-diacetylphloroglucinol (DAPG) that is active against the pathogen apart from aggressively colonizing the roots and competing for organic nutrients (Weller *et al*., 2002; Heydari and Pessarakli, 2010). In addition, Deuteromycetes (*Trichoderma* and *Gliocladium*), Firmicutes (*Bacillus* and related genera) and Proteobacteria (especially *Burkholderia* and *Pseudomonas*) are among the group of microorganisms that can contribute to soil suppressiveness (Raaijmakers *et al*., 2009).

While the microbiomes of a broad variety of disease-suppressive crop soils have been documented, the soil microbiome associated with oil palm BSR disease is not well established (Goh *et al*., 2020). Most research on Malaysian and Indonesian soil focused on the effects of the land-use change on soil microbiome in which comparisons were made between soils from the oil palm plantation, logged forest and rainforest (Kerfahi *et al*., 2014; McGuire *et al*., 2015; Tripathi *et al*., 2016; Wood *et al*., 2017; Tin *et al*., 2018). In light of this, Goh *et al*. (2020) has identified the soil prokaryotic and eukaryotic populations with low and high BSR disease incidence in Blenheim soil and Bernam soil respectively from an oil palm plantation in Perak, Malaysia. In Blenheim soil (low BSR disease incidence), prokaryotic and eukaryotic diversities were greater with the inclusion of a greater relative abundance of bacterial taxa linked with suppression of diseases. Besides, Khunaw *et al*. (2017) have studied the influence of indigenous soil microorganisms

on the suppression of *Ganoderma* in oil palm. It was found that sterilization of soil has eradicated the soil microorganisms, which in turn did not reduce or suppress the development of *G. boninense*, resulted in higher disease severity and the growth of the seedlings were affected. Hence, this confirmed the role played by soil microorganisms in the suppression of the disease. In another study conducted by Shyen Lo and Chong (2019), even though the microbial diversity in the low and high BSR incidences soil did not differ significantly, the microbes in low BSR incidences soil showed greater metabolic activity for utilization of the carbon substrate. It was concluded that the presence of *Ganoderma* affects the catabolic activity and negatively influenced the soil microbial activity.

2.5.2 Interaction between BCAs and the soil microbial community

While certain microorganisms have the capability to either directly or indirectly protect the plants against phytopathogens, their effectiveness is strongly affected by the rest of the microbial community (Berendsen *et al*., 2012). In order to have a significant effect on the pathogen, the antagonist (BCA) itself should be present in relatively high numbers (Raaijmakers *et al*., 1995). Commensals microorganisms in which they neither benefit nor harm the plant directly can also compete with the BCA and the pathogen in the rhizosphere. It has been reported that during competitive interactions with other bacteria, non-antagonistic soil bacteria can become antagonistic and contribute to the suppression of pathogenic and saprotrophic fungi (De Boer *et al*., 2007). The pathogen can also encounter the antagonistic activity of the biocontrol agent, as in the case of *F. oxysporum* and *P. fluorescens* in which the pathogen downregulates the production of antibiotic compound DAPG through the release of fusaric acid (Notz *et al*., 2002). This shows that microorganisms can influence each other one way or another, albeit indirectly. Therefore, although specific microorganisms can have a specific function in an

environment, the total microbial communities and their interactions will impact plant health (Berendsen *et al*., 2012) (Figure 2.5).

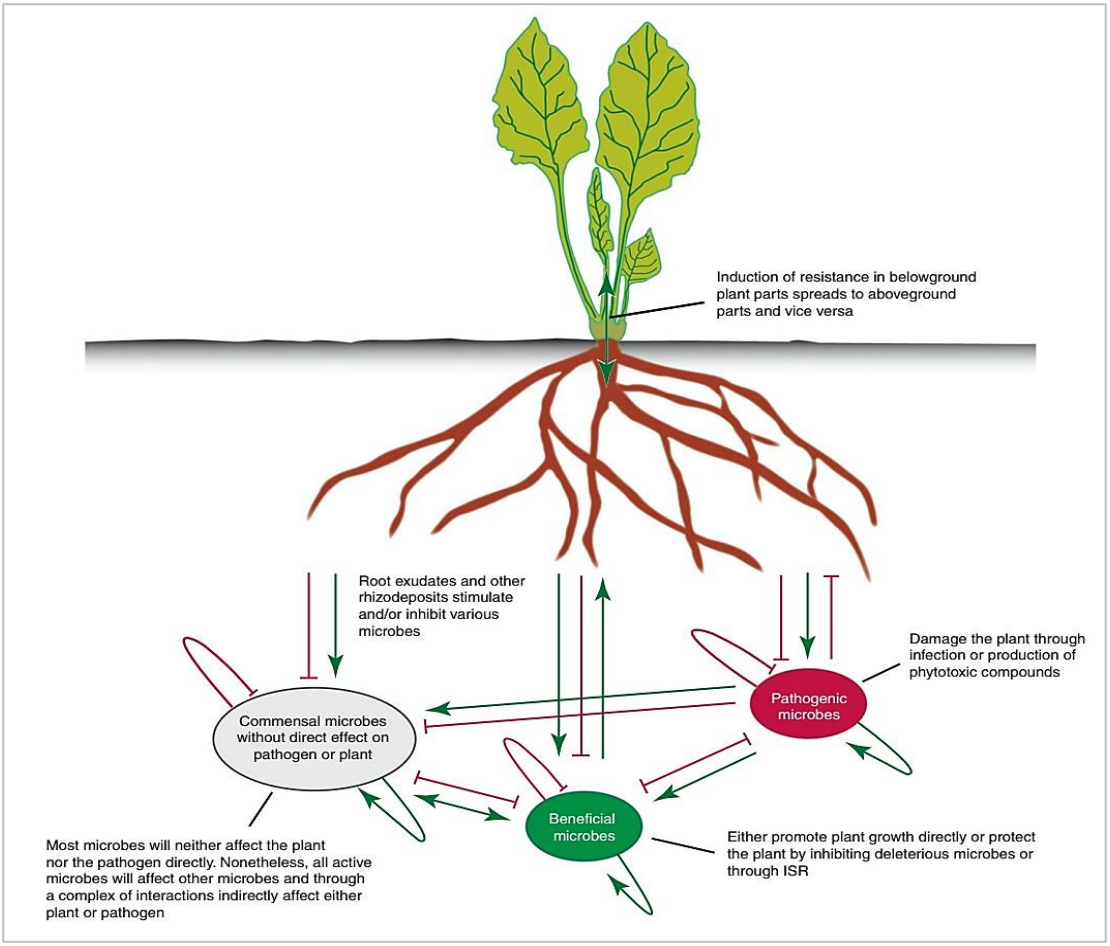


Figure 2.5 The biotic interactions in the rhizosphere. Plants have the capability to stimulate (green arrow) or inhibit (red blocked arrow) the microbiome in the rhizosphere via exudation of compounds. Vice versa, plant health can be affected by soil-borne pathogens. These deleterious microbes compete for resources and space with other microorganisms in the rhizosphere before infection. Meanwhile, beneficial microbes will restrict the development of the pathogen by producing biostatic compounds, absorption of the nutrients or inducing the plant's defence system. For the commensal microbes, as they occupy diverse ecological niches, they neither affect the plant nor the pathogen directly but they still likely to affect other organisms to some degree (Berendsen *et al*., 2012).

Since rapid colonization of the plant/host rhizosphere requires a high density of viable and effective BCA to be applied, this could lead to a transient disruption in the equilibrium of soil microbial communities. If essential native organisms are eliminated, shifts in the microbial composition can be unfavourable and hence affect subsequent crops growth (Trabelsi and Mhamdi, 2013). Nevertheless, ecosystem

resilience, which is affected by diversity and interactions between plants, soil, and microbes could buffer the change. Due to bacterial redundancy, the absence of such bacterial species does not alter the system from functioning, as various bacterial species may perform the same function (Kennedy, 1999; Nannipieri *et al*., 2003).

Many studies reported the interaction between *Trichoderma* spp. (as BCA) and soil microbial communities. Savazzini *et al*. (2008) showed that *T. atroviride* SC1 induced transient changes in soil microbes in the first two weeks after application, but over time it was found that environmental factors play a major role in the surveyed microbial communities as compared to the BCA treatment. In tobacco monoculture, *T. harzianum* helps to promote the growth of a particular bacterial species that changes the microbial structure (Gasoni *et al*., 2008) and also positively influenced the soil microbes which indirectly affecting the growth of Alfalfa (*Medicago sativa* L.) (Zhang *et al*., 2019). In contrast, the application of *T. atroviride* and *T. harzianum* did not cause any significant changes in the soil microbial communities (McLean *et al*., 2014; Ganuza *et al*., 2019). With regards to soil microbial community in an oil palm plantation, it was found that the fungal population increased after the application of BCAs-based product that consists of *Bacillus* spp. and *Trichoderma* spp. The BCAs might also induce the microbial community to an active state, as many new genus and species emerged which might be beneficial to the ecosystem and against phytopathogens (Alexander and Chong, 2014).

2.5.3 Approaches to studying disease suppressive soil

Historically, studies of soil microbial populations have been concentrated on the cultivation strategies by using a range of cultural media that intended to maximise the recovery of numerous microbial species. Nonetheless, with this method, only a small fraction (less than 0.1%) of soil microbial population was culturable (Hill *et al*., 2000). Another common culture-dependent approach is

known as community-level physiological profiles, where species of bacteria are classified on their use of numerous carbon sources. This technique is available commercially, known as BIOLOG® system and has been widely used for the identification of soil microbes (Garland, 1996; Hill *et al*., 2000). This method however, is suffering from the same bias problems associated with the culture plating approaches, hence making data interpretation difficult (Hill *et al*., 2000).

Furthermore, the composition of microbial communities can be estimated based on the extraction, quantification and recognition of molecules specific to microbial groups or certain microorganisms by culture-independent approaches. Among the molecules that can be targeted for such studies are nucleic acid and phospholipid fatty acids (PLFA) (Hill *et al*., 2000). PLFA is present in the cell membranes of microorganisms and can serve as essential markers of active microbial biomass, since it is rapidly metabolized after cell death. As PLFA are readily isolated from microbial cells, this provides access to a greater proportion of soil microflora as compared to the culture-dependant approach. When using these compounds to classify microbial populations, a major consideration is that particular fatty acids are indicative of specific classes of microorganisms. Even so, the signature PLFAs should be treated with caution because under all circumstances they might not be unique to certain communities and this may lead to the detection of a false community (Hill *et al*., 2000).

The use of ribosomal RNA (rRNA) genes as molecular markers was suggested by Carl Woese in the 1970s in conjunction with the Sanger sequencing method (Escobar-Zepeda *et al*., 2015). Advances in molecular techniques resulted in newer approaches to study microbial diversity, such as denaturing or temperature gradient gel electrophoresis (DGGE/TGGE), terminal restriction fragment length polymorphism (T-RFLP), real-time polymerase chain reaction (qPCR) and next-generation sequencing (NGS) (Trabelsi and Mhamdi, 2013; Escobar-Zepeda *et al*., 2015). For DGGE, the amplified DNA fragments were

separated based on denaturing gradient while TGGE is based on temperature gradient. Through analysis of bands that migrated separately, phylogenetic information on existing microbes and the structural changes in the population is acquired. Nevertheless, the DNA fragments to be analysed should be no more than 500 bp and often a single band do not imply a single strain of microbes being detected (Trabelsi and Mhamdi, 2013).

In a typical T-RFLP analysis, the gene of interest is amplified and later digested before the terminal restriction fragments (T-RFs) are detected by using DNA sequencer and used for analysis. This is a cost-effective and high-throughput approach for evaluation of the diversity and composition of microbial communities (Trabelsi and Mhamdi, 2013; Fredriksson *et al*., 2014). Even though NGS technologies have overtaken the fingerprinting methods (such as T-RLFP), several studies comparing these two techniques have demonstrated that the same conclusions can be achieved for the study of microbial communities, in terms of population composition and community dynamics (Fredriksson *et al*., 2014). Apart from that, qPCR is known to be a sensitive and rapid approach for quantifying the overall microbial population and also the expression of microbial genes (Trabelsi and Mhamdi, 2013). It is based on the detection of a reporter molecule in real-time, where fluorescence increases with the accumulation of PCR products over each amplification cycle (Boulter *et al.*, 2016).

Sanger sequencing technology has a significant influence on the early stage of microbial community research. It can acquire up to 96 sequences per run, with an average length of 650 bp which is sufficient to analyse phylogenetic markers (Escobar-Zepeda *et al*., 2015). In addition to Sanger sequencing, several newer platforms known as next generation sequencing (NGS) are able to sequence millions of DNA molecules with varying yields and sequence length in parallel. The first generation of these technologies is known as the 454 sequencing platform or 'pyrosequencing' (Margulies *et al*., 2005). This technology delivered a higher yield

of DNA molecules at a cheaper price, but with shorter read lengths as compared to Sanger sequencing. However, artificial insertions and deletions are the key limitation in 454 sequencing technology owing to long homopolymeric regions and therefore is now obsolete (Escobar-Zepeda *et al*., 2015). Meanwhile, the Ion Torrent platform is similar to 454 sequencing technologies but with a cheaper sequencing cost (Whiteley *et al*., 2012). Another popular NGS platforms are Illumina technologies (Escobar-Zepeda *et al*., 2015) and currently, there are third and fourth generation sequencing technologies such as PacBio RS (Fichot and Norman, 2013) and in situ sequencing (ISS) (Ke *et al*., 2016) respectively. One of the main advancement in the later sequencing generation is that DNA cloning vectors or bacterial hosts are not required for the preparation of DNA library, this further simplifies the library preparation and minimises the risk of contamination. Despite that, it should be taken into consideration that the data derived from these NGS technologies requires subsequent computational analysis (bioinformatics analysis) by using software or operative systems to make use of the metagenomic data. The larger the dataset produced, the greater the computing resources and more sophisticated analyses of bioinformatics are required (Escobar-Zepeda *et al*., 2015).

3 CHAPTER 3: GENERAL METHODOLOGY

3.1 EXPERIMENTAL DESIGN OF THIS STUDY

The overview of this research study can be seen in Figure 3.1. Generally, this study is structured into three experimental chapters (Chapter 4-6). **Chapter 4** focused on the mass production of *Trichoderma* spp. by using industrial bioreactors, followed by formulations with an inert carrier (biochar), mixing of *Trichoderma*-biochar (T-biochar) with organic fertilizer (Living Organic Fertilizer, LOF) and also the determination of the viability and shelf-life of *Trichoderma* spp. in the different formulations. Subsequently, in **Chapter 5**, this involved field applications at two locations (Palong and Tg Karang) of the bioorganic fertilizers formulated in Chapter 3. In the field, the treatments were divided into two bioorganic fertilizer treatments (FLOF and LOF) with different percentages of *Trichoderma* spp. (0, 5, 10 and 20%). Assessment of the soil microbial populations (*Trichoderma* spp., bacterial and fungal count), soil physicochemical properties (EC, pH and nutrients) were carried out. Apart from that, an evaluation of the BSR disease in oil palm (disease incidence, impacts on leaf nutrient and FFB yield) was also conducted. **Chapter 6** involved the molecular analysis of the oil palm rhizospheres at Palong (clayey laterite soil) and Tg Karang (peat soil) respectively. Assessments and characterization of the soil fungal communities were carried out through PCR and sequencing methods, while the population of the biocontrol agent (*Trichoderma* spp.) and the pathogen (*Ganoderma* spp.) were quantified via absolute quantification (qPCR).

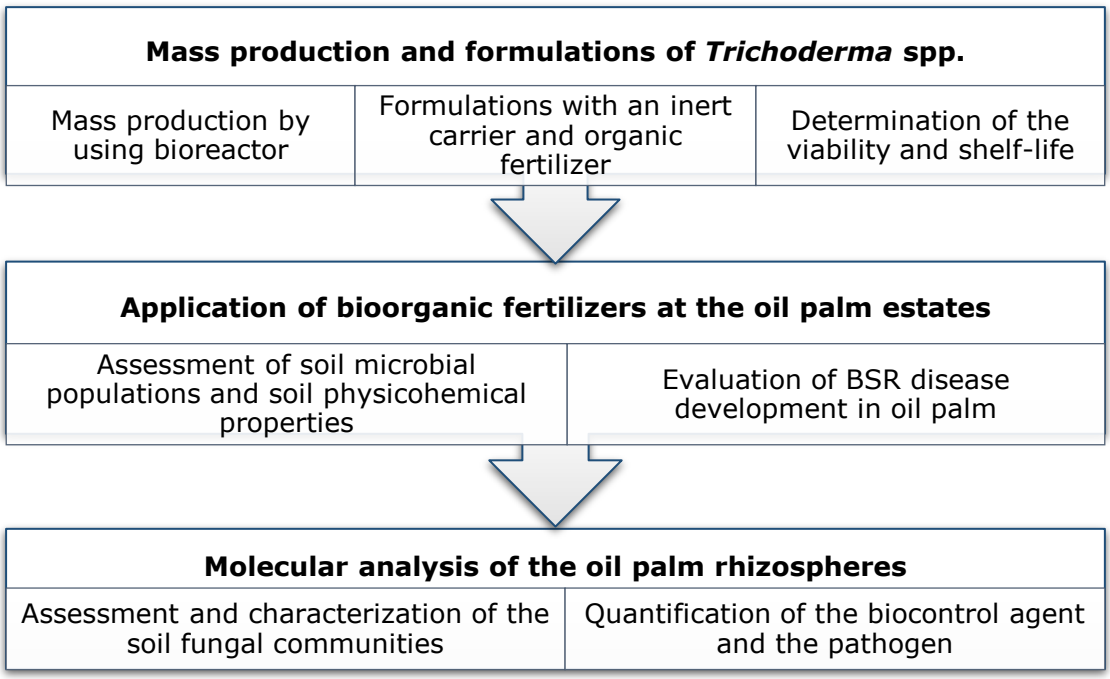


Figure 3.1 The general overview of the study.

3.2 SOURCES OF FUNGI

3.2.1 *Trichoderma* spp.

Three strains of *Trichoderma* spp. were used in this study, namely 4A, B2 and T1. Strains 4A and B2 were isolated from Balau Estate, Semenyih, Selangor by Dr. Christina Supramaniam (School of Biosciences, University of Nottingham Malaysia), while strain T1 was obtained from True Agrosolutions, Setia Alam, Selangor. Strain 4A and T1 were confirmed as *T. asperellum* (GenBank KM456217.1 and KY225680.1 respectively) and B2 was identified as *T. longibrachiatum* (GenBank KY225659) by sequencing of the internal transcribed spacer (ITS) region. *Trichoderma* strains 4A, T1 and B2 were selected as they exhibited promising antagonistic activity against *Ganoderma boninense* strain GBLS in *in vitro* study (Results were not shown). *Trichoderma* cultures were grown and maintained on potato dextrose agar (PDA; Oxoid, UK) by weekly subculturing of the fungi. A 5 mm^2 of mycelial plugs were transferred to new PDA plates and kept in the dark for a maximum of 7 days at room temperature of around 25 °C.

3.2.2 *Ganoderma boninense*

The pathogenic fungus, *Ganoderma boninense*, GBLS isolate was isolated by Dr. Christina Supramaniam from Lian Seng Oil Palm Estate, Johore, Malaysia. By sequencing of the ITS region, the GBLS isolate had been confirmed as *G. boninense* (GenBank KF164430.1). Similar to *Trichoderma* spp., GBLS cultures were maintained on PDA agar by weekly subculturing and kept at room temperature of around 25 °C.

Subcultures and maintenance of *G. boninense* and *Trichoderma* spp. were performed separately to prevent cross-contamination.

3.3 SOURCE OF BIOCHAR AND FERTILIZER

Biochar, Living Organic Fertilizer (LOF) and Fortified Living Organic Fertilizer (FLOF) were procured from Green Plant Organic Fertilizer Sdn. Bhd., Seremban, Negeri Sembilan, Malaysia. LOF contains 50% organic matter, 5.5% total N, P and K (nitrogen, phosphorus and potassium), 25% moisture content and pH between 7 to 9. Meanwhile, FLOF contains 40% organic matter, 25% moisture content and pH around 6.5 to 8.5. FLOF uses LOF as the base material and the N, P, and K formulation were adjusted to 5:5:10:2MgO (Magnesium oxide).

3.4 PREPARATION OF CULTURE MEDIA AND SOLUTIONS FOR BIOCHEMICAL ASSAYS

A weighing scale (Sartorius, CP124S) was used to measure media components and chemical solutions, prepared in laboratory glass bottles (Schott Duran, USA) of 100 ml, 500 ml or 1000 ml. Unless indicated otherwise, the culture media and buffer were autoclaved (Hirayama HVA-110, Japan) at 121°C for 15 minutes. The agar media was poured onto 9 mm Petri dishes in a Class II Biosafety Cabinet (ESCO, Singapore) at temperatures about 55-60 °C. The media was left to

cool and solidify before being used immediately or kept in the refrigerator at 4°C until use.

The pH values of culture media and chemical solutions were adjusted appropriately using a pH metre (Oakton pH 700, Cole-Parmer, USA) if required. Storage of all the chemical solutions was done according to the material safety data sheets (MSDS).

Potato dextrose agar (PDA) is one of the most commonly used media for the isolation and cultivation of fungi. PDA was prepared by adding 39.0 g of PDA (Oxoid, UK) powder into one litre of purified water.

Malt Extract Broth (MEB) is used for the detection, isolation and enumeration of fungi particularly yeasts and moulds. The liquid medium was prepared by weighing 17.0 g malt extract broth powder (Shanghai AB Food & Beverages Ltd, China) and dissolving it in one litre of purified water.

Nutrient agar (NA) is a general-purpose medium used for the cultivation of wide range of non-fastidious microorganisms. One litre medium was prepared by adding 28.0 g of NA (Oxoid, UK) powder into one litre of purified water.

Trichoderma Selective Medium (TSM) was formulated for selective growth of *Trichoderma* spp. TSM was prepared by adding 25.6 g of TSM (Himedia, India) powder into one litre of purified water. The medium was supplemented with 0.25 g of chloramphenicol and 1.40 ml of propamocarb (669.0 g of active ingredient per litre) (Williams *et al*., 2003).

Maximum Recovery Diluent (MRD) is an isotonic medium used for the recovery of microorganisms from a variety of sources. One litre medium was prepared by adding 9.5 g of MRD (Oxoid, UK) powder into one litre of purified water.

Further details on the preparation of chemical solutions can be found in Appendix 1.

4 CHAPTER 4: MASS PRODUCTION OF *TRICHODERMA* SPP. AND MONITORING OF THE PRODUCTS

4.1 INTRODUCTION

In a biocontrol study for plant diseases, the first step involves the identification of promising biological control agents (BCAs). Once suitable BCAs are identified, several factors need to be considered. These include the mass propagation methods, BCAs formulation and application in the field so that the product is stable and viable at the end of the process (Cumagun, 2014). Large-scale production of BCAs can be achieved through liquid and solid fermentation techniques. Solid fermentation involves the use of substrates whereas for submerged fermentation, microorganisms are grown in liquid media (Ramanujam *et al*., 2010).

Microbial biomass formulation is known to be a key step in advancing the commercial production of prospective products for biological control. An ideal formulation should have extended shelf life, is not phytotoxic to crops and should withstand adverse environmental conditions. The formulation requires mixing the active ingredients such as bacterial or fungal spores with inert material like surfactants and diluents so that the physical characteristics of the BCAs will be in desirable form. Besides, the viable nature of the BCAs should not be compromised by the chosen carrier material (Kumar *et al*., 2014).

Other than that, the viability of the BCAs and product storage conditions also play a role in the mass production of BCAs (Kumar *et al*., 2014). For *Trichoderma* specifically, there are various reports on the shelf life of different *Trichoderma* formulations stored at different temperatures. Formulation of the liquid paste of *T. asperellum* (Kolombet *et al*., 2008) and different combination of carriers (sawdust, carboxymethylcellulose (CMC), chitosan and talc powder) in formulations of *Bacillus subtilis*, *P. fluorescens* and *T. harzianum* (Abdel-Kader *et al.,* 2012) resulted in shelf-life up to 6 months at room temperature. Furthermore,

for all nine types of *Trichoderma*-based formulations, storage at room temperature retained viability up to only 6 months whereas the viability was up to 11 months when stored in the refrigerator (4 °C) (Rai and Tewari, 2016). Therefore, as the active ingredients in the product, the population of BCAs over time will influence the shelf life of the product. The population load of the antagonists will influence their effectiveness to control the plant pathogens (Panahian *et al*., 2012).

In this present study, different strains and species of *Trichoderma* were chosen as the biological control agent candidates. For the BCAs to be applied and delivered to the field for the control of BSR disease in oil palm, they were produced in a sequential process from small to large scale via shake flask method and industrial bioreactors. Exponentially grown *Trichoderma* spp. was combined with palm kernel shell (PKS) biochar as the carrier, before being added into two types of fertilizer known as Living Organic Fertilizer (LOF) and Fortified Living Organic Fertilizer (FLOF). The viability and stability of *Trichoderma* spp. in the products were monitored over time for up to 24 weeks.

4.2 MATERIALS AND METHODS

4.2.1 Physicochemical properties of biochar and organic fertilizers

The moisture content was determined by weighing out 50 g of samples (fresh weight) and oven-dried at 40-60 °C to a constant weight. The moisture content (MC) was calculated as shown below:

$$MC = \frac{\text{fresh weight} - \text{dry weight (constant)}}{\text{fresh weight}} \times 100$$

Electrical conductivity (EC) and pH were measured by preparing a 1:5 sample water suspension. EC of the samples was determined by using the EC meter (FieldScout 2265FS, Spectrum Technologies, USA) whereas the pH of the samples

was determined by using a pH meter (Oakton pH 700, Cole-Parmer, USA). Twenty grams of sample was weighed, and 100 ml of distilled water was added to dislodge soil particles. The resulting suspension was mechanically shaken for 1 hour followed by EC and pH measurements.

Other chemical properties of biochar and LOF can be found in Appendix 2 and Appendix 3, which were provided by Green Plant Organic Fertilizer Sdn. Bhd.

4.2.2 Production of Microbial Formulations for Stable BCA

4.2.2.1 Preparation of *Trichoderma* spp. seed culture

Initially, the seed culture of *Trichoderma* spp. was prepared using mycelial culture from 7-day old cultures grown on PDA. Three *Trichoderma* spp. BCA strains, 4A, T1 and B2 were grown on PDA to obtain the mycelial biomass. Mycelial plugs of each isolate were transferred to 2.5 L of malt extract broth (MEB) medium in 5 L Erlenmeyer flasks (Duran, Germany). The inoculated flasks were then agitated at 150 rpm and at room temperature for 18 hours on an orbital shaker (Stuart SSL1, Cole-Parmer, USA).

Subsequently, conidial suspension of *Trichoderma* spp. was used as the seed culture for mass production of the biological control agent. A 7-day old culture of each strain was grown on trays (34 x 34 x 3 cm) filled with malt extract agar (MEA; Shanghai AB Food & Beverages Ltd, China). The conidial suspension was prepared as described by Navaneetha *et al*. (2015) with modifications. Sterile distilled water was added to the culture trays and the surface fungi were dislodged using glass slides. The resulting suspension culture was filtered through four layers of sterile cotton gauze and the conidial concentration was determined by using a haemocytometer (Marienfeld Superior^{TM}, Germany). The cell concentration per millilitre (cells per ml) was calculated according to the following formula adapted from Hansen (2000):

$$\text{Cells per ml} = \text{the average count per square} \times \text{the dilution factor} \times 10^4$$

4.2.2.2 Large-scale production of *Trichoderma* spp.

Mass production of *Trichoderma* spp. was carried out using a 200 L bioreactor with a working volume of 150 L (Biostat D100, B. Braun, Germany) at the Bioprocessing and Biomanufacturing Research Centre, Universiti Putra Malaysia (BBRC, UPM) or using an 800 L bioreactor with a working volume of 250 L (LK 5755, APV Hills & Mills (M) Sdn Bhd) at Industrial Biotechnology Research Centre (IBRC), SIRIM Berhad. There were six rounds of production between November 2016 to November 2018. Rounds 1 and 3 were conducted at UPM, Serdang, Malaysia while rounds 2, 4, 5 and 6 were carried out at SIRIM, Shah Alam, Malaysia.

Trichoderma spp. cultures as described in Section 4.2.2.1, act as the source of inoculum for the production of microbial biomass. The bioreactor was inoculated with 10% (v/v) of mixed *Trichoderma* mycelial culture (rounds 1-2) or 1-2% (v/v) of conidial suspension of mixed *Trichoderma* spp. (rounds 3-6). Aerobic production was carried out for 18-24 hours with the temperature set between 28-30°C and the pH was maintained at pH 7 throughout the fermentation period. Agitation speed was between 80-100 rpm and dissolved oxygen concentration was kept above 10%. When necessary, an antifoam agent was added to suppress foaming in the tanks as foams impede oxygen uptake by living cultures.

During the harvesting of the *Trichoderma* spp. culture from the bioreactor, 10 ml of samples were taken and tested immediately for the estimation of the colony-forming units (CFU) on PDA via serial dilution method.

4.2.2.3 Bioformulations of *Trichoderma* spp.

Palm kernel shell (PKS) biochar is a by-product of the steam boiler that extracts crude palm oil from oil palm fresh fruit bunches. In this study, biochar was used as the carrier material for the formulation of *Trichoderma* spp. The biochar was autoclaved prior to mixing with the BCA at different ratios; 1:0.5 (B50%), 1:1 (B100%) and 1:2 (B200%) to get a non-contaminated and homogenous product.

Shelf life study for this product was carried out at every 4 weeks interval and continued up to 24 weeks. During the storage period, the products were kept in zip-lock bags at room temperature (25±2 °C).

The estimation of CFU of *Trichoderma* spp. in different formulations was done by suspending 10 g of the sample taken from triplicate bags of the product in 90 ml maximum recovery diluent (MRD; Oxoid, UK) and shaken at 150 rpm for 1 hour on an orbital shaker. Ten-fold serial dilution was carried out and plated on *Trichoderma* selective medium (TSM; Himedia, India). The plates were incubated at room temperature and single colonies were counted after 36 h.

4.2.2.4 Bioorganic fertilizer preparation

To determine the viability of *Trichoderma* spp. and for the mixing into fertilizer, *Trichoderma*-biochar (T-biochar) was mixed with LOF. This mixture was applied in the field for both LOF and FLOF treatments (Section 5.2.2). Customised chemical formulation (as recommended by the manufacturer) was supplemented for FLOF treatments but not LOF treatments in the field. The separate application of organic and inorganic components for FLOF treatments in the field was due to the reduction of T-biochar viability when mixed with straight chemicals. Different percentages of T-biochar were added into LOF and were mixed well (Table 4.1). The CFU of *Trichoderma* spp., bacteria and other fungi in bioorganic fertilizer were determined every 4 weeks for up to 24 weeks by plating on TSM, nutrient agar (NA; Oxoid, UK) and PDA that contained 250 µg/ml of streptomycin. The plates were incubated at room temperature and single colonies were counted after 36 h for *Trichoderma* spp. on TSM and between 3-5 days for other microorganisms on NA and PDA.

Table 4.1 Bioorganic fertilizer treatments with different percentages of *Trichoderma* spp.

Treatments	Percentages of *Trichoderma spp.* (%)
T1/T6	0
T2/T7	5
T3/T8	10
T4/T9	20

4.2.3 Statistical Analysis

Genstat (18th edition) was used for statistical analysis. There were three biological replicates for each treatment and for each biological replicate, three technical replicates were carried out. The data were analysed by using T-Test to compare the means between two groups and also by using one-way repeated measures analysis of variance (ANOVA) for significant difference between formulations/treatments if there were more than two groups. Tukey Multiple Comparison post-test was conducted to compare significant means at $P \leq 0.05$.

4.3 RESULTS AND DISCUSSION

4.3.1 Physical and chemical properties of biochar and organic fertilizers

The characteristics of biochar as a carrier and the fertilizers, LOF and FLOF are shown in Table 4.2. Between the two fertilizers, LOF had a higher moisture level at 19.3% as compared to FLOF which was 13.0%. The moisture level in biochar was at 9.0%. Biochar was alkali at pH 10 whereas both fertilizers were near neutral. For EC, both biochar and LOF had relatively low values at 3.75 and 6.10 dS/m respectively. However, EC for FLOF was very high in which it exceeded the maximum limit of the EC meter at 20.00 dS/m. This was due to the NPK chemicals added to FLOF by the manufacturer.

Table 4.2 Physical and chemical properties of biochar and fertilizers with ± standard error (SE).

Sample	Moisture level (%)	pH	EC (dS/m)
Biochar	9.0 ± 0.50	10.32 ± 0.03	3.75 ± 0.30
LOF	19.3 ± 0.53	7.08 ± 0.08	6.10 ± 0.13
FLOF	13.0 ± 0.31	7.83 ± 0.04	>20.00 ± 0

In this experiment, biochar was chosen as the carrier for mass production and formulation of *Trichoderma* spp. Biochar is produced through pyrolysis of the oil palm biomass. Other than that, biochar can be made from materials such as agricultural waste residues, wood, grasses and sewage sludge. Besides the starting material, other factors such as temperature and amount of oxygen present during the pyrolysis process can also affect the physicochemical characteristics of biochar (Antal and Grønli, 2003).

Successful mass production of *Trichoderma* spp. depends on the development of the formulations with suitable carriers that support the survival of this fungus for a considerable length of time (Kumar *et al*., 2014). There are different types of *Trichoderma* formulations that have been used for the management of plant diseases such as talc-based formulation, vermiculite-based formulation, wheat flour-kaolin, alginate prills, press mud and oil-based formulation. The carrier material used may be inert or a food base or combination of both and *Trichoderma* spp. can be formulated in many forms such as wettable powders, dust and granules (Ramanujam *et al*., 2010).

In this study, the use of biochar from oil palm waste is important as it contributes to the effective utilization of agricultural waste materials. The low moisture, alkali pH, high porosity and high organic matter content make biochar as a suitable carrier for fungi propagation (Appendix 2). Furthermore, the application of biochar to soil has been documented to increase soil organic content, neutralize the pH of acidic soils and improve soil microbial ecology (Sohi *et al*., 2010).

4.3.2 *Trichoderma* spp. count of the seed culture

There are three types of propagules that can be used for the formulation of *Trichoderma* spp. which are hyphae (mycelia), conidia and chlamydospores (Howell, 2003). Mycelia and conidia had been used as the seed culture for large-scale production of *Trichoderma* in this study. It was found that mycelial seed culture yielded around 4.00-5.00 log cfu/ml of *Trichoderma.* Whereas for the conidial suspension, *Trichoderma* strains 4A and T1 produced a similar amount of conidia at 7.70 to 7.80 log cells/ml, but strain B2 was slightly lower at 7.27 log cells/ml (Table 4.3).

Table 4.3 Average conidial concentration of different strains of *Trichoderma* spp. Values are mean ± SE.

