Endophytic Biocontrol Agents

For The Control of *Ganoderma boninense* In Oil Palm

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Abstract

This project aimed to investigate the potential of fungal endophytes as biocontrol agents against the oil palm pathogen *Ganoderma boninense*. The fungal endophytes selected in this study originated from banana plant (*Aspergillus calidoustous* BTF07, *Penicillium citrinum* BTF08, *Trichoderma asperellum* T2), *Mimosa pudica* (*Diaporthe phaseolorum* MIF01), and *Portulaca* weed (*Diaporthe phaseolorum* WAA02) and were found to have good inhibitory activity towards *G. boninense*. These endophytic isolates were then determined for their host specificity by performing calli test using tissue-cultured oil palm calli. Results showed that only isolate BTF08 has good growth promoting effects towards the oil palm host tissues, while other isolates did not show any impact on growth of oil palm calli. Defense mechanism of oil palm via lignification as a result of endophytic infection was determined by evaluating the extent of lignification via quantitative analysis and microscopic observation. Results revealed higher lignin production in endophyte-infected ramets while lignin levels in *G. boninense*-infected ramets were not significantly enhanced compared to ramets in control (no endophyte, no pathogen). Endophytic isolates were further examined for their production of volatile and non-volatile antifungal compounds towards *G. boninense.* These compounds were extracted and identified using SPME-GCMS and HPLC-NMR. It was discovered that volatile compounds such as 1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl), 3-Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthenecarbox amide, 1-methyl-4-(1-methylethyl), Benzene, 1-methyl-4-(1-methylethyl) and Benzothiophene-3-carboxylic acid,4,5,6,7-tetrahydro-2-amino-6-ethyl, ethyl ester were produced by these endophytes. Volatile compounds produced by T2 (i.e. 3-Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthenecarbox amide, 1,2-Dihydroanthra[1,2-d]thiazole-2, 6,11-trione, trans-4-(2-(5-Nitro-2-furyl)vinyl)-2-quinolinamine, 1,2-Benzenediol,

3,5-bis(1,1-dimethylethyl) and Benzoic acid, 5-methyl-2-trimethylsilyloxy) demonstrated the strongest inhibition towards this fungal pathogen. In addition, profiling of volatile compounds revealed that inhibition is attributed to the action of a single compound or the synergistic action of several compounds. Results also showed that endophyte BTF08 produced non-volatile compound with strong antifungal activity against *G. boninense* (MIC value of 100 μg/ml). This non-volatile compound was determined to be citrinin via bioassay-guided fractionation. In subsequent studies, isolate T2 and BTF08 were selected for biocontrol assessments. These isolates were evaluated for their efficacy as single or mixed treatment, by measuring vegetative growth of seedlings and observing oil palm disease severity after pathogen infection. It was found that the application of isolate T2, BTF08 or combination of both T2 and BTF08, significantly reduced disease symptoms of the infected oil palm seedling, where application of T2 recorded DSI value of 10, followed by BTF08 and combination of T2+BTF08 with DSI of 5 and 15, respectively. This suggested that isolate T2 and BTF08 have better plant protecting activity when applied as single inoculum, while the combination (T2 and BTF08) have a lower plant protecting activity, which may be attributed to competition among both endophytic isolates. In conclusion, BTF08 is the most suitable candidate in controlling *G. boninense*, attributed mainly to its good plant-growth promoting potential, antagonistic activity against *G. boninense* and induction of lignification in oil palm.

Thesis overview

This thesis comprised of 7 chapters with each chapter representing a manuscript submitted to the respective journals (except Chapter 1). Therefore, each chapter will contain its relevant parts on Introduction, Methods, Results, Discussion (or Results and Discussion, depending on journal), and a section on Conclusion. The thesis will begin with Chapter 1, which is a brief introduction to the threats of *Ganoderma boninense* to the oil palm industry and the benefits of endophytes as biocontrol agent against plant fungal pathogen. This is followed by Chapter 2-6, which illustrates the various key parts of the study. The References and Appendices complete the thesis.

Chapter 1 presents the introduction of the project which includes a brief introductory of the *Ganoderma* disease faced in oil palm industry and proposing a biocontrol method in solving this issue. In **Chapter 2,** the compatibility as well as host specificity of various endophytic fungi with oil palm is tested using a relatively new approach; via calli test. Prior to this test, the antifungal nature of the selected endophytes (production of antifungal compounds, mycoparasitism, colonizing the same ecological niche as plant pathogen) towards *G. boninense* was first established via dual culture test. The compatibility test ensued and was carried out by applying a relatively new rapid assay known as the endophyte-calli assay. This assay investigated the correlation between the host and the fungi, as well as the reaction of the host towards presence endophyte (and pathogen). It was discovered that BTF08 are compatible with oil palm calli with growth promoting properties, while T2 has good antifungal activity towards the pathogen. **Chapter 3** documents the evaluation on host response to endophyte (and pathogen) infection via lignification. The lignification process was examined via quantification using acetyl bromide assay and the changes to the cell wall (cell wall thickening) was observed via SEM. In

addition, histological staining of lignified plant cell wall using phloroglucinol-HCl further provided evidence of induced lignification as a response to endophyte infection. These suggested the potential of endophytic fungi as biocontrol agents to induce lignin production, which potentially served as a defence mechanism against *G. boninense*.

Chapter 4 explains the profiling of volatile compounds generated by endophytes to inhibit *G. boninens*e, while **Chapter 5** further characterizes the antifungal compounds in endophyte BTF08. The secondary volatile metabolites produced by endophytic fungi were entrapped using a solid-phase micro-extraction (SPME) technique, which was later subjected to Gas Chromatography-Mass spectroscopy (GC-MS) for analysis. The profiling of valuable major secondary volatile metabolites is possible through this approach, which gradually identifies compounds that may be responsible for growth of the host plant (oil palm) and protection against *G. boninense*. Among the many compounds, several such as 1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl), 3-Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthenecarbox amide, Benzene, 1-methyl-4-(1-methylethyl), and Benzothiophene-3-carboxylic acid,4,5,6,7-tetrahydro-2-amino-6-ethyl, produced primarily by T2, may be strong inhibitors of *G. boninense*. In addition, non-volatile metabolites for BTF08 (*P. citrinum*) were extracted and fractionated (using solvent), isolated via HPLC, and identified via NMR, to reveal the potent antifungal compound towards *G. boninense* as citrinin. Lastly, **Chapter 6** explores the efficacy of T2 and BTF08 applied as single and mixed treatments for the biocontrol of *G. boninense* (BSR incidence) in oil palm seedlings. BTF08 and T2 were selected as biocontrol candidates, primarily because both displayed antagonistic activities against *G. boninense* and demonstrated significant compatibility towards oil palm tissues (calli test). Both isolate T2 and BTF08 induced lignification as a response to endophyte infection, which serves as a protection against *G. boninense*. The

mycoparasitic nature of isolate T2 serves as good antagonistic mechanism against *G. boninense*, which T2 produced citrinin which is a potent antifungal compound against *G. boninense*.

In conclusion, isolate *P. citrinum* (BTF08) was discovered to have the most potential as biocontrol agent in preventing BSR cause by *G. boninense*. Inoculation with BTF08 showed plant growth promoting potential, antagonistic potential against *G. boninense*, induced lignin production in host and is highly compatible with oil palm.

General Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes an original paper published in a peer reviewed journal and four unpublished/submitted manuscripts for publication. The core theme of the thesis is endophytic biocontrol agents for the control of *Ganoderma* in oil palm. The ideas, development and writing of all the papers in the thesis were the principal responsibility of me (Cheong Siew Loon) under the supervision of Associate Professor Adeline Ting Su Yien and Dr. Cheow Yuen Lin within the School of Science.

Thesis chapter	Publication title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
Chapter 2	Characterizing antagonistic activities and host compatibility (via simple endophyte-calli test) of endophytes as biocontrol agents of *Ganoderma boninense*	Published	65% of analysis and interpretation of data, drafting and writing	1) Adeline Ting, 25% of writing and review of the publication 2) Cheow Yuen Lin, 10% of writing and review of the publication	No No
Chapter 3	Comparing lignification in oil palm ramets elucidated by endophytic and pathogenic infection	Unpublished/ submitted	65% of analysis and interpretation of data, drafting and writing	1) Adeline Ting, 25% of writing and review of the publication 2) Cheow Yuen Lin, 10% of writing and review of the publication	No No
Chapter 4	Profiling volatile compounds produced by endophytes to inhibit *Ganoderma boninense*	Unpublished/ submitted	65% of analysis and interpretation of data, drafting and writing	1) Adeline Ting, 25% of writing and review of the publication 2) Cheow Yuen Lin, 10% of writing and review of the publication	No No
Chapter 5	Isolation and characterization of antifungal metabolites of *Penicillium citrinum* against fungal pathogen *Ganoderma boninense*	Unpublished/ submitted	65% of analysis and interpretation of data, drafting and writing	1) Adeline Ting, 25% of writing and review of the publication 2) Cheow Yuen Lin, 10% of writing and review of the	No No

				publication	
Chapter 6	Exploring the efficacy of single and mixed treatment of different species of endophytic fungi as a potential biocontrol agent against *Ganoderma boninense* basal stem rot in oil palm	Unpublished/ submitted	65% of analysis and interpretation of data, drafting and writing	1) Adeline Ting, 25% of writing and review of the publication 2) Cheow Yuen Lin, 10% of writing and review of the publication	No No

Student signature: [redacted] **Date:** 16th October 2017

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: [redacted] **Date:** 16th October 2017

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List of Abbreviations

ANOVA	Analysis of variance
AUDPC	Area Under the Disease Progress Curve
BSR	Basal stem rot
CFU	Colony forming unit
DI	Disease incidence
DSI	Disease severity index
GC-MS	Gas chromatography–mass spectrometry
HPLC	High-performance liquid chromatography
HSD	Honestly Significant Difference
MIC	Minimum inhibitory concentration
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PIDG	Percentage of inhibition of diameter growth
PIRG	Percentage of inhibition of radial growth
SDW	Sterile distilled water
SEM	Scanning electron microscopy
SPME	Solid-phase micro-extraction

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Introduction and Literature Review

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Introduction and Literature Review

1.1 Oil palm physiology

Oil palm (*Elaeis guineensis* Jacq.) is categories as a monoecious plant that belongs to *Elaeis* genus of Palmae family (Abdullah and Wahid 2010). The seed consist of an endocarp with a single kernel most of the time. Multiple kernels may arise due to tri-carpellate abnormalities in the ovary but this case is rarely seen. The nut size varies across regions but averages at 2 – 3 cm with weigh spanning from 4 – 13 g (Corley and Tinker 2008). Fibres can be observed across the shell, with a plug of fibre ending at the germ pore to form a flat-like structure similar to the inner shell layout (Hussey 1958). The kernel shows layers of endosperm, which features as an oily greyish white substance and surrounded with dark-brown testa tangled in a mesh of fibres. The embryo lies within the endosperm and is separated from the germ pore by thin layers of endosperm cells, testa and the flat-like surface. These three structures is collectively known as the operculum, although separated (Corley and Tinker 2008). Seed germination occurs when the embryo forms the hypocotyl or in the case of Henry in La germination des grains d'Elaeisi (Henry 1951), the petiole part of the cotyledon. This then follows with emerging of the plumula and radicle via the ligule while the haustorium matures until it fills the nut cavity in a spongy formation (Arasu 1970). The seedling grows steadily, depending on its supply of nutrition from the endosperm for the first few weeks. These nutrients provided are mostly made out of fat and galactomannan, a type of carbohydrate which is preferred as utilization of energy over fat (Alang 1982). The removal of the seed occurs after 3 – 4 months, when the plumula has developed into cataphylls and substantial growth of the adventitious roots above the radicle-hypocotyl junction (Corley 1976, Corley and Tinker 2008).