Strains	Average conidial concentration (log cells/ml)
4A	7.87 ± 0.03
T1	7.70 ± 0.11
B2	7.27 ± 0.27

To control plant diseases, mixtures of different strains and combinations with other fungi or bacteria often provided more effective protection in comparison to individual application of *Trichoderma* (de Boer et *al*., 2003). Thus, a combination of the three strains of *Trichoderma* spp. had been practised in this study and they produced similar conidial concentration. This should have resulted in a similar growth ratio of each strain in the large-scale production. The combination of *T. viride* and *Rhizobium* in the seed treatment of chickpea significantly improved the shoot and root length, fresh and dry weight as well as the number of nodules per plant (Kumar and Upandhyay, 2003). In the management of maydis leaf blight of maize, it was found that the application of both *T. harzianum* and *T. viride* was effective in the control of disease rather than the application of individual microorganisms (Jha *et al*., 2004).

4.3.3 Large-scale production of *Trichoderma* spp. using industrial bioreactor

In this study, liquid state fermentation by using industrial bioreactor was chosen for the mass production of *Trichoderma*. From Figure 4.1, it can be seen that mycelial seed culture (4.95 log cfu/ml) produced a lower *Trichoderma* count in comparison to conidial seed culture (6.32 log cfu/ml) with all other growth parameters remaining the same and this was statistically different ($P<0.001$). This might be due to the low count of the mycelial seed culture itself (Section 4.3.2) which resulted in a low count of *Trichoderma* during large-scale production using bioreactor. Cumagun (2014) stated that the use of hyphae is less preferable because of its lack of dehydration tolerance whereas conidia and chlamydospores can tolerate adverse environmental conditions. Therefore, the use of conidia as the seed culture was a better option since it produced a higher cell density of 7.00 log cfu/ml as compared to the mycelial culture that required an inoculum of a minimum of 10% (v/v).

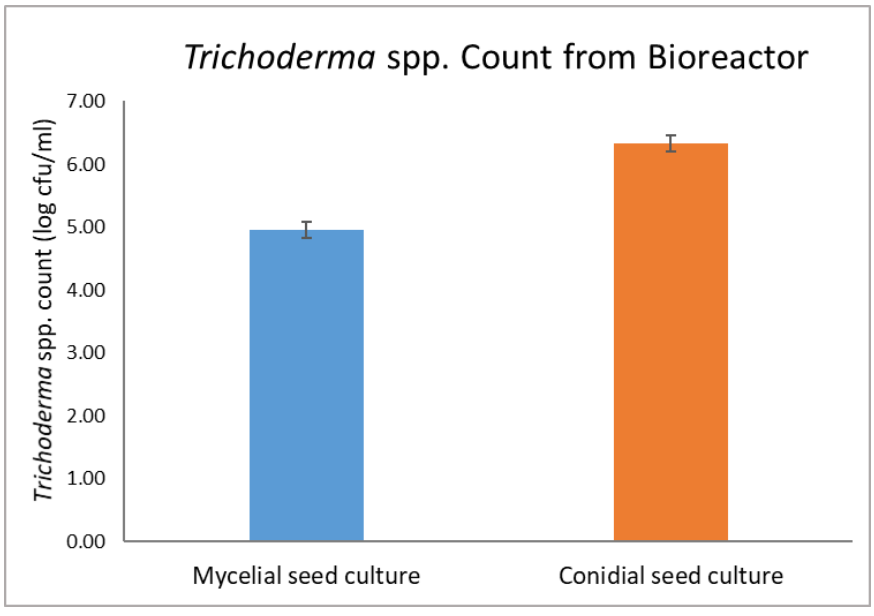


Figure 4.1 *Trichoderma* spp. cell density from the bioreactor using mycelia and conidia as inoculum. Conidial culture produced on average 6.32 log cfu/ml of *Trichoderma* spp. in comparison to mycelial culture (4.95 log cfu/ml).

In general, fungal biocontrol agents are usually mass-produced using solid fermentation technology (Khan *et al*., 2011). However, this requires a large amount of substrate, increases risk of contamination during fermentation and longer fermentation time. Hence, liquid fermentation is in favour compared to solid fermentation as mass multiplication of microorganisms can be achieved under axenic conditions within a short period of time (Khan *et al*., 2011).

4.3.4 Formulations and shelf-life of *Trichoderma* spp. using biochar as an inert carrier

Trichoderma that was produced on a large scale was added to an inert carrier, biochar to ease the handling or transportation processes and enable storage for a longer duration. Several formulations at B0%, B50%, B100% and B200% were tested at the beginning of the study (Production 2 and 3). *Trichoderma* spp. count showed a significant difference ($P<0.001$) between formulations and across time with B100% giving the higher *Trichoderma* count at around 6.00 log cfu/g (Figure 4.2A). Meanwhile, the quantity of *Trichoderma* spp. in B50% and B200% remained around 5.00 log cfu/g. For all formulations, *Trichoderma* spp. remained viable for 24 weeks (6 months). In Production 4, B100% was selected for the formulation of *Trichoderma* spp. with B0% as the control. At 0 week, the *Trichoderma* spp. count in B100% was 6.18 log cfu/g before gradually dropping and maintaining at 5.39 log cfu/g at week 20 and 24 (Figure 4.2B).

Subsequently, during Production 5, the same formulation was used but at this round, inoculations in autoclaved (B100%) and non-autoclaved biochar (Bu100%) was tested. There was no significant difference in both formulations and *Trichoderma* spp. was viable for 24 weeks with populations around 5.00-6.00 log cfu/g (Figure 4.2C). Although non-autoclaved biochar consists of other microorganisms (contaminants) (Appendix 4), it does not inhibit the growth of *Trichoderma* spp. (Figure 4.3). In production 6, Bu100% was again tested with

Bu0% as the control and the *Trichoderma* spp. count was comparable from the previous production in between 5.00-6.00 log cfu/g. Hence, non-sterile biochar is suggested as a carrier and is suitable for large-scale production whereby the cost of the process of autoclaving the biochar is eliminated.

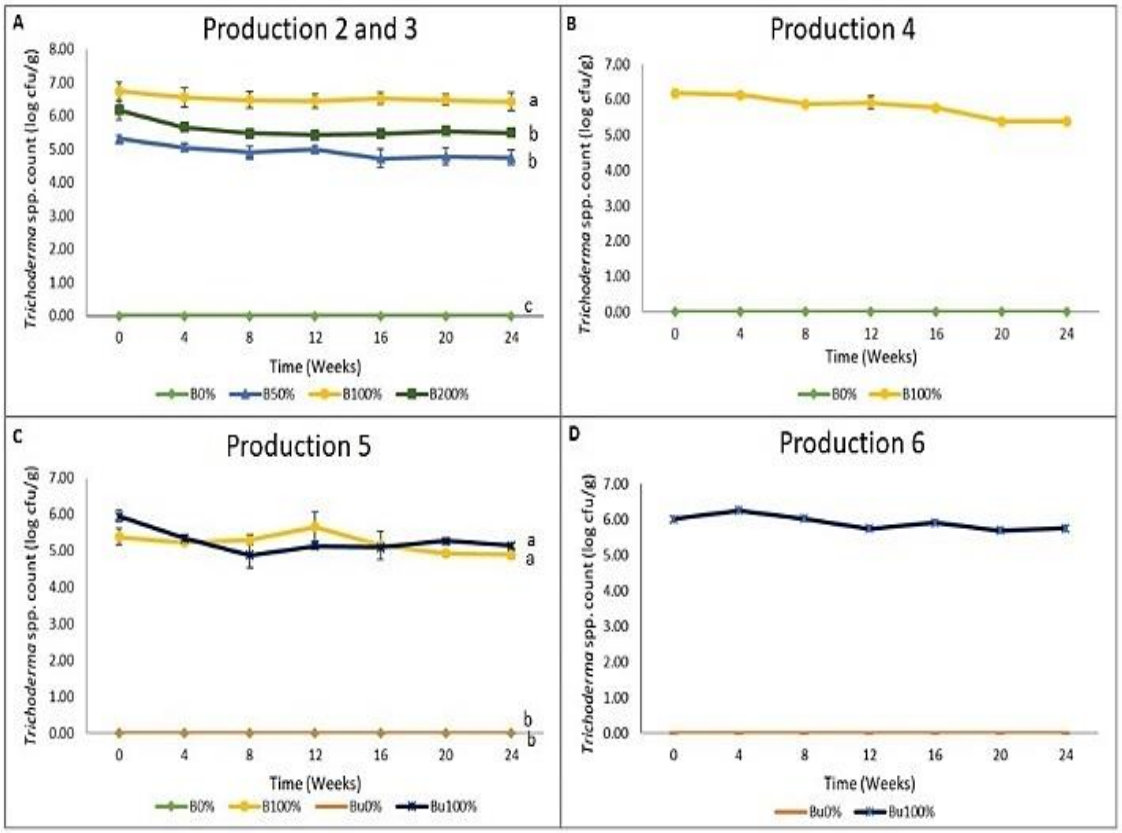


Figure 4.2 *Trichoderma* spp. count in biochar in different formulations for Production 2 and 3 (A), Production 4 (B), Production 5 (C) and Production 6 (D). Line with different alphabetic letters was significantly different at $P \leq 0.05$ by Tukey Multiple Comparison Test. Mean ± SE.

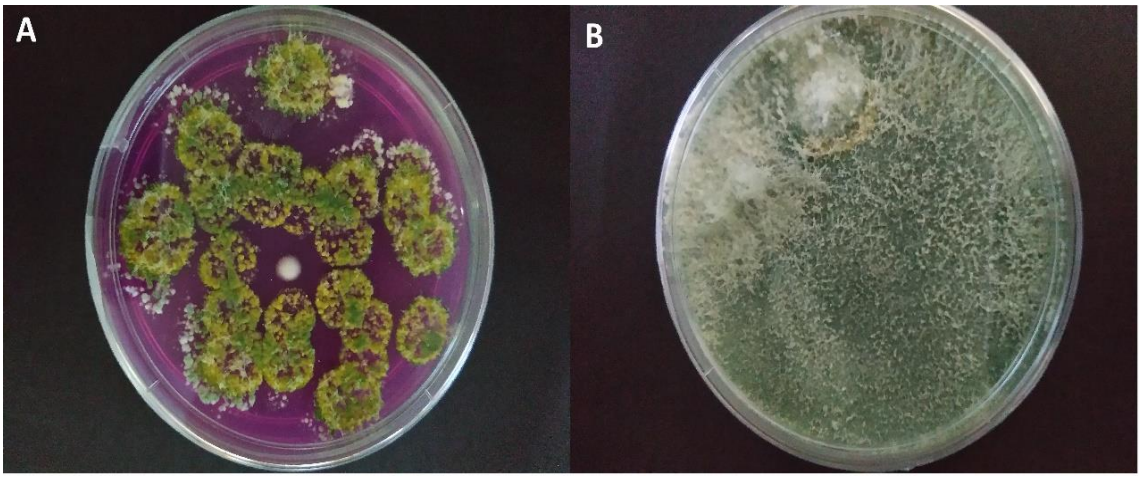


Figure 4.3 *Trichoderma* spp. on TSM plate (A) and PDA plate (B) from Bu100% sample.

For successful biocontrol activities, the shelf life of the formulated biocontrol agent product plays an important role. Kumar *et al*. (2014) stated that the use of organic food in biocontrol agent formulation will have a longer shelf life in comparison to inert or inorganic food bases. In this study, even though the population of *Trichoderma* spp. did not increase in biochar, the count remained stable over time in the inert carrier, biochar. Talc, kaolin, lignite and peat are the most commonly used carrier in the formulation of *Trichoderma* spp. (Ramanujam *et al*., 2010). However, Sankar and Jeyarajan (1996) reported that the viable propagules of *Trichoderma* spp. in talc formulation were reduced to 50% after 16 weeks of storage.

The carrier material, biochar has been used in formulations of microorganisms, particularly plant growth-promoting rhizobacteria (PGPR) such as *Burkholderia* sp. strain L2 and *Bacillus* sp. strain A30 (Tripti *et al*., 2017) and *Bradyrhizobium* sp. (Egamberdieva *et al*., 2017), whereas Ribera *et al*. (2017) incorporated *Trichoderma* spp. into biochar for control of wood decay fungi. Most of the formulations tested by these researchers remained viable for up to 6 months, similar to the shelf life of *Trichoderma* spp. formulations tested in this study. Among the characteristics that make it an ideal inoculum carrier include large surface area,

high internal porosity and the ability to adsorb organic compounds (Chun *et al*., 2004).

4.3.5 Microbial load in bioorganic fertilizer

4.3.5.1 Bacterial count in FLOF

For FLOF treatments (T1, T2, T3 and T4), it was found that fungi including *Trichoderma* spp. did not survive in Fortified Living Organic Fertilizer (FLOF) that has been added with chemical compounds. Only bacteria can be found in FLOF and it was significant between treatments ($P<0.001$). All treatments showed similar trends and fluctuate over time. At Week 4, the bacterial count was around 7.00 log cfu/g before dropping to around 5.50 log cfu/g in Week 24 (Figure 4.4). Additionally, the bacterial count in FLOF was found to be lower than in LOF (Figure 4.5A).

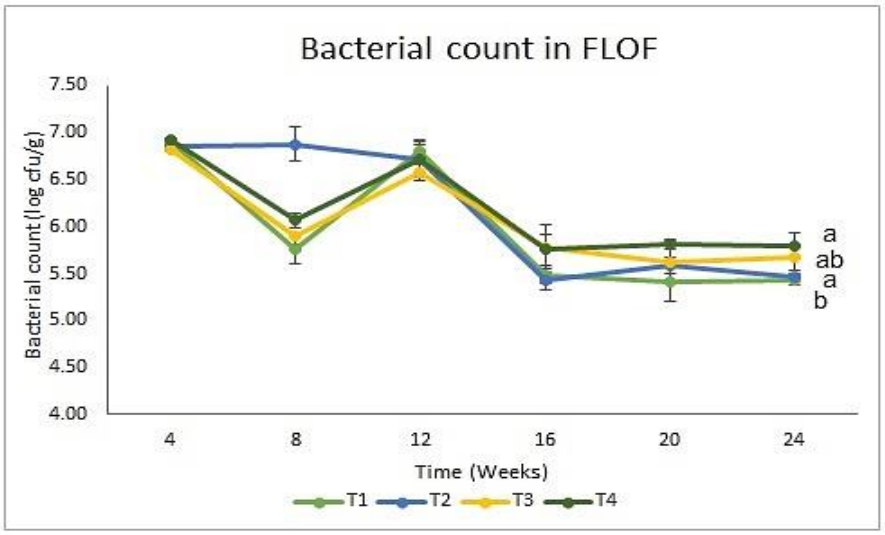


Figure 4.4 Bacterial count in FLOF for a period of 24 weeks. Line with different alphabetic letters was significantly different at $P \leq 0.05$ by Tukey Multiple Comparison Test. Mean ± SE.
T1:0% *Trichoderma* spp.; T2: 5% *Trichoderma* spp.; T3: 10% *Trichoderma* spp. and T4: 20% *Trichoderma* spp.

The reason that fungi did not survive in FLOF was probably due to high EC content (>20.00 mS/dm) (Table 4.2). EC is correlated with the salt concentration of a solution (salinity). Since FLOF has customized formulation of N, P and K, this

resulted in higher salinity. High concentrations of soluble salts will impact microbes by increasing the osmotic potential of water. This will draw water out of cells which may kill microbes through plasmolysis (Yan *et al*., 2015). For bacteria, they were able to tolerate high salinity and survived in FLOF.

4.3.5.2 Bacterial and fungal counts in LOF

For both bacterial and fungal counts in LOF (Figure 4.5), there were no significant differences between treatments ($P>0.05$). Generally, T1/T6 has a lower bacterial count (between 7.20-7.50 log cfu/g) as compared to other treatments (between 7.40-7.95 log cfu/g). The bacterial count was also decreased over time for T2/T7, T3/T8 and T4/T9 from 7.80 log cfu/g at Week 4 to between 7.40-7.60 log cfu/g at Week 24. In addition, fungal count fluctuated over time for all the treatments, where the fungal population in T1/T6 treatments increased sharply from 4.59 log cfu/g to 5.16 log cfu/g at Week 8 and maintained at around 5.00 log cfu/g until Week 24. The main fungus detected in T7, T8 and T9 was *Trichoderma* spp. whereas control (T1/T6) contained *Aspergillus* spp., *Penicillium* spp. and other fungi (Appendix 5). As the organic fertilizer (LOF) used in this study was a product of composting of oil palm's by-products, there were variations in the populations of bacteria and fungi present in the biofertilizer product. For this study, the presence of additional microorganisms is accepted because it is expected during the formulation of products on an industrial scale.

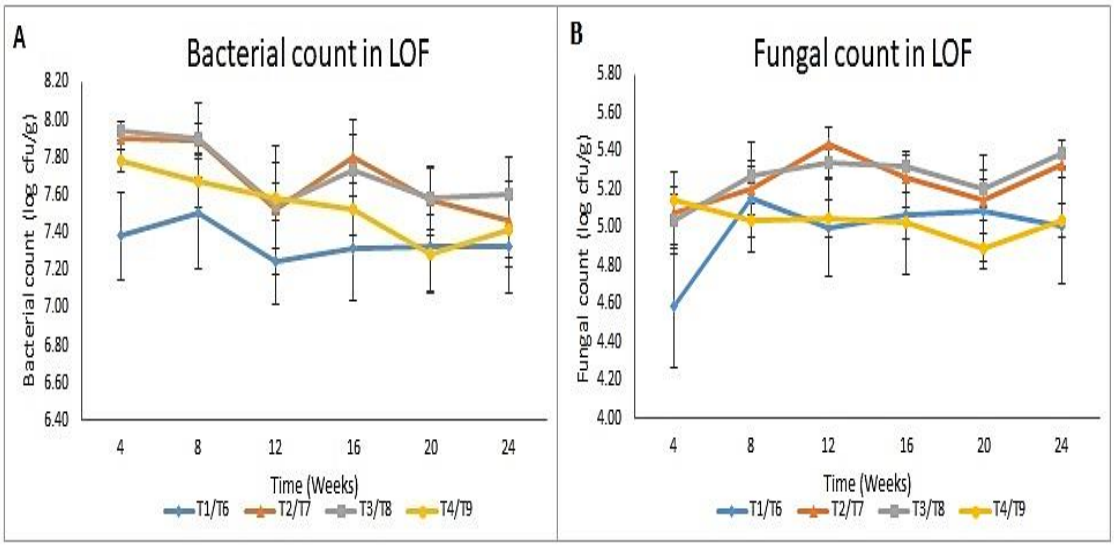


Figure 4.5 (A) Bacterial count and (B) Fungal count in LOF for a period of 24 weeks. Line with different alphabetic letters was significantly different at P ≤ 0.05 by Tukey Multiple Comparison Test. Mean ± SE.
T1/T6:0% *Trichoderma* spp.; T2/T7: 5% *Trichoderma* spp.; T3/T8: 10% *Trichoderma* spp. and T4/T9: 20% *Trichoderma* spp.

4.3.5.3 Shelf-life of *Trichoderma* spp. in LOF

Since FLOF was only able to support bacterial population and not fungi, T-biochar was added to LOF before the treatments were divided into two bioorganic fertilizer treatments for field trials (LOF and FLOF) (Section 5.2.2). The viability of *Trichoderma* spp. as candidate BCA in LOF was determined across time and it was found that there was a significant difference ($P<0.001$) between treatments T3/T8 (10% *Trichoderma* spp.) and T4/T9 (20% *Trichoderma* spp.) as compared to treatments T2/T7 (5% *Trichoderma* spp.). Both T3/T8 and T4/T9 showed a similar trend over time with *Trichoderma* spp. count of around 5.00 log cfu/g throughout the storage period (24 weeks), while T2/T7 dropped to 4.00 log cfu/g at Week 20 onwards (Figure 4.6). *Trichoderma* spp. remained viable for 24 weeks for all the treatments, similar to T-biochar formulations.

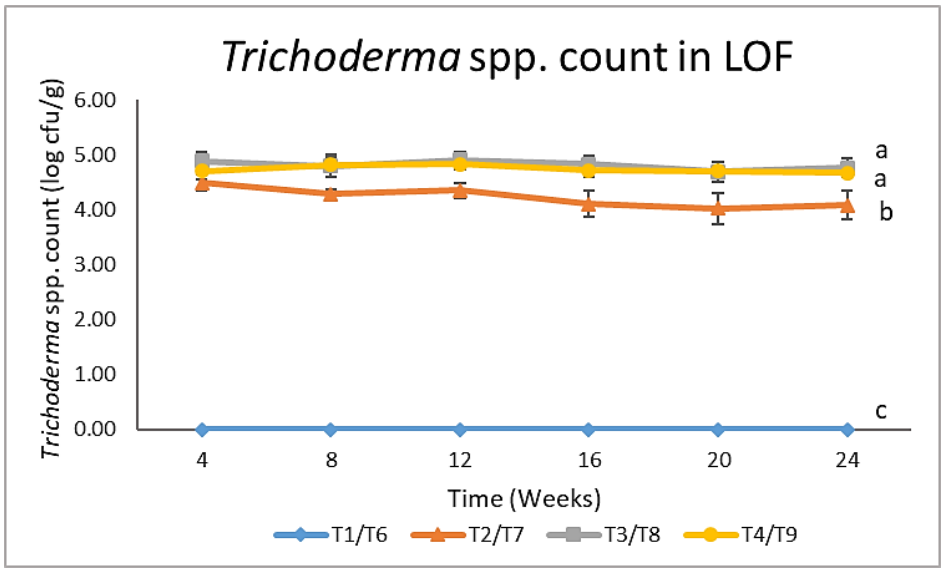


Figure 4.6 *Trichoderma* spp. count in LOF for a period of 24 weeks. Line with different alphabetic letters was significantly different at P ≤ 0.05 by Tukey Multiple Comparison Test. Mean ± SE.
T1/T6:0% *Trichoderma* spp.; T2/T7: 5% *Trichoderma* spp.; T3/T8: 10% *Trichoderma* spp. and T4/T9: 20% *Trichoderma* spp.

When *Trichoderma* spp. was incorporated into the fertilizer (LOF), it was expected that the count will increase before slowly decreasing over time. Yet, this was not the case as it was observed that in all treatments, even for T3/T8 and T4/T9 in which higher percentages of *Trichoderma* spp. were added in these two treatments, resulted in a similar count of *Trichoderma* spp. (log cfu/g). The presence of other microbes may create competition for *Trichoderma* spp. to multiply in the fertilizer. Wakelin *et al*. (1999) reported that in the presence of natural soil microflora, the growth of *Trichoderma koningii* was significantly reduced, while the growth was improved in soil sterilized with ethylene oxide and, to a lesser degree, in soil pasteurized through microwave treatment. Meanwhile, Bin *et al*. (1991) suggested that the presence of a large population of antagonistic bacteria in bulk soil inhibited a fungal biological control agent in certain restrictive circumstances, but the inhibition effect was diminished or suppressed when a large bacterial population was absent.

Another possible reason for the low count might be due to the physicochemical properties of LOF itself. As can be seen, the moisture content in LOF was 19.3% (Table 4.2) which is considered low for the optimum growth of *Trichoderma*. *Trichoderma* spp. is known to have low osmotolerance levels and water conditions have been shown to have a direct effect on *Trichoderma* activities such as spore germination, development of germ tubes and mycelial growth (Kredics *et al*., 2003). It was previously reported that in solid-state fermentation of *Trichoderma,* the ideal moisture level was between 60-80% to achieve optimum sporulation (Cavalcante et al., 2008; Mishra *et al*., 2015). Nonetheless, as *Trichoderma* spp. was still viable and the count was stable for up to 24 weeks (6 months), this means that the bioorganic fertilizer amended with *Trichoderma* spp. can be stored for that period of time before application without losing its quality with consistent populations of *Trichoderma* spp.

The introduction of some beneficial microbes into organic residues is termed bioorganic fertilizer (Cai *et al*., 2017). In this study, the fertilizer used was a product of composting of oil palm's by-product, including empty fruit bunch (EFB) fibre, decanter cake and palm oil mill effluent (POME). Later on, *Trichoderma* spp. was added into the matured compost as a biological control agent. *Trichoderma* strains were thought to be more effective in colonizing and enhancing plant growth if they are enriched in an organic substrate (e.g. applied as bioorganic fertilizer) rather than applied directly (Cai *et al*., 2015).

Currently, the farming sector in Malaysia is dependent on mineral, organic and compound chemical fertilizers (Chong *et al*., 2017). In oil palm plantations, fertilizer management constitutes the largest field cost item, which can go up to 85% of the total expenditure (Goh and Buloh, 2005; Silalertruksa *et al*., 2012). Organic fertilizers (LOF and FLOF) enhanced with *Trichoderma* spp. not only can provide nutrients but it is also anticipated to control and provide protection against *Ganoderma boninese* which is the causal agent for basal stem rot (BSR) disease.

Therefore, rather than utilizing another separate biocontrol agent or fungicide products to control this disease, plantation holders especially the smallholder can choose to apply one product which is the bioorganic fertilizer enhanced with *Trichoderma* spp. with dual functions in order to reduce expenses and overcome manpower shortage problem.

From this experimental chapter, the conidial suspension is a better candidate to be used as the seed culture for large-scale production of *Trichoderma* spp. through liquid state fermentation. This fermentation method can be achieved in a shorter period of time and less laborious in comparison to solid fermentation. Biochar was suggested to be a suitable carrier to store and transfer the *Trichoderma* spp. BCA into the fertilizer. *Trichoderma* spp. was observed to remain viable for 24 weeks in both the biochar carrier and biofertilizer (LOF) formulations.

5 CHAPTER 5: EVALUATION OF THE EFFICACY OF *TRICHODERMA* BIOORGANIC FERTILIZERS AT THE OIL PALM ESTATES

5.1 INTRODUCTION

Plant diseases are usually controlled by both chemical and biological applications and also by cultural practices. Nevertheless, the use of chemicals can be harmful to the environment and it might have undesirable effects on non-target organisms (Cook, 1993). One alternative to control plant diseases is through biological control agents. The term biological control in plant pathology refers to the use of microbial antagonists to inhibit or contain the diseases whereby biological control agents (BCAs) are referring to the organisms that suppress the pathogen (Cook, 1993). Most biocontrol researches focus on certain genera of bacteria (*Bacillus*, *Pseudomonas* and *Streptomyces*) and fungi (*Gliocladium*, *Paecilomyces* and *Trichoderma*) because they are easy to be cultured and mass-produced (Pal *et al*., 2006).

Microorganisms present in natural ecosystems have a role in reducing the potential of plant disease or damages caused by pathogens. Such beneficial microorganisms can either compete with the pathogens for food source, produce metabolites that impede the growth of pathogens or physically eradicate the pathogens by occupying the same niche (Cook, 1993). Some BCAs can even promote plant growth and induced resistance in the host plants. BCAs are generally formulated in different forms such as granules, dust and wettable powder (Schisler *et al*., 2004). In this study, the BCA (*Trichoderma* spp.) was carried by biochar agent and added into organic fertilizer (LOF) obtained from the composting of oil palm biomass to be applied in the oil palm plantations.

Though there is a lot of research on the use of various BCAs against *Ganoderma boninense* (Susanto *et al*., 2005; Nur Ain Izzati and Abdullah, 2008; Sapak *et al*., 2008; Sundram *et al*., 2008; Isma *et al*., 2014), these studies were limited to *in vitro* or greenhouse trials in which they were conducted in controlled

environmental conditions. Studies on the efficacy of BCAs to control *G. boninense* in actual field conditions were few and need to be further explored. Hence, this study aimed to evaluate the efficacy of BCA application in oil palm plantations (field condition) for the control of BSR disease, in terms of the presence of *Trichoderma* spp. in soil, microorganisms count in soil, soil chemical properties and nutrient content, and evaluation of *Ganoderma* BSR disease incidence in oil palm.

5.2 MATERIALS AND METHODS

5.2.1 Experimental sites and design

Field experiments were conducted from November 2016 to March 2019 at two different locations. The first location was at Paramount Estate, Bera, Pahang (Palong) owned by Havys Oil Mill Sdn. Bhd. (Coordinates: 2°53'41'' N, 102°39'40'' E) (Appendix 6) with oil palm of around 25 years old. The second location situated near Kampung Sungai Gulang-gulang, Tanjung Karang, Selangor (Coordinates: 3°25'08'' N, 101°12'53'' E (Block A); 3°23'58'' N, 101°11'51'' E (Block B); 3°23'39'' N, 101°11'29'' E (Block C)) (Appendix 7) which are owned by smallholder farmers with oil palm of around 8-10 years old. Each location consisted of 10 treatments (T1 to T10) with 3 replications (blocks) in complete block design (CBD) with 8 palms per treatment per block (A total of 24 palms per treatment in each location) (Appendix 8). The soil types at Palong were mainly clay and laterite whereas at Tg. Karang was peat soil.

5.2.2 Bioorganic fertilizer treatments

Two fertilizer types, LOF and FLOF were amended with T-biochar and applied in the field according to the following treatments:

Table 5.1 Bioorganic fertilizer applications at Palong and Tg Karang plantations. Modification to treatments T1 and T6 were made after the first year of plant health observations.

0-12 months (1st year)		**13-24 months (2nd year)**	
Treatments	**Percentage of *Trichoderma* (%)**	**Treatments**	**Percentage of *Trichoderma* (%)**
T1 (FLOF)	0	T1 (FLOF)	Control
T2 (FLOF)	5	T2 (FLOF)	5
T3 (FLOF)	10	T3 (FLOF)	10
T4 (FLOF)	20	T4 (FLOF)	20
T5 (FLOF)	GSP	T5 (FLOF)	GSP
T6 (LOF)	0	T6 (LOF)	Control
T7 (LOF)	5	T7 (LOF)	5
T8 (LOF)	10	T8 (LOF)	10
T9 (LOF)	20	T9 (LOF)	20
T10 (LOF)	GSP	T10 (LOF)	GSP

GSP= Grower's standard practice

The amount of FLOF and LOF that were applied in the field was 3 kg and 5 kg per palm per application respectively as recommended by the manufacturer. From 0-12 months (1st year), for T1 (FLOF) and T6 (LOF), the fertilizers were amended with biochar that contained 0% *Trichoderma*. However, from 13-24 months (2nd year), these treatments were changed to FLOF and LOF treatments alone without the addition of T-biochar. Even though there was a slight modification in the treatments, there were no changes in the overall monitoring and assessments in the field. The application of biofertilizers was tailored to three times per year by circle broadcast around the rhizosphere of the palm (approximately 3 feet distance from the stem).

5.2.3 Soil sampling and analyses

Soil sampling was done according to the treatments (T1-T10) at the two trial locations by digging into the depth of 30 cm by using augers and the soil was

sampled every 4 months. Soil samples were taken at three different spots per row in the field (per treatment) and pooled to yield one composite sample representing the treatment. The soil samples were stored at 4°C prior to microbiological analyses.

Estimation of the microbial populations was done as described in Section 4.2.2.4. A suspension of 10 g of soil in 90 ml MRD was prepared. The soil suspension (3 ml) was then diluted serially at ten-fold and used to estimate bacterial, fungal and *Trichoderma* spp. populations by spread plate dilution method. NA was used for bacterial isolation, PDA with 250 µg/ml of streptomycin was used for fungal isolation whereas TSM was used for *Trichoderma* spp. estimation.

For determination of the soil electrical conductivity (EC) and pH, soil samples were dried in the oven for 3 days at 60 °C. After the soil was dried, it was ground and sieved through 2.0 mm mesh. Soil EC was determined by using an EC meter (FieldScout 2265FS, Spectrum Technologies, USA) whereas soil pH was determined by using a pH meter (Oakton pH 700, Cole-Parmer, USA) by preparing 1:5 soil water suspension. A total amount of 20 g of dry soil was weighed and 100 ml of distilled water was added to the soil. The soil suspension was shaken for 1 h at 90 rpm on an orbital shaker (Stuart SSL1, Cole-Parmer, USA) followed by EC and pH measurements.

To evaluate the nutrient status of the soil (nitrogen, phosphorus and potassium), the dried and sieved soil samples were sent to Applied Agricultural Resources Sdn. Bhd. (AAR), Selangor, Malaysia for analysis.

5.2.4 Evaluation of BSR disease in oil palm

Evaluation Of The Efficacy of LOF/FLOF with BCAs

Name : _____ Treatment Row: _____
Date : _____ Block: _____

Symptom	Palm							
	1	2	3	4	5	6	7	8
Ganoderma spp. fruiting body on trunk or base								
Drooping fronds (more than 50% of fronds)								
1-5 Drooping fronds								
Mature fronds –brown								
Mature fronds –green								
>3 unopened fronds (spear)								
Spear fronds –brown								
Spear fronds –green								
Root – brittle								
Root – strong								
Healthy Oil Palm								
Presence of fruit bunches								
Comments								

Figure 5.1 Field evaluation form for BSR disease based on 12 visual symptoms for oil palms at the two locations, modified from Supramaniam (2016).

Each palm in the sampling plots was monitored and evaluated for symptoms of BSR disease by using field evaluation form (Figure 5.1) as described by Supramaniam (2016). The data obtained from field evaluation was processed and the palms were scored for BSR severity on a scale of 0-4 based on the method of Abdullah *et al*., (2003) and Lelong *et al*., (2010) with modifications (Table 5.2).

From these data, the disease incidence (DI) was calculated according to Campbell and Madden (1990):

$$DI\ (\%) = \frac{\text{Number of palms identified as diseased}}{\text{Number of palms per treatment set}} \times 100\%$$

Table 5.2 Scoring scale and their corresponding signs and symptoms of infection for BSR disease of oil palm.

Disease class	Description
0	Strong roots, no wilting fronds, healthy palms.
1	Strong roots, 1-5 wilting fronds, desiccation of fronds
2	Brittle roots and 50% wilting fronds, desiccation of fronds
3	*Ganoderma* fruiting body is present, brittle roots and 50% wilting fronds
4	Death of the plant

Disease class	Palm Appearance/Symptoms
0	
1	

2	
3	
4	

Figure 5.2 Illustration of the condition of the oil palm under different disease classes. Description of the individual disease class is described in Table 5.2.

5.2.5 Leaf nutrient status of the palms

To determine the nutrient contents and track any nutrient deficiencies in palms, sampling of the palm's leaves was carried out. Leaf 17 was sampled as recommended by Pushparajah (1994) and Woittiez *et al*. (2016). Leaf sampling was conducted at Palong site only due to the availability of trained/skilled workers. Processing of the leaves samples was conducted according to Woittiez *et al*. (2016). The leaves were dried in an oven at 60 °C for a few days and ground before sending them to AAR Sdn. Bhd for analysis.

5.2.6 Statistical analysis

Genstat (18th edition) was used for statistical analysis. There were three biological replicates for each treatment and for each time point. For each biological replicate, three technical replicates were carried out. All data were statistically analysed by using two-way repeated measures analysis of variance (ANOVA) except for plant disease incidence (DI) which the data were analysed using Generalized Linear Model (GLM). The post-hoc test was carried out using Least Significant Difference (LSD) or Tukey Multiple Comparison Test where appropriate. Transformation was carried out for the data that doesn't fit the normality assumption. Pearson's correlation coefficient test was used to evaluate the relationships between the soil biological parameters (bacterial, fungal and *Trichoderma* spp. populations) and soil pH with the soil electrical conductivity (EC).

5.3 RESULTS AND DISCUSSION

5.3.1 Presence of *Trichoderma* spp. in the soil as a biological control agent

The population pattern of *Trichoderma* spp. at Palong were irregular between treatments at each sampling point of 6, 12, 16, 20 and 24 months. From Figure 5.3, there was a significant difference between treatments and across time ($P<0.001$) in which T3 and T9 resulted in the highest population of BCA with an average of 5.00 log cfu/g of *Trichoderma* spp. Apart from T5, all the other control treatments (T1, T6 and T10) have lower *Trichoderma* populations (between 3.00-4.00 log cfu/g). Despite there was no *Trichoderma* application in T5, it still consisted of a high population of *Trichoderma* spp. This might be because Palong had a higher population of *Trichoderma* at the start of the experiment (0 month) at around 4.64 log cfu/g.

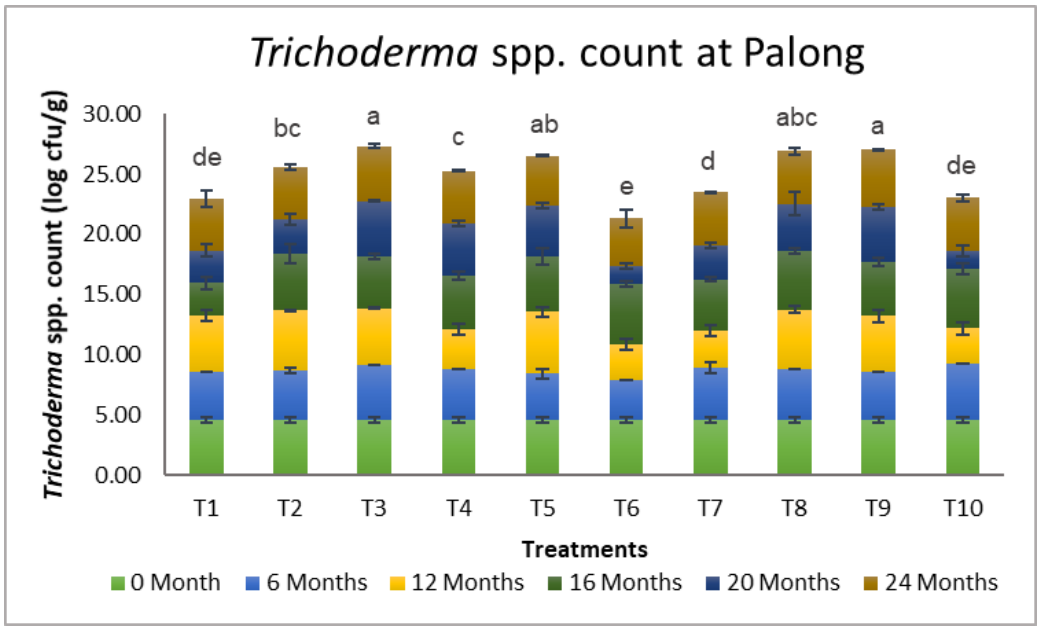


Figure 5.3 Stacked bar chart of *Trichoderma* spp. count at Palong for treatments T1 to T10 for a period of 24 months. Column with different alphabetic letters was significantly different at $P \leq 0.05$ by LSD test.

T1: 0% *Trichoderma* in FLOF (1st year)/FLOF control (2nd year); T2: 5% *Trichoderma* in FLOF; T3: 10% *Trichoderma* in FLOF; T4: 20% *Trichoderma* in FLOF; T5: GSP; T6: 0% *Trichoderma* in LOF (1st year)/LOF control (2nd year); T7:

5% *Trichoderma* in LOF; T8: 10% *Trichoderma* in LOF; T9: 20% *Trichoderma* in FLOF; T10: GSP.

Tg. Karang showed a similar trend as at Palong, in which *Trichoderma* spp. counts fluctuated for all the treatments over time (Figure 5.4). It was found that there was a significant difference between treatments and across time ($P<0.001$) in which T2 and T9 had the highest populations of *Trichoderma* at around 4.00 log cfu/g followed by T3 at 3.70 log cfu/g. On the contrary, T5 had the lowest *Trichoderma* spp. with an average count of 2.59 log of cfu/g. In general, Tg Karang had lower *Trichoderma* spp. count in comparison to Palong and at the start of the study, *Trichoderma* spp. count was around 2.00 log cfu/g. Over time, *Trichoderma* counts showed increment for all the treatments at Tg Karang treatment plots.

Based on the results from Palong and Tg Karang, treatment T9 (20% *Trichoderma* in LOF) had the highest *Trichoderma* spp. count at both locations, indicating that the formulation resulted in a higher *Trichoderma* spp. populations. As both locations have different soil types and soil structures, this might affect the colonization and survivability of *Trichoderma* spp. in these two areas. As Palong already had a high *Trichoderma* spp. count in the beginning, application of *Trichoderma* bioorganic fertilizer might not have a significant effect on the population of the BCA. In contrast, Tg Karang had resulted in a higher *Trichoderma* count at the end of the field trial and the increase may be contributed by the application of the treatments. Therefore, 20% of *Trichoderma* in biofertilizer could be the minimum amount for field application (threshold value) and this should be increased in the future for the control of BSR disease. This is because biocontrol agents should be applied at population thresholds or even at higher rates than the threshold value for effective suppression of soil-borne pathogens (Yang *et al*., 2011).

There is not much information available on *Trichoderma*'s population dynamics, particularly on its survivability and proliferation in the local ecosystem

with respect to types of soil, soil depth and crop history. As the application of the biofertilizers was around the rhizosphere of the palm and its main mode of action is by mycoparasitism and possibly by antibiosis, the effectiveness of *Trichoderma* as BCA will be depending on its ability to disperse and colonize roots (Sariah *et al*., 2005). Longa *et al.* (2009) investigated *Trichoderma atroviride* SC1 survivability and dispersion following application to vineyard soil in Italy. The findings of that study showed that the fungus was still detected at a high concentration 18 weeks after inoculation and interestingly, *T. atroviride* SC1 spreads up to 0.4 m vertically and 4.0 m horizontally from the inoculation point. Therefore, this showed that *Trichoderma* has the ability to move from a treated row to an untreated row which had been observed in this study.

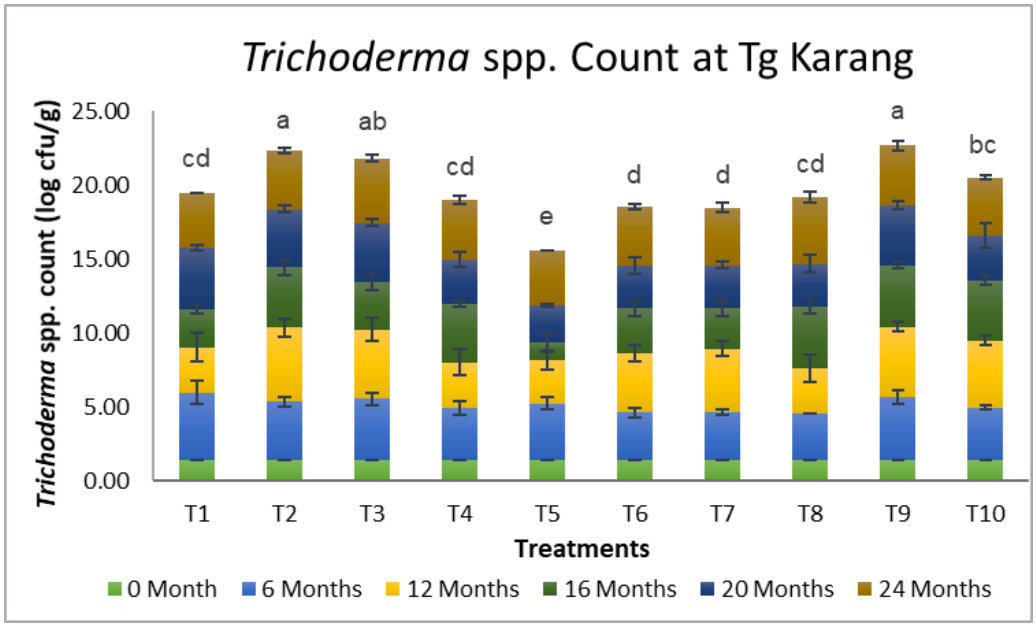


Figure 5.4 Stacked bar chart of *Trichoderma* spp. count at Tg Karang for treatments T1 to T10 for a period of 24 months. Column with different alphabetic letters was significantly different at P ≤ 0.05 by LSD test.
T1: 0% *Trichoderma* in FLOF (1st year)/FLOF control (2nd year); T2: 5% *Trichoderma* in FLOF; T3: 10% *Trichoderma* in FLOF; T4: 20% *Trichoderma* in FLOF; T5: GSP; T6: 0% *Trichoderma* in LOF (1st year)/LOF control (2nd year); T7: 5% *Trichoderma* in LOF; T8: 10% *Trichoderma* in LOF; T9: 20% *Trichoderma* in FLOF; T10: GSP.

In a study to determine the population dynamics of *Trichoderma* in compost-amended and fumigated soil, *Trichoderma hamatum* T382 was shown to be higher in soil supplemented with compost and the BCA in which the population remained stable between 2.90-3.30 log cfu/g. *T. hamatum* maintained below 1.00 log cfu/g in other soil treatments (compost-amended, fumigated and untreated soil). Interestingly, for indigenous *Trichoderma* spp., the population was greater in fumigated soil (up to 60-fold) as compared to the soil from any other treatments (Leandro *et al*., 2007). This showed that type of soil plays a role in the ability of *Trichoderma* to colonize an area. Introduction or addition of new *Trichoderma* spp. to soil were able to remain stable in the soil although the count was low. This might be similar to the results from Palong and Tg. Karang in which *Trichoderma* spp. strains 4A, T1 and B2 became stable in the soil but this needs to be investigated further and the interaction with indigenous *Trichoderma* spp. might also affect the survivability of these three strains in the soil.

Sariah *et al*. (2005) concluded that *Trichoderma* spp. abundance was not considerably different between the oil palm and non-oil palm ecosystems and the two upper horizons (0-30 cm and 30-60 cm) supported higher *Trichoderma* populations (between 3.00-4.00 log cfu/g). At Palong and Tg. Karang, the counts of *Trichoderma* spp. in some of the treatments can reach up to 5.00 log cfu/g, which was higher than the findings from the previous studies (Sariah *et al*., 2005).

5.3.2 Bacterial and fungal populations in the soil

Population density of microorganisms at Palong and Tg. Karang was collected at 0, 6, 12, 16, 20 and 24 months. From Figure 5.5, it was observed that the bacterial counts for all treatments were similar from both locations, which were between 6.00-7.00 log cfu/g. The bacterial count was insignificant ($P \geq 0.05$) at Palong and Tg Karang plantations across the treatments.

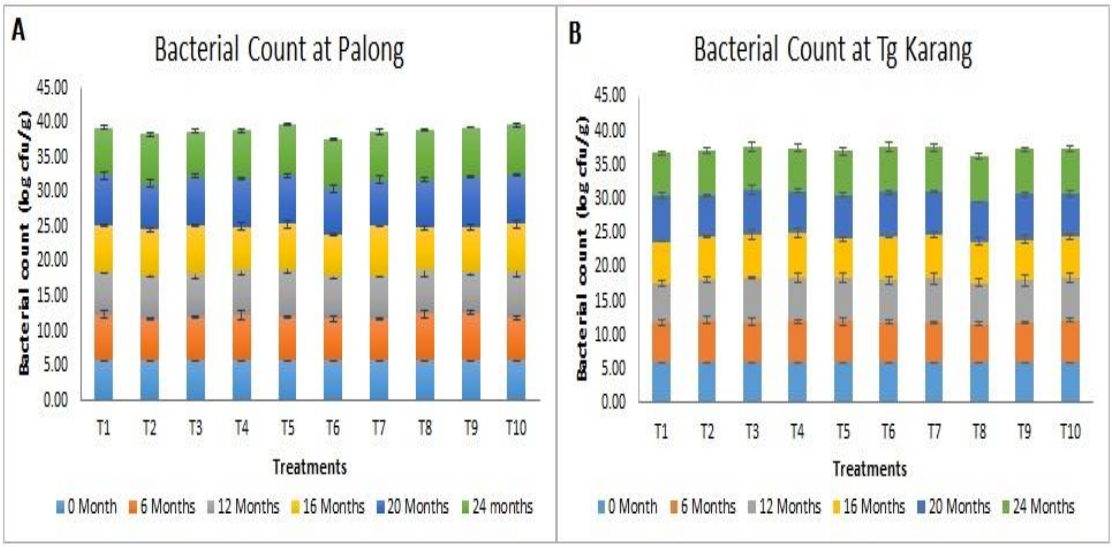


Figure 5.5 Stacked bar chart of the bacterial population at (A) Palong and (B) Tg Karang for treatments T1-T10 for a period of 24 months.

T1: 0% *Trichoderma* in FLOF (1st year)/FLOF control (2nd year); T2: 5% *Trichoderma* in FLOF; T3: 10% *Trichoderma* in FLOF; T4: 20% *Trichoderma* in FLOF; T5: GSP; T6: 0% *Trichoderma* in LOF (1st year)/LOF control (2nd year); T7: 5% *Trichoderma* in LOF; T8: 10% *Trichoderma* in LOF; T9: 20% *Trichoderma* in FLOF; T10: GSP.

Soil is made up of different components such as sand, clay, silt and organic matter, this contributes to a wide range of microhabitats for different types of microorganisms (Garbeva *et al*., 2004). Fertile soil contains a high bacterial population and served as an indicator of good soil quality (Van Beelen and Doelman, 1997). In addition, the presence of bacteria in soil samples was determined by fresh organic matter content rather than the total carbon content in soil (Kubat *et al*., 1999).

In a previous study, the counts of microorganisms were monitored at 11 localities on arable lands made up of different soil types. It was found that the average count of total bacteria was 6.75 log cfu/g and there were no significant differences in the bacterial count between these localities (Popelářová *et al*., 2008). Masirah *et al*. (2005) sampled soil in the young oil palm area at Universiti Putra Malaysia (UPM) randomly and the results showed that bacteria colony count at

around 6.60 log cfu/g. The results from the current study were comparable to previous studies as the bacterial count from both locations of this study was similar.

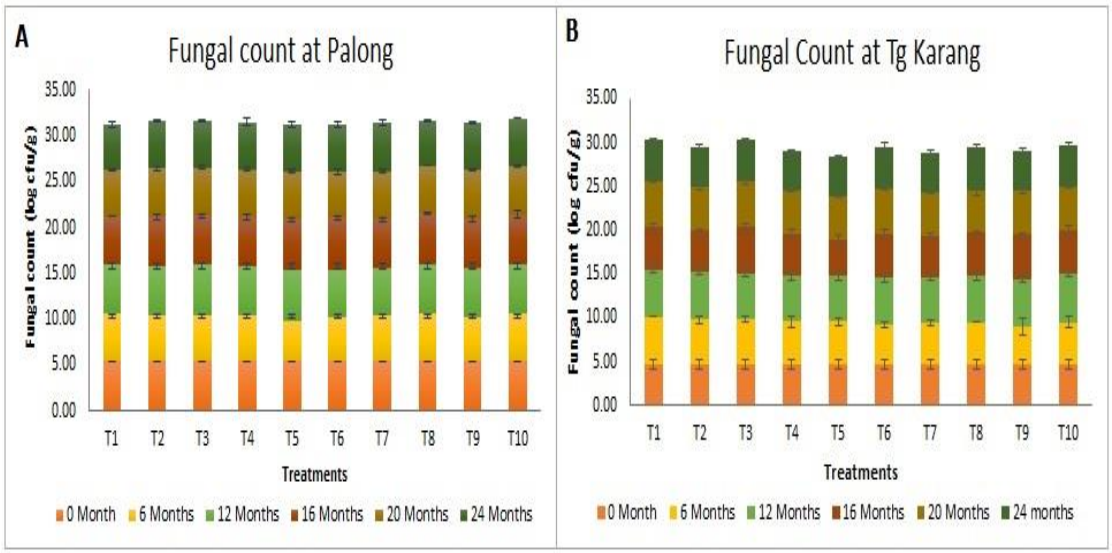


Figure 5.6 Stacked bar chart of the fungal population at (A) Palong and (B) Tg Karang for treatments T1-T10 for a period of 24 months.
T1: 0% *Trichoderma* in FLOF (1st year)/FLOF control (2nd year); T2: 5% *Trichoderma* in FLOF; T3: 10% *Trichoderma* in FLOF; T4: 20% *Trichoderma* in FLOF; T5: GSP; T6: 0% *Trichoderma* in LOF (1st year)/LOF control (2nd year); T7: 5% *Trichoderma* in LOF; T8: 10% *Trichoderma* in LOF; T9: 20% *Trichoderma* in FLOF; T10: GSP.

From Figure 5.6A, it was observed that the fungal populations of all *Trichoderma* treatments at Palong had similar counts at each sampling time at around 5.00-6.00 log cfu/g. In general, the fungal count was the highest at 12 and 16 months for most of the treatments (except for T1, T4 and T7) in comparison to 0, 6, 20 and 24 months. At Tg. Karang (Figure 5.6B), the fungal counts increased over time up to 12 months for the majority of the treatments except for T6, T7 and T9. The fungal count was the highest at 12 months before the counts decreased at 16, 20 and 24 months in all of the treatments. The fungal population at Tg. Karang was slightly lower than at Palong, between 4.00-5.00 log cfu/g. Statistical analysis

showed that there was no significant difference (P≥0.05) in fungal count between the treatments and across time for both Palong and Tg. Karang.

In a study by Alexander and Chong (2014) at an oil palm plantation in Sabah, they found out that the initial fungal population was around 2.00 log cfu/g. After the application of BCAs-based products containing *Trichoderma* spp. and *Bacillus* spp., the fungal population showed an increment between 2.00-4.00 log cfu/g. In another study, isolation of microbial colony from young oil palm plantation at Universiti Putra Malaysia (UPM) showed that the fungal count was around 5.40 log cfu/g (Masirah *et al*., 2005). On the contrary, Nur-Hanani *et al*. (2020) reported that at different time points, the fungal population was the highest at the oil palm plantation (5.57 log cfu/g) in comparison to a secondary forest in Sarawak, Malaysia in June 2013 and this difference were found to be associated with time and location. In general, the population density of fungi usually ranges from 3.00-6.00 log of cfu/g (Atlas and Bartha, 1998). Therefore, the population density of fungi at Palong and Tg. Karang was well within this range and comparable to other studies.

One of the main concerns of the introduction of BCAs against fungal pathogen is the impacts of these BCAs on the non-target indigenous soil microorganisms, in which biodiversity and/or abundance of these microorganisms might be altered (Brimner and Boland, 2003). Nevertheless, it was not the case in this study as the bacterial and fungal counts at both locations remained similar and stable throughout the studied period as compared to the counts at the beginning of the study. In the soil of oil palm plantation, the microbial community has been constantly affected by agricultural activities such as the application of chemicals (fungicides, fertilizers, pesticides, etc). These activities have detrimental effects on beneficial soil microorganisms which leads to an imbalanced equilibrium in soil and weaken the soil microbial community (Raaijmakers *et al*., 2009). This causes the emergence of soil-borne pathogens (Steenwerth *et al*., 2005) such as *G. boninense*

that causes BSR disease in oil palm. For this reason, it is necessary to maintain healthy and stable soil microbial communities to suppress phytopathogens.

5.3.3 Chemical properties of the soil

5.3.3.1 Soil pH

Soil pH is a measurement of soil acidity, it was tested at both Palong and Tg. Karang oil palm plantations. The differences of soil pH at both locations were insignificant between treatments. At Palong, T3 and T7 had the lowest pH value at 3.8 and 3.9 respectively whereas T5 was the highest at pH 4.1. Other treatments had a pH value of around pH 4.0 (Figure 5.7A). Meanwhile, soil pH at Tg Karang did not differ much between treatments and the soil acidity level was generally at around pH 5.1 (Figure 5.7B). Differences between the soil pH might be influenced by chemicals leaching, acidic parent materials, degradation of organic matter and rainfall. These can leach calcium out from the soil which lowers the pH level and increase soil acidity at Palong (Havlin *et al*., 2013).

Goh and Chew (1997) came up with the classification of soil nutrient status for oil palm plantation and one of their parameters is to measure the soil pH (Appendix 9). From Appendix 9, the soil at Palong and Tg Karang plantations were classified in low and high category respectively for pH. It is important to consider the pH value of the soil as it provides a rough indicator of nutrients availability within the soil. As reported by Mutert (1999), the topsoil (0-30 cm) collected from eight types of soil widely used for oil palm in Southeast Asia were all acidic, with pH less than 5.0. It was also found that N, available P and K content were either low or very low in six of them while Mg was also low in four of the soil types when evaluation for fertility parameters was conducted. Therefore, as the soil becomes more acidic, some nutrients may become less available (P, Mg and Calcium (Ca)) whereas other elements such as aluminium (Al) and manganese (Mn) can become

more available and might reach toxic levels for plants (Aini *et al*., 2014; Neina, 2019).

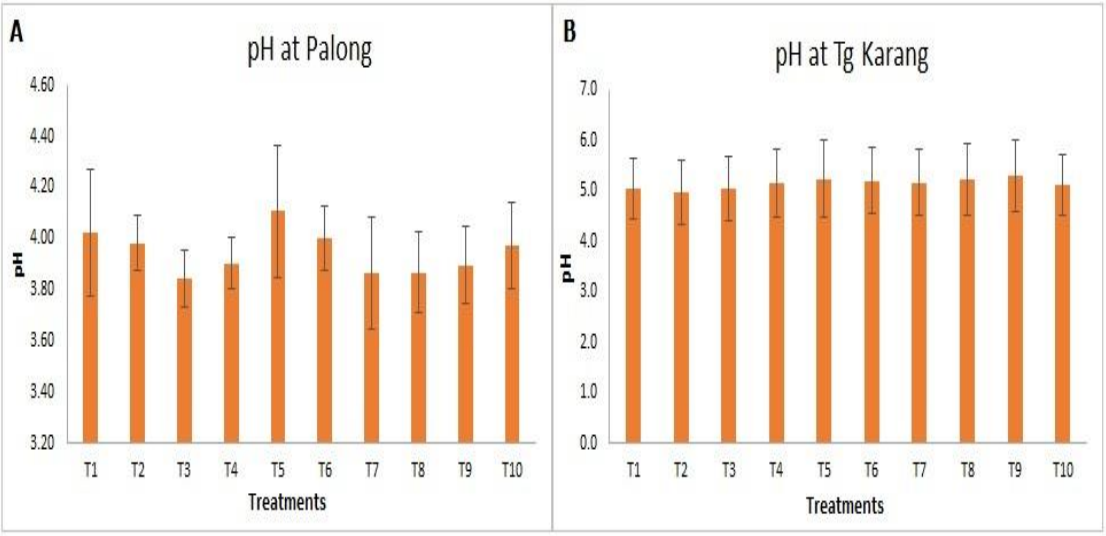


Figure 5.7 Average pH at (A) Palong and (B) Tg Karang according to the different treatments (T1 to T10). Average pH reading was pH 3.9 at Palong and pH 5.1 at Tg. Karang.
T1: 0% *Trichoderma* in FLOF (1st year)/FLOF control (2nd year); T2: 5% *Trichoderma* in FLOF; T3: 10% *Trichoderma* in FLOF; T4: 20% *Trichoderma* in FLOF; T5: GSP; T6: 0% *Trichoderma* in LOF (1st year)/LOF control (2nd year); T7: 5% *Trichoderma* in LOF; T8: 10% *Trichoderma* in LOF; T9: 20% *Trichoderma* in FLOF; T10: GSP.

In addition, oil palm grows well at soil pH 4.3-6.5 and is moderately tolerant to acidic soil. However, if soil pH drops too low (below pH 3.0), oil palm will experience hyperacidity and the palms will become stunted (Shamshuddin *et al*., 2014). For soil microorganisms, it was found that in arable soil, the diversity and relative abundance of bacteria were positively correlated to soil pH, whereas the fungal diversity was correlated weakly to soil pH and the fungal relative abundance was not influenced by soil pH (Rousk *et al*., 2010). Sariah *et al*. (2005) determined the pH of oil palm soil and non-oil palm soil in Teluk Intan, Perak and the results showed that soil pH in these two types of soil ranged between pH 3.0-4.0. In addition, *Trichoderma* spp. favour slight acidic soil, in which they are able to grow in a wide range of pH from 2.0 to 6.0 with the optimum range in between pH 4.6-6.8 (Kumar *et al*., 2014).

5.3.3.2 Soil electrical conductivity (EC)

Soil electrical conductivity (EC) is an estimation of salt content or chemical supply in soil and is related to soil salinity. The soil EC contents were not statistically significant (P>0.05) for all the treatments at both locations and generally, the EC was higher at Tg Karang in comparison to Palong. The EC did not differ much in Palong and ranged between 0.06 to 0.09 dS/m for all the treatments (Figure 5.8A). In contrast, the EC fluctuated across the treatments at Tg Karang and T1 and T2 had the lowest EC value at 1.14 and 1.10 dS/m respectively. Other treatments had EC values between 1.20 to 1.40 dS/m (Figure 5.8B).

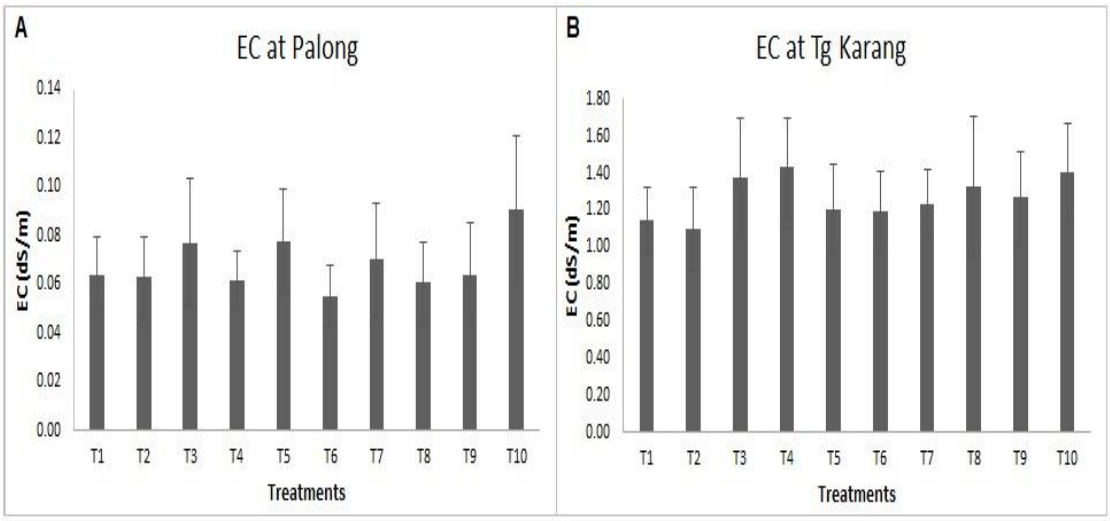


Figure 5.8 Average EC at (A) Palong and (B) Tg Karang according to the different treatments (T1 to T10).
T1: 0% *Trichoderma* in FLOF (1st year)/FLOF control (2nd year); T2: 5% *Trichoderma* in FLOF; T3: 10% *Trichoderma* in FLOF; T4: 20% *Trichoderma* in FLOF; T5: GSP; T6: 0% *Trichoderma* in LOF (1st year)/LOF control (2nd year); T7: 5% *Trichoderma* in LOF; T8: 10% *Trichoderma* in LOF; T9: 20% *Trichoderma* in FLOF; T10: GSP.

For oil palm plantation, the recommended soil EC is within 0.1-4.0 dS/m (Mohidin *et al*., 2015). The electrical conductivity of soil is determined by the moisture contents of soil particles. Sand is low in conductivity, silt has average soil conductivity and clay soil is high in conductivity. Hence, the EC in the soil is strongly correlated to soil moisture, texture, particle sizes, temperature and organic matter content (Grisso et al., 2009; Aminudin *et al*., 2018). This can explain the differences

in the soil EC contents at Palong (laterite and clay soil) and Tg. Karang (peat soil) plantations of this study, as both locations have different soil types and profiles. Bai *et al*. (2013) demonstrated that with rising water content, saturation level and dry density, the EC of lateritic soil increased. Meanwhile, the EC of peat soil was shown to be between 0.1-10 dS/m (Aminudin *et al*., 2018). In a study by Asadi and Huat (2009), it was found that the electrical conductivity of the highly decomposed peat was higher in comparison to normal peat soil that was less decomposed. However, as the organic content increased, the electrical conductivity decreased in both highly- and very slightly- decomposed peats.

Besides, soil salinity can affect soil microorganisms through osmotic effect and specific ions effect (Yan *et al*., 2015). When the osmotic (more negative) ability of soil water increases, this removes water from cells that can kill microorganisms and plant roots by plasmolysis. The low osmotic potential also makes it more difficult to extract water from the soil for roots and microorganisms (Oren, 1999). Additionally, certain ions such as Na^+, Cl^- and HCO_3^- HCO_3^- can be detrimental to many plants at high concentrations (Yan *et al*., 2015). Hence, it is important to have a balanced level of soil salinity for better plant growth and optimum microbial activities.

There was a negative and weak correlation between the soil pH and EC levels from Palong plantations in this study (Table 5.3). Meanwhile, the soil pH, bacterial and *Trichoderma* populations were weakly and positively correlated with the soil EC at Palong. The only significant correlation at Palong plantation was between the fungal population and soil EC (P=0.046). At Tg Karang, soil pH was positively correlated to soil EC, but the bacterial population was negatively correlated and these correlations were significant (P=0.009 and P=0.047 respectively). In contrast, the population of *Trichoderma* spp. at Tg. Karang was not influenced by soil EC as there was no correlation between these two parameters.

Table 5.3 Correlations between soil parameters and electrical conductivity (EC) at Palong and Tg Karang.

Location	Soil Parameters	Electrical conductivity (EC)			
		Pearson R	R^2	*P-value*	*Summary*
Palong	pH	-0.035	0.001	0.806	ns
	Bacterial population	0.145	0.021	0.311	ns
	Fungal population	0.280	0.079	0.046	*
	Trichoderma spp. population	0.242	0.058	0.087	ns
Tg Karang	pH	0.365	0.133	0.009	**
	Bacterial population	-0.280	0.078	0.047	*
	Fungal population	0.106	0.011	0.461	ns
	Trichoderma spp. population	-0.020	0.0004	0.888	ns

ns = non significant, * significant at P > 0.01, ** significant at P < 0.01

From the correlation analysis, higher soil EC influenced the soil pH at Tg Karang plantation, in which an increase in soil EC resulted in the increase of soil pH or vice versa. Depending on the types of soil and agricultural land use, there were a number of authors reported the positive correlation between soil EC and pH previously (Ranjbar and Jalali, 2016; Bogunovic *et al*., 2017), while others reported the negative correlation between the two parameters (Romic *et al*., 2012; Aini *et al*., 2014; Mohd Aizat *et al*., 2014*)*.

Other than that, due to the different salinity levels at both locations, the fungal population and bacterial population were affected at Palong and Tg Karang plantations respectively. There are numerous environmental factors that can influence soil microbial communities and these had been investigated by Lozupone and Knight (2007) and Lauber *et al*. (2008), where they suggested that the salinity level and soil texture were the major factors. Interestingly, *Trichoderma* spp. population might not be affected by soil electrical conductivity at both locations (P>0.05 from the correlation analysis in Table 5.3). Being a free-living fungus that can be found in all types of soil and other natural habitats (Siddiquee *et al*., 2010), it is important for *Trichoderma* to adapt to most of the biotic and abiotic stresses.

Some species of *Trichoderma* were found to be tolerant to increasing osmotic pressure (halotolerant) (Gal-Hemed *et al*., 2011), soil temperature (0-50 °C) and pH (Al-Ani, 2018). These features are crucial for *Trichoderma* spp. to be served as a biological control agent as they could have a better stress tolerance level against the other soil phytopathogens (Kredics *et al*., 2004).

5.3.3.3 Soil nutrient content

Soil nutrients including nitrogen, phosphorus and potassium contents were evaluated from 2017 to 2019 at Palong and Tg. Karang plantations. These are the essential macronutrients that played important roles in the growth and development of plants. The soil analysis results were interpreted using Goh and Chew (1997) methods in this study (Appendix 9). Based on Figure 5.9, the nitrogen (N) contents throughout the experiment at Palong plantations were between 0.07-0.09%. The N content was considered to be relatively low in the year 2018 and very low in the year 2017 and 2019. Phosphorus (P) contents in soil showed the same trend as nitrogen contents. The highest amount of total P and available P were recorded in the year 2018 (162.3 mg/kg and 13.2 mg/kg respectively), which fell in the low category (Appendix 9) whereas the soil P contents in the year 2017 and 2019 were very low. In contrast, potassium (K) content in the year 2017 was low at 0.1 $cmol^{+}$/kg before it raised up 0.47 $cmol^{+}$/kg in the year 2019, which was in the very high category (Figure 5.9).