This coincides with the start of photosynthesis from its first leaves, indicating no dependency towards the endosperm anymore. The continuous growth of the stem shows a swollen stem base, with no visible intermodal elongation (Corley 1976) with little height growth in the first three years. The early years of growth shows a wide stem base in the shape of an inverted cone structure, where the primary adventitious roots will originate from. Once reaching adulthood, the stem serves as a supporting structure as well as the vascular organ for the plant. It consists of rich amounts of fibrous phloem sheaths, with sclerotic parenchymal cells as its main support (Corley 1976). Storage tissues are in loosely packed in the central area of the vascular bundle, where silica and starch containing cells are found in abundance. The maturation of the leaf however is extremely slow, but in mature plants, shows linear leaflets on each side of stalk, hence pinnate. The amount of leaflets produced annually ranges from 30 – 40 within 2 – 4 years of growth, with decline shortly after the 7th year of growth (Corley and Tinker 2008). In mature palms, countless primary roots originate from the bole, with its extensive root system depending on the surrounding soil (Purvis 1956). Most researchers have agreed on four classes of roots; primary, secondary, tertiaries and quaternaries. The primary roots show an outer epidermis and highly lignified hypodermis encircling the cortex filled with air spaces. The cortex consists of the vascular cylinder with highly lignified endodermis keeping the bundle and medulla from collapsing. A point should be made on the presence of pneumatodes on the aerial and underground roots. Their function is unknown but many have postulated a soil ventilation purpose (Corley and Tinker 2008). The difference in inflorescence can be observed separately in both male and female. In brief, the female inflorescence is arranged around the spikelet spirally subtended by a cavity and bract, at which these bracts form into sharp spines. The male inflorescence however is placed on longer peduncles when compared to female while containing

non-spiky cylindrical spikelets. The fruits take approximately six months for pollination to occur and developed to maturity. The fruit comprises of an oily outer layer known as the pericarp, with a single seed (kernel), which also contains oil (Basiron and Weng 2004, Corley and Tinker 2008).

1.2 The oil palm industry in Malaysia

It is a fact that the Malaysian oil palm industry has been acknowledged to be significant in its role from the stance of economic for its contribution to the profitable export trades (Basiron, 2007). Furthermore, Malaysian contribution to the global oil and fat was at 11%, with 27% for exports worldwide (MPOC, 2014). Hence, due to the volumes of profits earned, the nation is blanketed with oil palm plantation in order to meet the escalating demands at the global level. As such, the total area of oil palm plantation, which had been 54, 000 hectares in 1960, witnessed a whopping increment in 2009 that hit 4.69 million hectares with a 10.06% growth at the annual rate (MPOC 2014).

Nevertheless, the growth of the oil palm industry is threatened by the spread of Basal Stem Rot (BSR) disease caused by *Ganoderma boninense* (*G. boninense*). This disease is rampant in oil palm plantations since the last three decades (Chong et al., 2017). *G. boninense* is soil-borne in nature and grows on oil palm roots and stumps as it depends on tissues of the oil palm as source of food. Within the initial stage of disease spread, basidiomata of *G. boninense* would start growing at the basal stem or the roots of the oil palm. This white-rot fungi spreads and destroy tissues rapidly, damaging half of the basal stem within a short period of time. Symptoms include a decrease in the fruit size, discolourization of the palm leaves, necrotic tissues, wilting, yellowing or dead leaves, growth of fungi on the surface of the rotting wood, gummosis, as well as stems with internal discoloration (Chong et al., 2017). As a result, the oil

palm suffers from water reticence and nutrient deficiency, which eventually lead to water distress and malnutrition (Chong et al., 2017).

G. boninense decreases the longevity of oil palm, thus reducing the production of oil palm. In fact, up to 80% of oil palm plants can be easily infected by *G. boninense* before reaching 10 years of age (Chong et al., 2017). The spread of disease amongst younger palms (up to two years old) is common, with at least 30% infection (Chong et al., 2017). Palm trees that are infected experience decrease in production by 0.2% in the initial stage, and this percentage could increase up to 24.1% within the next five years. As time passes without any treatment, the oil palm tree can be severely infected and die without producing any fruit (Chong et al.,2017).

1.3 Ganoderma infection

In Malaysia, a sizable size of most plantations was infected by *Ganoderma* which is considered specifically a disease of oil palm (Flood et al., 2002). Initially thought as an infection attacking only older palm, it is now considered a threat after detection in younger palms planted in place of coconut or older palms (Turner, 1981). Hence, the extend of which *Ganoderma* affects oil palm is largely disastrous due to the versatility in infecting oil palm across different age strata. This alarming condition is term “Basal Stem Rot” disease (BSR) although in retrospect, initial visible symptoms include unopen fronds and undeveloped “spears” towards the crown (Turner, 1966). Such visible deterioration indicates severe damage of the root and stem system, in which water uptake is compromised. In older palms, younger leaves would turn from olive green to yellow in color and progressively die from the tip. At the base of the stem, the bole would turn black in coloration with visible growth of *Ganoderma* sp. fruiting body, which may be accompanied with exudation of gum (Corley and Tinker, 2008). Physiologically, peripheral tissues remain unaffected although hard, while presence of mycelium can be found throughout

the tissue including roots. The cortex of the plant also turns brownish; indicating the process of decay has initiated with presence of sporophores as well (Corley and Tinker, 2008). Initiation of infection appears to originate from root contact in *Ganoderma*-infected soil, with studies supporting this theorem (Navaratnam and Chee, 1965, Lim et al., 1992, Sariah et al., 1994, Breton et al., 2006). However, it is noted that BSR is not exclusive to *Ganoderma* where other root-defiling basidiomycete conditions of perennial crops and trees spread via vegetative growth (Rees et al., 2009). Examples include colonization of living roots by *Heterobasidian annosum* which initiate originate from root stumps of dead pines (Woodward et al., 1998). Nevertheless, this theorem is not without argument, where primary infection of BSR via root system would be hard for *G. boninense* to infect oil palm plantation due to extreme heterogeneity of *G. boninense* in soil (Miller et al., 1999). BSR is also considered a "silent killer", as symptoms on leaves were only seen at later stages of the disease.

The route of infection spans from root to spore infection, although these methods varies in credibility and is highly debatable. In root infection, BSR apparently arise from contaminated soil inoculum which was in contact with other infected roots (Rees, Flood et al. 2009). Trials were done by using *Ganoderma*-cultured wooden blocks placed in contact with oil palm seedlings, which leads to typical pathological changes of BSR (Navaratnam and Chee 1965). Furthermore, the extent of possible soil contamination was shown where seedlings planted in proximity of infected stumps became infected as well (Hasan and Turner 1998). Origins of soil contamination can be contributed by contaminated debris or windrows (Virdiana, Hasan et al. 2010) as well as poor management of landfill of leftover boles (Hasan, Foster et al. 2005). Another side note to consider is the direct contact of infected seedlings or mature palm root with non-infected plants. Root infection containing basidiomycete diseases can spread even by

vegetative growth, seen in *Armillaria mellea* and *Heterobasidion annosum* (Woodward, Stenlid et al. 1998). Colonization arising from *G. boninense* primarily occurs at unwounded roots, which progresses into the cortex layer (Rees, Flood et al. 2009). However, infection through the vascular bundle may offer another alternative route of infection, although such studies have yet to prove this case.

Method of infection of *Ganoderma* requires close or even direct contact with palm roots of successful parasitic colonization to occur. This event suggests crucial characteristics of *Ganoderma*, where it is primarily a weak pathogen which requires a large inoculum to infect its host as well as being less competent against surrounding saprotrophic micro-environment (Cooper, Flood et al. 2011). This is proven where it is unable to obtain nutrient source from surrounding soil or organic debris, although growth of *Ganoderma* was observed when exposed towards sterilized nutrients from soil (Rees, Flood et al. 2007). Hence, the possibility of *Ganoderma* build-up in frond debris or soil must occur within diseased stump or root prior to infecting new plants. From the point of view, it is highly unlikely that upper stem rot could occur from this mechanism as infected debris in contact with fronds is rarely observed. However, several studies have shown that *G. boninense* was actually spread via basiodiospores rather than direct contact of roots onto infected debris. Molecular analysis of *G. boninense* obtained from two different sources from Malaysia and Papua New Guinea plantations showed diverse variation in chromosomal content, largely in alleles, somatic (or vegetative) and mitochondrial DNA markers (Miller, Holderness et al. 1999, Pilotti, Sanderson et al. 2004). Such genotypic differences could have occurred during sexual recombination, followed by spreading of spores. This contingency seems true in studies showing BSR in new plantations with no *G. boninense* inoculum present (Sanderson, Pilotti et al. 2000). The chromosomal structure of *G. boninense*,

which is tetrapolar with polymorphous alleles and heterothallic at the crossbreed loci also favors outcrossing to ensure transfer of resilient traits in the next inoculum (Pilotti, Sanderson et al. 2002). With diversified molecular alteration occurring at every generation, it would be hard to gauge the estimate change of characterization from one generation to another. Multiple infections via spores and root route could yield different mtDNA RFLP results and somatic congeniality within the same plant as shown in gel electrophoresis studies (Rees, Flood et al. 2012). Root infections occurring intramurally showed development shift (Rees et al., 2009). The antecedent event occurs in both the stem base and root cortex, in which a biotrophic phase involving colonization of host cells by hyphae which is similar to various ascomycetes like *Colletotrichum* spp. (Perfect et al., 1999). An antipathetic necrosis-like stage then follows which leads to cell wall degradation of the host plant with pigmented mycelium within the host tissues and larger pigmented lesions on the external roots, appearing as tough pseudosclerotia (Cooper et al., 2011). Penetration of cell wall to invade into the vascular bundle requires multiple wall-degrading enzymes to breakdown lignin, cellulose and suberin. These three components coat the outermost tissue and makes up the recalcitrant polymers which are actively secreted by plants. Enzymes secreted by *G. boninense* were found to specifically target lignin and other biological polymers (Rees et al., 2009). The characteristics of BSR can then be seen as white "patches" on roots which is apparent where lignin is oxidized by these enzymes (Adaskaveg et al., 1990, Rees et al., 2012). Subsequent invasion results in minute puncture or holes across the cell wall layers, which is caused by loss of electron density within the middle lamella, with little decadence of S1 and S2 polysaccharide components of the cell wall layers. It was noted that starch content decreased rapidly in oil palm seedlings during early stages of infection where it seem that starch could be another source of carbon and energy for *G. boninense* during BSR. However, more

study is needed to elucidate the exact bio-mechanism of *G. boninense* in infiltrating the bole tissues of oil palm seedlings such as molecular studies of early host-pathogen relationship.