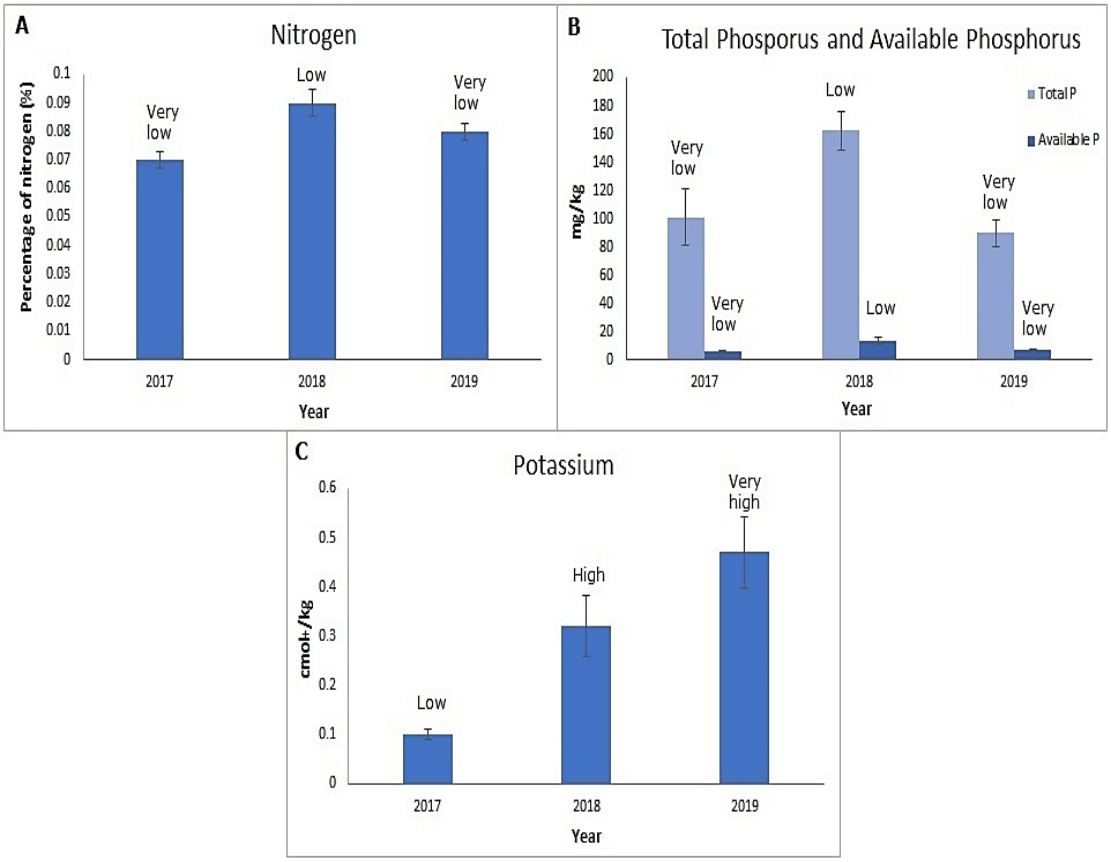


Figure 5.9 Soil nutrient content, (A) Nitrogen, (B) Total and available phosphorus and (C) Potassium at Palong in the year 2017, 2018 and 2019. The N, P and K content were interpreted using Goh and Chew (1997) methods (Appendix 9).

From the year 2017 to 2019, N and P contents showed the same trends at Palong plantation, where both nutrient contents were the highest in the year 2018. This was due to the fertilization regime at Palong (Appendix 11) in which organic fertilizer (LOF), fortified fertilizer with customized N, P, K and Mg content (FLOF) and potassium chemical fertilizer (muriate of potash, MOP) were applied at varying amount each year. Previous study has shown that oil palm requires large quantities of nutrients, particularly potassium (K), nitrogen (N), phosphorus (P), magnesium (Mg) and boron (B) (Sundram *et al*., 2019). Potassium notably affects bunch weight and bunch number (yield) and oil palm with K deficiency have been reported to produce less oil significantly (Naqiuddin *et al*., 2020). Other than that, potassium deficiency in oil palm has also been associated with BSR disease, cercospora leaf spot, vascular wilt disease and other physiological disorders that resulted in the

bunch and plant failure (Rankie and Fairhurst, 1999). The high amount of soil potassium at Palong plantation in the year 2018 and 2019 can be attributed to the extra application of K in the form of muriate of potash (MOP). According to Goh and Chew (1997), when the N, P and K status are in the very low or low category, the palms might experience symptoms of nutritional deficiency and it is recommended to raise the fertilizer rate for N and P in Palong. In contrast, as K was in the high/very high category, the application of K fertilizer should be reduced.

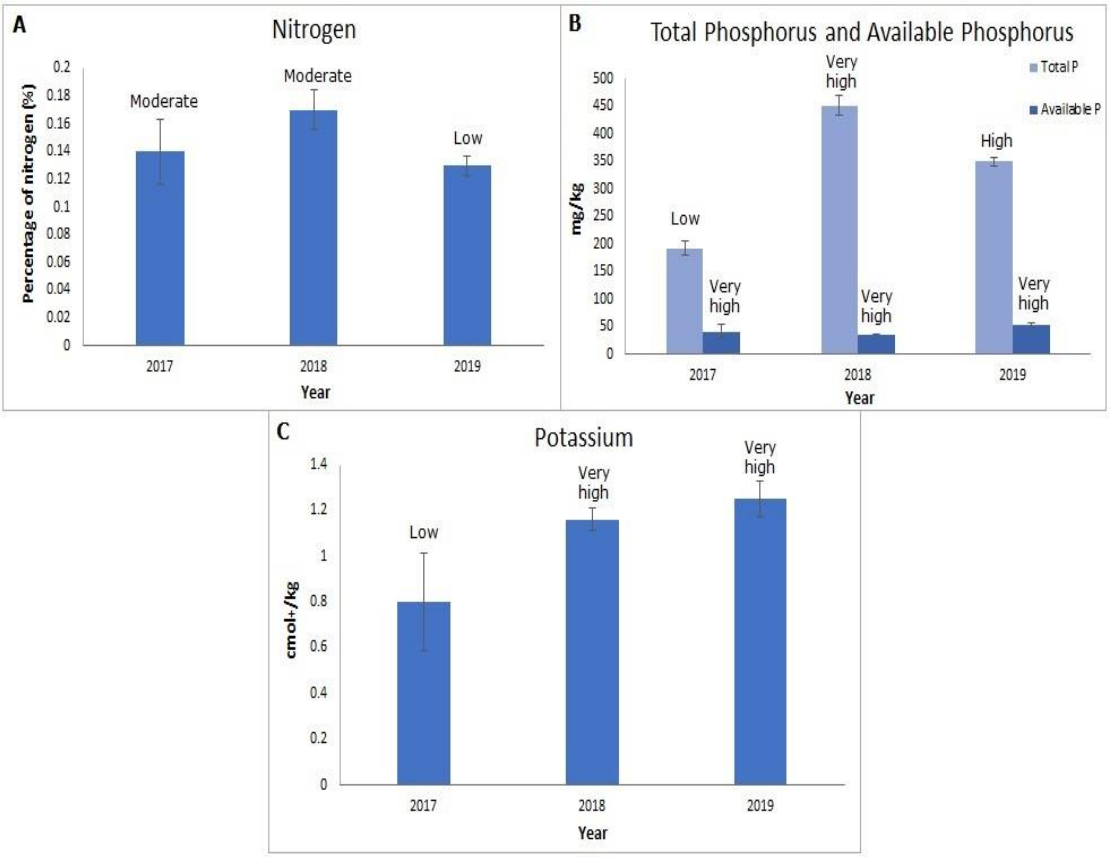


Figure 5.10 Soil nutrient content, (A) Nitrogen, (B) Total and available phosphorus and (C) Potassium at Tg Karang for the year 2017, 2018 and 2019. The N, P and K content were interpreted using Goh and Chew (1997) methods (Appendix 9).

At Tg Karang, soil nitrogen content was recorded at 0.14% in the year 2017 before increasing to 0.17% in the year 2018 and later dropping to 0.13% in the year 2019 (Figure 5.10). For phosphorus, even though total P was low in the year 2017 (192.3 mg/kg), this doesn't affect the contents of available P, which were

high for all the years and total P contents had doubled in the year 2018 and 2019. This may due to the presence of phosphorus solubilizing microorganisms (PSMs) that help to solubilize soil P and make it available for plant uptake (A. Sharma *et al*., 2012). It was suggested that PSMs might be lacking at Palong plantation and hence resulted in a lower level of available P in the soil. Depending on the types of soil, total P contents may vary and is influence by organic matter content, parent materials, climatic conditions and degree of fertilization in the plantation (Salem *et al*., 2014). Similarly, soil potassium level at Tg Karang plantation was initially low in the year 2017 (0.8 $cmol^{+}/kg$), before it increased to 1.16 and 1.25 $cmol^{+}/kg$ in the year 2018 and 2019 respectively.

Typically, fertilisation rates in smallholder oil palm plantations differ considerably based on the monetary resources and distance available to fertilizer suppliers (Dislich *et al*., 2017). Based on the description of the plantation owner at Tg Karang, LOF and FLOF were applied before and during the field trial together with other chemical fertilizers such as urea and ammonium chloride/sulphate (N fertilizer), rock phosphate (P fertilizer), MOP (K fertilizer) and kieserite (Mg fertilizer) (S.T. Yeo, personal communication, August 10, 2018). Therefore, the differing trends in N, P and K content in soil at Palong and Tg Karang were influenced by the fertilization regime at both locations.

For optimum crop production, the cultivation of oil palm demands a huge amount of fertilizers to complement the natural supply of soil nutrients, increase soil fertility and compensate for nutrients loss in the soil through leaching or uptake by the harvested products. Previous studies had indicated that balanced use of fertilizers will provide a better resistance in crop plants towards biotic and abiotic stresses such as drought, cold, pests and diseases (Sundram *et al*., 2019). Soil nutrient contents influence the progression of plant diseases and this may be linked to the effects of different nutrients on the enzyme production by pathogens (Dordas, 2008; Paterson, 2019). Hence, it is important for oil palm to be supplied

with balanced nutrition as this will play an essential role in plant resistance towards diseases especially BSR disease as well as sustainability in oil palm plantation. As oil palm is planted in diverse soil types, optimum design of fertilizer management should be practiced to complement the nutrient requirements in different soil types (Sundram *et al*., 2019).

5.3.4 Evaluation of BSR disease in oil palm

5.3.4.1 Disease incidence (DI)

The occurrence of plant diseases can be defined as the number of diseased plant units (visibly) in relation to the total number of plants evaluated (Madden and Hughes, 1995). Statistical analysis using Generalized Linear Model (GLM) showed no significant difference between treatments at each time point. From Table 5.4, at Palong plantation, disease incidence (DI) was recorded in treatments T1, T5 and T6 (non-*Trichoderma* treatments) and T2, T3 and T4 (FLOF-*Trichoderma* amended treatments). Treatments T7 to T10 (LOF-*Trichoderma* amended treatments) were relatively free from diseases. For T1, T5 and T6, the occurrence of disease was at 12 months onwards, whereas T2 and T3 only showed disease incidence at the end of the study which was at 20 and 24 months. Interestingly, in treatment T4 (FLOF amended with 20% *Trichoderma* spp.), it was noted that there were diseased palms at the start of the experiment with DI of 4.2 % and the DI in this treatment increased to 8.3 % at 6 months. However, after 6 months of treatment, there was no more disease incidence recorded in T4 palms, which suggested that the palms from this treatment might have recovered from BSR disease.

Generally, the disease incidences at Palong were low (below 13.0%) considering that the palms were at the end of their economic life (>25 years). Although *Ganoderma* also attacks young oil palm, it is thought that the disease incidence is greatest and more common in older palms. This is because, during their lifetime, older palms are more likely to be damaged/injured and may be regularly

exposed to the disease (Hushiarian *et al*., 2013). However, the disease and disease symptoms in plants may not necessarily become worse due to rapid wound healing, natural wood resistance or antagonistic interaction between microorganisms (Hushiarian *et al*., 2013). As Palong had high microbial populations (Figure 5.3, Figure 5.5A and Figure 5.6A), this might contribute to the suppression of the disease. This is supported by Khunaw *et al*. (2017) in which the presence of soil microorganisms was found to suppress *G. boninense* development in oil palm seedlings. Furthermore, a higher diversity of microorganisms is closely linked to the suppression of pathogenic diseases in plants (Rees *et al*., 2012).

Table 5.4 Disease incidence (%) at 0, 6, 12, 16, 20 and 24 months for 10 different treatments at Palong plantation.

Treatment	Disease Incidence at Palong (%)					
	0 Month	**6 Months**	**12 Months**	**16 Months**	**20 Months**	**24 Months**
T1	0	0	0	4.2	12.5	12.5
T2	0	0	0	0	0	4.2
T3	0	0	0	0	4.2	8.3
T4	4.2	8.3	0	0	0	0
T5	0	0	0	4.2	4.2	4.2
T6	0	0	4.2	4.2	4.2	4.2
T7	0	0	0	0	0	0
T8	0	0	0	0	0	0
T9	0	0	0	0	0	0
T10	0	0	0	0	0	0

T1: 0% *Trichoderma* in FLOF (1st year)/FLOF control (2nd year); T2: 5% *Trichoderma* in FLOF; T3: 10% *Trichoderma* in FLOF; T4: 20% *Trichoderma* in FLOF; T5: GSP; T6: 0% *Trichoderma* in LOF (1st year)/LOF control (2nd year); T7: 5% *Trichoderma* in LOF; T8: 10% *Trichoderma* in LOF; T9: 20% *Trichoderma* in FLOF; T10: GSP.

At Tg Karang, disease incidence was observed and recorded in all of the treatments (Table 5.5). Statistical analysis using Generalized linear model (GLM) showed that the treatments were significantly different at 20 and 24 months (P=0.048 and P=0.028 respectively). The DI in treatment T8 and T10 were

relatively high at the start of the experiment (12.5%) followed by T4 and T6 (8.3%) and T9 (4.2%). During the experimental periods, the DI in Treatments T2, T4, T6, T8, T9 and T10 had increased to between 16-30%, where the highest DI was recorded in T8 (29.2%) and T10 (25.0%) at 24 months. Meanwhile, T3 and T7 resulted in low DI at 4.2% at 16-24 months and 6-12 months respectively. There was no more occurrence of disease for palms in T7 at 16 months onwards, which was assumed that all the diseased palms might have recovered from BSR disease.

Besides, types of fertilizer also had a significant effect on DI (P=0.005), as it appeared that LOF treatments (T6-T10), except for T7, had recorded high incidences of *Ganoderma* attack, especially at the end of the field trial at 24 months in comparison to FLOF treatments (T1-T5). LOF is a pure plant-based organic fertilizer that contains high organic matter (OM) and low in N, P and K contents. It might not be suitable to be applied on peat soil. This is because peat soil already contained a high amount of OM, between 20-50% as compared to other soil types with around 5% of OM and this makes peat soil the vital storehouse of organic carbon (Uddin *et al*., 2019). As more organic matter/carbon was added to the soil through the application of LOF, this might favour the growth of *Ganoderma*. This was in agreement with the findings of Qudsia *et al*. (2017), in which high soil OM and N contents favour the growth and reproduction of pathogenic fungi in soil samples of cucumber plantation. In contrast, Widiastuti *et al*. (2018) reported that the carbon content in Mamuang soil (oil palm estate with wet soil) was highly correlated with *Ganoderma* sp., where the high carbon content resulted in a lower rate of attack by *Ganoderma* sp. Despite that, it is suspected that the *Ganoderma* sp. attack rate might be influenced by the form of carbon compound instead of the total amount of carbon in soil (Widiastuti *et al*., 2018). For this reason, FLOF may be more suitable for application at Tg Karang plantation or other plantations made up of peat soil. Since FLOF fertilizer has customized N, P and K formulations, these

nutrients (along with the additional application of other macro/micronutrients) will be vital to meet the nutrient requirements of oil palm.

Table 5.5 Disease incidence (DI) at 0, 6, 12, 16, 20 and 24 months for 10 different treatments at Tg Karang plantation.

Treatment	Disease Incidence at Tg Karang (%)					
	0 Month	**6 Months**	**12 Months**	**16 Months**	**20 Months**	**24 Months**
T1	0^{a}	0^{a}	4.2^{a}	12.5^{a}	8.3^{ab}	8.3^{abc}
T2	0^{a}	0^{a}	8.3^{a}	12.5^{a}	16.7^{ab}	16.7^{abc}
T3	0^{a}	0^{a}	0^{a}	4.2^{a}	4.2^{ab}	4.2^{bc}
T4	8.3^{a}	4.2^{a}	12.5^{a}	12.5^{a}	20.8^{a}	16.7^{abc}
T5	0^{a}	4.2^{a}	0^{a}	4.2^{a}	$8.3a^{b}$	8.3^{abc}
T6	8.3^{a}	8.3^{a}	20.9^{a}	8.3^{a}	16.7^{ab}	16.7^{abc}
T7	0^{a}	4.2^{a}	4.2^{a}	0^{a}	0^{b}	0^{c}
T8	12.5^{a}	4.2^{a}	29.2^{a}	20.8^{a}	25.0^{a}	29.2^{a}
T9	4.2^{a}	4.2^{a}	0^{a}	12.5^{a}	16.7^{ab}	20.8^{ab}
T10	12.5^{a}	4.2^{a}	8.3^{a}	16.7^{a}	16.7^{ab}	25.0^{a}

Means in a column followed by different alphabetic letters were significantly different ($P<0.05$).
T1: 0% *Trichoderma* in FLOF (1^{st} year)/FLOF control (2^{nd} year); T2: 5% *Trichoderma* in FLOF; T3: 10% *Trichoderma* in FLOF; T4: 20% *Trichoderma* in FLOF; T5: GSP; T6: 0% *Trichoderma* in LOF (1^{st} year)/LOF control (2^{nd} year); T7: 5% *Trichoderma* in LOF; T8: 10% *Trichoderma* in LOF; T9: 20% *Trichoderma* in FLOF; T10: GSP.

From the field studies at Palong and Tg. Karang plantations, diseased palms at varying levels of severity were observed. Symptoms include unopened spear leaves, the skirt-like appearance of the leaves, bole creation on the base of the trunk and the presence of *Ganoderma* fruiting bodies (Figure 5.2). These are the typical symptoms of BSR disease (Abdul Razak *et al*., 2004). Besides, the rate of BSR disease infection in oil palm depends on how contagious the disease is and the distribution pattern of the pathogen. The oil palm roots can come into contact with *Ganoderma* inoculums, basidiospore and secondary inoculum in the shape of an alternative house/host (Turner, 1981). Based on the compatibility studies of *Ganoderma* spp. collected from the same area or field may have different origins.

Hence, mycelial growth might not be the only method of disease transmission between palms (Miller, 1995).

Papavizas (1981) suggested that *Trichoderma* spp. need to reach an effective magnitude of 6.00 log cfu/g in the soil whereas biocontrol activity of *Trichoderma* spp. has been reported at populations between 5.00-7.00 log cfu/g in growing medium (Krause *et al*., 2001). The fact that the population of *Trichoderma* spp. in this study was only around 4.00-5.00 log of cfu/g may contribute to the inefficient biocontrol activity in the field and high DI, especially at Tg Karang plantation. Apart from that, it has been shown that there was a positive correlation between *Trichoderma* population and palms age to disease incidence and the soil profiles (Sariah, 2003). Sariah *et al*. (2005) reported that even though the population of *Trichoderma* was observed to be higher in the BSR infected area (DI>30%) than in other fields sampled within the oil palm ecosystem, the occurrence of BSR disease was still high. The soil in their study consisted of *Trichoderma* spp. population below 6.00 log cfu/g, thus an opportunity may exist for the phytopathogen to become prevalent in that area.

Table 5.6 Distribution of diseased palms and palms that have recovered in control and *Trichoderma* treatments at Palong and Tg Karang plantations.

Location	Treatment	No. of palms with disease (Percentage)	No. of palm recovered
Palong	Control treatments (T1, T5, T6, T10)	5 (5.2%)	0
	Trichoderma treatments (T2, T3, T4, T7, T8, T9)	5 (3.5%)	2
Tg Karang	Control treatments (T1, T5, T6, T10)	16 (16.7%)	0
	Trichoderma treatments (T2, T3, T4, T7, T8, T9)	30 (20.8%)	5

T1: 0% *Trichoderma* in FLOF (1st year)/FLOF control (2nd year); T2: 5% *Trichoderma* in FLOF; T3: 10% *Trichoderma* in FLOF; T4: 20% *Trichoderma* in FLOF; T5: GSP; T6: 0% *Trichoderma* in LOF (1st year)/LOF control (2nd year); T7:

5% *Trichoderma* in LOF; T8: 10% *Trichoderma* in LOF; T9: 20% *Trichoderma* in FLOF; T10: GSP.

Overall, the number of palms with the disease at Palong plantation was low. Out of 96 palms in the control treatments (T1, T5, T6 and T10), 5 palms (5.2%) were diseased. Meanwhile, out of 144 palms in *Trichoderma* treatments (T2, T3, T4, T7, T8 and T9), only 5 palms (3.5%) were infected with *G. boninense*. In comparison, the number of palms with the disease increased 3-6 folds at Tg Karang, about 16 palms (16.7%) for control treatments and 30 palms (20.8%) for *Trichoderma* treatments. Similarly, for control treatments at both locations, there were no palms that recovered from BSR disease based on the DI in this study. For *Trichoderma* treatments, there were 2 palms that recovered from Palong plantation, while at Tg Karang plantation, there were 5 palms that showed no symptoms of the disease at the end of the experiment.

During field observation, all the palms were individually monitored and assessed for symptoms of BSR disease. Therefore, it was possible to identify the palms that were infected and the disease progression of these palms was recorded during every field visit. It was noticed that there was no more growth of new fruiting body on the root/stem regions of some of the recovering and recovered palms in *Trichoderma* treatments while the old fruiting bodies of *Ganoderma* eventually dried up and fell off from the palms. There was also the formation of new roots on these recovering and recovered palms (Figure 5.11). However, it was also observed that in several palms with severe cases of *Ganoderma* infection, *Ganoderma* fruiting bodies had spread to the upper stem area, the roots and stems were brittle and usually, there were holes at the root base area (Figure 5.12).



Figure 5.11 One of the recovered palms at Palong. (A) Growth of new roots at the root area of the palm. (B) Close-up of the root area. Old *Ganoderma* fruiting bodies can be seen (red arrows). Photos were taken on 11 December 2018.



Figure 5.12 Severe cases of *Ganoderma* infection at Tg Karang. (A) Hole formation at the base/stem area. The fruiting bodies can be seen on the inside and outside of the trunk (red arrows). (B) The development of multiple fruiting bodies and these had progressed towards the upper part of the stem. Photos were taken on 5 March 2019.

As observed that only the palms from *Trichoderma* treatments that had recovered in this study, it was possible that *Trichoderma* had induced or enhanced the defence responses in oil palm, but further investigation is required to support this hypothesis. There were reports that shown *Trichoderma* had induced local and

systemic resistance against a variety of phytopathogens in plants (Harman *et al*., 2004). Musa *et al*. (2018) reported that a mixture of *Trichoderma* spp. treatment had improved plant defence enzymes activity significantly, namely polyphenol oxidase (PPO) and peroxidase (PO) as well as total phenolic content (TPC) in response to *G. boninense* infection in oil palm. However, it was suggested that *Trichoderma* was only effective for palms at the early stage of *Ganoderma* infection and could not cure palms that were severely infected (Abdullah *et al*., 2003). Meanwhile, there are other crucial defence mechanisms utilized by oil palm against the phytopathogen, such as the strengthening of the cell wall, pathogenesis-related proteins production and antifungal secondary metabolites (Ho *et al*., 2016).

Different types of soil may affect the spread of BSR disease in oil palm. In this study, Palong and Tg. Karang plantations are made up of different soil types (clayey laterite soil and peat soil respectively), this might explain the variations seen in the DI and the recovery rate of the palms at both locations. In Selangor, Malaysia, the BSR incidence was the second-highest at 30.96% on peat (Typic Fibric Tropohemist) soil after the Selangor-Kangkong (Aeric Tropic Fluvaquent-Typic sulfuric tropic fluvaquent) soil series (49.4%) (Parthiban *et al*., 2016). Nonetheless, there were reports that showed a high BSR disease incidence in inland soils (Khairudin, 1990), laterite soil (Benjamin and Chee, 1995) and peat soils (Rao, 1990). This suggests that BSR disease poses a threat in oil palm plantations across different soil types and it is necessary to further investigate the roles of different soil types in influencing the level of disease in the oil palm fields. In addition, the oil palm plantation at Tg Karang is located at the coastal area in this study, the plantation had previously been cultivated with coconut palms based on the description by the plantation's owner (S.T. Yeo, personal communication, August 10, 2018). It has been reported that the coconut palms can act as the source of inoculum for *Ganoderma* spp. to infect oil palm. When replanting was done on land previously cultivated with coconut or oil palm, the disease emerged earlier, between

12-24 months after planting. However, the disease was more common when palms were 4-5 years old. When palms reach 15 years old, disease incidence could be up to 40-50% *(*Kannan *et al*., 2017).

5.3.4.2 Leaf nutrient content

Treated oil palm leaves were sampled in the year 2018 and 2019 from Palong plantation and were analysed to determine the nutritional status of the palms. The N, P and K contents in leaf were interpreted according to von Uexküll and Fairhurst (1991) (Appendix 10), where the nutrients were categorized as deficiency, optimum or in excess levels. From Figure 5.13, leaf nutrients were at 2.74%, 0.18% and 1.22% respectively for N, P and K in the year 2018 before dropping to 2.30% N, 0.15% P and 0.87% K in the year 2019. The leaf nutrients were optimum in both years except for N in the year 2019 which was close to deficiency. In general, leaf N, P, and K contents were higher in the year 2018 as compared to the year 2019 at Palong plantation. This observation had complimented the results of soil nutrient contents as recorded in Figure 5.9 and was related to the fertilization regime (Appendix 11). Although the soil N and P contents were either in the low or very low category, this was the opposite with the leaf N and P contents, suggesting that the N and P nutrients in the soil were taken up by the palms.

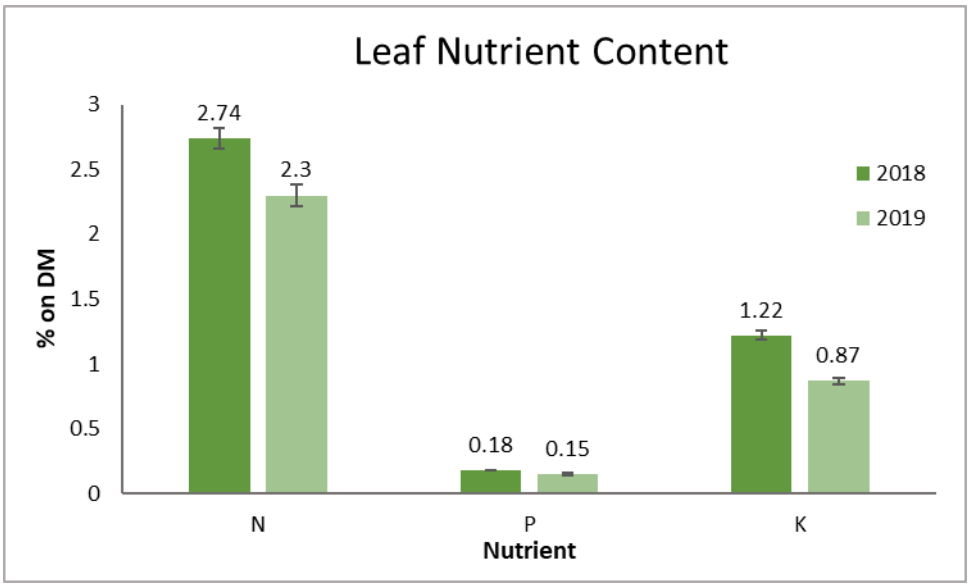


Figure 5.13 Leaf N, P, and K content at Palong for the year 2018 and 2019. The N, P and K content were interpreted according to von Uexküll and Fairhurst (1991) (Appendix 10).

As oil palm is a heavy feeder, it is necessary to supply an adequate amount of nutrients for its growth and yield. Often, nutrients are lost or removed through harvesting of the fruit bunches, immobilization in the trunk, leaching and run-off. It is therefore necessary to regularly track and monitor the soil nutrient status and leaf nutrient concentration of oil palm plantations (Behera *et al*., 2016; Woittiez *et al*., 2017). More importantly, many reports that suggested proper macro and micronutrient supply to oil palm were essential to control BSR disease (Nur Sabrina *et al*., 2012; Tengoua *et al*., 2014; Rahamah Bivi *et al*., 2016; Rebitanim *et al*., 2020).

Throughout the years, a variety of variables have been reported to affect BSR disease emergence and progression in oil palms, such as soil quality, nutrient contents, age of the palm, soil pH and temperature as well as planting techniques (Parthiban *et al*., 2016). Yet, it is unclear which of these variables have more influence on BSR development. These parameters may also be the factors that influence the survival of *Trichoderma* spp. In this study, the field trials were conducted at two different locations with different soil types and ages of palms.

Different estate management might play a greater and more significant role in BSR development, in which the independent smallholder estate at Tg Karang located on peat soil has a higher incidence of *Ganoderma* attacks. Another concern is that this proved that the BSR disease has no longer targeted older palms only as reported earlier since the oil palms at Tg Karang in this study were younger (around 10 years old). As Palong had higher *Trichoderma* and fungal count (4.00-5.00 log cfu/g and 5.00-6.00 log cfu/g respectively) in comparison to Tg Karang, this may play a role in the control of BSR disease as the higher population of beneficial microbes were able to suppress the pathogen. This contributed to lower disease incidence at Palong plantation.

There were very few fundamental studies on the suppression of BSR disease in the field as most of the researches was conducted based on evaluation at the nursery stage (Rebitanim *et al*., 2020). Field trials were influenced by greater extraneous variables and climatic conditions as compared to the nursery experiments and these factors may greatly affect the health and growth of the plants as well as the survival and proliferation of the biological control agent. Nonetheless, for successful biological control, *Trichoderma* spp. has to adapt and survive in the ecosystem and their population must be more than the targeted pathogens. Future application of *Trichoderma* treatments should be more than 20% while the impact of environmental conditions (uncontrollable variables) should also be studied further. In addition, the choices of fertilizer application (either LOF or FLOF) should be based on different soil types and properties.

6 CHAPTER 6: DETECTION AND QUANTIFICATION OF FUNGAL COMMUNITIES IN THE RHIZOSPHERES OF GANODERMA INFECTED MATURE OIL PALMS AT PALONG AND TG KARANG

6.1 INTRODUCTION

Soil microorganism plays a major role in controlling plant diseases. Soil microbiomes are related to plant disease in ways that they can stimulate the production of plant growth hormones, induce plant resistance towards pathogens, produce antibiotics or compete with the pathogens for the nutrients (Berendsen *et al*., 2012). The oil palm industry in Malaysia is under threat from soil-borne phytopathogens *Ganoderma boninense*, which is the causal agent for basal stem rot (BSR) disease. Most soil-borne pathogens are difficult to manage by traditional approaches through the use of resistant host cultivars and synthetic fungicides (Weller *et al*., 2002).

Since the management and control of plant diseases by the use of microorganisms is a natural, environmentally safe and chemical-free strategy, the inclusion of beneficial microbes to establish disease-suppressive soil is preferred for controlling plant pathogens (Penton *et al*., 2014). In contrast to the surrounding soils, disease suppression in plants is highly influenced by greater microbial diversity in the suppressive soil (Cook and Baker, 1983; Rees *et al*., 2012). Microbial interaction plays an important part in the soil microbiome and the interaction between different microbial groups is essential in influencing the functionality and stability of the ecosystem (Zhou *et al*., 2011). Besides, it also should be noted that the size and function of microorganisms are influenced by soil types. Currently, there are not many studies done on the role of soil microbial community on the suppression of *G. boninense* in oil palm with regards to types of soil.

While the oil palms at Palong were planted on laterite soil, the palms at Tg Karang were on peat soil in which from the previous chapter (Chapter 5), the occurrence of BSR disease was very high. The role of soil types on disease incidences was not yet fully understood or reported. The clayey peat soil that has high water retaining ability and poor drainage might favour BSR disease (Parthiban *et al*., 2016). BSR disease on peatlands is a major problem due to higher severity and occurs sooner (8 years) than on mineral land. The use of biological control agents has become the focus for the control of BSR disease, yet the development of this method has been limited by peatlands characteristics which differ from mineral soils (Supriyanto *et al*., 2020). Nevertheless, through the use of Geographical Information System (GIS), it was found that the distribution of BSR disease to be unrelated to the type of soil series (Parthiban *et al.*, 2016).

It has been suggested that the absence of indigenous soil microorganisms allowed *G. boninense* to thrive in the oil palm seedlings' roots and this affected the growth of the seedlings (Khunaw *et al*., 2017). Besides, the soil fungal community has been reported to increase in the presence of *T. harzianum* and *Glomus etunicatum* while *G. boninense* adversely affected the density of soil fungal community in the soil of oil palm seedlings (Alizadeh *et al*., 2011). While recognizing the significance of microbes in maintaining the soil environment, there is minimal research on the capacity of soil microbial diversity in the suppression of oil palm BSR disease. Understanding the nature of soil-borne diseases in relation to the rhizosphere's microbial composition would also be crucial to progress in the area of biological control

Hence, the aim of this experimental chapter was to investigate the microbial communities in the palm rhizospheres at both locations with different soil types. The fungal community structures especially *Trichoderma* spp. (as the biological control agent) and other fungal species were explored in relation to palms with different symptoms (healthy, asymptomatic and symptomatic). *Trichoderma* spp.

as well as the pathogen, *G. boninense* were also quantified via real-time Polymerase Chain Reaction. Most of the researches reported the detection of *G. boninense* in diseased oil palm root tissue but the detection of this pathogen in the soil has not yet been reported. The emphasis was on the fungal communities since this group of soil organisms plays a crucial part in the soil ecosystem as mutualists, decomposers and pathogens. It is hypothesized that the shift in the fungal community, in which a decrease in beneficial fungi will enable potential pathogen such as *G. boninense* to become prevalent. This in turn affects the soil and plant health.

6.2 MATERIALS AND METHODS

6.2.1 Collection of the rhizosphere samples

The oil palm at Palong and Tg Karang plantation were monitored for a period of 28 and 24 months respectively and *Ganoderma*-infected palms had been identified for the sampling of the rhizospheres. These palms are classified as symptomatic (S; *Ganoderma*-fruiting body was present throughout observation up until 28 months) and asymptomatic (AS; *Ganoderma*-fruiting body was detected and disappeared at some point during observation). Healthy palms (H) in which the palms did not show any typical symptoms of BSR disease, no wilting of fronds and looked healthy were chosen as the control. There were at least three biological replicates for each category and a summary of the palms that were sampled is shown in Table 6.1.

Table 6.1 Descriptions of the palms at Palong and Tg Karang plantations in which the rhizosphere samples were taken. H: healthy, AS: asymptomatic and S: symptomatic.

Location	Palm Label	Sample number	Symptom	Description
Palong	PACtrl	31	H	Healthy palm at Palong (Block A)
	PBCtrl	32	H	Healthy palm at Palong (Block B)
	PCCtrl	33	H	Healthy palm at Palong (Block C)
	PA3T4	1	AS	Fruiting body at 0 month and 6 months only
	PB6T3	7	AS	Fruiting body at 20 months to 24 months, disappear at 28 months
	PC2T4	10	AS	Fruiting body at 6 months only
	PB3T1	3	S	Fruiting body appeared at 16 months to 28 months
	PB7T2	5	S	Fruiting body appeared at 24 months to 28 months
	PB8T3	8	S	Fruiting body at 28 months (new case)

Tg Karang	KACtrl	37	H	Healthy palm at Tg Karang (Block A)
	KBCtrl	38	H	Healthy palm at Tg Karang (Block B)
	KCCtrl	30	H	Healthy palm at Tg Karang (Block C)
	KA6T3	13	AS	Fruiting body at 20M only
	KA6T4	14	AS	Fruiting body at 6M only
	KC1T6	27	AS	Fruiting body at 20M only
	KC6T7	28	AS	Fruiting body at 12M only
	KB1T2	17	S	Fruiting body appeared at 20M to 24M
	KB2T3	18	S	Fruiting body at 16M and 24M only
	KB8T10	22	S	Fruiting body at 24M
	KC4T4	25	S	Fruiting body appeared at 12M to 24M

*Example= PA3T4, P= Palong; A= Block A, 3= Tree number 3; T4= Treatment 4

Sampling of the rhizosphere was done by using soil augers at depth of 10 cm according to McGuire *et al*. (2015) and Yeoh *et al*. (2016). Three rhizosphere samples were taken around the palm and pooled together. In the field, soils were kept on ice and immediately stored at -20°C in the laboratory prior to DNA extraction.

6.2.2 DNA extraction from the soil

Total DNA from soil samples was extracted from 0.25 g (fresh weight) of soil by using DNeasy Power Soil Kit (QIAGEN Inc., USA) according to the manufacturer's instructions with modifications in which all the centrifugation steps were carried out at 10 °C. DNA purity and yield were determined spectrophotometrically by using Nanodrop One^{c} (Thermo Fisher Scientific).

6.2.3 Extraction of DNA from *Ganoderma boninense* and *Trichoderma* spp.

G. boninense, GBLS isolate was grown on PDA to obtain the mycelial biomass. Fourteen days old GBLS mycelial plugs were transferred into 25 ml of MEB in 50 ml centrifuge tubes. The inoculated tubes were then agitated at 120 rpm and at room temperature for 7 days on an orbital shaker. The mycelial culture was harvested by centrifugation at 10 000 g for 20 mins (Eppendorf, 5810R). The supernatant was discarded and the pellet was air-dried followed by grinding in liquid nitrogen. The same step was carried out for *Trichoderma* spp. strains 4A, T1 and B2 (Tmix) but by using 7-days old culture grown on PDA plates.

DNA extractions were carried out for *G. boninense* and *Trichoderma* spp. according to Góes-Neto *et al*. (2005) and Goh (2016) by using Cetyltrimethylammonium bromide (CTAB) buffer-based extraction method. Firstly, 500 µl of CTAB extraction buffer (100 mM Tris HCl at pH 8.0, 1.4 M NaCl, 2% CTAB, 20 mM EDTA and 1% PVP) was warmed up to 65 °C before being added to 1.5 ml microcentrifuge tube containing 0.05 g ground mycelial DNA. The mixture was incubated overnight before being centrifuged down at 10 000 g for 10 mins. The supernatant was homogenized with chloroform:isoamyl alcohol (24:1), precipitated with 0.5 volumes of isopropanol and 0.3 volumes of 3 M sodium acetate and washed three times by using 70% ice-cold ethanol. The DNA pellet was suspended in 30 µl nuclease-free water and 100 µg/ml RNase A. The tube was further incubated at 37 °C for 1 hour before the DNA samples were stored at -20°C until further use.

6.2.4 Polymerase Chain Reaction (PCR) and DNA sequencing of fungal communities in the soil

For detection of *Trichoderma* spp., PCR master mix was prepared in 25 µl reactions and consisted of the following components: 1X Green GoTaq® Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.25 U GoTaq® Flexi DNA polymerase, 500

nM forward and reverse primers, 50-60 ng DNA samples and nuclease-free water. For detection of *Trichoderma* spp., primer pair TITS5F and TITS5R were used whereas the universal primer pair of ITS1-F and ITS2 were used to amplify the first internal transcribed spacer region (ITS1) of the fungal rRNA gene.

Table 6.2 The sequence of the forward and reverse primers that were used to detect *Trichoderma* spp. and fungal populations in rhizosphere samples.

Target	Primer design
***Trichoderma* ITS** (Saravanakumar *et al*., 2016)	Forward primer, TITS5F-5'-CCT GTC CGA GCG TCA TTT CA-3' Reverse primer, TITS5R-5'-TGG GTG TTT TAC GGA CGT GG-3'
Fungal ITS (Gardes and Bruns, 1993; Bellemain *et al*., 2010)	Forward primer, ITS1-F-5'-CTT GGT CAT TTA GAG GAA GTA A-3' Reverse primer, ITS2-5'-GCT GCG TTC TTC ATC GAT GC-3'

For detection of *Trichoderma* spp. in the soil, PCR was conducted by using Tmix DNA as the positive control and nuclease-free water as the negative control. The PCR Thermocycler (Eppendorf, Mastercycler Gradient) was programmed according to the following conditions: Initial denaturation at 94°C for 2 mins, followed by 40 cycles of 94 °C for 30 s, 57 °C for 1 min and 72 °C for 1 min. A final extension was performed at 72°C for 10 mins.

Meanwhile, detection of the fungal population was carried out by using nuclease-free water as the negative control. The PCR conditions were as follow: 94 °C for 3 mins for initial denaturation followed by 35 cycles of 94 °C for 45 seconds, 50 °C for 1 min and 72 °C for 90 s of annealing and extension of the target gene. The final extension was carried out at 72 °C for 10 mins.

After PCR amplification, 10 µl of PCR products were loaded onto 1.5% agarose gel that was stained with 4.0 µl of SYBR® Safe (Invitrogen, US) in 1x Tris-Borate-EDTA (TBE) buffer. A volume of 4 µl of 100 bp DNA ladder (GeneDireX, US) was used as a DNA marker. The gel was run for 42 mins at 80 V and images of the

bands were captured using a UV transilluminator connected to the Gel Doc™ XR imaging system (Bio-Rad, US).

The PCR products were sent to First Base Laboratories Sdn Bhd, Seri Kembangan, Malaysia for sequencing. Sequences were analysed using Applied Biosystems Sequence Scanner Software 2.0 and were aligned with the Basal Local Alignment Search Tool (BLAST) program to search for the closest matches in the National Center for Biotechnology Information (NCBI) GenBank Database.

6.2.5 Quantification of *G. boninense* and *Trichoderma* spp. in the rhizospheres through real-time Polymerase Chain Reaction (qPCR)

The quantity of *Ganoderma boninense* and *Trichoderma* spp. in the rhizosphere samples were quantified via absolute quantification in qPCR. DNA of rhizosphere samples were diluted in nuclease-free water so that the final concentration of DNA templates that will be used in qPCR amplification was 10 ng/ µl. A standard curve of pure GBLS DNA was prepared through 10-fold dilution series (1×10^{1}, 1×10^{0}, 1×10^{-1}, 1×10^{-2}, and 1×10^{-3} ng/µl). The qPCR master mix was prepared in a total volume of 20 µl in low-profile PCR tube strips (Labcon, US). Each reaction mixture contained 1X SensiFAST SYBR® No-Rox Mix (Bioline, UK), 400 nM of GbF and GbR primers (Table 6.3), 1 µl DNA template of rhizosphere samples and each of the GBLS DNAs that were serially diluted for the standard curve and nuclease-free water. For non-template control (NTC), it contained the same mixtures except that 1 µl of nuclease-free water was added instead of DNA template. The PCR tube strips were briefly spin down by using a mini centrifuge (Heathrow Scientific, US) to ensure that the reaction mixture was well mixed.

Real-time PCR amplifications were conducted using CFX Connect Real-Time PCR Detection System (Bio-Rad, US). Thermal cycling conditions were programmed at 95 °C for 3 mins for polymerase activation, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. The melt curve temperature profile was set for 1 cycle at 95

°C for 10 s, 65 °C for 5 s and 95 °C for 5 s. There were three individual reactions (technical replicates) for each rhizosphere biological sample and the standard curve and the assays were repeated thrice. The quantitation cycle (Cq) in which fluorescence can be detected was calculated by Bio-Rad CFX Manager software (Bio-Rad, US). A standard curve was obtained by plotting the Cq values against the log starting quantity of each 10-fold dilution series of GBLS DNA. By using correlation analysis, interactions between Cq value and DNA concentration were analysed. Therefore, the abundance of *G. boninense* in the rhizosphere samples was determined by comparing the Cq values to the crossing point values of the linear regression line of the standard curve.

The same protocol was applied to quantify *Trichoderma* spp. in the rhizosphere samples but using 10-fold dilution series of Tmix as the standard curve and specific primers to detect *Trichoderma* (Table 6.2).

Table 6.3 The sequence of the forward and reverse primers that were used to detect *Ganoderma* DNA.

Target	Primer design
***Ganoderma* ITS** (Supramaniam, 2016)	Forward primer, GbF-5'-TTG ACT GGG TTG TAG CTG-3' Reverse primer, GbR-5'- GCG TTA CAT CGC AAT ACA-3'

In order to ensure that the qPCR products were of the expected size, the samples were loaded onto the wells of 1.5% agarose gel in 1X TBE buffer at 80 V for 42 minutes. The DNA bands were visualized using the Gel Doc™ XR imaging system.

6.2.6 Statistical analysis

Genstat (18th edition) was used for statistical analysis. The data were statistically analysed by using analysis of variance (ANOVA) and Tukey Multiple Comparison test was used to detect significant differences between treatments ($P \leq 0.05$). Transformation was carried out for the data that doesn't fit the normality

assumption while non-parametric data were analysed using the Kruskal-Wallis test. Pearson's correlation coefficient test was used to evaluate the relationship between the quantity of *Trichoderma* spp. and *Ganoderma* spp. in the palm rhizospheres.

6.3 RESULTS AND DISCUSSION

6.3.1 Molecular detection and sequencing of fungal communities in the palm rhizospheres

6.3.1.1 *Trichoderma* spp. at Palong plantation

By using specific primers for *Trichoderma* (TITS5F and TITS5R), *Trichoderma* ITS DNA was successfully detected in all the rhizosphere samples in which all the bands were of similar size with the positive control (~139 bp) (Figure 6.1). This result was consistent with the past finding (Saravanakumar *et al*., 2016), where *Trichoderma* was amplified in infected cucumber root by using the same primer. *Trichoderma* spp. that were present in the soil samples might be the same species of the Tmix (*T. asperellum* and *T. longibrachiatum*) or other *Trichoderma* spp. that were ingenious in the soil.

The PCR products that were amplified from the *Trichoderma* ITS region were sequenced and analysed with the BLAST program. From BLAST, the E-values of all the samples were between 4e-32 to 1e-67, indicating that database matches of very high quality (smaller E-value will give a better match). Other than that, the percent identity was around 86.92% to 99.32% in which the most common species found in all the symptoms were *T. asperellum, T. viride, T. hamatum* and *T.* sp. (Figure 6.2). There were more *Trichoderma* species found in healthy samples (8 species) with *T. harzianum* being the unique species while *T. asperolloides* and *T. yunnanense* were shared between healthy and asymptomatic samples. In addition, *T. effusum* was common between the healthy and symptomatic samples and *T. reesei* was unique to symptomatic samples. Interestingly, asymptomatic samples

did not have any unique *Trichoderma* and they also did not have any similarities with symptomatic samples.

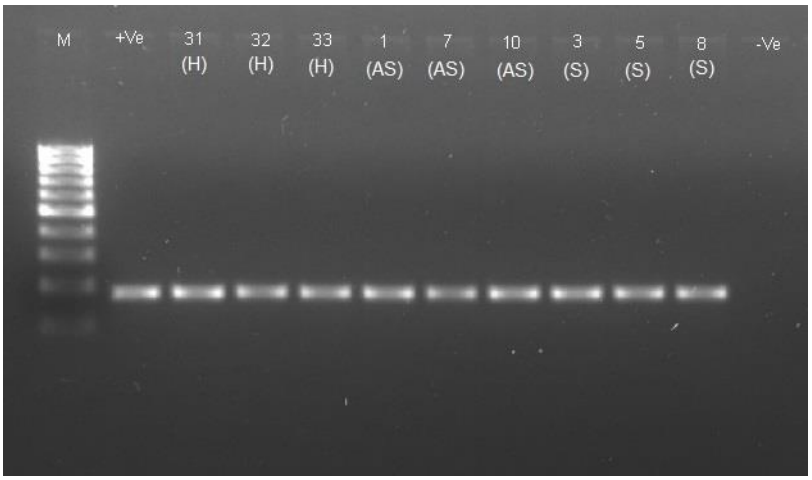


Figure 6.1 PCR amplification of *Trichoderma* DNA according to sample number and symptoms (H, AS, S) at Palong. M: 100 bp ladder; +Ve: Tmix DNA; -Ve: Nuclease-free water.

Furthermore, *T. longibrachiatum* (B2) which was applied as bioorganic fertilizer was not detected in any of the samples. This was in contrast with *T. asperellum* (4A and T1) which was the dominant *Trichoderma* species found, regardless of the symptoms. The absence of B2 indicated that the strain/species might not be able to adapt and survive in the oil palm rhizosphere even though it showed promising results against *Ganoderma in vitro* (Results are not shown). *T. reesei* found in symptomatic samples is phylogenetically very close to *T. longibrachiatum* while *T. effusum*, *T. reesei* and *T. longibrachiatum* belong to *Longibrachiatum* clade (Plessis *et al*., 2018; Kubicek *et al*., 2019). It is also possible that *T. longibrachiatum* was in the rhizosphere but was not detected since the primers used were not species-specific and sometimes sequence-based approaches did not give correct identification due to genetically closest neighbouring species (Devi *et al*., 2012). To improve *Trichoderma* detection and identification especially for 4A, T1 and B2 which were applied as biofertilizer, specific primers should be designed and/or conserved sequences of target genes such as *translation*

elongation factor-1α (*tef1*) and ribosomal subunit (rpb2) (Devi *et al*., 2012; Jaklitsch and Voglmayr, 2015) can be used besides the ITS region. This is because, it is of special importance to track the fate, behaviour and population dynamics of *Trichoderma* strains released as biocontrol agents into the environment (Kredics *et al*., 2018).

Isolation and characterization of *Trichoderma* from the oil palm ecosystem based on DNA polymorphism revealed that *T. harzianum* was the most common isolates found, followed by *T. virens, T. koningii* and *T. longibrachiatum* (Sariah *et al*., 2005). In Indonesia, endophytic *Trichoderma* was isolated from oil palm roots and from 20 isolates, the three most promising isolates against *Ganoderma* were identified as *Trichoderma reesei* strain RHa, *Trichoderma asperellum* isolate F1 and *Trichoderma asperellum* strain Q1 through DNA sequencing of 5.8S-ITS region (Haryadi *et al*., 2019). Cummings *et al*. (2016) collected root samples from more than 58 genera including *Elaeis guineensis* (oil palm), from a range of habitats in Malaysian Borneo. The most often isolated species were *T. asperellum*, *T. asperelloides*, *T. harzianum* and *T. virens*, according to tef1 analysis. Other species present were *T. afroharzianum*, *T. guizhouense*, *T. reesei* and *T. strigosum*.

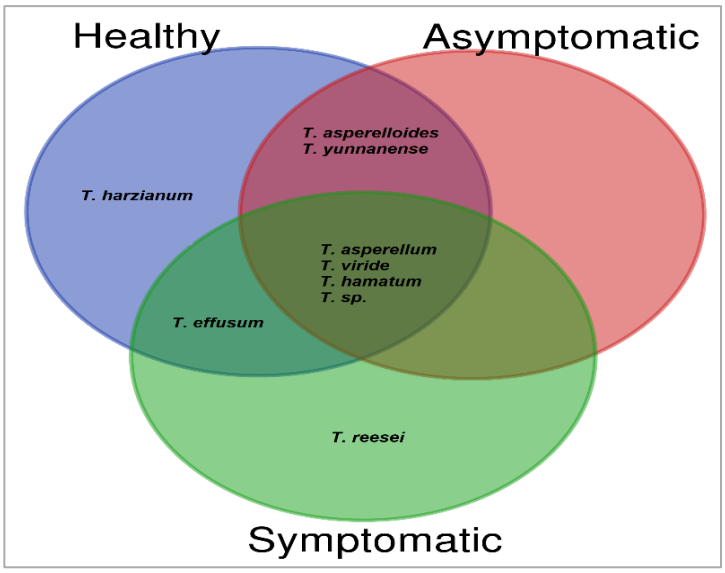


Figure 6.2 Venn diagram of the *Trichoderma* spp. found between samples of different symptoms at Palong. From the BLAST program, the E-values were between 4e-32 to 1e-67 and the percent identity was around 86.92% to 99.32%.

From the previous studies, it showed that generally, the most common *Trichoderma* spp. found in oil palm plantations were *T. harzianum*, *T. asperellum* and *T. virens.* In this study, the most frequently encountered species in all the samples was *T. asperellum* which can either be indigenous in the soil or the biological control agent added through the application of bioorganic fertilizer. It is considered to be mainly distributed in tropical regions with rare occurrences in temperate regions (Cummings *et al*., 2016). Except for *T. harzianum*, *T. viride* and the *Longibrachiatum* clade mentioned above, all the *Trichoderma* species belong to *Hamatum*/*Asperellum* clade (Qin and Zhuang, 2016). Since *T. harzianum* was only found in healthy samples, this indicated that there might be a shift in *Trichoderma* population/species which enabled *Ganoderma* to become prevalent or the presence of the pathogen itself alters the dynamics of *Trichoderma* in asymptomatic and symptomatic samples. Ros *et al*. (2017) showed that the changes in the bacterial

rhizosphere community of pepper seedlings were due to infection by *Phytophthora nicotianae* and not attributed to the *Trichoderma* strain.

6.3.1.2 *Trichoderma* spp. at Tg Karang plantation

Trichoderma was successfully detected in the oil palm rhizospheres by using DNA samples extracted directly from the soil. From Figure 6.3, all the bands of different intensities were of the same size as the positive control (Tmix DNA). Since the negative control had no signal/band, the presence of *Trichoderma* in any of the samples was not due to contamination or false-positive DNA fragments.

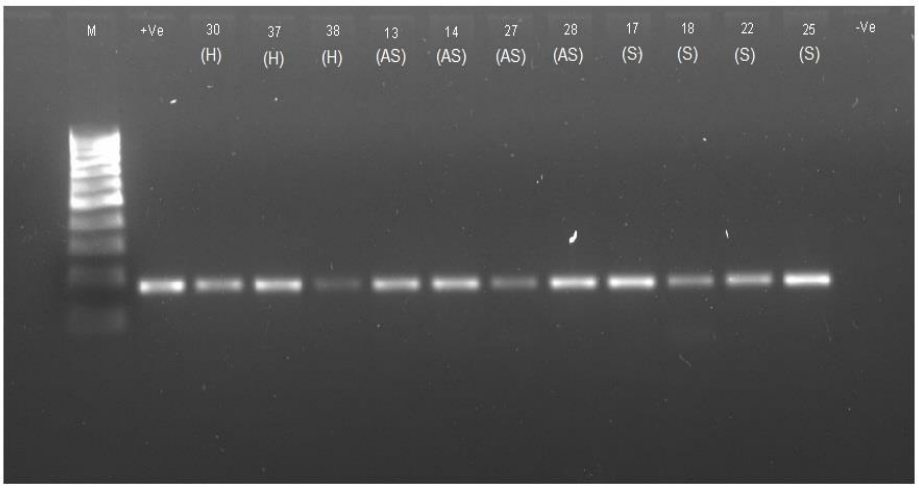


Figure 6.3 PCR amplification of *Trichoderma* DNA according to sample number and symptoms (H, AS, S) at Tg Karang. M: 100 bp ladder; +Ve: Tmix DNA; -Ve: Nuclease-free water.

Although the size of the PCR products among the samples was approximately the same, the sequence similarity nor the phylogenetic relationship may not be fully represented. Analysing tools such as random amplified polymorphic DNA (RAPD), amplification length polymorphisms (RAFLP) or genomic sequence analysis (BLAST, phylogenetic tree, etc.) may further discern the genomic sequence (Haryadi *et al*., 2019). By using BLAST program, it was found that the percent identity for all the samples was very high, between 95.33% to 100% and

the E-value between 3e-59 to 1e-68. From Figure 6.4, *T. asperellum*, *T. viride*, *T. hamatum* and *T.* sp. were found in all three symptoms whereas *T. yunnanense*, *T. asperelloides* and *T. paucisporum* were common between asymptomatic and symptomatic samples. *Trichoderma* populations in asymptomatic and symptomatic samples were very similar to each other while only healthy samples possessed unique species of *Trichoderma* (*T. effusum* and *T. reesei*). As in Palong samples (Figure 6.2), *T. longibrachiatum* was absent in all the samples.

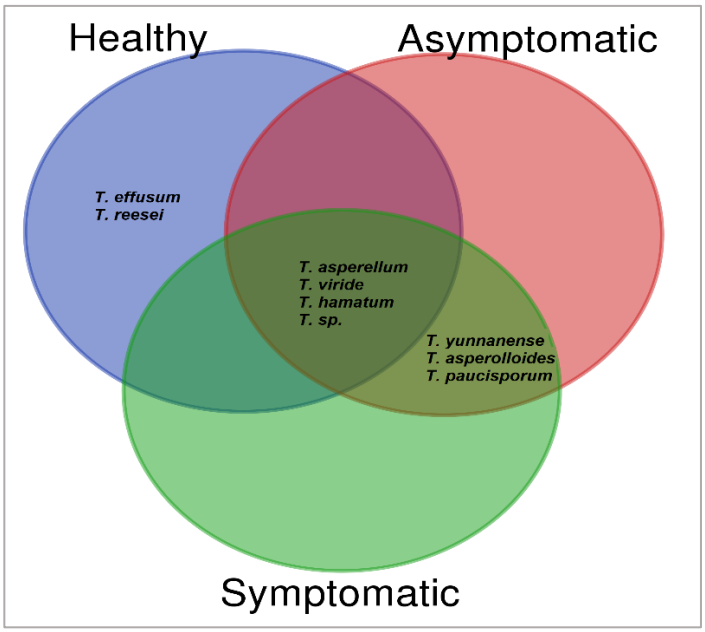


Figure 6.4 Venn diagram of the *Trichoderma* spp. found between samples of different symptoms at Tg Karang. From the BLAST program, the E-values were between 3e-59 to 1e-68 and the percent identity was around 95.33% to 100%.

Similar *Trichoderma* species were found at both Tg Karang and Palong (Section 6.3.1.1), but with different distribution between the different symptoms. Since only the healthy samples possessed unique species, further investigation needs to be conducted on whether these play a role in suppressing pathogens such as *Ganoderma* and/or enhance the plant defence response. This is in agreement with the findings of Cummings *et al*. (2016) in which *T. asperellum* was found in the rhizosphere of oil palm and in contrast with the findings of Joseph (2000) where

T. *harzianum* was the prominent species isolated from the palm rhizosphere in Malaysia. Other than that, in a study to determine the fungal community associated with peat soil in Sarawak, Malaysia, it appeared that *Hypocrea atrovidis* was generally associated with areas planted with oil palm and logged-over forest. *Hypocrea* is teleomorphs of *Trichoderma* where *Trichoderma* themselves have *Hypocrea* as anamorphs (Siti Ramlah *et al*., 2016). Since *T. asperellum* was the main *Trichoderma* species detected at Tg Karang plantation (peat soil), it might be the biocontrol agent added through bioorganic fertilizer. This might explain the CFU count at Tg Karang in which the population was low at the beginning of the field trial (0 month) and increased after application and became stable over time (Section 5.3.1).

6.3.1.3 Other fungal communities at Palong plantation

The universal primers used for fungal amplification were ITS1-F and ITS2. These primer pairs will amplify the ITS1 region and the expected size of the amplicons will be between 250-600 bp (Bokulich and Mills, 2013; Hoggard *et al*., 2018). Unlike ITS2, there are 3 restriction sites in the ITS1 region which mean there is more divergence observed in ITS1 as compared to ITS2. Hence, it is suggested to design an experiment based on ITS1 sequences (Hushiarian *et al*., 2013). From Figure 6.5, agarose gel electrophoresis showed that all the samples were amplified with variations in the ITS regions and the size of the bands was mostly around ~300 bp.

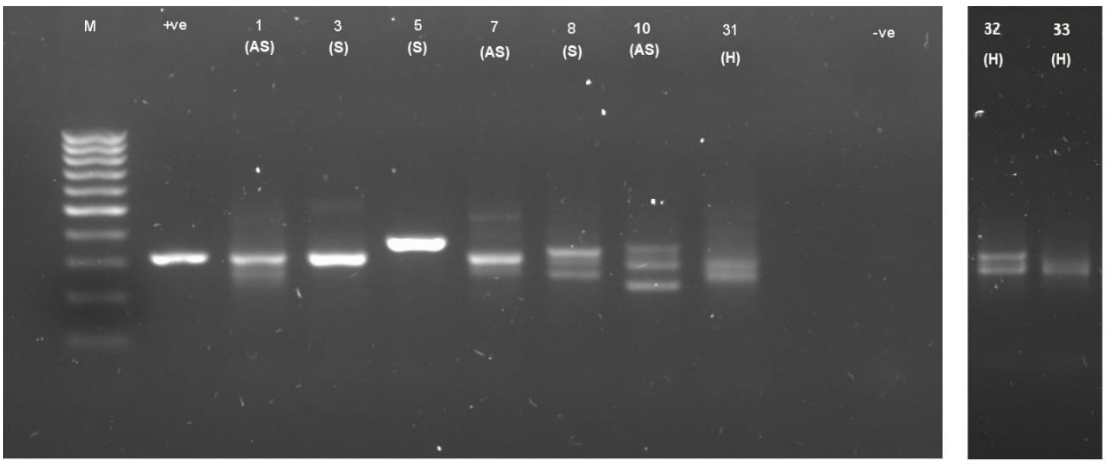


Figure 6.5 PCR amplification of the fungal communities in the rhizosphere of oil palms with different symptoms at Palong. M: 100 bp ladder; +Ve: GBLS DNA; -Ve: Nuclease-free water.

By using the BLAST program, the cut-off for E-value was set to 1e-05 for all the samples (Kerfeld and Scott, 2011). E-value is the first parameter that can be used for a quality filter for the BLAST search result and the E-value is not a fixed value as it depends on the length of the query sequences and the size of the database. A BLAST hit with an E-value of less than 1e-50 indicates very high-quality database matches, whilst an E-value of less than 0.01 indicates a reasonable homology match (Scholz, 2008). Little is known about the differences of soil microbes between healthy and infected soils with *Ganoderma boninense*. For this reason, the possible genera obtained from BLAST that was present in healthy, asymptomatic and symptomatic samples were listed in Table 6.4. Healthy samples were very similar to each other, with 8 common genera (*Aspergillus*, *Clonostachy*s, *Corallomycetella*, *Fusarium*, *Metarhizium*, *Monocillium*, *Thelonectria*, *Trichoderma*). This was followed by asymptomatic samples with 4 common genera namely *Diplodia*, *Ganoderma*, *Lasiodiplodia* and *Penicillium* whereas symptomatic samples were very dissimilar with each other with only *Trechispora* as the common genus.

Besides *Lasiodiplodia* and *Exophiala*, *Aspergillus* was also shared between healthy and asymptomatic samples. This is supported by the findings of Goh *et al*. (2013) where *Aspergillus* was detected in healthy and diseased (*Ganoderma*

boninense infected) soil of oil palm. *Aspergillus* species are widespread in the environment with some being pathogen and toxin producers (Goh, 2013) while several species of *Penicillium* and *Aspergillus* secrete extracellular enzymes for hydrolysis of lignocellulosic biomass such as oil palm fruit bunch (Shahomlail *et al*., 2013). For *Lasiodiplodia*, it has been recovered from common spear rot (CSR) lesion of oil palm leaves and was classified as a weak pathogen. Also, it has been associated with another disease of oil palm such as seedling anthracnose that is caused by *Lasiodiplodia theobromae* (Suwandi *et al*., 2012).

By using universal primers for fungal identification, *Trichoderma* was detected in healthy samples but not in asymptomatic and symptomatic samples. This was in contrast with the use of *Trichoderma*-specific primers (Section 6.3.1.1) in which the target fungus was detected in all the samples of the 3 symptoms. Since universal primers have a broad range, the ITS region of any fungi present will be amplified. *Trichoderma* might have been masked by other fungi with higher populations, thus lowering its chance of being amplified/detected by the primers. Besides *Trichoderma*, the genus *Clonostachys* and specifically *Clonostachys rosea* was also detected in healthy samples. It is a necrotrophic mycoparasitic fungus used as biological control agent and has been reported to control diseases caused by *Botrytis cinerea*, *Bipolaris sorokiniana*, *Fusarium graminearum*, *Fusarium culmorum*, *Alternaria* spp. and *Sclerotinia sclerotiorum* (Nygren *et al*., 2018). Recently, *C. rosea* had been discovered from the trunk tissues of oil palm infected with *G. boninense* and its potential as an antagonist against *Ganoderma* has been evaluated (Goh *et al*., 2020a).

Table 6.4 List of fungal genera found in samples of different symptoms at Palong based on the BLAST Program and NCBI GenBank Database.

Healthy	Asymptomatic	Symptomatic
Acrodontium	*Alanphillipsis*	*Auricularia*
Aspergillus[*#]	*Aplosporella*	*Clavulina*
Beuveria	*Aspergillus*[#]	*Cortinarius*
Clonostachys[*]	*Barriopsis*	*Entoloma*

*Corallomycetella**	*Botryosphaeria*	*Flammulina*
Cordyceps	*Byssochlamys*	*Litschauerella*
Endomelanconiopsis	*Cyphellophora*	*Magnaporthiopsis*
Exophiala#	*Dextricocystis*	*Phacidium*
*Fusarium**	*Diplodia**	*Pseudobaeospora*
Graphium	*Emmia*	*Rodocybe*
Lasiodiplodia#	*Exophiala*#	*Scytinopogon*~
Mariannaea	*Ganoderma**	*Stereum*
*Metarhizium**	*Irpex*	*Subulicystidium*~
*Monocillium**	*Lasiodiplodia**#	*Symbiotaphrina*
Neofusicoccum	*Macrophomina*	*Termitomyces*
Paraisaria	*Mucoharknessia*	*Trechispora**~
Phiosalosphora	*Penicilium**	*Tricholoma*
Placynthium	*Plialophora*	*Tubulicium*
Sarea	*Pseudevernia*	
Staurothele	*Scytinopogon*~	
*Thelonectria**	*Sphaeropsis*	
Tolypocladium	*Subulicystidium*~	
*Trichoderma**	*Taloromyces*	
Trimatostroma	*Tetracladium*	
Zythia	*Trametes*	
	Trechispora~	

* Denotes common genera between samples of the same category

# Denotes common genera between Healthy and Asymptomatic samples

~ Denotes common genera between Asymptomatic and Symptomatic samples

The genus *Metarhizium,* particularly *Metarhizium anisopliae* has been used as a biopesticide to control rhinoceros beetle (*Oryctes rhinoceros*) which is a serious pest of oil palm in Malaysia (Moslim *et al*., 2013). The presence of *Metarhizium* in Palong may be due to its application by the estate management in which it can be applied as a spore solution by blanket spraying, the use of auto dissemination trap for the release of infected adult beetles and artificial breeding site (ABS) technique. Nevertheless, the choice of application will be influenced by localities, areas of infestation and available facilities at the plantation (Moslim *et al*., 2013). Apart from this, the genus *Monocillium* has also been reported to parasitize nematode eggs. *Monocillium gamsii* sp. nov. and *Monocillium bulbillosum* colonized and destroyed the cereal cyst nematode *Heterodera filipjevi*. There are currently eighteen species

in this genus and can be found in soil, plant material, building material and also from other fungi (Ashrafi *et al*., 2017).

For the genus *Fusarium,* the most frequently encountered species from the BLAST search was *Fusarium oxysporum*. This was similar to the study conducted by Brinkmann *et al*. (2019) in which the species was the most abundant pathogen identified in an oil palm plantation, thus supporting the idea that oil palm monoculture encourages the proliferation of such species. The genus in general can either act as saprophytes, decomposers or plant pathogen (Karim *et al*., 2016). In oil palm, *Fusarium oxysporum* f. sp. *elaeidis* (Foe) is known to cause vascular wilt disease. Although it is endemic in Africa, it is an anomaly that the disease is not prevalent in Southeast Asia. It is speculated that the absence of the disease in Malaysia is due to natural suppression by microbial soil components, acting directly or indirectly through the plant defence system (Cooper and Rusli, 2014). It is also interesting that *Ganoderma* was detected in asymptomatic samples but not symptomatic. This showed that even though *Ganoderma* was not seen physically as a fruiting body on the oil palm stem, it still persists in the soil. For symptomatic samples, perhaps *Ganoderma* is more concentrated in the palm, degrading the lignin component of the wood as compared to in the soil though this should be investigated further.

Another compelling finding was for the genus *Trechispora* in which it was dominant in symptomatic samples and was also found in asymptomatic samples. Previous studies reported that *Trechispora* was found in the rhizosphere of coffee and wheat and was also abundant in the rhizospheric soil of *Panax ginseng* (Sharma-Poudyal *et al*., 2017; Tan *et al*., 2017; Fulthorpe *et al*., 2020). Very little is known about this genus but like most basidiomycetes in Agaricales, they produce enzymes that break down wood and lignins (Sharma-Poudyal *et al*., 2017). *Trechispora* spp. are generally regarded as soil-inhabiting saprophytic fungi and were found to have high relative abundance in hot and dry conditions (Fulthrope *et*

al., 2020). It is unclear what role *Trechispora* play in the palm rhizosphere but probably they are opportunistic fungi that feed on decaying roots and stems of the palm after being broken down by *Ganoderma* spp.

It should also be noted that one of the symptomatic samples was dominated by the genus *Termitomyces*. It is a paleotropical, edible mushroom that forms an obligate symbiotic or mutualistic association with the fungus-feeding termites under the family Macrotermitinae (Isoptera). Species of *Termitomyces* are also able to degrade and decompose lignin so that the termites can utilize the cellulose more efficiently. The termites themselves (Macrotermitinae) can be found exclusively in Africa and Southeast Asia (Sathiya Seelan *et al*., 2020). It has been reported that *Termitomyces* were common and abundant in the oil palm plantation (Treu, 1998; Seephueak *et al*., 2018) and this seems to be the case at Palong since many termite nests were spotted near and around oil palm at the plantation. Nevertheless, the abundance of *Termitomyces* in *Ganoderma* diseased palm indicated that there is a lack of microbial diversity in the rhizosphere and this might contribute to the emergence of the pathogen.

From these results, there was an obvious shift in the fungal community from healthy to asymptomatic and to symptomatic samples. Asymptomatic samples were in between and shared 3 genera with both healthy and symptomatic whereas there was no overlapping of genus between healthy and symptomatic, indicating that they were very different from each other. There were also fewer genera found in symptomatic samples. The deviation of the fungal community in asymptomatic and symptomatic samples should be investigated further as to whether these changes allowed *Ganoderma* to thrive or *Ganoderma* itself influences the soil microbial community structure. In the microbial rhizosphere of pepper (*Capsicum annuum* cv. Lamuyo), infection by *Phytophthora nicotianae* shifted the bacterial population, but fungi were unaffected by the pathogen and were dependent on the type of substrate. (Ros *et al*., 2017). On the contrary, Alizadeh *et al*. (2013)

observed that the association of oil palm seedlings with *G. boninense* resulted in a substantial reduction in soil microfungal density. At 126 dpi (days postinoculation), the soil microfungal community drops drastically and reaches negligible levels. Hence, this showed that *Ganoderma* has the capability to influence and reduce the soil fungal communities.