1.4 Control measures for Basal Stem Rot (BSR) Disease

Control of BSR disease has been attempted using several approaches, primarily through the use of physical and chemical approaches. The physical approach includes soil mounding, trunk surgery, clearing of infected stumps, isolation of infected trees, and good land preparation methods. On the other hand, fungicides are used for chemical approach.

The following discusses the various control measures attempted. Soil mounding, which refers to a physical approach used in many plantations, is a commonly used technique. This technique uses soil to be heaped around the trunk of palm trees up to 75 cm in height and 1 m radius. Research revealed that this technique has been widely acknowledged by planters/growers as effective, especially for encouraging healthy root growth. This technique does not halt the spread of BSR (Soepena, Purba & Pawirosukarto, 2000). The other method is through the use of trunk surgery. Trunk surgery refers to the elimination of tissues that are dead or *basidiocarps* with a chisel or a mechanical back-hoe. This technique is only viable for plantations that are of small scale, and is ineffective for cases of palm trees with deep decay. Deep decay penetrates deep into the trunk, and the tissue is severely injured when trunk surgery is performed (Utomo & Niepold, 2000). Other than that, the method of physical clean clearing has also been attempted. Clean clearing refers to the placing of collapsed oil palm trunks along the rows of old palms (Hushiarian et al., 2013). This approach does not require intense labour, thus is relatively cost effective. However, this technique does not prevent the contact of roots among young and growing trees with those of collapsed, diseased plants (source of inoculum). The collapsed trees

also become a breeding place for rhinoceros beetles, which are pests that can severely damage healthy trees (Hushiarian et al., 2013). Another method, isolation of infected plants, has also been used, but with limited success. Isolation refers to a method that is employed to halt the spreading of the disease by placing all infected trees into ditches and troughs. As such, there is less contact between infected trees and healthy palm trees. This method is not favoured by planters, as this technique demands rigorous labour and incurs high cost (Sariah & Zakaria, 2000).

The chemical approach has also been attempted, typically involving the use of fungicide-based treatment. In fact, most oil palm plantations adopt the use of chemicals to reduce BSR incidence in the field. The fungicide is applied via hand-knock pressure, so as to minimise loss of chemical during treatment. It has been shown that injection of hexaconazole mixed in 10 litres of water via hand-knock pressure successfully limited, to a certain extent, the disease incidence in healthy palm trees as the palm trees subsequently yield fruit bunches after 5 years (Al-Obaidi et al., 2014). Nonetheless, manpower and expensive chemicals (e.g. bromoconazole, triadimefon, carboxin, carbendazim, and methfuroxam) are required for this method, which is not economically feasible (Shamala et al., 2006).

To address the limitations arising from physical and chemical control measures, the biological control approach is studied. This approach uses beneficial microorganisms as biocontrol agents, as they have been known to render the following benefits; increased drought tolerance (Arechavaleta et al., 1989), deterrence of insect herbivores (Breen, 1994), and protection against fungal pathogen (Clarke et al., 2006). Biological control for *Ganoderma* has been studied by Soepena et al. (2000), in which they found that the application of endophytic biocontrol agent displayed 70% effectiveness in constricting the spread of *Ganoderma* disease.

Typical mechanisms of biocontrol include production of volatile antifungal compounds (Mumpuni et al., 1998), production of non-volatile compounds (Cooney et al., 1997, Siddiquee et al., 2009), and mycoparasitism by mycoparasites such as *Trichoderma* (Abdullah et al., 2007).

1.5 Endophytes as Biocontrol Agents

Endophytes are bacteria or fungi that spend part of their life cycle in a plant but they do not cause any disease symptoms to the plants. Endophytes are highly-diverse although they are typically grouped into clavicipitaceous (C-endophytes) and non-clavicipitaceous (NC-endophytes) endophytes. C-endophytes infect grasses while NC-endophytes infect vascular plants, gymnosperms and angiosperms (Nair & Padmavathy, 2014). Rodriguez et al. (2009) documented the many benefits conferred by endophytic biocontrol agents to their host plants. For example, endophytes are know for growth stimulation in plants, increment in nutrient uptake, growth inhibition of pathogens in plants, reduction in disease symptoms in plants, as well as enhanced tolerance in plants towards harsh environments. Hoyos-Carvajal et al. (2009) documented the role of endophyte *Trichoderma* in inducing growth stimulation in beans. Endophyte *Neotyphodium coenophialum* was found promoting the mineral uptake in tall fescue plant (Malinowski et al., 2000). Arnold et al. (2003) found that fungal endophytes limit pathogen damage in trees by mediating plant defence. Root colonizing endophyte *Pirifomospora indica* was documented to stimulate the expression of drought stress related genes in *Arabidopsis* (Sherameti et al., 2008).

To date, the study on endophytes for the control of *Ganoderma boninense* is limited to endophytes isolated from healthy oil palms (Zaiton et al., 2006). Rashyeda et al. (2016) investigated 582 endophytic bacteria as biological control agents against the *Ganoderma* disease found in oil palm. As a result, three of the endophytic bacteria examined in the study were

discovered to be potential biological control agents, with high percentage of inhibition of radial growth (PIRG) for both dual culture and culture filtrate tests. They include *P.* a*eruginosa* GanoEB1, *B.* c*epacia* GanoEB2, and lastly, *P. syringae* GanoEB3. Bivi et al. (2010) found *P. aeruginosa* as well, to have potential to inhibit *Ganoderma*. Endophytic fungal isolates are equally important. Naidu et al. (2015) found non-pathogenic *hymenomycetes* were effective against *G. boninense*. The isolates were identified as *Pycnoporus sanguineus*, *Trametes lactinea*, and *Grammothele fuligo* with 84, 82, and 81% inhibition rates, respectively. Researches have also discovered endophytic fungi *Phlebia* GanoEF3 having good potential to inhibit *G. boninense* (Nurrashyeda et al., 2012). The findings obtained from the study showed that the *Phlebia* GanoEF3 powder had not only managed to restrict the development of BSR disease, but also inhibited the spread of *Ganoderma* disease in oil palm. Both bacteria and fungal endophytes are known to be able to demonstrate production of antifungal compounds, secreting lytic enzymes, enhanced plant defense and stimulating plant secondary metabolites production for the control of *G. boninense* oil palm (Gao et al., 2010).

The use of endophytes as biocontrol agents is however, met with several challenges. Initially, soil and the microbiota were identified as limiting factors to endophyte growth and establishment in the field (Handelsman & Stabb, 1996). In recent years, new theories have emerged on the possible host-endophyte-pathogen interaction as a limiting factor. Chow, Rahman, and Ting (2017) conducted a study to examine this host-endophyte-pathogen relationship and discovered that some endophytes colonized the host plant upon inoculation, but were not proliferating in the host tissues, which may have implicated biocontrol activities. Nevertheless, this study pursued the research on various endophytes and their role as biocontrol agents for the control of *G. boninense*.

1.6 Research objectives

This particular research proposes fungal endophytes as potential biocontrol agents for the control of *G. boninense*. As such, these strains were tested against the primary oil palm pathogen, *G. boninense*, in order to determine their antagonistic activities. The specific objectives outlined for this study are listed as follows:

1. To determine the antifungal potential of endophytic fungi and their inhibitory mechanisms
2. To investigate the endophyte-host plant compatibility
3. To screen volatile and non-volatile compounds produced by selected endophytic fungi
4. To test the most promising endophytic fungi for their biocontrol and growth promoting properties

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Chapter 2

Characterizing antagonistic activities and host compatibility (via simple endophyte-calli test) of endophytes as biocontrol agents of *Ganoderma boninense*

Overview

This part of the study aimed to propose fungal endophytes as potential biocontrol agents against basal stem rot in oil palm caused by *G. boninense*. One of the most important aspects of endophytic biocontrol agent is host compatibility. Host incompatibility may induce strong responses such as the production of tannin and cell death, which may be a form of induced host resistance. Host compatibility is a good indication of the biocontrol efficacy of fungal endophytes in oil palm, where compatible biocontrol agent may increase effectiveness while reducing detrimental side effects. Therefore, this study was conducted to address the host compatibility of endophytes tested, including *Aspergillus calidoustous* BTF07, *Penicillium citrinum* BTF08, *Trichoderma asperellum* T2, *Diaporthe phaseolorum* WAA02, *Diaporthe phaseolorum* MIF01 to inhibit BSR caused by *G. boninense*. The experiment was carried out by first establishing the antifungal activities of selected fungi using dual culture test, in order to gauge the antagonistic activity exerted by the fungus towards *G. boninense*. We then carried out compatibility test via rapid endophyte-calli assay. The use of this assay allows the study of host-fungal interaction and provides an understanding on how the host reacts towards fungal infection. This serves as an indication of the compatibility between endophytic fungi and oil palm host. Result from dual culture test showed all endophytic fungi have antagonistic effect towards *G. boninense* with isolate BTF07 (*A. calidoustous*) demonstrating the strongest inhibition towards *G. boninense* (PIRG value of 49.55%), followed by T2 (*T. asperellum*), WAA02 (*D. phaseolorum*), MIF01 (*D. phaseolorum*) and BTF08 (*P. citrinum*) with 47.75%, 39.64%, 36.04%, and 13.51%, respectively. Results also showed that only BTF08 had growth-promoting effects towards the host tissues and endophyte BTF07, T2, WAA02 and MIF01 displayed good antagonistic activity against the pathogen. In conclusion, host compatibility can be deduced using calli and ramets

inoculation. Isolate BTF07, T2, WAA02 and MIF01 are potential candidates to be developed as biocontrol agents.

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Characterizing antagonistic activities and host compatibility (via simple endophyte-calli test) of endophytes as biocontrol agents of *Ganoderma boninense*

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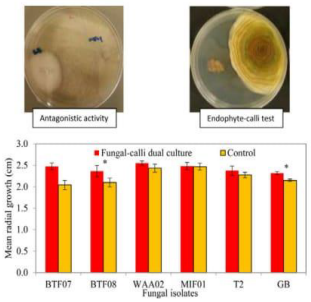
HIGHLIGHTS

- Endophytes inhibited pathogen via inhibitory compounds and competitive exclusion.
- BTF08 is compatible with host-plant with growth promoting effects towards calli.
- Calli produced substances that stimulated growth of BTF08 and GB.
- Endophyte-calli assay results are validated by endophyte-ramet test.
- *P. citrinum* BTF08 and *T. asperellum* T2 have most potential as biocontrol agents.

GRAPHICAL ABSTRACT



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ABSTRACT

This study characterized the antagonistic activities of five fungal endophytes (*Aspergillus calidoustous* BTF07, *Penicillium citrinum* BTF08, *Trichoderma asperellum* T2, *Diaporthe phaseolorum* WAA02, *Diaporthe phaseolorum* MIF01) and evaluated their endophyte-host compatibility with the host plant (oil palm). The antifungal activities of the endophytes towards *Ganoderma boninense* (GB) were first established using the dual culture test, revealing antagonistic nature of endophytes via production of non-volatiles, volatiles and competitive exclusion. Endophyte-host compatibility was then assessed using a simple but rapid endophyte-calli dual-culture assay, and results validated using endophyte-ramet test. Results revealed that endophytes elicited different responses in oil palm calli. BTF08 had growth promoting effects towards the host tissues with the highest calli weight (1013 mg) obtained, while BTF07 appeared to inhibit calli (1006 mg) leading to browning and necrosis. This endophyte-calli test also revealed the influence of calli on endophyte growth. Isolates BTF08 and GB benefited from host association, with increased radial growth (2.36 cm and 2.31 cm, respectively) compared to growth in the absence of calli (2.10 cm and 2.15 cm, respectively). Endophytes and GB were also isolated from host tissues, suggesting compatibility and ability to colonize host tissues (root, stem, leaf). This suggested the reliability of the endophyte-calli test as a rapid assay to provide an insight on the endophyte-host compatibility.

1. Introduction

Endophytes are microorganisms which colonize the internal plant tissues without causing any disease symptoms (Arnold,

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2007). In recent years, endophytes have been studied for various purposes such as for their plant growth regulating properties, increased tolerance towards environmental changes, antagonistic properties towards pathogens and in triggering induced-host resistance in plants (Arnold, 2007; Duijff et al., 1997; Pleban et al., 1995; Porras-Alfaro and Bayman, 2011; Sturz et al., 1998). Several of these applications (e.g. biological control, tolerance to abiotic stress) require the introduction of endophytes into "new" host plants (Postma and Rattink, 1992; Thomas and Upreti, 2014). Under these circumstances, endophytes are isolated from one host plant and purposefully inoculated into a different/new host plant to produce the desired effect. The association and interaction between the introduced endophyte with the "new" host plant will gradually determine their symbiotic response (mutualistic, antagonistic or synergistic). It is hypothesized that the interaction between endophytes and host could either lead to subsequent proliferation or inhibition of the endophytes in the host plant. This ultimately influences their expression of beneficial characteristics when present in the host plants.

It is therefore paramount that the compatibility between endophyte and the host is investigated, particularly for antagonistic endophytes with biocontrol potential. Several profound findings were reported, which include the following; (i) endophyte colonization in plants are achieved by artificial inoculation of conidia, ascospores and mycelium through wounding of the plant tissues (Lahrmann et al., 2013; Leuchtmann, 1993); (ii) colonization frequency varies in different parts (leaf, stem, root and flower) (Masumi et al. (2015); (iii) and possible tissue-specificity traits in certain endophyte genera such as the detection of *Stemphylium* and *Aspergillus* in leaf tissues, *Ulocladium*, *Drechslera* and *Curvularia* from stem tissues, and *Cylindrocarpon* from root tissues of *Thymus* sp., respectively (Masumi et al. (2015). The endophyte-host compatibility is also said to be influenced by chemical recognition between host and fungi (Ride, 1992; Tyler, 2002). Chapela et al. (1991) reported that endophytic *Xylariaceous* species responds to certain chemical compounds in the host plants such as monolignol glucosides, which influences ascospore germination. Thus, the function of chemical molecules produced by host tissues is observed to have a certain degree of influence on the growth and proliferation of endophytes in the host tissues. It is also hypothesized by Dupont et al. (2015) and Eaton et al. (2010) that introduced endophytes could elicit recognition mechanisms in the new host plant to either accept or reject endophyte colonization.

To date, none of the studies were on commercially important agronomic crops such as oil palm as studies on endophytes and oil palm mostly focused on endophyte-growth promoting effects (Anuar et al., 2015) and biocontrol (Sundram et al., 2015), not on the endophyte-host compatibility. Hence, the endophytes tested were potential biocontrol agents of *Ganoderma boninense*, a pathogen causing devastating loss to the oil palm industry (Rajesh et al., 2014). These endophytic isolates (*Aspergillus calidoustous* BTF07, *Penicillium citrinum* BTF08, *Trichoderma asperellum* T2, *Diaporthe phaseolorum* WAA02, *Diaporthe phaseolorum* MIF01) were selected as they belong to genera with known antifungal properties (producers of inhibitory compounds, mycoparasitic action, competitive-exclusion) (Akrami et al., 2011; Ting et al., 2009a,b, 2012, 2010; Yu et al., 2010). In addition to antagonistic properties, it is highly desirable that these endophytes also have good compatibility with the host plant. This study was therefore conducted to define the compatibility of the endophytes with oil palm using a rapid assay; the endophyte-calli assay.

The endophyte-calli assay is a simple endophyte-host model based on the *in vitro* dual culture concept, using endophyte and oil palm calli co-inoculation for rapid observation of their interactions. This model used the calli of oil palm because a calli cell mimics the actively-dividing cells of the cambium tissues of a plant (Hendry et al., 1993; Nawrot-Chorabik, 2013). This report is the first to present the combination of calli-test model and colonization studies as useful assays to determine endophyte-host compatibility.

2. Materials and methods

2.1. Culture establishment

Fungal endophytes were first isolated from various host plants; isolate BTF07, BTF08 and T2 from stem tissues of *Musa* sp. (Ting et al., 2009b, 2012; Ting and Jioe, 2016), isolate WAA02 from roots of *Portulaca* sp. (Ting et al., 2009b, 2010), and isolate MIF01 (Ting et al., 2010) from roots of *Mimosa pudica*. The endophytes were identified as *Aspergillus calidoustous* BTF07 (GenBank accession No. KT329189), *Penicillium citrinum* BTF08 (GenBank accession No. KT964566), *Trichoderma asperellum* T2 (GenBank accession No. KT964564) and two isolates of *Diaporthe phaseolorum* MIF01 (GenBank accession No. KT964565) and WAA02 (GenBank accession No. KT964567). These isolates demonstrated good biocontrol potential; with *T. asperellum* (T2), *P. citrinum* (BTF08), *D. phaseolorum* (WAA02) showing complete overgrowth towards *Ganoderma boninense* under metal-stress (Ting and Jioe, 2016). Other researchers have also identified the biocontrol activities of these isolates against another pathogen (*Fusarium oxysporum*), establishing the antifungal potential of the selected endophytes (Akrami et al., 2011; Ting et al., 2009a,b, 2012; Yu et al., 2010). The pathogen, *G. boninense* was obtained from Prof. Dr. Sariah Meon, Universiti Putra Malaysia. All fungal cultures (endophytes and pathogen) were maintained on Potato Dextrose Agar (PDA) (Merck) at room temperature (25 ± 2 °C).

2.2. In vitro screening for antagonistic activity

In vitro screening for antagonistic activity of the selected endophytes against *G. boninense* was evaluated by dual culture assay. Antagonistic activity of endophytes towards *G. boninense* were evaluated through dual culture test (Albert et al., 2011; Meon, 1998). Control plates were established by co-inoculating *G. boninense* with PDA agar plug. All tests were conducted with three replicates and incubated at room temperature. The diameter of *G. boninense* was recorded. The inhibition percentage was recorded after incubating for a week. Inhibition of *G. boninense* by endophyte was calculated based on Percentage of Inhibition of Radial Growth (PIRG):

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

where R1: radial growth of *G. boninense* co-inoculated with plain agar plug (control), and R2: radial growth of *G. boninense* co-inoculated with fungal endophytes.

2.3. Calli-endophyte dual culture test

The dual culture test was adopted from Peters et al. (1998), where petri dishes were first filled with 25 ml callus multiplication medium (Murashige and Skoog medium supplemented with 2 mg 2,4-dichlorophenoxyacetic acid, 5 mg 6-Benzylaminopurine, and 7 g phytoagar) (Murashige and Skoog, 1962). In our study, we used two-week old oil palm calli (supplied by Applied Agricultural Resources Sdn. Bhd.) and they were transferred onto the agar. Mycelial plug of 7-day old endophyte culture was then co-inoculated onto the agar at an equi-distance of 2 cm from the periphery of the plates. For observation of calli growth without the influence of endophytes, plain agar plugs (instead of endophyte

mycelial plug) were used to establish the control plates. For the observation on endophyte growth, control was established by inoculating endophytes on agar plates in the absence of calli. Three replicates were prepared for each calli-endophyte challenge. The growth of endophyte (radial growth, cm) and calli (fresh weight, mg) were recorded over the next 14 days. Measurements were discontinued when endophyte overgrow the calli or when the callus made contact with the endophyte. The endophyte growth and difference in calli weight was compared to control (endophytes cultured without presence of calli). Therefore, from this calli-endophyte test, two main observations were determined; firstly, the influence of calli on endophyte growth, and the influence of endophyte on calli growth.

2.4. Determination of endophyte/pathogen colonization extent

Four-week old tissue-cultured oil palm ramets (supplied by Applied Agricultural Resources Sdn. Bhd.) were used for the colonization study. Seven treatments were designed (BTF07, BTF08, MIF01, WAA02, T2, GB and control). The endophytes (or pathogen GB) were inoculated separately (100 ml, 10^6 cfu/ml) to each ramet by soil drenching. Sterile distilled water was applied for ramets in control. Isolation was performed at 2, 6, 12, 24 and 36 h after inoculation according to Taylor et al. (1999), where surface-sterilization was first performed on the ramets, followed by sectioning into leaf, stem and root tissues (1 cm in length), and the tissues were plated and incubated on PDA. The re-isolation was recorded by observing the presence of fungi (endophyte and pathogen) from the tissue sections on PDA. From this colonization test, the colonization pattern (presence, preference and rate) of the endophytes (and pathogen) were determined.