There were many studies on microbial community effects including suppressive soil that provide defence and protection against soil-borne pathogens, making them unable to establish themselves or to persist in the soil or the plant. The result from this study was in agreement with Wei *et al*. (2020) in which the microbial community structure in the rhizosphere of rusty-root infected *Panax ginseng* was significantly different than healthy ones. The fungal diversity was also lower in rusty-root-affected soils in comparison to healthy soil. In addition, Wang *et al*. (2018) concluded that the micro-environment shift from "healthy" to "ill" in the rhizosphere of *Rehmannia glutinosa* was probably due to the imbalance of the microbial community, especially the increment of potential pathogens. This resulted in the occurrence of *R. glutinosa* root rot.

The fungal genera found in each category were grouped into the two most dominant phyla, Ascomycota and Basidiomycota based on the number of hits from BLAST. From Figure 6.6, the majority of fungal sequences recovered from healthy samples belonged to Ascomycota with 66 hits. This was followed closely by asymptomatic with 59 hits while there were only 2 hits for symptomatic. Although it is not significant for Ascomycota, the number of hits for Basidiomycota was significant with symptomatic samples with the highest hits (81) followed by asymptomatic and healthy samples (29 and 1 respectively). It is clear that healthy samples were dominated by Ascomycota and asymptomatic samples showed the same trend while it was the opposite for symptomatic samples which have more Basidiomycota.

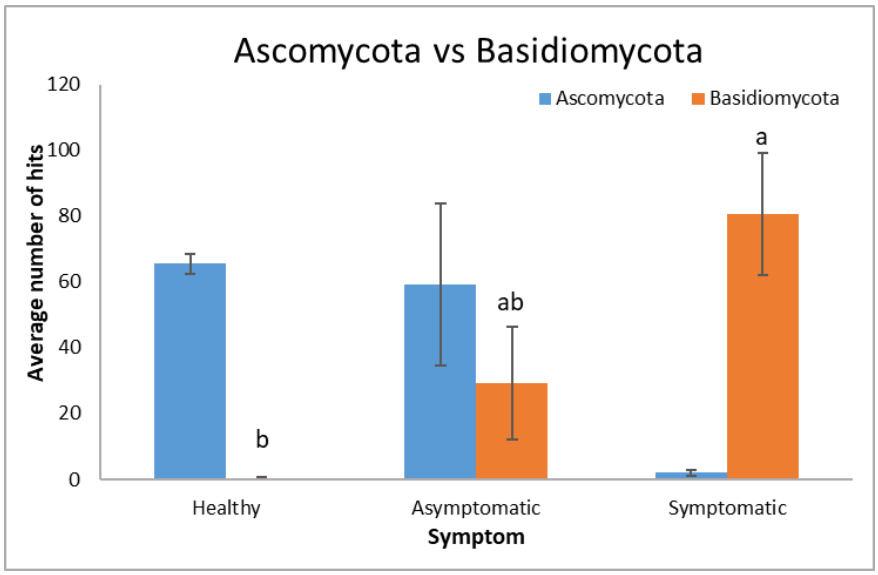


Figure 6.6 Average number of hits for Ascomycota and Basidiomycota according to the symptoms for samples at Palong based on the BLAST program and NCBI GenBank Database. Error bars are the standard errors of the mean for the three replicates. Column with the different alphabetic letters was significantly different at P≤0.05 by Tukey Multiple Comparison Test.

The findings for healthy and asymptomatic samples mirrors that of Kerfahi *et al*. (2014) who found that Ascomycota was more abundant in the oil palm plantations compared to forest soils. Meanwhile, the population of Basidiomycota was the highest in the unlogged forest, moderate in the logged forest and the lowest in oil palm plantations. In the primary forest and regenerating forest, the unique fungal community was dominated by the Basidiomycota (64% and 53% respectively), however, only 21% of unique taxa from Basidiomycota was found in the oil palms soils (McGuire *et al*., 2015). Similarly, Brinkmann *et al*., (2019) reported that Basidiomycota had the second-highest relative abundance across all-land use systems (oil palm plantations, rubber plantations, rubber agroforest and secondary rainforest) and their abundances varied where the highest was in the rainforest and the lowest was in oil palm plantation.

From these studies (Kerfahi *et al*., 2014; McGuire *et al*., 2015; Brinkmann *et al*., 2019), it can be seen that generally, Ascomycota was abundant in oil palm plantation while it was the opposite for Basidiomycota. The scarcity of a large

amount of coarse woody debris, mostly contributed by branches or roots of forest trees may lead to the low abundance of Basidiomycota in oil palm soils. On the contrary, high abundance of Ascomycota may be attributed to the organic matter from oil palm leaves and roots, as well as the herbaceous weedy layer that emerges underneath the palms, rendering the soil relatively low in lignin but abundant in nutrients, which favours the growth of ascomycetes (Kerfahi *et al*., 2014). Nonetheless, the abundance of Basidiomycota in symptomatic/diseased soil might provide an indicator in the shift of the fungal communities and probably result in the increase in phytopathogens such as *Ganoderma* spp. Also, the fact that symptomatic palms have more basidiomycetes means that the oil palm is being broken down since this group has the ability to break down relatively recalcitrant substrates (Kerfahi *et al*., 2014). It should be noted that for asymptomatic samples, the abundance of the two phyla was like healthy samples though basidiomycetes were a bit higher, it was still lower than symptomatic. Most probably when the palms were infected by *Ganoderma*, basidiomycetes were high in the soil. However, as the plant fight back through its defence mechanism and the beneficial soil microbes also help to suppress the pathogen, this halted the progression of disease in the asymptomatic palms. Variations in the fungal structure and composition between the samples/symptoms can be caused by multiple abiotic and biotic factors that have yet to be measured for BSR disease, so further research and analysis would be essential.

6.3.1.4 Other fungal communities at Tg Karang plantation

By using the universal primer pair, the ITS1 regions of all the samples were successfully amplified and sequenced. Gel electrophoresis showed that the PCR products were between 250-300 bp (results not shown), a result that has been reported by others (Bokulich and Mills, 2013; Hoggard *et al*., 2018).

The PCR products that were sequenced were then analysed using the BLAST program. Similar to Palong samples (Section 6.3.1.3), the cut-off for the E-value

was set to 1e-05. From BLAST, the genera that were present in healthy, asymptomatic and symptomatic samples were presented in Table 6.5. It was found that the samples were different from each other (unique) even for samples under the same category. The genus, *Marasmius* was shared between asymptomatic and symptomatic samples while *Rhodotorula* was shared between healthy and symptomatic samples. In addition, *Hortaea* was shared between healthy and asymptomatic samples.

Many species of *Marasmius* and *Marasmiellus* were mentioned in the literature as being the pathogen for palms, sugar cane, maize, turfgrass (seashore paspalum) and taro (*Colocasia esculenta*) (Tamur *et al*., 2018). *Marasmius palmivorus* has been identified as the cause of fruit bunch rot of oil palm (Chung, 2012; Sutarta *et al*., 2015). However, it was later grouped under the genus *Marasmiellus*, thus known as *Marasmiellus palmivorus* based on its close morphology to *Marasmiellus* species such as *Marasmiellus semiutus* and *Marasmiulles troyanus*. The genus was also revised by Wilson and Desjardin (2005) and the pathogen was tentatively accepted as *Marasmiellus palmivorus* (Sharples) Desjardin comb. prov. until further phylogenetic analysis is conducted for a more accurate identification (Pong *et al*., 2012). Bunch rot is not a concern in plantations with up-to-date pruning of old fronds and good harvesting practices (Chung, 2012). Nevertheless, both *Marasmius* and *Marasmiellus* were detected in asymptomatic samples in which they are basidiomycetes under the family Marasmiaceae. In a recent report, *Marasmiellus palmivorus* was confirmed as the pathogen that caused bunch rot disease in oil palm in West Malaysia and also was suggested to have the potential to infect any plant parts such as the seeds and roots (Almaliky *et al*., 2012).

Table 6.5 List of fungal genera found in samples of different symptoms at Tg Karang based on the BLAST program and NCBI GenBank Database.

Healthy	Asymptomatic	Symptomatic
Amylosporus	*Acidomyces*	*Amauroderma*
Boletus	*Fusarium*	*Bacidia*
Ceratobasidium	*Hortaea*#	*Ganoderma*
Coprinellus	*Hygrocybe*	*Grammothele*
Coprinopsis	*Lecanostictopsis*	*Lasionectria*
Devriesia	*Leptodiscella*	*Leucosporidium*
Hortea#	*Marasmiellus*	*Marasmius*~
Inocybe	*Marasmius*~	*Natantispora*
Lentinus	*Mucoporon*	*Ophiostoma*
Microporus	*Mycoleptodiscus*	*Phylloporia*
Neodevriesia	*Mycosphaerella*	*Porogramme*
Occultifur	*Neosonderhernia*	*Remispora*
Papiliotrema	*Palawania*	*Rhodotorula*^
Passalora	*Paramycosphaerella*	*Tinctoporellus*
Piloderma	*Phyllosticta*	
Pluteus	*Ramichloridium*	
Pseudocercospora		
Psythyrella		
Rhizoctonia		
Rhodotorula^		
Russula		
Septoria		
Tormentella		
Tricholoma		
Zasmidium		

# Denotes common genera between Healthy and Asymptomatic samples
^ Denotes common genera between Healthy and Symptomatic samples
~ Denotes common genera between Asymptomatic and Symptomatic samples

The genus *Rhodotorula* is a member of the basidiomycetous yeasts that occur naturally in soil and air and can be readily isolated. Most species of this genus are non-pathogenic while there were also reports of some species being regarded as emerging yeast pathogen which cause dermatophytoses and are referred to as rhodotorulosis (Biswas *et al*., 2001). One species in particular, *R. glutinis* which was found in the healthy samples is capable of synthesizing various metabolites useful in industries, such as enzymes, carotenoids and lipids. There was also a study that

shown *R. glutinis* was able to utilize and remove chemical oxygen demand (COD) from palm oil mill effluent (POME) that was used as fermentation media and this in turn resulted in improved biomass, lipid content, and carotenoid production of *R. glutinis* (Saenge *et al*., 2011). Besides, *R. mucilaginos* was found to improve the hyphal length, hyphal growth and root length colonization of the arbuscular mycorrhizal fungi *Glomus mosseae* in soybean and *Gigaspora rosea* in red clover (Fracchia *et al*., 2003).

Another yeast, *Hortaea werneckii* found in healthy and asymptomatic samples is the most halotolerant fungus known. It can grow in a salinity range of up to 30% NaCl and tolerate up to 2 M concentrations of the chaotropic salt $MgCl_2$ (Zalar *et al*., 2019). *Hortaea* belongs to the black yeast group, under Capnodiales order and Ascomycota phylum. *H. werneckii* can be found in different marine habitat and soil as an endophyte such as in mangrove plants (Elsayed *et al*., 2016; Zalar *et al*., 2019). As Tg Karang in general and the oil palm estate specifically are situated near the sea (Straits of Malacca) (Appendix 7A), it is not surprising that *Hortaea* was found in the plantation since the soil salinity might favour its growth.

The list of these genera have been reported from peatlands; *Boletus*, *Fusarium*, *Hygrocybe*, *Inocybe*, *Leucosporidium*, *Piloderma*, *Pluteus*, *Rhizoctonia*, *Rhodotorula*, *Russula*, *Septoria*, *Tricholoma* (Thormann and Rice, 2007). Among these, interestingly *Boletus*, *Inocybe*, *Russula*, *Piloderma* and *Tricholoma* which were found in healthy samples are ectomycorrhizal (ECM) fungi that form mutualistic symbioses with many tree species and are known to be important organisms in carbon and nutrient cycles in forest ecosystems. Moreover, these ECM fungi (except *Boletus* and *Piloderma*) have been reported to be found in North Selangor Peat Swamp Forest and oil palm plantations with various management systems planted on peat swamp forests in Selangor (Shuhada *et al*., 2020). Given the unusual morphology of the root systems of the oil palm and also results from experimental studies, it seems that oil palm is fairly dependant on mycorrhizal for

nutrition. McGuire *et al*. (2015) reported that the oil palm soils had the lowest relative abundance of ECM fungi while the primary and regenerating forests had a similar relative abundance. The abundance of several ECM genera was influenced by the strategy employed, either by being early-successional or late-successional. *Russula* and *Boletus* were found to be late-successional in some ecosystems and *Inocybe* spp. are thought to be early-successional fungi (McGuire *et al*., 2015).

The genus, *Fusarium* found in the asymptomatic sample is one of the most frequently isolated genus in tropical peatlands. Species like *F*. *oxysporum*, *F*. *solani*, *F*. *proliferatum*, *F*. *verticillioides* and *F*. *semitectum* were successfully isolated from peat soil in Perak and Pahang (Karim *et al*., 2016). Another genus, though it is not the pathogen of oil palm is *Ophiostoma* which was found in symptomatic sample. There are currently about 40 taxa described from this genus and most of them are associated with different bark beetle species. Many of the *Ophiostoma* species produce spores in sticky droplets that can easily adhere to their insect vectors' exoskeletons. Members of this genus encompass plant-associated species with differing degrees of pathogenicity. They are the causal agent for the blue-stain in freshly exposed sapwood while certain highly virulent species caused the death of the trees in both natural and managed forest ecosystems (Jankowiak *et al*., 2019).

The causative agent for BSR disease, *Ganoderma* was detected in the symptomatic sample by sequencing the ITS region. One sample in particular, was dominated by *Ganoderma* with *G. boninense* was at the top of the BLAST search result. This demonstrated that the detection of *Ganoderma* from the soil is possible, as a lot of investigations involved the use of infected oil palm tissue and not soil. One of the most common genera found in tropical peat was *Ganoderma* while the abundance of this genus was also high from peat soil sampled in Selangor (Omar *et* al., 2012; Shuhada *et al*., 2020). Other than that, the white-rot genera of *Perenniporia*, *Phlebia* and *Ganoderma* which have the ability to degrade lignin was found in greater abundance in mineral soil, disturbed peat soil and natural peat

swamp forest soil in Indonesia. It has also been documented that disturbed peat soil contains a large number of pathotrophs, indicating that disruption of the natural peat forest may result in changes in microbial diversity and the emergence of potential plant diseases (Liu *et al*., 2020). Supriyanto *et al*. (2020) determined that the level of the water table, pH and age of oil palm influenced the severity of *Ganoderma* disease in peatlands. However, further research is necessary to understand why peat biological factors (dominant vegetation, antagonistic bacteria and fungi) did not correlate with *Ganoderma* attacks intensity.

Since there were no common genera found within samples of the same category, it was difficult to pinpoint any fungi that may play an important role in the palm rhizospheres, though it was worth mentioning the genera that may be beneficial or pathogenic. This may be due to the location of the blocks in which Block B and C were situated along the same road but were separated by buildings, other smallholder plantations and different vegetation while Block A was situated at the other side of the main road (Appendix 7B). There were also high disturbances as the plantations were situated near the road and this might affect the palms and the soil microbial community. In spite of that, it was clear that the ECM fungi were missing from asymptomatic and symptomatic samples and there was also a shift in the fungal communities and fewer genera detected as compared to healthy palm rhizospheres. As a result, this might contribute to the emergence of *Ganoderma*. As mentioned by Raaijmakers *et al*. (2009), to develop a parasitic association with plants, pathogens should therefore engage with the diverse rhizosphere populations and this in turn will have an effect on the infection’s outcome

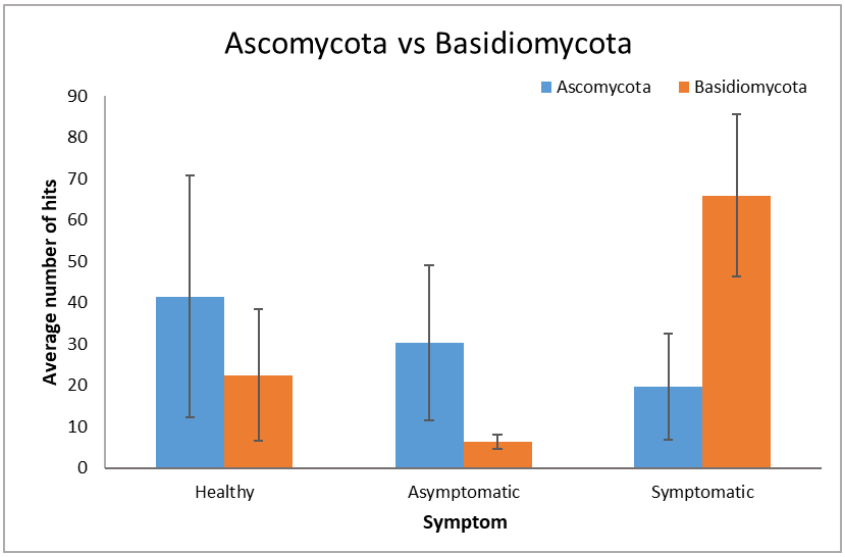


Figure 6.7 Average number of hits for ascomycetes and basidiomycetes according to the symptoms for samples at Tg Karang based on the BLAST program and NCBI GenBank Database. Error bars are the standard errors of the mean for the three replicates.

Based on Figure 6.7, the average number of hits for Ascomycota showed the same trend as at Palong plantation (Figure 6.6) in which healthy rhizosphere samples has the highest hits (42 hits) followed by asymptomatic samples while the lowest was symptomatic samples (30 and 20 hits respectively). In contrast, Basidiomycota was the highest in symptomatic samples with 66 hits, intermediate in healthy (23 hits) and the least in asymptomatic (6 hits). Even though the average number of hits were not significant for both Ascomycota and Basidiomycota, the results for healthy and asymptomatic samples were in agreement with Siti Ramlah *et al*. (2016) in which Ascomycota was the most commonly found fungal division from deep peat forest and peat cultivated with oil palm in Sarawak. Liu *et al*. (2020) reported that the most abundant phylum was Ascomycota (40.5% of the total sequence), across the three sites investigated which were mineral soil, disturbed peat soil and natural peat swamp forest soil in Indonesia. This was followed by Basidiomycota at 18.8% while Zygomycota and Glomeromycota made up only 0.01% of the total sequence. In addition, about 601 fungal species from peatlands have been described globally where the largest group with 276 species (46%) was

Ascomycetes. Basidiomycota was the second largest group with 243 species or about 40%.

From these reports, it was obvious that Ascomycota was the dominant phyla found in peat soil. A greater change of the microbial community was observed in a rubber plantation in comparison to an oil palm plantation when the existing peatland forest was converted to agriculture (Nurulita *et al*., 2016). Furthermore, it has been concluded that the changes in soil microorganisms in which decreased in beneficial microbes and increment of harmful microbes contributed to higher disease incidence in ramie plants (*Boehmeria nivea* L. Gaud) (Zhu *et al*., 2018). In this study, only symptomatic samples had higher Basidiomycota than Ascomycota. Similar to Palong plantation, this indicated a shift in the fungal community, thus the soil is not suppressive to *Ganoderma*. Moreover, healthy samples seemed to have higher Basidiomycota than asymptomatic even though this was still less than the Ascomycota. This might be a sign that changes in the fungal structure were happening in the rhizosphere of healthy samples and this might provide the opportunity for *Ganoderma* to infect the palms as seen in the next section (Section 6.3.2.4) where the quantity of *Ganoderma* was considered high for healthy samples.

6.3.2 Absolute quantification of *Trichoderma* spp. and *Ganoderma* spp. DNA in the palm rhizospheres

6.3.2.1 Quantification of *Trichoderma* spp. DNA at Palong plantation

In absolute quantification, a standard curve is constructed by plotting the quantitation cycle (Cq) values against the log quantity of a dilution series of known DNA samples. This is crucial since the standard curve contains details on the efficacy of the amplification primers as well as the amount of genes of interest present in the unknown samples (Boulter *et al*., 2016). The slope of the qPCR assay

was -3.659 and the amplification efficiency (E) was 87.6% with a correlation coefficient (R^2) at 0.987 (Figure 6.8). The E value was slightly lower than the acceptable range of 90-110% in which E=100% corresponds to a slope of -3.32. This follows the assumption that the template doubles after each cycle during exponential amplification (Svec *et al*., 2015). There are many factors that can influence the PCR efficiency including the sample matrix. Since the samples were extracted from soil, they may contain inhibitors and other interfering substances from the samples (Svec *et al*., 2015).

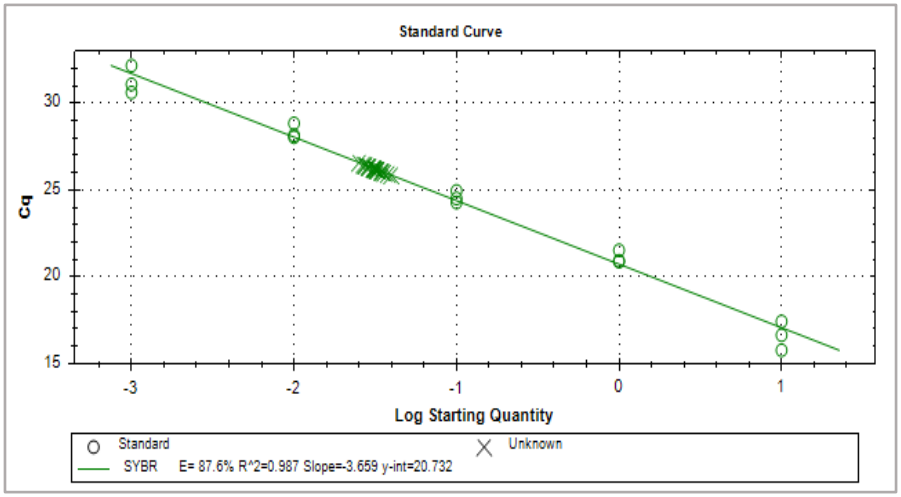


Figure 6.8 Quantification of *Trichoderma* spp. in healthy, asymptomatic and symptomatic palms at Palong plantation using Tmix as the standard. Linear regression of log starting quantity versus quantitation cycle (Cq) gave the standard curve, and this was used to calculate template concentrations of the samples (Unknown).

The specificity of the qPCR assay was confirmed by melting curve analysis (Figure 6.9). A single peak was observed at 89 °C, which means that the qPCR assay has produced a single, specific product. This is a straightforward and simple method for the characterization of the reaction products and therefore lessens the need for gel-electrophoresis which is a time-consuming process (Dorak, 2007).

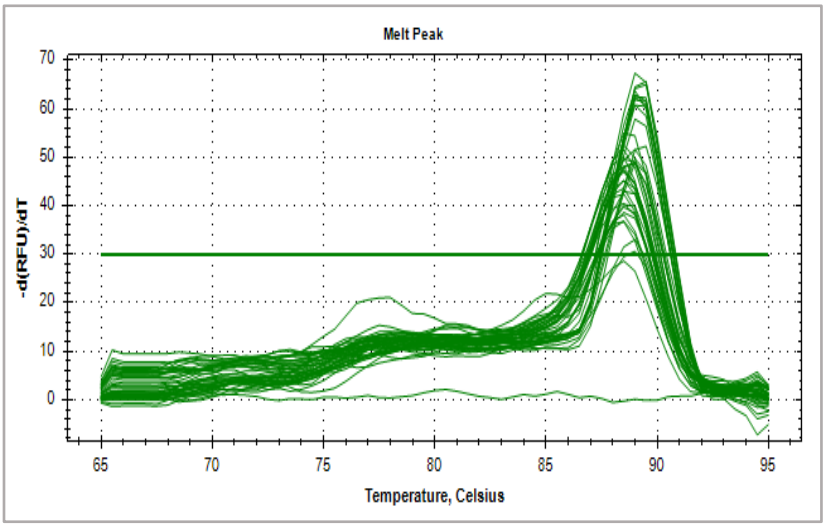


Figure 6.9 Melting curve analysis of Tmix DNA and the samples at Palong to determine the specificity of the qPCR reactions.

Real-time PCR was able to quantify the amount of *Trichoderma* DNA in the rhizosphere of the palms of different symptoms. From Figure 6.10, there was little variation in the DNA quantity in which symptomatic samples have the highest *Trichoderma* DNA at 0.0300 ng/µl. This was followed by healthy and asymptomatic samples at 0.0263 and 0.0240 ng/µl respectively. Analysis of variance showed that there was no significant difference (P>0.05) between the symptoms.

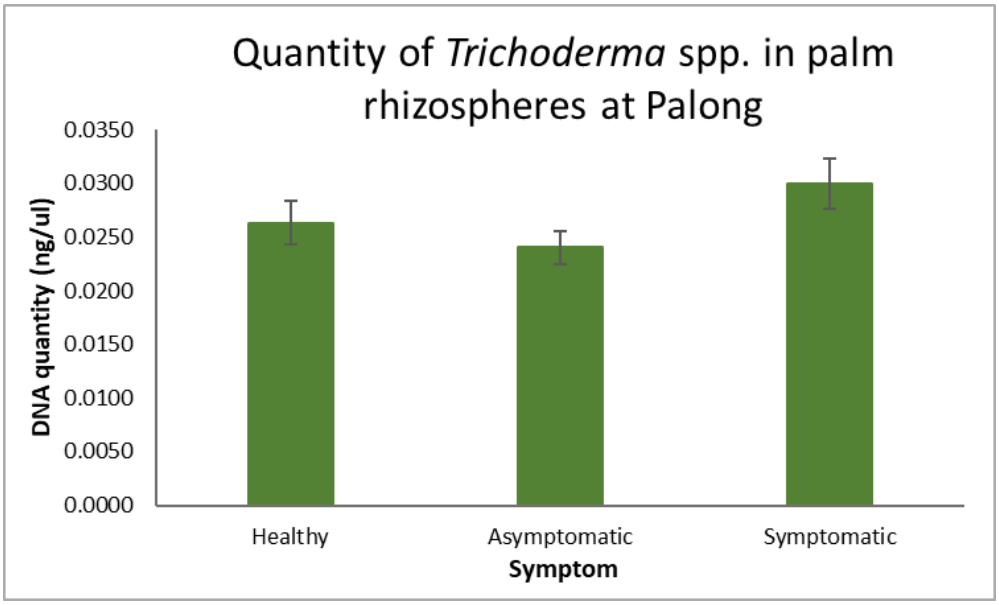


Figure 6.10 Quantity of *Trichoderma* spp. in palm rhizospheres of different symptoms at Palong plantation. SE of three replicate readings was represented by the error bars.

Although PCR amplification of *Trichoderma* DNA was able to detect and confirm the presence of *Trichoderma* spp. (Section 6.3.1.1), quantification of *Trichoderma* spp. was only possible through real-time PCR amplifications. It has been widely recognized as the most sensitive method to quantify minute amounts of nucleic acids (Svec *et al*., 2015). qPCR amplification has been used to detect *Trichoderma atroviride* SC1 (Savazzini *et al*., 2008) and *T. guizhouense* (Y. Zhang *et al*., 2019) abundance in soil. In addition, *T. afroharzianum, T. harzianum and T. gamsii* were quantified from the rhizosphere of durum wheat by using qPCR assay in the presence of *Fusarium pseudograminearum* that causes crown rot (Stummer *et al*., 2020).

6.3.2.2 Quantification of *Trichoderma* spp. DNA at Tg Karang plantation

From the PCR amplification, the standard curve revealed a linear relationship (R2= 0.985) between the log values of *Trichoderma* DNA and the quantitation cycle (Cq) (Figure 6.11). This showed that as the quantity of DNA templates increased, the Cq value decreased. The slope of the qPCR assay was -4.107 and the amplification efficiency (E) was 88% in which the E value was close to that of Palong samples (Figure 6.8).

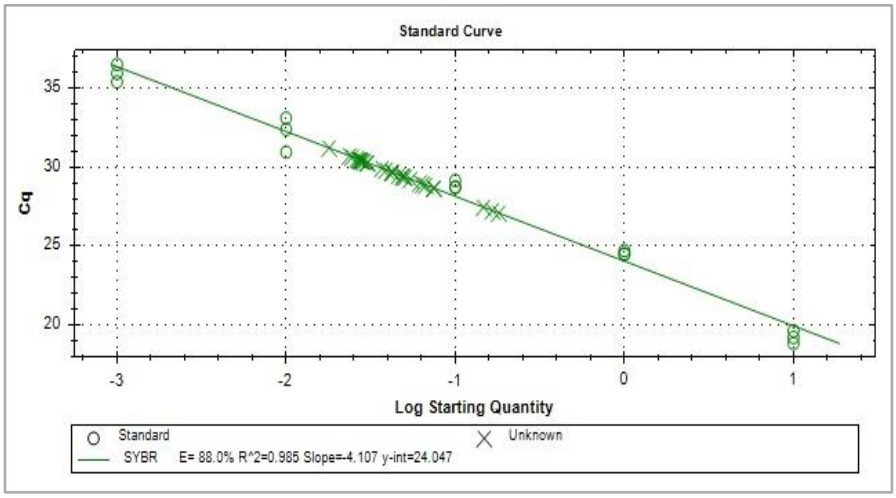


Figure 6.11 Quantification of *Trichoderma* spp. in healthy, asymptomatic and symptomatic palms at Tg Karang using Tmix as the standard. Linear regression of log starting quantity versus quantitation cycle (Cq) gave the standard curve, and this was used to calculate template concentrations of the samples (Unknown).

For the melting curve analysis (Figure 6.12), there were sharp fluorescent peaks at 89 °C, indicating that a single product was amplified by using the primers TITS5F and TITS5R.

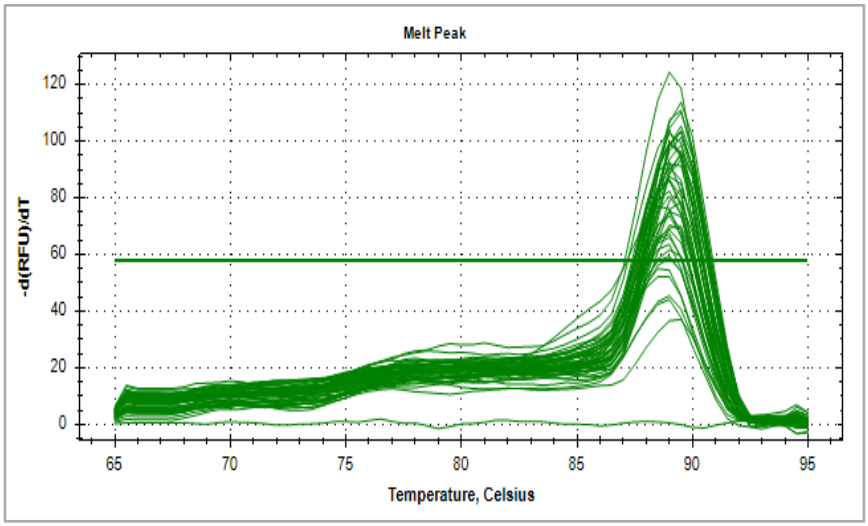


Figure 6.12 Melting curve analysis of Tmix DNA and the samples at Tg Karang plantation to determine the specificity of the qPCR reactions.

Quantification of the amount of *Trichoderma* in the rhizosphere of the palms of different symptoms showed an increasing trend in which symptomatic samples has the highest DNA quantity at 0.0342 ng/µl. *Trichoderma* DNA quantity in asymptomatic samples was 0.0285 ng/µl followed by healthy samples at 0.0228 ng/µl (Figure 6.6). Although there was no significant difference (P>0.05) between the different symptoms, the quantity of *Trichoderma* in palm rhizospheres at Tg Karang was similar to Palong (between 0.020-0.035 ng/µl) (Figure 6.10). This showed that *Trichoderma* was successfully quantified at different locations with different soil types.

In a study conducted by López-Mondéjar *et al*. (2010), the authors utilized the qPCR-based method to quantify *T. harzianum* and extrapolated the data to fungal biomass values. It was found that the correlation between the DNA quantity and biomass was 0.76. Thus, this technique might be useful for more accurate

quantification of fungi in the soil especially for the detection of the biological control agent.

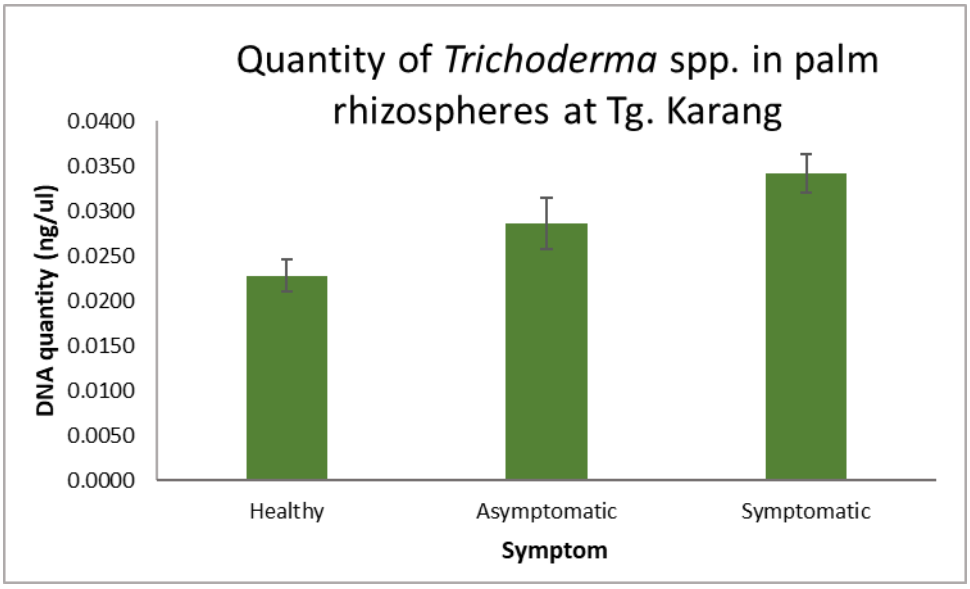


Figure 6.13 Quantity of *Trichoderma* spp. in palm rhizospheres of different symptoms at Tg Karang plantation. SE of three replicate readings was represented by the error bars.

6.3.2.3 Quantification of *Ganoderma* spp. DNA at Palong plantation

The efficiency of the quantification of *Ganoderma* DNA was at 82.4% and the slope was -3.831. Meanwhile, there was a linear relationship between log values of GBLS DNA and the quantitation cycle (R^2= 0.959) (Figure 6.14), illustrating that increment in DNA quantity resulted in the decrease of the Cq value.

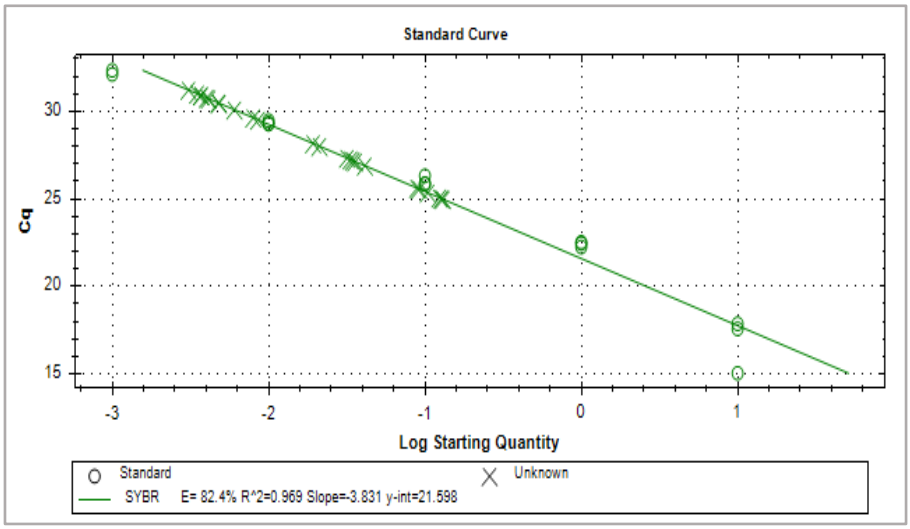


Figure 6.14 Quantification of *Ganoderma* spp. in healthy, asymptomatic and symptomatic palms at Palong using pure GBLS DNA as the standard. Linear regression of log starting quantity versus quantitation cycle (Cq) gave the standard curve, and this was used to calculate template concentrations of the samples (Unknown).

From the melting curve analysis (Figure 6.15), there were two peaks that have been detected at 85.5 °C and at around 88 °C respectively in which the latter peak was due to non-specific PCR product. The physical closeness of primer pairs at mismatched sites and concentrations of primers, template and non-template DNA can lead to non-specific amplification (Ruiz-Villalba *et al*., 2017). Moreover, since primers with higher Tm values are usually more efficient at binding to DNA templates, the low melting temperatures (Tm) of the primers (GbF: 51.9 °C and GbR: 50.3 °C) used in this study might contribute to non-specific amplification. This reduces the probability of binding to non-target sequences (Chung 2004; Goh 2016). There was also another peak at 73 °C contributed by no-template controls (NTCs), which is an indication of the presence of primer-dimers as it exhibits a lower melting temperature than the amplicon. The formation of primer-dimers in NTCs was due to the abundance of primer and absence of template and this decreases the PCR efficiency (E) (Dorak, 2007).

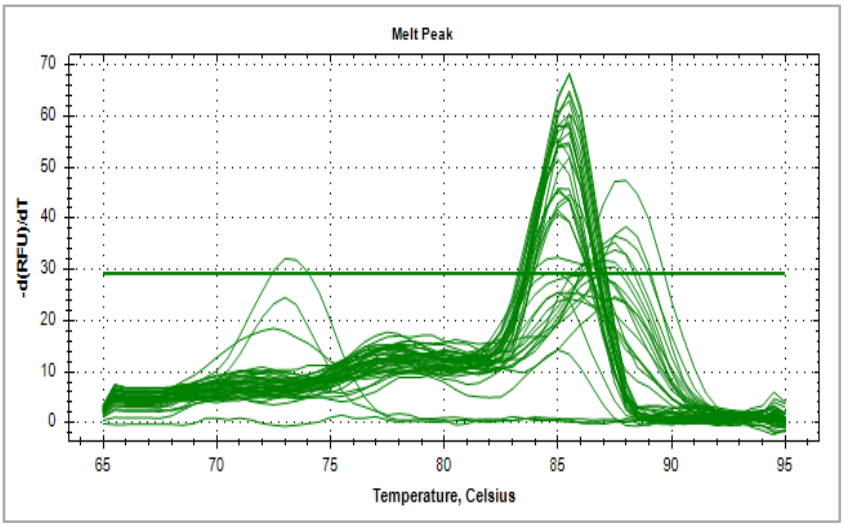


Figure 6.15 Melting curve analysis of pure GBLS DNA and the samples at Palong to determine the specificity of the qPCR reactions.

Gel electrophoresis confirmed the presence of bands of the expected size of around 167 bp for all the samples and also non-specific PCR products at around 400-500 bp for some of the samples (Figure 6.16). In this study, the primers used targeted the ITS fragment specific to *Ganoderma* spp. Nonetheless, even with well-designed primers, there is always a possibility of amplification of non-specific products or formation of primer-dimers (Dorak, 2007). Since the samples were extracted from soil, the presence of PCR inhibitors such as humic acids and tannins, as well as the presence of a high amount of DNA from other microorganisms in the soil might affect the PCR amplification (Y. Zhang *et al*., 2019). It should be noted that the intensity, in terms of thickness and brightness of the bands of different samples/symptoms were different. This suggests that the quantity of the fungal DNA was different and this was reflected in the Cq value.

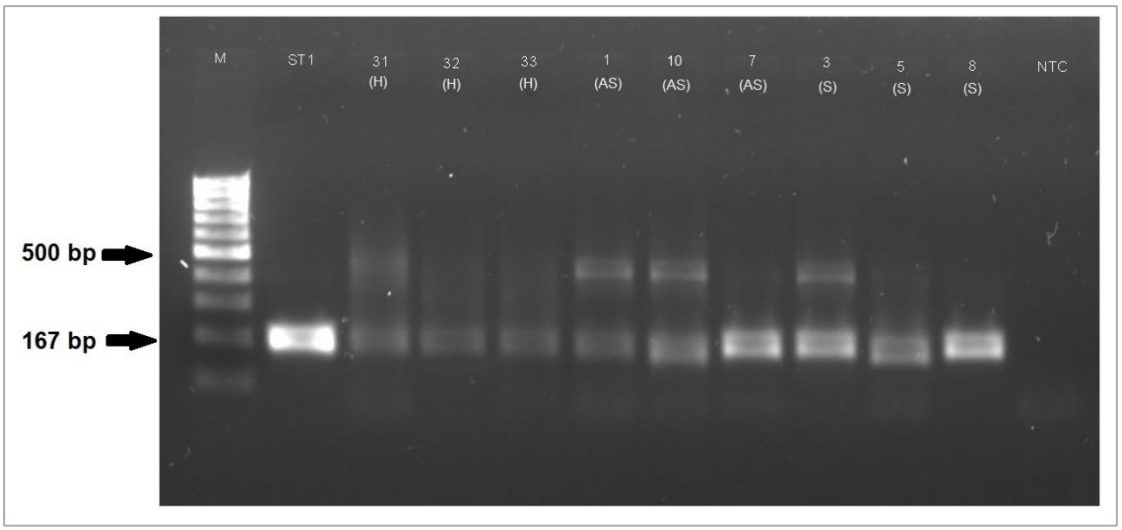


Figure 6.16 Confirmation of *Ganoderma* spp. at Palong plantation from the qPCR reactions. M: 100 bp ladder; ST1: GBLS DNA as a positive control; NTC: Nuclease-free water.

Quantification of *Ganoderma* DNA showed that healthy samples have the lowest amount at 0.0072 ng/µl whereas *Ganoderma* DNA in asymptomatic and symptomatic samples was ten times higher at 0.0775 ng/µl and 0.0724 ng/µl respectively (Figure 6.17). Although this was not significant, it provided a useful insight into which diseased palms, either the fruiting body is present (symptomatic) or absent (asymptomatic) have higher *Ganoderma* in the soil as compared to healthy palms. The presence of *Ganoderma* in healthy samples/palms might be due to the fact that it is also a natural decomposer of log and stump and had been found on trees in natural and plantation forest in Peninsular Malaysia (Zakaria *et al*., 2009). The practice of leaving oil palm fronds and empty fruit bunches to decompose between the rows of palms in the plantation may also attract this fungus (Treu, 1998).

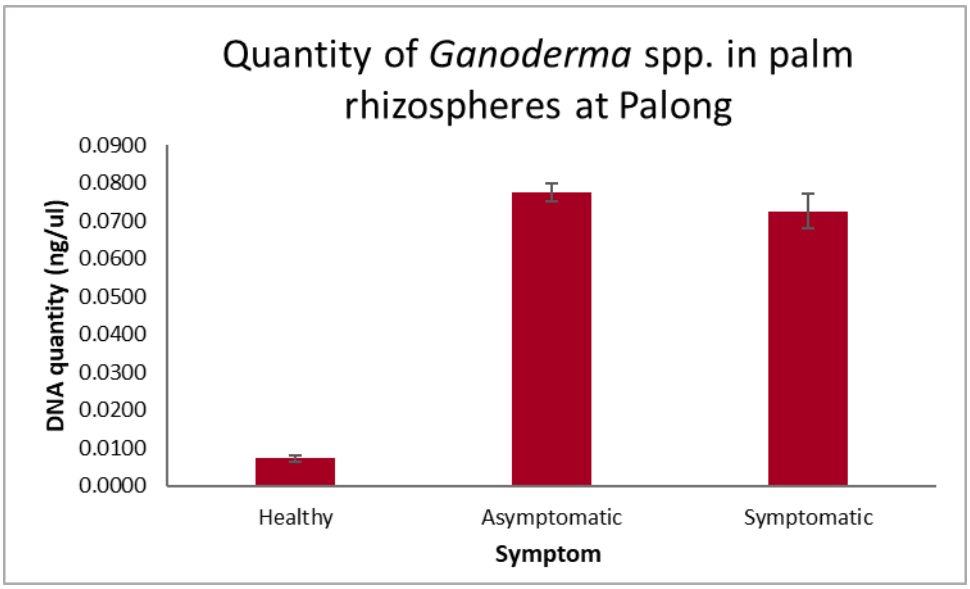


Figure 6.17 Quantity of *Ganoderma* spp. in palm rhizospheres of different symptoms at Palong plantation. SE of three replicate readings was represented by the error bars.

Absolute quantification of *Ganoderma boninense* through real-time Polymerase Chain Reaction in oil palm tissue had been carried out before (Goh 2016, Supramaniam 2016) but not from soil. This is the first report that has attempted to quantify *Ganoderma* spp. from the rhizosphere of palms with different symptoms that have been monitored for 28 months before being sampled. In a study by Supramaniam (2016), GBLS DNA in infected plantlets was the highest at day 14 before declining sharply at day 35 in which the plants were showing severe symptoms even with reduced GBLS quantity. However, on day 42, the pathogen may have overcome the plant/host defences or killed the tissue which enabled it to grow extensively and resulted in the increase of GBLS quantity. In a similar study, it has been reported that GBLS DNA quantity was positively correlated to disease severity index (DSI) in oil palm plantlets in which increment in GBLS DNA resulted in the increase of DSI by 53.92% (Goh, 2016).

For asymptomatic samples, even though the quantity of *Ganoderma* was high in the rhizosphere (higher than symptomatic), there was no fruiting body observed and the plants showed no signs of infection. In fact, the palms were

deemed to have recovered from the disease since the fruiting body was not seen/disappeared for more than a year and there was the growth of new roots (Figure 5.11). It is possible that asymptomatic palms utilized numerous defence mechanisms such as reinforcing of the physical barriers (e.g. lignin and cellulose), production of antimicrobial compounds (phytoalexins) and pathogenesis-related (PR) protein synthesis (e.g. chitinases) against *Ganoderma* (Naher *et al*., 2012). Meanwhile, as a BCA, it has been reported that *T. harzianum* helped to improve the nutrition status and transport of nutrients in oil palm (Ho *et al*., 2016). Nevertheless, since the quantity of *Ganoderma* is still high in the rhizosphere, there is a possibility for the disease to become prevalent again when the plant defence system failed or when there are changes/decline in beneficial microbes that help to suppress the pathogen.

There are several methods developed for detection of *Ganoderma* in infected palm such as isolation by using *Ganoderma* Selective Medium (GSM), polyclonal antibodies, ITS-DNA-PCR, volatile compounds measurement and analysis of fungal ergosterol quantity (Hushiarian *et al*., 2013; Tan *et al*., 2013). Other recent techniques being explored are Hyperspectral reflectal data, E-nose and tomography (Hushiarian *et al*., 2013). While these methods are very useful for *Ganoderma* detection, the focus is on the infected palm tissues. By the time the basidiomata/fruiting body becomes apparent, the disease is already well-establish and it might be too late to treat the infected palm. Since *Ganoderma* is a soil-borne pathogen, the priority should be on detecting the fungus in the soil. One alternative approach that focuses on soil testing is through the use of wireless sensor networks (WSNs) in which abnormal or anomalous data from monitoring of soil composition can reveal any changes in oil palm soil at the early stage (Zamry *et al*., 2015).

6.3.2.4 Quantification of *Ganoderma* spp. DNA at Tg Karang plantation

For the quantification of *Ganoderma*, the slope of the qPCR assay was -4.055 and the amplification efficiency (E) was 76.4% with a correlation coefficient (R2) at 0.987 (Figure 6.18).

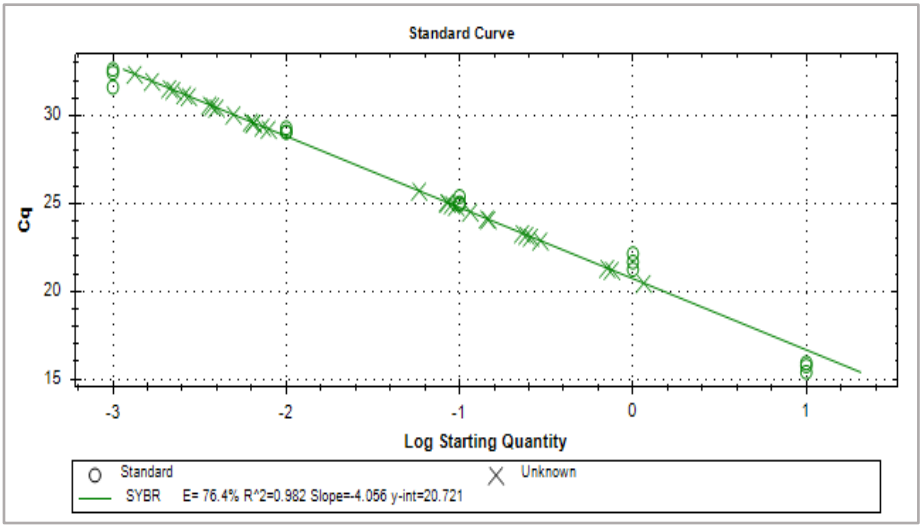


Figure 6.18 Quantification of Ganoderma spp. in healthy, asymptomatic and symptomatic palms at Tg Karang plantation using pure GBLS DNA as the standard. Linear regression of log starting quantity versus quantitation cycle (Cq) gave the standard curve, and this was used to calculate template concentrations of the samples (Unknown).

Melting curve analysis showed that there were several peaks detected with the main qPCR products at 85.5 °C and non-specific amplification at around 72-74 °C (Figure 6.18), which is most likely due to primer-dimer since it has a lower melting temperature than the desired amplicon.

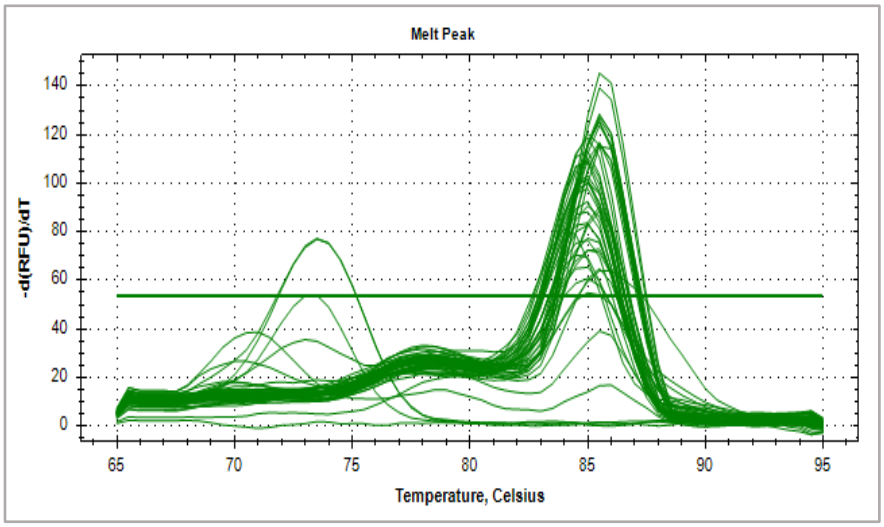


Figure 6.19 Melting curve analysis of pure GBLS DNA and the samples at Tg Karang plantation to determine the specificity of the qPCR reactions.

For *Ganoderma* ITS DNA, the expected size is ~167 bp (Goh, 2016; Supramaniam, 2016). From gel electrophoresis, all the samples tested showed the presence of a band indicating the presence of *G. boninense* in the rhizospheres (Figure 6.19). The brightness and thickness of the bands were different especially for asymptomatic and symptomatic samples which indicated that the quantity of *Ganoderma* DNA was higher in those samples.

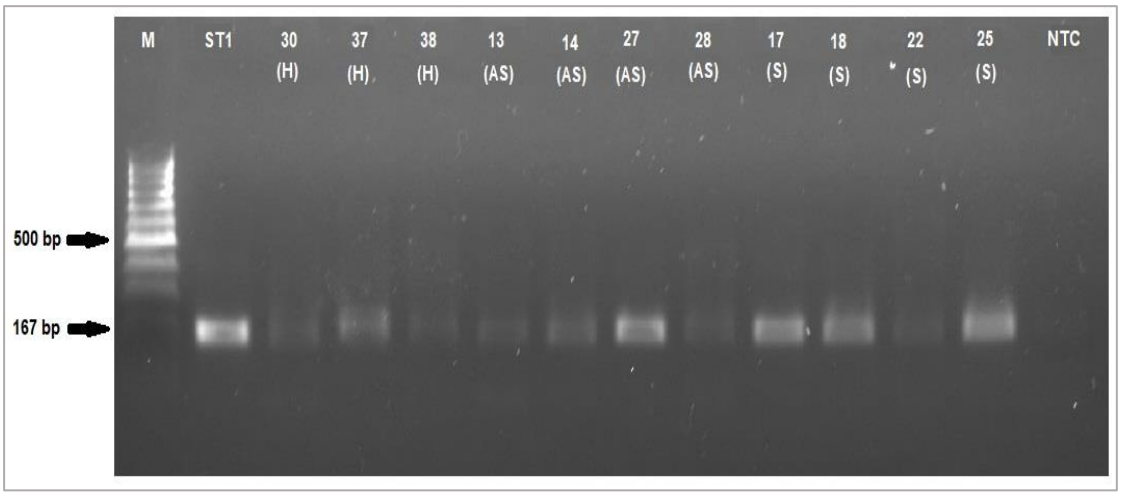


Figure 6.20 Confirmation of Ganoderma spp. at Tg Karang plantation from the qPCR reactions. M: 100 bp ladder; ST1: GBLS DNA as a positive control; NTC: Nuclease-free water.

Ganoderma DNA showed the same pattern as Palong samples (Figure 6.17) in which asymptomatic samples had the highest DNA quantity (0.4497 ng/µl) followed by symptomatic and healthy samples at 0.3414 ng/µl and 0.0414 ng/µl respectively (Figure 6.21). Although there was no significant difference (P>0.05) in the DNA quantity among the different symptoms, it should be noted that *Ganoderma* quantity in symptomatic and asymptomatic samples was 8-10 times higher than healthy samples. Another concerning trend is that in general, the pathogen level in palm rhizospheres at Tg Karang was 10-fold higher than *Trichoderma* as the biological control agent (Figure 6.13) as well as the *Ganoderma* quantity at Palong (Figure 6.17). This might explain the high disease incidence (DI) and the high percentage of palm with the disease at Tg Karang as compared to Palong (Section 5.3.4). There is a high probability that the healthy palms will eventually succumb to the disease since Ganoderma quantity was high or the palms may already be infected but the symptoms do not appear. Therefore, real-time PCR might be an excellent technique for early detection of *Ganoderma* in the soil so that symptomless palms which were deemed healthy in this study can be detected and treated earlier.

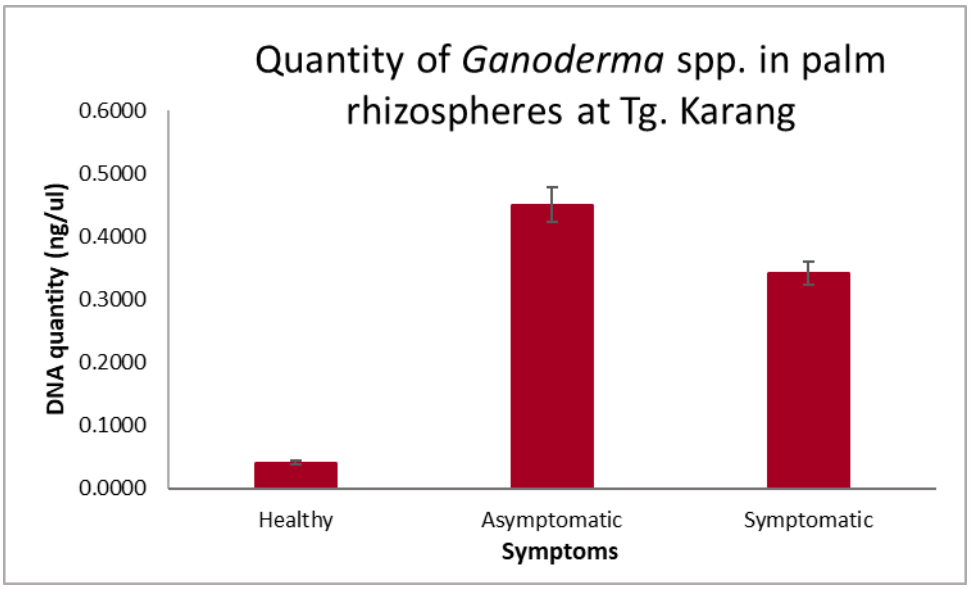


Figure 6.21 Quantity of *Ganoderma* spp. in palm rhizospheres of different symptoms at Tg Karang. SE of three replicate readings was represented by the error bars.

Currently, the most common way of detecting BSR disease is based on foliar symptoms and basidiomata/fruiting body appearance at the base of the infected stem (visual assessment). The palms were already in the final stages of infection by the time visible signs emerged, and the fungus typically has destroyed half of the basal tissues (Soepena *et al*., 2009). Besides, one of the drawbacks of visual assessment is that this technique only gives a measure of the extent of the symptoms of the disease but not for the extent of colonization of pathogen (Brouwer *et al*., 2003). From observation in the field, there were also a few instances where palms without any symptoms falling over. Further inspections revealed the presence of *Ganoderma* fruiting body inside the stem or around the exposed roots (Appendix 12). This demonstrated that *Ganoderma* was the causal agent that causes the degradation of the palm's root and stem.

In a study conducted by Lievens *et al*. (2006), quantification of phytopathogens such as *Fusarium solani*, *Rhizoctonia solani* and *Pythium ultimum* in soil cultivated with various crops were conducted via qPCR. Half of the ten soil samples tested were found to harbour *P. ultimum*, with only two of them showing disease symptoms. From these, the soil sample with the highest *P. ultimum* quantity (8.32 ng/g) was established as the principal cause of the disease. The remaining four samples were associated with *R. solani* with DNA concentrations between 0.12-1.31 ng/g and the corresponding crops showed typical *Rhizoctonia* symptoms. In contrast, *F. solani* was found not to be related to any observed symptoms even though it was detected in four of the samples. For BSR disease, since *Ganoderma* is the main pathogen of oil palm, its presence will likely cause disease to the plant and therefore a high level of the pathogen in the soil as at Tg Karang plantation should be a major concern for the growers. Correlation analysis between *Trichoderma* spp. and *Ganoderma* spp.

In this study, it has been proven that detection and quantification of *Ganoderma* in the soil can be achieved via real-time Polymerase Chain Reaction.

For future analysis, more samples are needed so that a baseline value can be established and therefore *Ganoderma* quantity in the soil can act as an indicator of soil/oil palm health and the probability of the palm being infected so that treatment can be conducted earlier. However, it is also important to improve the DNA extraction method to get good quality DNA for downstream processing and minimise the risk of primer-dimer formation and amplification of non-specific products.

6.3.2.5 Correlation analysis between *Trichoderma* spp. and *Ganoderma* spp.

From correlation analysis, there was a weak, negative relationship between *Trichoderma* spp. and *Ganoderma* spp. (Table 6.6). Even though the correlation was not significant (P=0.332), it seems that the increase in *Trichoderma* resulted in the decrease of *Ganoderma* and vice versa. This might be the case for healthy samples in which *Trichoderma* quantity was higher than *Ganoderma* (Figure 6.10 and Figure 6.17), thus keeping the pathogen in check by competing for space and nutrients or suppressing it through mycoparasitism. This restricts the pathogen's ability for root colonization (Sariah *et al*., 2005).

Table 6.6 Correlation between the quantity of *Trichoderma* spp. and *Ganoderma* spp. in the palm rhizospheres at Palong and Tg Karang plantations.

Location	**Fungi**	***Ganoderma* spp. DNA**			
		Pearson R	R^2	*P-value*	*Summary*
Palong	***Trichoderma* spp. DNA**	-0.395	0.156	0.332	ns
Tg. Karang		0.584	0.341	0.06	ns

ns = non-significant

Meanwhile, at Tg. Karang site, there was a moderate, positive relationship between *Trichoderma* spp. and *Ganoderma* spp. (Table 6.6). The relationship was almost significant (P=0.06) in which the increase of *Ganoderma* spp. resulted in

the increase of *Trichoderma* spp. By referring to *Ganoderma* and *Trichoderma* DNA quantity in the rhizospheres (Figure 6.13 and 6.21), it is hypothesized that *Trichoderma* is not able to suppress *Ganoderma* due to the fact that the pathogen level was way too high for the biocontrol agent (*Ganoderma* quantity was ten-fold higher than *Trichoderma*). *Trichoderma* might grow and proliferate extensively but could not keep up with *Ganoderma*, hence this provided the opportunity for the pathogen to colonize the rhizosphere and infect the palms.

Most BCAs have a threshold level required for efficient biocontrol, and the initial BCA population may affect the level of biocontrol (Paulitz, 2000). It has been proposed that the degree of disease suppression by a biocontrol agent is determined by the density of the agent and the pathogen, the agent's capacity to suppress the pathogen and the population of the pathogen that is potentially impacted by the agent (Johnson, 1994).

In a study to determine rhizosphere colonization by *T. harzianum* SQR-T037 against Fusarium wilt of cucumber, it was found that increasing application rate of SQR-T037 (10^5-10^6) has significantly reduced the population of *F. oxysporum* regardless of biocontrol population treatments. The population of the pathogen in the rhizosphere was between 2.15-2.24 $\log_{10}$ ITS copies and the population should be no more than 3.30 log10 ITS copies per gram dry root for successful biocontrol (Yang *et al*., 2011). Hence, it should be a similar case for the control of *Ganoderma* at the oil palm estate in which the *Trichoderma* population must exceed the population of the pathogen. This is because the biocontrol agent's ability to suppress disease is directly proportional to its population density (Yang *et al*., 2011).

From this experimental chapter, there was a shift in the fungal community structure in terms of the genera detected and the proportion of Ascomycota and Basidiomycota in healthy, asymptomatic and symptomatic palms at both locations.

Similar to Palong plantation, healthy peat soil in Tg Karang was dominated by Ascomycota and unlike Palong, the fungal population was unique even for samples from the same category. There was a clear pattern in which unhealthy soil/diseased plant had higher Basidiomycota as compared to Ascomycota and this indicated that the soil might be dominated by phytopathogens such as *G. boninense* and other opportunistic basidiomycetes that breaks down the lignin content of the palm. Hence, this study presented the changes in the soil rhizospheres with regards to *Ganoderma* infection and future detection methods can be developed based on the soil microbial community so that healthy soil can be distinguished from potentially diseased/unhealthy soil. Additionally, the use of next-generation sequencing (NGS) should be considered since this method may provide a more direct approach to detecting and identifying microbial taxa.

7 CHAPTER 7: GENERAL DISCUSSIONS AND CONCLUSION

Oil palm produces the highest oil yield by using the least amount of land, therefore is the world's most productive oil crop. Malaysia is the second-largest palm oil producer and a major exporter of palm oil. Nevertheless, the present problem in the oil palm industry is the prevalence of BSR disease, in which the causal agent is *Ganoderma boninense*. The challenges in treating this disease are due to the absence of obvious external symptoms in infected palms before the advanced stage and the infected palms might not be able to respond to any treatment provided when it comes to this point (Rahamah Bivi *et al*., 2016). There is no adequate control for BSR in the field to date, since there is no effective fungicide available for successful control of BSR.

Thus, in this study, biological control method using *Trichoderma* spp. (strains 4A, T1 and B2) was explored and formulations of *Trichoderma* with carrier (biochar) and organic fertilizer (LOF) have been developed **(Chapter 4)**. *Trichoderma* was successfully mass-produced by using industrial bioreactors, with conidia as the seed culture resulted in a higher CFU of *Trichoderma* in comparison to mycelia as the seed culture. Also, only 1-2% of conidial inoculum was needed for the mass production as compared to mycelial culture (10%), which reduced the cost and time needed for the preparation of seed culture. Moreover, through liquid fermentation in a controlled environment, *Trichoderma* biomass can be rapidly produced with minimal contamination rate (Cumagun, 2014; de Rezende *et al*., 2020).

Initially, inert carrier (biochar) was autoclaved prior to mixing with *Trichoderma* spp. to ensure sterility of the materials. Subsequently, non-autoclaved biochar was used, which gave similar results in term of the *Trichoderma* counts (cfu/ml) and viability (*Trichoderma* spp. remained viable up to 24 weeks) as compared with the autoclaved biochar. The *Trichoderma* spp. used in this study

were aggressive competitors, able to outgrow the contaminants (bacteria and fungi) and was the dominant species found on PDA plates. Therefore, the use of non-autoclaved biochar (possible with the presence of contaminants) was not affecting the quality of *Trichoderma* formulations, since *Trichoderma* spp. was able to thrive in the formulations. Biochar used in this study as the carrier material was also ideal since it is originated from oil palm materials. *Trichoderma*-biochar was only added to LOF during formulation as *Trichoderma* spp. did not survive in FLOF, due to the chemical contents in FLOF. For field trial, treatments were divided into two types of bioorganic fertilizers (LOF and FLOF) in which for FLOF treatments (T2-T4), the chemicals were applied separately around the rhizosphere of the oil palms after two weeks of application of the bioorganic fertilizer consisting of *Trichoderma* spp. It was found that the *Trichoderma* spp. in LOF was also viable up to 24 weeks with populations around 5.00 log cfu/g for T3/T8 and T4/T9. The results were comparable with the previous study of Purwanto *et al*. (2017) in the formulation of *T. harzianum* with biochar and organic matter in which *Trichoderma* was viable up to 24 weeks with the counts around 5.00-6.00 log cfu/g. Despite that, future works should be focused on increasing *Trichoderma* spp. populations during the mass production and formulation so that a higher population of BCAs can be delivered to the field.

The use of *Trichoderma* spp. against *Ganoderma boninense* for the control of BSR disease *in vitro* and under greenhouse conditions (Susanto *et al*., 2005; Nur Ain Izzati and Abdullah, 2008; Sundram *et al*., 2008; Angel *et al*., 2016; Musa *et al*., 2018; Muniroh *et al*., 2019) were promising, but their effectiveness under the field conditions still requires further investigation. Hence, to evaluate the performance of *Trichoderma* bioorganic fertilizer, field trials were conducted at two oil palm plantations; 1) Paramount Estate, Pahang (Palong) which is owned by a private company and 2) Tg. Karang, Kuala Selangor which is owned by smallholder farmers. The age of the palms at Palong was 25 years old at the start of the field

trial (clay and laterite soil), while the palms at Tg Karang was between 8-10 years old (peat soil). Bioorganic fertilizers were applied every 4 months (3 times per year) for 24 months together with the assessments of soil microbial communities, soil physicochemical properties and evaluation of BSR disease in oil palm **(Chapter 5)**.

The patterns of *Trichoderma* populations at both Palong and Tg Karang were irregular between treatments at each sampling time, with an average of 5.00 log cfu/g at Palong throughout the experimental period. Meanwhile, the *Trichoderma* populations at Tg Karang increased from 2.00 log cfu/g at the start of the experiment to 4.00-5.00 log cfu/g, which indicated that the increment was most likely due to the application of the bioorganic fertilizers. At both locations, T9 treatment (20% *Trichoderma* in LOF) was significantly different and had the highest *Trichoderma* spp. populations, suggesting that this formulation should be recommended for field application with 20% *Trichoderma* as the threshold value. *Trichoderma* spp. populations at both locations in this study were higher than the one reported by Sariah *et al*. (2005) in an oil palm plantation in Perak, Malaysia whereas Oskiera *et al*. (2017) reported that the indigenous *Trichoderma* population in their study to be 3.00 log cfu/g before increasing to 5.00 log cfu/g after application of bio preparations containing *Trichoderma*.

In most agricultural systems, it appears that the indigenous *Trichoderma* populations are at low level with little fluctuation in size (Lundquist *et al*., 1999). This may be demonstrated by how agricultural systems are managed in which there are frequent applications of huge volumes of chemicals in the form of fertilizer, pesticides, herbicides or fungicides in the oil palm plantations which might be detrimental to soil microorganisms such as *Trichoderma.* Therefore, the challenge is to increase the *Trichoderma* population to 6.00 log cfu/g as suggested by Papavizas (1981) for efficient biocontrol activity in the soil system. Nevertheless, as there was no significant difference in the bacterial and fungal population as well as in the EC and pH of the soil, this suggests that the application of *Trichoderma*

bioorganic fertilizers does not influence soil biological and chemical properties. Also, the soil and probably the leaf nutrient contents were directly correlated with the fertilization regimes at both estates. One of the limitations faced at Tg Karang (smallholder plantations) during this study was that the leaf sampling could not be carried out due to the difficulty in finding a skilled worker to identify the leaf no. 17, in which it was the suggested frond/position to be sampled from mature oil palm (more than 6 years). This is because this particular frond will give a more accurate estimation of the plant's nutrient concentration as compared to other fronds (Pushparajah, 1994; Woittiez *et al*., 2016).

In this study, the field evaluation form for BSR disease based on 12 visual symptoms (12-point evaluation criteria) was modified from Supramaniam (2016). This enabled the scoring of the palm (from Scale 0 to 4), with Scale 0 indicated a healthy palm and Scale 4 represented a dead palm due to BSR disease. At Palong, palms from T7-T10 were free of disease. The palms at Palong had lower DI (below 13.0%) and comparable yield to the younger palms at Tg Karang. This might be due to the higher microbial populations found at Palong. In contrast, Tg Karang recorded high disease incidences, especially for T8 and T10. This might be contributed by the already high DI at the start of the experiment (12.5%) and *Trichoderma* bioorganic fertilizers applied were no longer able to control the progression of the disease. Additionally, this study provided evidence that LOF might not be suitable to be applied on peat soil due to the chemical properties (high carbon content) of the soil itself. This is supported by Qudsia *et al*. (2017), in which pathogenic fungi were positively correlated with soil OM in cucumber fields.