2.5. Statistical analysis

All experiments were performed in triplicate, where one ramet constitutes a replicate. The data was analyzed using Statistical Package for the Social Sciences (SPSS) version 20.0. Means were compared using the Tukey-Kramer multiple comparison test (Honestly Significant Difference, HSD, $P < 0.05$), or paired T-test ($P < 0.05$ for paired-comparisons) where relevant.

3. Results

3.1. Antagonistic activity

The dual culture test revealed that all endophytic isolates inhibited the growth of *G. boninense*. Isolate BTF07 (*A. calidoustous*) demonstrated the strongest inhibition towards *G. boninense* (PIRG value of 49.55%), followed by T2 (*T. asperellum*), WAA02 (*D. phaseolorum*), MIF01 (*D. phaseolorum*) and BTF08 (*P. citrinum*) with 47.75, 39.64, 36.04 and 13.51%, respectively (Fig. 1). Inhibition was predominantly due to the fast-growing trait (T2), competitive exclusion (WAA02, MIF01, T2), production of volatile inhibitory compounds (inhibition zone observed) (BTF08), and mycoparasitism (T2) of the respective endophytes.

3.2. Influence of calli (host plant) on fungal growth

All of the endophytes (BTF07, BTF08, T2, WAA02, MIF01) and pathogen tested (GB), were able to grow on the callus multiplication medium when cultured with calli of oil palm. As such, the results were considered valid, as growth of endophytes was not comprised in any manner. BTF08 (*P. citrinum*) was the only endophyte, which showed excellent compatibility with calli, as significant increase in radial growth was detected when co-inoculated with the calli (2.36 cm), compared to solitary endophyte growth (control) (2.10 cm) (Fig. 2). On the contrary, the growth of the other endophytes (BTF07, T2, WAA02, and MIF01) was not induced by the presence of calli (Fig. 2). Interestingly, the pathogen (GB) growth was positively influenced by the presence of calli, with radial growth of 2.32 cm, compared to solitary pathogen growth (control) with 2.15 cm (Fig. 2). The positive influence of calli on endophyte (BTF08) and pathogen (GB) growth suggested that calli may produce metabolites (diffused into the agar) which could promote growth of specific fungi (BTF08 and GB).

3.3. Influence of endophytes on calli growth

The isolate *P. citrinum* (BTF08) formed a synergistic association with host calli, as presence of BTF08 positively influenced calli growth. A significant increase in fresh weight of calli was detected when co-inoculated with BTF08 (1012 mg fresh weight). On the contrary, association of calli with T2 (*T. asperellum*) and MIF01 (*D. phaseolorum*) appeared to have implicated the growth of oil palm calli, detected by the decrease in fresh weight of calli with 1001 and 1000 mg, respectively, compared to 1007 mg in control (Fig. 3). For isolate BTF07 (*A. calidoustous*), although the growth of calli was not inhibited (1007 mg, similar to control), necrosis of calli was detected when endophyte and calli were in close proximity (1 mm apart). The association of calli with *G. boninense* revealed GB has no detrimental impact on calli, with 1008 mg non-necrotic calli weight recorded.

3.4. Endophyte/pathogen colonization study in ramets (in planta)

Colonization occurred progressively from roots, spreading gradually to other plant tissues (root, stem and leaf). This is because the root tissues are the site of inoculation, thus was naturally the first plant tissue to harbor endophytes/pathogen. The rate of colonization differs for all endophytes and pathogen. Endophytes (BTF07, BTF08, T2, WAA02, MIF01) colonized faster when compared to the pathogen (*G. boninense*). This is based on endophyte/pathogen re-isolation from various plant parts at 2, 6, 12, 24 and 36 h after inoculation, whereby the presence of endophytes on that particular incubation period on specific plant part illustrated the colonization efficacy and preference of the fungal isolate. Comparison among endophytic isolates revealed that T2 demonstrated the most rapid colonization activity through successful colonization of root and stem tissues within 2 h after inoculation (Table 1). The remaining isolates (BTF08, MIF01 and WAA02) required at least 6 to 12 h for successful colonization in the host (Table 1). Endophyte *P. citrinum* BTF08 and pathogen GB presumably has the poorest colonization rate, with both isolates requiring at least 36 h for successful colonization in roots (Table 1). Endophytic *A. calidoustous* (BTF07) and pathogen (GB) were found only in the root tissues, suggesting they are compatible with the host but have specific colonization preference. The other endophytes (BTF08, MIF01, WAA02 and T2) do not indicate any colonization preference and can be re-isolated from the whole plant (root, stem and leaf) (Table 1). Our findings also revealed that our selected endophyte isolates could colonize the host (oil palm ramets) asymptomatically.

4. Discussion

4.1. Endophyte antagonistic activities

This study discovered that all endophytes tested here have antagonistic effects towards the pathogenic *G. boninense*, as revealed in the dual culture assay. Some endophytes (MIF01, WAA02 and T2) showed better antagonistic properties compared

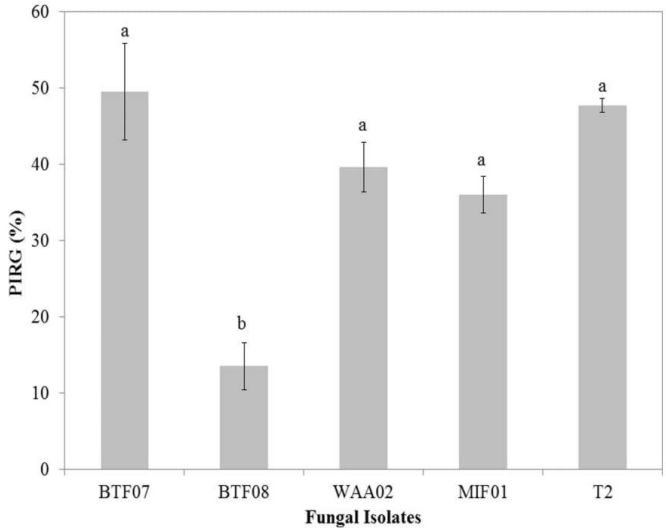

Fig. 1. Mean percentage inhibition of radial growth (PIRG, %) of five isolates. Means with the same letters between isolates are not significantly different at $HSD_{(0.05)}$. Bars indicate standard error of means (±SE).

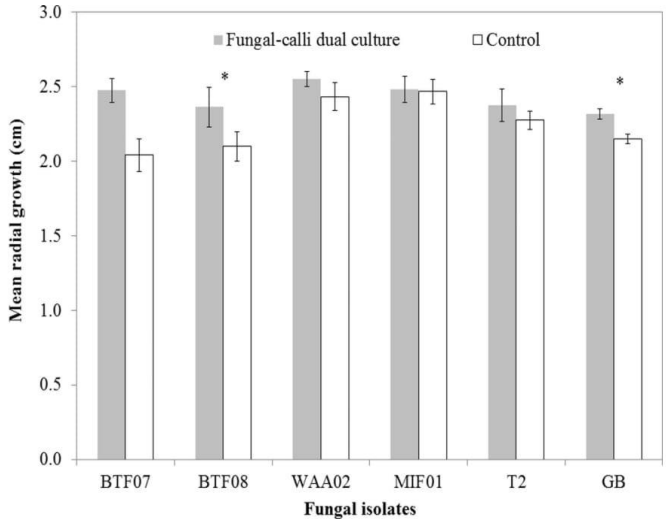

Fig. 2. Mean radial growth of fungal mycelium in calli-fungal dual culture. "∗" indicates significant differences based on T-test between treatment and control ($p < 0.05$). Bars indicate standard error of means (±SE).

to others (BTF08) in inhibiting the growth of *G. boninense*. This is presumably due to the mycoparasitic trait of endophytes (for isolate T2), and their ability to execute competitive exclusion (for isolate WAA02, MIF01 and T2) and production of volatile inhibitory compounds (for isolate BTF08 and T2). Isolate T2 demonstrated competitive exclusion via overgrowth and mycoparasitism, typical of the genus *Trichoderma* (Lo, 1998; Shafawati and Siddiquee, 2013; Ting and Jioe, 2016). *Trichoderma* spp. are known to produce cell-wall degrading enzymes such as β-1,3-glucanase, N-acetylglucominidases, chitinase, which are responsible for the degradation of mycelium (Qualhato et al., 2013; Ting and Chai, 2015). *A. calidoustus* (BTF07) inhibited the growth of *G. boninense* by competitive exclusion against *G. boninense*. This could be attributed to antifungal compounds produced as well, as a recent study by Rodrigues de Carvalho et al. (2016), reported that *A. calidoustus* secretes yellow soluble pigments into agar, consisting of antifungal compounds ophiobolin K and 6-epi-ophiobolin. Conversely, the inhibited growth of *G. boninense* by isolate BTF08 appeared slightly inferior when compared to other endophytes (MIF01, WAA02 and T2), despite the formation of a clear inhibition zone between BTF08 and *G. boninense*. The antifungal compounds produced by BTF08, a *P. citrinum* species, are presumably citrinin, dihydrocompactin and the antifungal protein PcPAF (Lam et al., 1981; Wakana et al., 2006; Wen et al., 2014). Both *D. phaseolorum* isolates (WAA02, MIF01) demonstrated similar antifungal activities towards GB despite originating from two different host plants; MIF01 from roots of

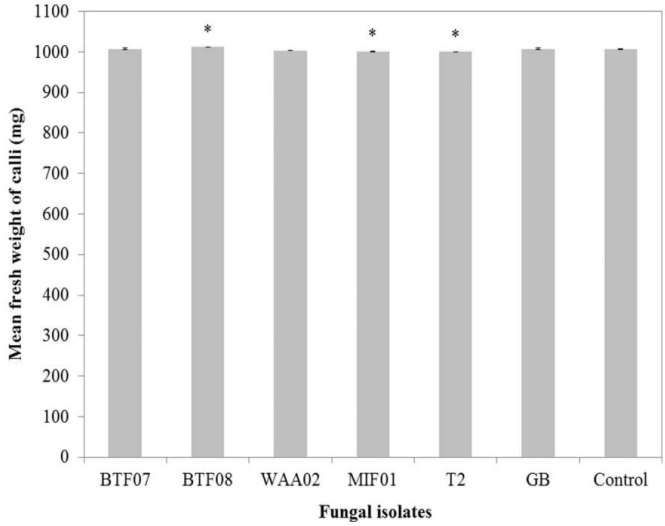

Fig. 3. Fresh weight of plant calli after incubation on MS medium in calli-fungal dual culture. "*" indicates significant differences based on T-test between treatment and control ($p < 0.05$). Bars indicate standard error of means (±SE).

Table 1
Recovery of endophytes (BTF07, BTF08, MIF01, WAA02, T2) and pathogen (GB) from various plant tissues at different incubation intervals.

Isolates	Plant tissues	Incubation period (h)				
		2	6	12	24	36
GB	Leaf	–	–	–	–	–
	Stem	–	–	–	–	–
	Root	–	–	–	–	–
BTF07	Leaf	–	–	–	–	–
	Stem	–	–	–	–	–
	Root	–	–	–	–	+
BTF08	Leaf	–	+	+	+	+
	Stem	+	–	+	+	+
	Root	–	+	+	+	+
MIF01	Leaf	–	–	–	–	–
	Stem	–	+	–	+	+
	Root	–	+	+	+	+
WAA02	Leaf	–	–	–	–	+
	Stem	–	–	–	+	+
	Root	–	–	+	+	+
T2	Leaf	–	–	–	+	+
	Stem	+	+	+	+	+
	Root	+	+	+	+	+

"+" indicates the presence of fungal colonization; "–" indicates the absence of fungal colonization.

Mimosa pudica (Ting et al., 2010) and WAA02 from roots of *Portulaca* sp. (Ting et al., 2009b, 2010; Ting and Jioe, 2016). Antifungal compounds from *D. phaseolorum* however, were most likely fungistatic as *G. boninense* co-inoculated with *D. phaseolorum* retained its viability and was able to grow when transferred onto fresh PDA (Ting and Jioe, 2016).

4.2. Fungal growth in calli dual culture test

The findings from this study suggested that growth of endophytes is presumably stimulated by the presence of compounds produced by the host calli. Compatibility with calli suggests compatibility with cambium tissues, which are common entry points and colonization sites for endophytes. Positive endophyte (BTF08) growth in the direction of calli was detected and statistical evidence (T-test) indicated significant effect of calli promoting the growth of these fungal isolates. The positive influence of calli on endophyte/pathogen growth suggested that calli may produce metabolites (diffused into the agar), which could promote growth of specific fungi (BTF08 and GB). Plant cells, in the form of cell cultures, are known to produce a wide variety of compounds such as alkaloids, terpenoids, steroids, saponins, phenolics, flavonoids and amino acids, which may be responsible to stimulate growth of endophytes (Vanisree et al., 2004). Other similar observations include growth stimulation of *Cryptodiaporthe hystrix* with the presence of its host calli, *Acer macrophyllum* (Sieber et al. (1990); and the stimulated growth of the grass endophytes *Atkinsonella hypoxylon* and *A. texensis* with callus of its host (Lu and Clay, 1994). Both authors (Lu and Clay, 1994; Sieber et al., 1990) agreed that growth of endophytes with host calli correlates to host-compatibility. In our study, the fungal growth-promoting phenomena were observed only in BTF08 and GB, compared to other endophytes (BTF07, T2, MIF01, WAA02). Dual-culture performed with GB further provided insights into the pathogen-host association,

in comparison to endophyte-host association. Our results are suggestive that BTF08 and GB are compatible to the host plant, with host plant possibly producing substances that induce growth of BTF08 and GB. On the contrary, BTF07, T2, MIF01, and WAA02 has no growth-promoting or adverse effect from interactions with the host plant.

4.3. Influence of endophytes on calli growth

The increase of calli growth from the effect of growth-promoting metabolites originating from the endophyte *P. citrinum* (BTF08) suggested active callogenesis activity. These metabolites may include substances such as hormones being secreted by endophyte into the agar. Although this study did not characterize the substances involved, a similar study conducted by Khan et al. (2008) showed production of plant growth-promoting hormone (gibberellins) by *P. citrinum* towards growth of waito-c rice and *Atriplex gemelinii* seedlings. On the contrary, endophytes MIF01 and T2 may produce substances that slowed the growth of oil palm calli (passive callogenesis). Other studies have suggested that endophytes secrete metabolites into the media during dual culture, and these metabolites were found to be toxic to plant calli, irrespective of its host material (host or non-host) (Peters et al., 1998; Sieber et al., 1990; Sirrenberg et al., 1995). In our present study, oil palm calli exposed to isolates (BTF08, WAA02, MIF01, T2 and GB) showed no signs of cell necrosis. This would indicate that these isolates probably did not produce any harmful compounds that implicates calli, or that the calli may have adaptations to the substances produced by the isolates. On the contrary, isolate BTF07 caused necrosis in oil palm calli. Although no further test was carried out to examine this toxic compound, *A. calidoustous* has been reported to cause harm in adult plants where phosphorus deficiency is initiated in plants, affecting growth of roots and the absorption of soil nutrients (Duan et al., 2013). We postulate the necrotic effect on oil palm calli is due to nutrient deficiency. However, this does not rule out several other factors that may be responsible for the necrotic effect, which include growth rejection of calli and secretion of toxic metabolites by BTF07. This study reveals that fungal-calli dual culture can be used as a preliminary screening for the selection of potential biocontrol compatible non-native fungal strain to oil palm.

4.4. Endophyte colonization study

A separate test detecting colonization activities of endophytes and GB inoculated to tissue-cultured ramets, further validates the endophyte-host compatibility test results derived from the calli model. In this present work, we found that each fungal isolate (endophytic, pathogenic) have unique colonization behavior and possibly tissue (root, stem, leaf) preference as well. Colonization of endophytic fungi is non-symptomatic and this is observed in our study consistently for all endophytic isolates. Tissue-cultured ramets were used to ensure the endophytes or pathogen was the only isolates to colonize the host plant (oil palm ramets) as ramets were propagated in sterile-condition. Results from this study suggested that fungal colonization in oil palm ramets was influenced by the colonization rate and preferred plant parts. These observations explain that the endophytes are sporadically host-specific in their endophytic behavior, as the selected endophytes were isolated from several different hosts. Similar results were seen in other endophytes such as *Piriformospora indica* (Rai et al., 2001). In this study, endophytes that showed higher colonization rates were isolates that have been reportedly found in oil palm in other studies such as *Trichoderma* (Mejía et al., 2008). This presumably explains the higher colonization rate in oil palm ramets as observed for *T. asperellum* (T2). Mejía et al. (2008) also discovered that good colonizers usually showed less antifungal activity than the slower growing isolate. Indeed, our finding on *P. citrinum* (BTF08) concurred with Mejía et al. (2008), where slow-growing BTF08 showed good antifungal activity but inferior colonization rate compared to other fast-growing endophytes. Nevertheless, *T. asperellum* (T2) was an exception, as this fast-growing endophyte was a good colonizer, with relatively good antagonistic activity against GB. We could also conclude that both isolates of WAA02 and MIF01 spreads throughout the oil palm after primary introduction of fungal isolate via the root system.

Colonization preference was clearly exhibited by endophyte BTF07 and pathogen GB. Both only colonized root tissues, which is the primary site of inoculation. Our inability in re-isolating these fungal strains from other plant tissues (stem and leaf) may relate to the fungus' niche colonization tissue preferences, which are the roots. Endophyte BTF07 and pathogen GB occurred exclusively in below-ground tissue (roots), which can be categorized as a highly localized infection. Growth responses of endophytes were different in both ramets and calli, although inoculated within the same hosts at different growth stages. The difference in response between the two can be explained by reduced capacity of resistance in younger growth stage, which in this case, the calli stage. Conversely, endophytic fungi inoculated at ramet stage could have the advantage of developing their resistance towards toxic metabolites produced by the endophytic fungi.

5. Conclusions

Our present study revealed host compatibility can be deduced using calli and ramets inoculation.By combining calli and ramet study, we were able to measure the various response of oil palm to different endophytic fungi/pathogen in different growth stages of the plant. This will be useful in future studies of biological control of pathogens using host-compatible endophytic fungi where our findings will serve as a basis in exploring the interaction of other antagonistic endophytic fungi to develop strategies in biocontrol against plant pathogens. From our observations, T2 and BTF08 showed promising biocontrol properties (*in vitro*) to inhibit *G. boninense* and have the potential for further studies in application.

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Chapter 3

Potential of endophytic fungi in inducing lignification in oil palm ramets, a defense mechanisms towards pathogenic *Ganoderma boninense*

Overview

Apart from the exceptional compatibility displayed with host plants (Chapter 2), the capability of endophytic biocontrol agent in inducing lignification among host plants would appear beneficial as it reflects a disease resistance mechanism in plants. With that, the next level of this research is exploration of lignin-induction capability among endophytic fungal isolates (BTF07, BTF08, MIF01, WAA02, and T2) in oil palm ramets, and later, a comparison of these with the pathogenic *Ganoderma boninense*. Initially, an inoculation process was carried out for seven weeks on fungal isolates via soil drenching method. Upon inoculation of seven weeks, the content of lignin was quantified using acetyl bromide assay. The lignified plant cells were stained with phloroglucinol-HCl to validate lignin accumulation. In addition, the Scanning Electron Microscope (SEM) was employed to observe any cellular changes that may occur. The findings revealed higher lignin production with endophytic fungi infection, whereas pathogenic GB induced lignin content in an insignificant manner among the infected ramets. This signified that oil palm ramets had the ability to elicit some defence response via cellular lignification upon infection caused by endophytic fungi (BTF07, BTF08, MIF01, WAA02, and T2) indicating a less susceptible attribute among ramets towards endophytic infection. Nevertheless, ramets infected with GB failed to exert defence response via lignification, thus highlighting the susceptibility of oil palm towards pathogenic GB infection. Furthermore, *G. boninense* has been commonly known as white-rot basidiomycete that attacks the lignin part of the host, which leads to cell exposure, thus escalating the plant's susceptibility towards the process of decomposition generated by other fungal strains. In short, the potential of inducing lignin production was displayed by the endophytic fungi, which functioned as a defence mechanism against the lignin-degrading *G. boninense*.

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Potential of endophytic fungi in inducing lignification in oil palm ramets as a defense mechanism towards pathogenic *Ganoderma boninense*

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Abstract

This study investigated the response of lignin accumulation upon infection induced by endophytic and pathogenic fungi oil palm ramets. This research incorporated the *G. boninense* pathogen to compare the 5 endophytic fungi (BTF07, BTF08, MIF01, WAA02, and T2) in order to identify the best biocontrol agents by quantifying the lignin produced upon endophytic fungi infection, with the aim to control the disease spread by *G. boninense* pathogen in oil palm. Lignification of oil palm ramets were induced by inoculating endophytic fungi via soil drenching. Lignification was then assessed using acetyl bromide assay for quantification of lignin content. Lignin accumulation was stained with phloroglucinol-HCl. In addition, cellular changes were observed using Scanning Electron Microscope (SEM). Results revealed that infection by endophytic fungi induced higher lignin production in ramets (ranging from 332.71 mg g^{-1} cell wall to 379.47 mg g^{-1} cell wall), but pathogenic GB did not induce higher lignin content in infected ramets (276.22 mg g^{-1} cell wall). Histochemical assay confirmed lignin deposition in oil palm tissues with the detection of alteration of plant cell with the observation of cell wall apposition. SEM observation showed cell wall modification and wall apposition upon endophytic (and pathogenic) infection, suggesting oil palm ramets had the ability to elicit some defence response via cellular lignification upon infection caused by endophytic fungi (BTF07, BTF08, MIF01, WAA02, and T2). This suggested the potential of inducing lignin production by the endophytic fungi, which may functioned as a defence mechanism against the lignin-degrading *G. boninense*.