The percentages of palms with BSR disease for both control treatments (16.7%) and *Trichoderma* treatments (20.8%) at Tg Karang were higher than at Palong (5.2% and 3.5% respectively) and this mirrors the DI. Interestingly, at both locations, only palms from *Trichoderma* treatments recovered and showed no more disease symptoms. The effects of *Trichoderma* application can be seen on individual

palms although the impact might not be apparent on the overall treatments. It is possible that *Trichoderma* parasitizes and competes with *Ganoderma* for space and nutrients and also enhances plant defence responses as reported in the literatures (Harman *et al*., 2004; Waghunde *et al*., 2016; Musa *et al*., 2018; Sood *et al*., 2020). Further researches are needed to understand the mode of action of *Trichoderma* and its interactions with the plant, *Ganoderma boninense* and the soil microbial communities in the oil palm estates. Oil palm roots can be sampled and isolation of pure *Ganoderma* and *Trichoderma* can be done using culture media as well as molecular detection via PCR or qPCR. In addition, defence enzyme activities such as PO and PPO can be investigated by using the leaf samples and this will provide an insight into the defence mechanisms employed by oil palm in the field.

Field experiments and evaluation were influenced by climatic conditions and extraneous variables. There are various reports on BSR disease in different types of soil which are alarming, but it was found that through Geographical Information System (GIS), BSR disease distribution was not correlated with the soil series in Selangor, Malaysia (Parthiban *et al*., 2016). The majority of soil in agriculture was originally forest soil, such as in Palong; whereas Tg Karang soil was from coconut plantation. As mentioned by Parthiban *et al*. (2016), the spread of BSR was largely limited to coastal areas, which may be attributed to oil palm planting in the previous coconut stands. This was in agreement with the findings of this study where Tg Karang plantation recorded a higher incidence of BSR disease. It has been reported that BSR disease emerged much earlier, between 12-24 months after replanting on land that has been planted with coconut or oil palm previously; whereas the disease only occurred when the palms were around 10-12 years old when replanting from jungle land or rubber plantation (Kannan *et al*., 2017). Thus, the contrasting disease incidences at both Palong and Tg Karang could be attributed to their cropping history, in which coconut palms became the source of inoculum for

Ganoderma spp. This makes it difficult to control and manage BSR disease with the application of bioorganic fertilizer alone at Tg Karang plantation.

Therefore, proper management and sanitation are important before planting or replanting oil palms. Planters/growers should follow the initiative introduced by Malaysian Palm Oil Board (MPOB) known as Integrated *Ganoderma* Management (IGM). IGM includes sanitation during replanting, application of BCAs and fertilizer with beneficial/trace nutrient elements (Norman *et al*., 2019). For future work, the bioorganic fertilizer can be applied into the planting hole during replanting of new palms as a prevention method for the root disease problem. Surveys or observations for pests and diseases can be done one month after planting. The frequency of application of the bioorganic fertilizer can also be increased from 3 times per year to 6 times per year. This will improve the distribution of *Trichoderma* in the soil and reduces the development of pathogenic microbes.

In this study, *Trichoderma* populations in the rhizospheres of oil palm with different symptoms were investigated (**Chapter 6**). *T. asperellum* was present in all samples, indicating that it might be from the bioorganic fertilizers applications (strains 4A and T1) or the indigenous population in the soil. However, *T. longibrachiatum* (strain B2) was not detected, suggesting that this strain is not rhizosphere competent. This shows the importance of field experiments as the data/results from the *in vitro* and nursery evaluation will not guarantee the same outcome for field trials due to uncontrollable variables from the environment. At Palong and Tg Karang, similar *Trichoderma* spp. were present but with varying distribution patterns between the symptoms. Interestingly, only soil samples from healthy palms possessed unique species of *Trichoderma* with *T. harzianum* at Palong, *T. effusum* and *T. reesei* at Tg Karang. Even though further analysis needs to be conducted to understand the role of these species of *Trichoderma* in the soil samples, this study provided an insight into the *Trichoderma* populations in the palm rhizospheres. This further supports studies conducted by Joseph (2000),

Sariah *et al.* (2005) and Cummings *et al*., (2016) to identify the *Trichoderma* spp. present in Malaysia generally and the oil palm plantations specifically.

Apart from that, this study has identified the fungal populations in the palm rhizospheres with different symptoms by targeting the ITS region **(Chapter 6)**. Soil samples of healthy palms from Palong were very similar to one another, with 8 common fungal genera and the presence of biological control agents (*Trichoderma* spp. and *Clonostachys rosea*). This was followed by soil samples from asymptomatic and symptomatic oil palms, with 4 and 1 common genera respectively. The results were contradicted with Tg Karang soil samples where there were no common genera found between samples of the same category. Even so, ectomycorrhizal fungi (ECM) of different genera were observed to be present in healthy soil samples. Both locations revealed the presence of various genera with only *Fusarium* as the common genus. This is due to soil properties such as texture, pH, salinity and other characteristics which leads to the development of a unique microbial community in each soil environment (Zwolinski 2007). Besides, it is clear that the microbial communities in asymptomatic and symptomatic palms were different from the healthy ones, which indicated that the changes/disruption of the fungal communities might leads to the establishment of *Ganoderma* spp. in soil or vice versa. The rhizospheres of symptomatic palms also had less fungal genera, even with the presence of opportunistic fungi such as *Trechispora* and *Ophiostoma*.

Studies conducted by Kerfahi *et al*. (2014), McGuire *et al*. (2015) and Brinkmann *et al*. (2019) showed that tropical rainforests were dominated by Basidiomycota and conversion of these rainforests into oil palm plantation resulted in the abundance of Ascomycota. For peat forest and oil palm plantation cultivated on peat soil, it was reported that Ascomycota was the most abundant phylum (Siti Ramlah *et al*., 2016; Liu *et al*., 2020). In this study, Ascomycota was the most abundant in the rhizospheres of healthy and asymptomatic palms at Palong and Tg. Karang and this was in agreement with previous studies conducted by Kerfahi *et*

al. (2014), McGuire *et al*. (2015), Siti Ramlah *et al*. (2016), Brinkmann *et al*. (2019) and Liu *et al*., (2020). Conversely, Basidiomycota was abundant in the rhizospheres of symptomatic palms. This showed that there were changes in the fungal communities when palms were infected by *Ganoderma* spp. and this leads to the presence of other opportunistic basidiomycetes that feeds on decaying roots and stems of the palm. This is an interesting discovery as this is the first research describing the changes in the fungal communities over the course of BSR disease. In light of this, for future work, it would be valuable to explore other taxonomic groups such as Bacteria and Archaea during the occurrence of BSR disease as this would give more information about the microbial communities and their interaction with regards to BSR disease. Besides, the research would be crucial to understand the shift in microbial populations and the contributing factors. While Sanger sequencing used in this study is a good choice for microbe identification, perhaps the use of NGS for future studies will be appealing since it allows deep sequencing of amplicons and the capability to sequence multiple samples (Escobar-Zepeda *et al*., 2015).

This work has determined that qPCR could be useful to quantify *Trichoderma* spp. and *Ganoderma* spp. populations in the soil. Quantification of *Trichoderma* in the soil had been conducted by many researchers (Savazzini *et al*., 2008; Yang *et al*., 2011; Zhang *et al*., 2019; Stummer *et al*., 2020), but to the best knowledge of the author, it was the first time *Ganoderma* spp. was being quantified from the soil by using the qPCR method. *Trichoderma* spp. quantity was similar in the soil samples at Palong and Tg Karang (between 0.020-0.035 ng/µl). For *Ganoderma*, the general trend was that the populations in rhizospheres of asymptomatic and symptomatic palms were about ten times higher than the rhizospheres of healthy palms which reflects the health status of the palm. However, it was concerning that the *Ganoderma* quantity at Tg Karang was 10-fold higher than *Trichoderma* and the *Ganoderma* quantity at Palong. This may prove that there was a high

Ganoderma inoculum due to the planting of coconut previously and might explain the high DI at Tg Karang, thus resulting in inefficient biocontrol activity of *Trichoderma* towards BSR disease at the smallholder plantations.

Other than that, correlation analysis revealed that there was a negative relationship between *Trichoderma* and *Ganoderma* at Palong. Meanwhile, at Tg Karang, it was a positive relationship between the BCA and the pathogen. Negative interactions indicated that there was a decline in the pathogen population concomitant with an increase in the biocontrol agent population (Paulitz, 2000). This should be the ideal and targeted interaction between the BCA and the pathogen as *Trichoderma* should antagonize *Ganoderma* for successful control of the disease. On the contrary, a positive relationship at Tg Karang was due to the high *Ganoderma* population, as *Trichoderma* needs to proliferate extensively in order to keep up with the pathogen. The results obtained in this study were important as they indicated the need to establish a link between *Ganoderma* DNA concentrations and its threshold levels so that detection and treatment of disease can be conducted earlier. Furthermore, the threshold level of *Trichoderma* DNA should also be taken into account for effective biocontrol activity. At the moment, qPCR has been used for the detection and quantification of pathogens of various plants such as *F. solani*, *P. ultimum*, *R. solani* and *Verticillium dahliae* in tomato (Lievens *et al*., 2006), *Pyrenophora tritici-repentis* and *Parastagonospora nodorum* in wheat (Abdullah *et al*., 2018), *A. flavus* in maize (Mitema *et al*., 2019) and *Colletotrichum camelliae* in tea (He *et al*., 2020). Hence, the use of qPCR should be the way forward in the detection and control of BSR disease since it can provide a rapid and accurate quantification of pathogen and can replace the traditional pathogen isolation and quantification methods.

The results of this work on developing *Trichoderma* bioformulations and evaluation of the bioorganic fertilizers under field conditions for the control of BSR disease could further improve the understanding of biological control agents and

the factors that contributed to the success of biocontrol. Additionally, this study shed light on the changes in the fungal communities in palm rhizospheres over the course of BSR disease. Interaction between the biological control agent and the pathogen also highlights the importance of soil microorganisms in the suppression or progression of plant disease. This could contribute positively to the oil palm industry in detecting, controlling and managing BSR disease.

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APPENDICES

Appendix 1. DNA extraction

1a: 5 M Sodium chloride (NaCl): 29.22 g NaCl (w/v) (R&M Chemical, USA) was dissolved in dH_2O and volume was made up to 100 ml. The solution was autoclaved before use.

1b: 1 M Tris-hydrochloric acid (HCl): 15.76 g Tris-HCl (1^{st} Base, Singapore) was dissolved in dH_2O and the volume was made up to 100ml.

1c: 10% Cetyltrimethylammonium bromide (CTAB): 10.00 g of CTAB (w/v) was dissolved in dH_2O and the volume was made up to 100 ml.

1d: 0.5 M Ethylenediaminetetraacetic acid (EDTA) (pH 8.0): 18.16 g EDTA (w/v) (Promega, USA) was dissolved in dH_2O. The pH was adjusted to 8.0 by using 1 N sodium hydroxide (NaOH) and the volume was adjusted to 100 ml. The solution was autoclaved before use.

1e: 5% Polyvinylpyrrolidone (PVP): 5.80 g PVP (w/v) (Acros Organics, USA) was dissolved in dH_2O and the volume was made up to 100 ml.

1f: 3 M Sodium acetate (NaAc) (pH 5.0): 24.61 g NaAc (w/v) (SYSTERM®, Malaysia) was dissolved in dH_2O. The pH was adjusted to 5.0 with glacial acetic acid and the volume was made up to 100 ml prior to autoclaving.

1g 1X Tris-Borate-EDTA (TBE) buffer: 100 ml of 10X TBE (v/v) (Vivantis, USA) was mixed with 900 ml dH_2O.

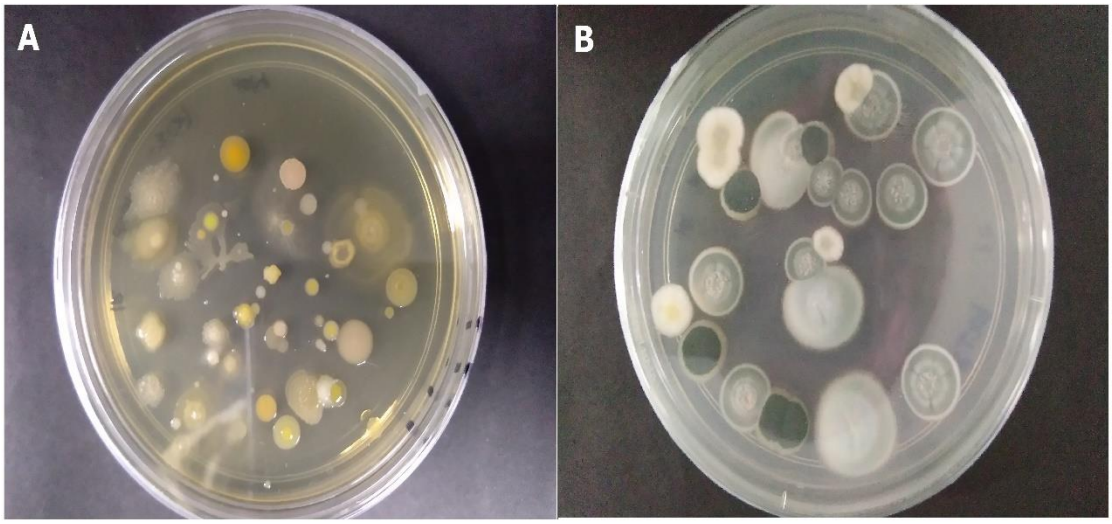
1h: 1.5% Agarose gel: 1.50 g agarose (w/v) (1^{st} Base, Singapore) was added to 100 ml of 1X TBE buffer. The solution was microwaved to dissolve the content and was cooled down briefly before adding 4 µl of SYBR™ safe DNA gel stain (Invitrogen, USA). The molten gel was poured onto a gel tray with a comb.

Appendix 2. Chemical properties of biochar.

Sample Reference	Lab Reference	Type of fertilizer	% on sample												pH (5 % Solution)
			Moisture	N	Total P_2O_5	Citric acid soluble P_2O_5	K_2O	MgO	CaO	B_2O_3	Organic Matter by Loss on Ignition	Organic Carbon by Loss on Ignition			
BIOCHAR	FE18/GPOF3		11.9	0.56	0.27		1.21				92.4	53.6	on wet basis		10.24
				0.64	0.31		1.38				93.3	54.1	on dry basis		

Appendix 3. Chemical properties of LOF.

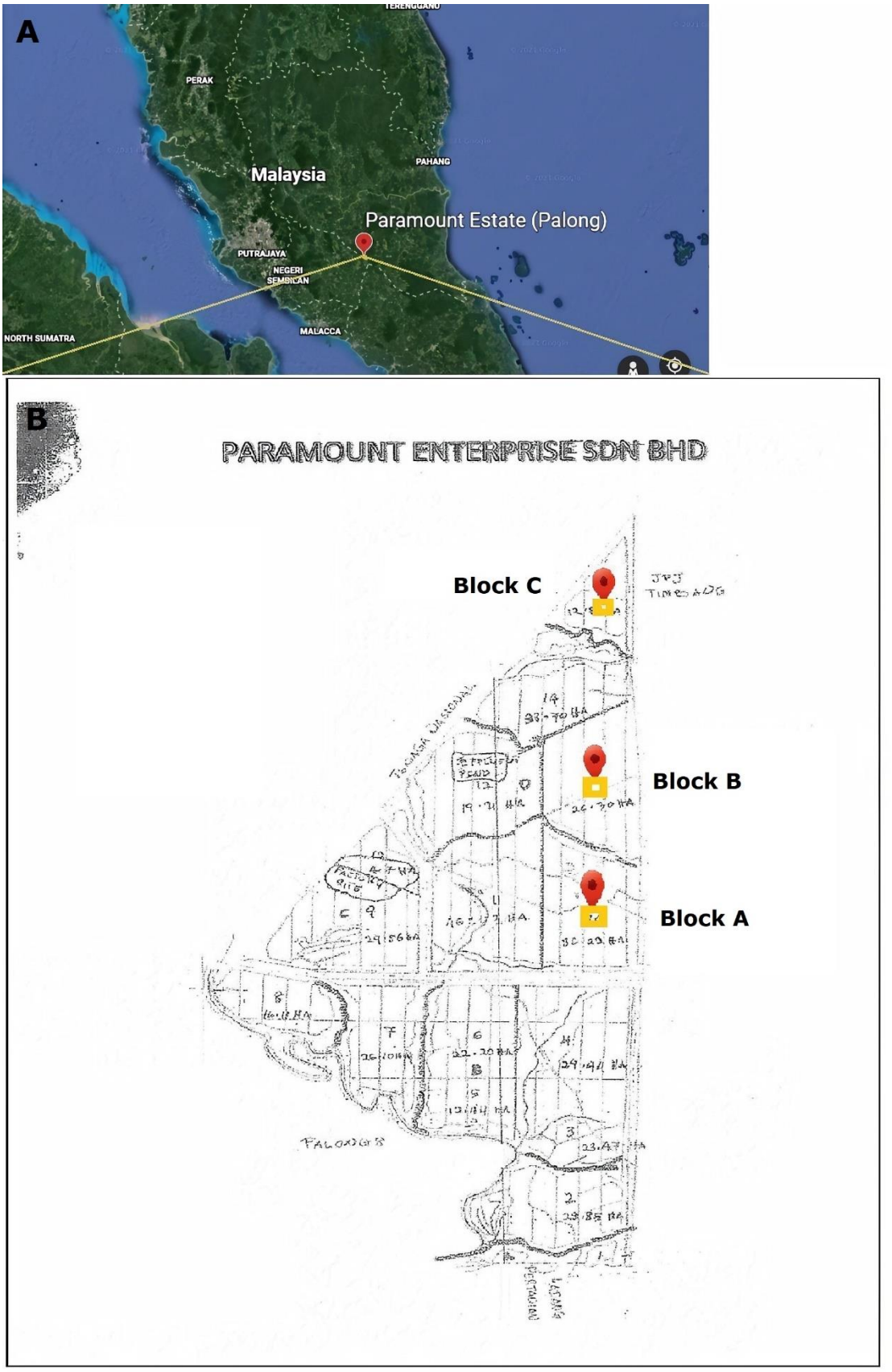
Test Description	Unit	Result(s)	Method or Equipment Used
Nitrogen (as N)(Dry basis)*	%w/w	1.4	MS417:1994
Nitrogen (as N)(As is basis)*	%w/w	1.0	MS417:1994
Phosphorus (as P2O5)(Dry basis)*	%w/w	0.9	MS417:1994
Phosphorus (as P2O5)(As is basis)*	%w/w	0.6	MS417:1994
Potassium (as K2O)(Dry basis)*	%w/w	4.5	MS417:1994
Potassium (as K2O)(As is basis)*	%w/w	3.1	MS417:1994
Total Organic Matter*	%w/w	46.0	MS 417: Part 8:1997
Organic Carbon*	%w/w	27.1	MS 417: Part 8:1997
pH (10%)*		9.3	pH meter
Moisture*	%w/w	30.6	Oven (2 hours, 105 degrees Celsius)
Arsenic (as As)	mg/kg	2.9	AOAC 971.21, 17th Ed & Hydride Generator
Lead (as Pb)	mg/kg	1.7	AOAC 972.23, 17th Ed
Cadmium (as Cd)	mg/kg	1.0	AOAC 973.34, 17th Ed
Mercury (as Hg)	mg/kg	ND <0.01	AOAC 971.21, 17th Ed
Nickel (as Ni)*	mg/kg	0.3	MY/STP/009 based on AOAC 968.08, 17th Ed
Chromium (as Cr)*	mg/kg	1.3	ICP-OES



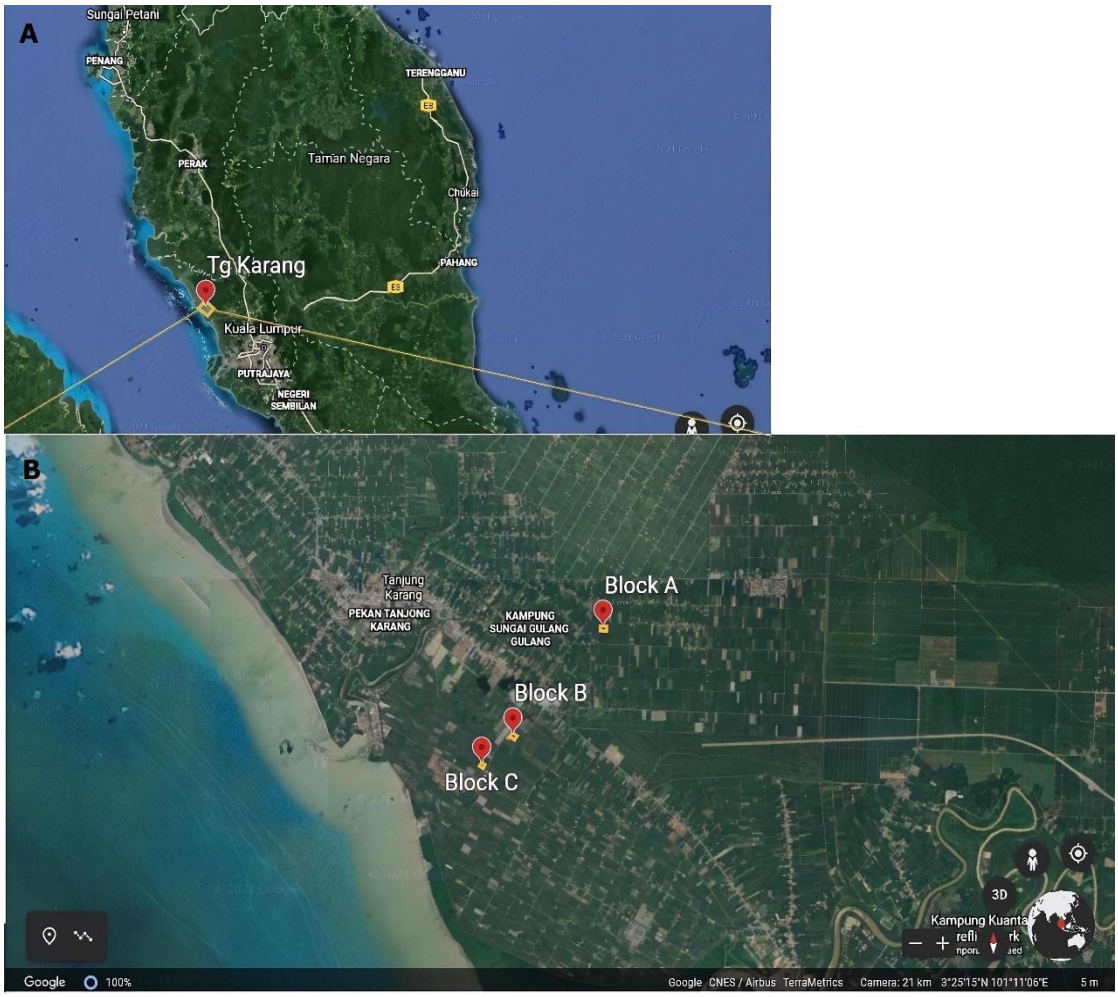
Appendix 4. Bacterial (A) and Fungal (B) colonies on NA and PDA plates respectively from Bu0% sample.



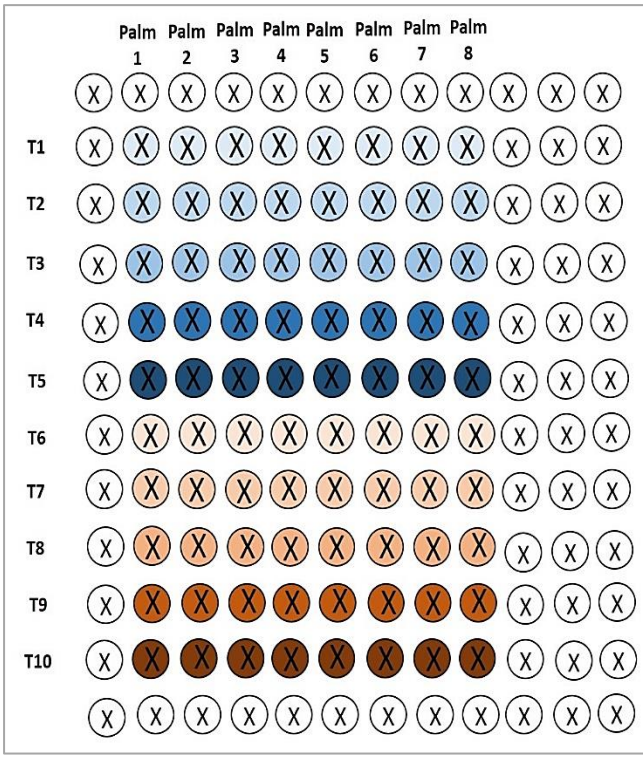
Appendix 5. Fungi that can be seen from LOF (T1/T6) on a PDA plate.



Appendix 6. Location of Paramount Estate (Palong) in Bera, Pahang, Malaysia. (A) Geographic location. (B) Aerial view of the experimental plots.



Appendix 7. Location of the smallholder plantations in Tg Karang, Selangor, Malaysia. (A) Geographic location. (B) Aerial view of the experimental plots.



Percentages of *Trichoderma* spp. (%) in FLOF and LOF			
Treatment	FLOF	Treatment	LOF
T1	Control	T6	Control
T2	5	T7	5
T3	10	T8	10
T4	20	T9	20
T5	GSP	T10	GSP

Control = Fertilizer application alone (FLOF/LOF)
GSP = Grower's standard practice

Appendix 8. General layout of the treatment plan at Palong and Tg Karang plantations. The experimental design was Complete Block Design (CBD) with 3 replications (Block A, B and C). Each block consisted of 10 treatments (T1 to T10) with 8 palms per treatment. Different coloured dots represent palms under different treatments.

Appendix 9. General classification table of soil nutrient status for oil palm (Goh and Chew, 1997).

Property	Units	Very low	Low	Moderate	High	Very High
pH	-	<3.5	4.0	4.2	5.5	>5.5
Total N	%	<0.08	0.12	0.15	0.25	>0.25
Total P	mg/kg	<120	200	250	400	>400
Available P	mg/kg	<8	15	20	25	>25
Exchangeable K	$cmol^+/kg$	<0.08	0.20	0.25	0.30	>0.30
Deficiency	-	Likely	Possible	-	-	Induced
Hidden hunger	-	-	-	Likely	-	Possible
Response to fertilizers	-	Definite	Likely	Possible	-	Possible

Appendix 10. Nutrient concentration in leaf 17 in old palms (over 6 years of planting) (von Uexküll and Fairhurst, 1991).

Nutrient	Units	Deficiency	Optimum	Excess
N	% DM	<2.3	2.40-2.80	>3.00
P	% DM	<0.14	0.15-0.18	>0.25
K	% DM	<0.75	0.90-1.20	>1.60

Appendix 11. Manuring programme in the year 2016-2019 at Paramount Estate (Palong).

2016

MANURING 2016			Total NPK Nutrient (KG/Palm)		
MONTH	**FERTILIZER**	**QUANTITY**	**N**	**P**	**K**
JAN - APR	ORGANIC MIXTURE (16+12+0+5)	5 KG 3 KG	0.075 0.48	0.05 0.36	0.175 -
APR-JUNE	MIXTURE (16+12+0+5)	4 KG	0.64	0.48	-
AUG-SEPT	MOP	4 KG	-	-	2.40
	TOTAL	**16KG/Palm**	**1.195**	**0.89**	**2.575**

2017

MANURING 2017			Total NPK Nutrient (KG/Palm)		
MONTH	**FERTILIZER**	**QUANTITY**	**N**	**P**	**K**
JAN - FEB	ORGANIC + MIXTURE (5+5+10+2)	5 KG	0.25	0.25	0.50
APR-JUNE	MOP (K, 60%)	2 KG	-	-	1.20
JUNE-NOV	ORGANIC (12/12/0/2)	5 KG	0.60	0.60	-
	TOTAL	**12 KG/Palm**	**0.85**	**0.85**	**1.70**

2018

MANURING 2018			Total NPK Nutrient (KG/Palm)		
MONTH	FERTILIZER	QUANTITY	N	P	K
MAC-APR	ORGANIC + MIXTURE (12/12/17/2)	5 KG	0.60	0.60	0.85
SEPT - DEC	MOP	3 KG	-	-	1.80
	TOTAL	8 KG/Palm	0.60	0.60	2.65

2019

MANURING 2019			Total NPK Nutrient (KG/Palm)				
MONTH	FERTILIZER	QUANTITY	N	P	K	MgO	ORGANIC MATTER
JAN	CFC MIXTURE (12/6/22/3+TE)	3 KG	0.36	0.18	0.66	0.09	-
MAY	ORGANIC	10 KG	0.15	0.10	0.35	0.13	5.00
SEPT	MOP	3 KG	-	-	1.80	-	-
	TOTAL	13KG/Palm	0.51	0.28	2.21	0.22	5.00



Appendix 12. (A) One of the falling palms at Tg Karang. *Ganoderma* fruiting body (red circle) was seen at the exposed root area. (B) Close-up of the basidiomata. Photos were taken on 25 January 2019.

Appendix 13. Statistical analysis

13a: One-way repeated measures ANOVA of *Trichoderma* spp. count in biochar in different formulations

Production 2 and 3

Source of variation	d.f.	s.s.	m.s.	*F* test	*P* value
Rep.Subject stratum					
Treatments	3	496.533	165.511	144.52	<.001
Residual	9	10.3072	1.14524	40.26	
Rep.Subject.Time stratum					
d.f. correction factor 0.5478					
Time	6	1.81015	0.30169	10.61	<.001
Time.Treatments	18	1.12768	0.06265	2.2	0.039
Residual	72	2.0479	0.02844		

(d.f. are multiplied by the correction factors before calculating F probabilities)

NB: d.f. = degrees of freedom; s.s. = sum of the squares; m.s. = mean square

Production 5

Source of variation	d.f.	s.s.	m.s.	*F* test	*P* value
Rep.Subject stratum					
Treatments	3	372.2911	124.097	3397.11	<.001
Residual	6	0.21918	0.03653	0.55	
Rep.Subject.Time stratum					
d.f. correction factor 0.4135					
Time	6	0.76006	0.12668	1.92	0.166
Time.Treatments	18	1.46639	0.08147	1.24	0.33
Residual	48	3.16155	0.06587		

(d.f. are multiplied by the correction factors before calculating F probabilities)

NB: d.f. = degrees of freedom; s.s. = sum of the squares; m.s. = mean square

13b: One-way repeated measures ANOVA of bacterial count in FLOF

Source of variation	d.f.	s.s.	m.s.	*F* test	*P* value
Rep.Subject stratum					
Treatments	3	0.50335	0.16778	7.92	0.017
Residual	6	0.12716	0.02119	1.05	
Rep.Subject.Time stratum					
d.f. correction factor 0.4122					
Time	5	20.26665	4.05333	200.82	<.001
Time.Treatments	15	2.67544	0.17836	8.84	<.001
Residual	40	0.80737	0.02018		

(d.f. are multiplied by the correction factors before calculating F probabilities)

NB: d.f. = degrees of freedom; s.s. = sum of the squares; m.s. = mean square

13c: One-way repeated measures ANOVA of *Trichoderma* spp. count in LOF

Source of variation	d.f.	s.s.	m.s.	*F* test	*P* value
Rep.Subject stratum					
Treatments	3	296.7516	98.9172	250.55	<.001
Residual	12	4.73767	0.39481	6.81	
Rep.Subject.Time stratum					
d.f. correction factor 0.3589					
Time	5	0.53998	0.108	1.86	0.176
Time.Treatments	15	0.58667	0.03911	0.68	0.656
Residual	80	4.63491	0.05794		

(d.f. are multiplied by the correction factors before calculating F probabilities)

NB: d.f. = degrees of freedom; s.s. = sum of the squares; m.s. = mean square

13d: Two-way repeated measures ANOVA of *Trichoderma* spp. count at Palong

Source of variation	d.f.	s.s.	m.s.	*F* test	*P* value
Block.Subject stratum					
Fertilizer	1	2.1522	2.1522	13.86	0.002
Concentration	4	12.0277	3.0069	19.36	<.001
Fertilizer.Concentration	4	3.6186	0.9046	5.82	0.003
Residual	18	2.7956	0.1553	0.86	
Block.Subject.Time stratum					
d.f. correction factor 0.6335					
Time	5	38.125	7.625	42.43	<.001
Time.Fertilizer	5	14.5691	2.9138	16.21	<.001
Time.Concentration	20	17.1451	0.8573	4.77	<.001
Time.Fertilizer.Concentration	20	30.154	1.5077	8.39	<.001
Residual	100	17.9723	0.1797		

(d.f. are multiplied by the correction factors before calculating F probabilities)

NB: d.f. = degrees of freedom; s.s. = sum of the squares; m.s. = mean square

13e: Two-way repeated measures ANOVA of *Trichoderma* spp. count at Tg Karang

Source of variation	d.f.	s.s.	m.s.	*F* test	*P value*
Block.Subject stratum					
Fertilizer	1	0.0605	0.0605	0.34	0.567
Concentration	4	5.6297	1.4074	7.9	<.001
Fertilizer.Concentration	4	14.7905	3.6976	20.74	<.001
Residual	18	3.2085	0.1782	0.72	
Block.Subject.Time stratum					
d.f. correction factor 0.6363					
Time	5	136.2655	27.2531	110.64	<.001
Time.Fertilizer	5	6.8644	1.3729	5.57	0.002
Time.Concentration	20	11.241	0.562	2.28	0.016

Time.Fertilizer.Concentration	20	22.3093	1.1155	4.53	<.001
Residual	100	24.6313	0.2463		

(d.f. are multiplied by the correction factors before calculating F probabilities)

NB: d.f. = degrees of freedom; s.s. = sum of the squares; m.s. = mean square

13f: Generalized linear model analysis of disease incidence (DI) at Tg Karang

20 Months

Change	d.f.	deviance	mean deviance	deviance ratio	approx chi pr
+ Fertilizer	1	0.152	0.152	0.15	0.696 ns
+ Concentration	4	2.57	0.643	0.64	0.632 ns
+ Fertilizer.Concentration	4	9.578	2.394	2.39	0.048 *
Residual	20	35.459	1.773		

ns = non significant, * significant at $P > 0.01$, ** significant at $P < 0.01$, *** significant at $P < 0.001$)

24 Months

Change	d.f.	deviance	mean deviance	deviance ratio	approx chi pr
+ Fertilizer	1	1.733	1.733	1.73	0.188 ns
+ Concentration	4	2.754	0.688	0.69	0.6 ns
+ Fertilizer.Concentration	4	10.854	2.714	2.71	0.028 *
Residual	20	35.64	1.782		

ns = non significant, * significant at $P > 0.01$, ** significant at $P < 0.01$, *** significant at $P < 0.001$)

Fertilizer effect (all time points)

Change	d.f.	deviance	mean deviance	deviance ratio	approx chi pr
+ Fertilizer	1	7.897	7.897	7.9	0.005 **
Residual	178	287.227	1.614		

ns = non significant, * significant at $P > 0.01$, ** significant at $P < 0.01$, *** significant at $P < 0.001$)

13g: One-way ANOVA of average number of hits for Basidiomycota at Palong

Source of variation	d.f.	s.s.	m.s.	*F* test	*P* value
Symptoms	2	102.152	51.076	22	0.007
Residual	4	9.288	2.322		

NB: d.f. = degrees of freedom; s.s. = sum of the squares; m.s. = mean square