Keywords: Endophyte; G.boninense; Induce response; lignin; pathogen

3.1 Introduction

In nature, plants comprise of three key polymeric constituents, which are cellulose, hemicellulose, and lignin (Higuchi, 1997). Lignin, which is the main building block of the plant's secondary cell wall, is a heterogeneous three-dimensional phenolic polymer generated from oxidative polymerization of monolignols (Boerjan et al., 2003; Vanholme et al., 2010).

In fact, the process of lignification becomes a mechanical resistance in plants against degradation caused by many pathogens due to its chemical intricacy that is linked with formation of peroxidase-mediated cross-links with carbohydrates (hemicellulose, pectin, and callose) (Minor, 1991; Guillén et al., 2005). Besides, Lange et al., (1995) pointed out that lignin induced by microbial elicitor (*Rhizosphaera kalkhoffii*) in spruce exhibited lignin defence, but not lignin for plant development. In fact, the plant species is composed of varying compositions of monomeric lignin during its development phase, namely, phenylpropanoids *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S). For instance, the lignin component of wood gymnosperm consists of 98-99% of guaiacyl that originates from coniferyl alcohol. The angiosperm lignin is made up of guaiacyl and syringyl units, in which syringyl derives from coniferyl alcohol (Lange et al., 1995). Developmental pathways regulate developmental lignin where lignin monomers are polymerized to form lignin in the plant system in normal development (Whetten and Sederoff, 1995). Lignin functions as the defence system when triggered by defence responses, in which the CCoA-OMT enzyme seeps into methylation of both caffeoyl-COA and 5-hydroxyferuloyl-COA during the process of monolignol biosynthesis (Whetten & Sederoff, 1995). Lignin progression only occurs when the growth adheres to normal conditions. Nonetheless, defence lignin is only present when a certain plant is undergoing some environmental strain or phyto-pathological infection, where

the defence lignin is developed biosynthetically using simpler subunits (Kärkönen & Koutaniemi, 2010).

Plant defense system is activated by fungal infection (pathogenic and non-pathogenic) (Redman et al.; 1999, Gao et al., 2010; Harman et al., 2004). This defense system triggers pathogen-related (PR) gene expression and lignification (Greenberg and Yao, 2004). Defensive lignification can be mounted through activation of plant peroxidases and laccases, through oxidative coupling of three p-hydroxycinnamyl alcohols (coniferyl, p-coumryl and sinapyl alcohols) (Espiñeira et al., 2011, Boudet et al., 1995, Barceló, 1997). However, wood decaying fungi especially white-rot fungi such as *Ganoderma boninense* could effectively depolymerized and mineralized lignin, hence dampening plant defense response (Guillén et al., 2005).

Since past these years, the endophytic fungi have been discovered to display a significant potential that retards the process of degrading lignin in host plants due to infection by pathogens (Paterson et al., 2002). As for colonizing patterns, the endophytic fungi tend to colonize both the intercellular and intracellular parts of the host without any vivid symptoms (Rodriguez et al., 2009). Existing literature also depicts that endophytic fungi in host plants initiated defence response through deposition of lignin at an escalated level in gaining protection from fungal pathogenic infection (Redman et al., 1999; Gao et al., 2010; Harman et al., 2004). With that, this study investigated the capability of endophytes to induce lignification in oil palm ramets, which functions as a mechanism of defence response. Pathogen *G. boninense* had been incorporated in this study so as to compare the process of lignification stimulated upon infection by pathogen.

This study hypothesized that excessive lignin alteration in oil palm may be beneficial, especially in promoting a delay for the process of lignin degradation or infection caused by *G. boninense* pathogen. Although the impact of infection caused by *G. boninense* upon lignification

in oil palm has been widely reported and documented, studies pertaining to oil palm response towards asymptomatic endophytic fungi are rather scarce. As such, this study investigated the response of lignin accumulation upon infection induced by endophytic and pathogenic fungi oil palm ramets. This research incorporated the *G. boninense* pathogen to compare the four selected species of endophytic fungi in order to identify the best biocontrol agent(s) by quantifying the lignin produced upon endophytic fungi infection, with the aim to control the disease spread by *G. boninense* pathogen in oil palm.

3.2 Materials and methods

3.2.1 Culture establishment

The selected fungal endophytes for this study had been isolates BTF07 and BTF08 (Ting et al., 2012, 2009; Ting & Jioe, 2016), WAA02 (Ting et al., 2009; 2010), and MIF01 (Ting et al., 2010) and were deposited in Monash Malaysia Microbiology Laboratory culture collection. These fungal isolates were *Aspergillus calidoustous* BTF07 (GenBank accession no. KT329189), *Penicillium citrinum* BTF08 (GenBank accession no. KT964566), as well as two isolates of *Diaporthe phaseolorum* MIF01 (GenBank accession no. KT964565) and WAA02 (GenBank accession no. KT964567). *G. boninense* (GB) pathogen was collected from Universiti Putra Malaysia. All the fungal cultures (both endophytes and pathogen) were cultivated on Potato Dextrose Agar (PDA) (Merck) at room temperature (25 ± 2°C). Mycelial plug from PDA had been inoculated into Potato Dextrose Broth (PDB) with an incubation period of 14 days at room temperature (25 ± 2°C). Mycelial biomass was harvested and homogenized with sterilized distilled water and adjusted (100 ml, 10^6 cfu/ml) inoculum.

3.2.2 Inoculation of oil palm ramets

Some four-week old tissue-cultured ramets of oil palm (supplied by Applied Agricultural Resources Pte. Ltd) had been tested in this research, whereby they were subjected to treatment with the selected endophytes (T2, BTF07, BTF08, MIF01, and WAA02), as well as pathogen (*G. boninense*). First, the ramets of the oil palm had been washed with sterile distilled water before they were transplanted into a pot. Next, the fungal inoculum (100 ml, 10^6 cfu/ ml) was inoculated into the pot via soil drenching technique. Sterile distilled water (100 ml) was used instead of inoculum for the control treatment. The incubation process performed on the oil palm ramets was continued for a total of seven weeks.

3.2.3 Determination of lignin content in oil palm ramets

Some plant tissues (roots, stems, and leaves) weighing 1 g had been collected and grounded in 40 ml of 95% ethanol. Next, the homogenate was centrifuged at 1000 x *g* for 5 min. After that, the pellets were washed thrice with 40 ml 95% ethanol followed by two washes of 40 ml ethanol and hexane mixture (ratio of 1:2 of ethanol:hexane). This mixture was then incubated at 70℃ for 30 min in 1 ml of 25% acetyl bromide in acetic acid. Once the mixtures were cooled down to room temperature (25 ± 2°C), reaction substrate (0.9 ml of 2 M NaOH, 0.1 ml of 7.5 M hydroxylamine hydrochloride) was added into the mixture. The total volume was topped up to 10 ml with acetic acid. The mixtures were centrifuged at 1000 x *g* for 5 minutes before its absorbance were measured at 280 nm (Fukuda and Komamine, 1982; Sasaki et al., 1996). A standard curve was generated with alkali lignin (Sigma-Aldrich) and results were expressed as mg g^{-1} cell wall.

For histochemical assay, the staining was performed using phloroglucinol-HCl to determine the presence of lignin in both the oil palm ramets inoculated with fungal isolates and control (without fungal infection). Leaf, stem and root section were stained with 0.5 ml 1% (w/v)

phloroglucinol in 70% ethanol. After staining, plant sections were observed under light microscope at 100x magnification. A burgundy-red stain indicated the presence of lignin in cell wall while plant sections with no lignin content remain colorless. The stain was effective and useful for observation of lignin deposition within the plant tissue This is because; the stain only reacts with cinnamaldehyde found in lignin, thus resulting in cationic chromophore that appears as burgundy-red on tissues with lignin detected (Beardmore et al., 1983; Soukup, 2014).

3.2.5 Scanning electron microscopy (SEM) of oil palm ramets

Several plant tissues (leaves, stems, and roots) were tested for SEM observation. These plant tissues; both inoculated and control ones, were dissected (1 cm) with a sterile blade. Next, all the samples were immersed in 5 ml of 3 % gluteraldehyde in 0.1 M phosphate buffer at pH 7.4 overnight. The tissue samples were washed thrice with 5 ml of phosphate buffer for 30 min and were dehydrated in a series of 5 ml ethanol dehydration washes beginning from 20%, 40%, 60%, 80%, 90%, 95%, and eventually, 100% ethanol. Later, the tissues were immersed in each concentration for 20 min and dried in a desiccator. Sample was placed on a metal stub and coated with gold using a sputter coater. Lastly, the specimens were observed by using the variable-pressure SEM (VP-SEM Hitachi S3400N-II)**.**

3.2.6 Statistical analysis

All the experiments were performed in triplicates, in which one ramet constituted a replicate. Besides, the mean data retrieved were analysed by using the Statistical Package for Social Sciences (SPSS) version 20.0. Furthermore, the mean values were compared by using the Tukey-Kramer multiple comparison test (Honestly Significant Difference, HSD, $P<0.05$).

3.3 Results

3.3.1 Lignin content in oil palm ramets

The ramets that were inoculated with endophyte BTF07 displayed the highest lignin production (379.47 mg g^{-1} cell wall), which had been significantly higher than that of pathogenic GB. Furthermore, the findings revealed that other endophytic isolates (BTF08, MIF01, WAA02, and T2) also did induce lignin generation in oil palm ramets, with lignin contents of 348.06 mg g^{-1} cell wall, 332.71 mg g^{-1} cell wall, 347.47 mg g^{-1} cell wall, and 358.22 mg g^{-1} cell wall, respectively (Figure 1). In addition, it was noted that the content of lignin in the tested ramets did not differ significantly for endophytic treatments. In fact, all the endophytic infections induced significantly higher lignin production in oil palm ramets, in comparison to pathogenic GB infection (Figure 1). The GB pathogen failed in adjusting the production of lignin in ramets, which displayed closer result of the control, whereby the concentration of lignin was 280.99 mg g^{-1} cell wall in GB treated ramets and 276.22 mg g^{-1} cell wall in control ramets (Figure 1). The findings further exhibited that oil palm ramets possessed the ability to initiate a defence response via cellular lignification towards endophytic fungi (BTF07, BTF08, MIF01, WAA02, and T2) infections, indicating that the ramets were indeed less susceptible to endophytic infection. Other than that, those ramets infected with GB failed to exemplify any hint of significant lignification in the tested oil palm ramets, pointing out the susceptibility of oil palm tissues towards pathogenic infection, particularly GB.

3.3.2 Histochemical observation of lignin deposition

Some amount of lignin deposition had been observed in tissues of fungal inoculated ramets (endophytes and pathogen) at the epidermal cells around penetration area. Furthermore, deposition of lignin was also found in the oil palm ramets of stem and roots, as indicated by the

concentrated burgundy-red tint (Figures 2B and 2C) observed at the epidermal layer at the tip of the roots (Figure 2B), as well as endodermis and pericycle tissues of the ramets that were infected by fungi (Figure 2C). In a similar manner, deposition of lignin was noticed at the vascular bundle (xylem and phloem) of the stem (Figure 2C) and leaves (Figure 2D). Lignin deposition was absent for all plant parts (leaf, stem, and root), particularly in the ground tissue, which includes all tissues that are neither dermal nor vascular. Ground tissue can be divided into three types based on the nature of the cell walls (parenchyma cells, collenchyma cells and sclerenchyma cells). This shows that the defence mechanism (lignification) was only generated in parts of plants that was strongly vital for survival (xylem and phloem), which possess the function to transport both water and nutrients throughout the plant system. It was discovered that both xylem and phloem had the highest nutrient concentration, in comparison to ground tissues, thus being more susceptible to infections brought by pathogens. In precise, the deposition of lignin revealed that infection caused by endophytic fungi generated lignification in oil palm, which has a protective role against infections caused by microbial.

3.3.3 Scanning electron microscopy (SEM) for oil palm ramets

The findings generated from SEM illustrated cellular alterations in the ramets of oil palm. Besides, all parts of the plant displayed some modifications in the cellular morphology, especially after fungal inoculation (BTF07, BTF08, MIF01, WAA02, T2, and GB) was performed. In fact, a hypha structure had been noted at the surface of the root in the palm ramets (Figure 3), further proving the successful fungal colonization. Besides, colonization of fungal was also observed at the inner cortex of the root primarily because the root tissue exhibited wall apposition on both endodermis and pericycle (Figure 3), whereas no changes had been notated in the control (Figure 3). The fungal hypha colonised the inner cortex of the root ramets (Figure 3).

SEM results of the control ramets failed to hint any altered cellular morphology in the root tissue, dictating the absence of fungal colonization and wall apposition (Figure 3). A rather dense hypha structure was noted for ground tissue of ramet stem, along with wall apposition (Figure 3); whereas the control ramets indicated no hypha colonization or any cell wall alteration (Figure 3). Thehypha structure was also noticed in the tissue of leaf hypodermis and phloem (Figure 3). Wall apposition was observed on the phloem for the infected oil palm ramets (Figure 3). Meanwhile, the tissues of control ramet (root, stem, and leaf) exhibited absence of any type of protuberance, wall apposition, or fungal hypha (Figure 3). In short, the SEM imaging vividly illustrated the alterations in plant tissue upon fungal infection, in comparison to the control sample. This finding is especially useful when evaluating the extent of fungal infection in plants and the morphological modifications as a result of GB infection. In addition, the structural defence reaction, for instance, wall apposition and modifications, suggests development of lignin and other cell wall elements. Changes in cellular reflect induced resistance and signify that lignification does play a significant role in plant defence against infection caused by fungi.

3.4 Discussion

This particular research verified the pathogenicity of *G. boninense*, as well as the potential of endophytic fungi as biocontrol agents after performing a series of lignification examinations in oil palm ramets upon infection due to fungi. After being infected by *G. boninense*, changes that were significant had been noted in the lignin content of oil palm ramets. This notion strongly indicates the benefits of *G. boninense* in by-passing the defence mechanism in oil palm host. Several reports have published that *G. boninense* could produce lignin-degrading enzymes, for example, laccases, manganese peroxidase, and oxidases (Paterson et al., 2008) which may degrade the induced lignin produced during fungal inection, as well as other

compounds like veratryl alcohol (Valc) and lignin peroxidase, which portray the tendency to interfere with lignin polymerization (Goh et al., 2014). Results showed lignin content of *G. boninense* infected ramets were similar when compared to uninoculated control ramets. The fungus Ganoderma produces unspecific ligninolytic enzymes consisting of peroxidases and laccases that can catalyze the oxidative process of cell wall degradation in oil palm

In this present research, the tested endophytic fungi (BTF07, BTF08, MIF01, and WAA02) displayed significant lignin production in oil palm ramets, in comparison to that of the control. In fact, lignin offers mechanical resistance to its host and protects the cellulose from biological dilapidation (Guillén et al., 2005; Paterson et al., 2008). As such, this study postulates that increase in lignin production found in oil palm, especially after beneficial endophytic fungi (BTF07, BTF08, MIF01, WAA02, and T2) inoculation, may function as a barrier that protects and hampers *G. boninense* invasion into oil palm tissue. Research showed redirection of tryptophan in transgenic potato plant through expression of tryptophan decarboxylase cause the decrease of lignin in potato tubers, thus increases the susceptibility of the plant being infected by *Phytophthora infestans*, illustrating the importance of lignin in defence (Wu et al., 1997). Researcher develops a type of transgenic corn with increase lignin content which showed greater resistance to infection, hence reducing susceptibility to fungi (Saxena et al., 2001). Presently, some research has been carried out to alter the lignin structure via the modification of hemicelluloses (Bindschedler et al., 2007). However, edophytic fungi may also altered lignin content in oil palm with several added benefits towards the plant such as decrease the space to disallow penetration of *G. boninense* into oil palm tissue, but also escalate competitions of ecological niche and nutrition. Hence, infections induced and spread by pathogen can be successfully impeded (Gao et al., 2010).

This research also validated the ability displayed by endophytic fungi in inducing defence response in oil palm ramets through the mechanism of lignification. The function of endophytic fungi in becoming an effective stimulator of host defence via lignification (*A. calidoustous* BTF07, *P.citrinum* BTF08, as well as two isolates of *D. phaseolorum* MIF01 and WAA02) has yet to be established to date.

This study portrayed that deposition of lignin was highly concentrated in the epidermis and pericycle parts of the roots due to the inoculation of fungi that took place at the roots, in which the soil drenching technique was performed to inoculate the samples. Paterson et al., (2009) claimed that the process of lignification in plant cells that occurs due to infection reflects the defence response of plant to lessen and halt spread of pathogen. As such, this study demonstrated that the induced lignin deposition found in the pericycle area refers to the impact of induced-resistant that hindered the fungal isolates from further penetrating into the xylem tissue in roots, which can influence water uptake in the plant system if infected. Similarly, deposition of lignin was also observed in the vascular tissue of oil palm ramets. The lignin reinforced-vascular tissue may also fortify and function as waterproof to cell walls (Albersheim et al., 2010). Thus, based on the findings retrieved from this study, the endophyte-infected oil palm ramets had been proven to generate a defence response by reinforcing the cell wall integrity at vascular tissue via lignification that eventually blocks water source to fungi, which then hampers the colonization of pathogenic fungal strain in oil palm ramets. The response was indced by the inoculation of endophytc fungi (BTF07, BTF08, MIF01, WAA02, and T2), which may reinforced the plant tissue against further pathogenic *G. boninense* infection.

The findings depicted from SEM illustrated the occurrence of deposition matrix at both the epidermal layer and vascular bundle of the fungal-infected ramets (both endophytic and

pathogen) with the newly developed wall apposition. Furthermore, Lewis and Yamamoto (1990) asserted that these appositions had been comprised of lignin and callose. In addition, polymerisation of lignin took place simultaneously with the formation of peroxidase-mediated cross-links with hemicellulose, pectin, and callose (Minor, 1991; Wi et al., 2005). Interestingly, the findings retrieved from SEM exhibited that the plant induced defence response (wall apposition) even prior to fungal infection, regardless of either pathogenic or endophytic infection. Nonetheless, a different picture was found with lignin quantification, as escalation of lignin was noted in endophytic-infected ramets alone. The dense hypha structures had been notated in the cellular structure of leaf tissues, in which fungal concentration was found to be the highest. In fact, leaves tend to become a preference for endophytic fungi colonization due to the readily large surface area, thin wall, and rich in nutrients (Chareprasert et al., 2006; Huang et al., 2008). These attributes were not in line with oil palm ramets, in which the concentrated hypha structures had also been noted at the roots (Figure 3A).

Prior researches that investigated isolates BTF07, BTF08, MIF01, WAA02, and T2 revealed their capability in generating some volatile compounds so as to hamper the growth of fungal pathogens (Ting et al., 2010). As such, the endophytic isolates (BTF07, BTF08, MIF01, WAA02, and T2) exemplified antifungal characteristics when *in vitro* dual culture plate assay was carried out (Ting et al., 2009). In fact, endophyte BTF08 displayed the ability to generate inhibitory metabolites against pathogenic fungal strain (Ting et al., 2009), whereby this particular isolate had been proven to induce host resistance in monocots (Ting et al., 2012). Isolates BTF07, MIF01, and WAA02 had been documented as fast colonizer and inhibited growth of pathogen by spreading all over and colonizing the growing space (Ting et al., 2009). Other than that, isolate T2 refers to a type of mycoparasitic fungus (Ting & Jioe, 2016).

Therefore, these endophytic fungi indeed can function as an environmental cue for oil palm ramets, as investigated in this study, to produce lignin that induces defence response towards 'immunizing' the oil palm ramets against further spread of fungal infection. This study proposes that the biocontrol potential exhibited by oil palm compatible endophytic fungi from various host species may indeed be a solution to control *G. boninense*. These compatible endophytes could be applied to colonize plant tissues, besides stimulating protection to the plant in a multi-genic manner (antifungal metabolites, space competition, and induced lignification), whereby pathogen (*G. boninense*) would find it difficult to overcome such resistance, hence projecting towards the progression of long term biocontrol schemes.

3.5 Conclusion

This study had proven the primary function of lignification as a defence mechanism against fungal infection (endophyte and pathogen). *G. boninense* did not induce higher lignin content in oil palm ramets due to its lignolytic properties. The endophytic isolates (BTF07, BTF08, MIF01, WAA02, and T2) demonstrated stimulation of lignification in oil palm ramets. Hence, lignification, an induced defence response in oil palm ramets, could be beneficial for future studies towards developing effective biocontrol agents against *G. boninense*. With that, the use of endophytic fungi as a lignin-inducing agent can turn into a future treatment technique against lignin-degrading pathogen (*G. boninense*). Future studies can look into the compatibility between oil palm isolates and endophytic fungi from other varying host species, which may be responsible for massive lignin production in compromised oil palm, thus further harnessing this ability to turn into a biocontrol strategy against *G. boninense*.

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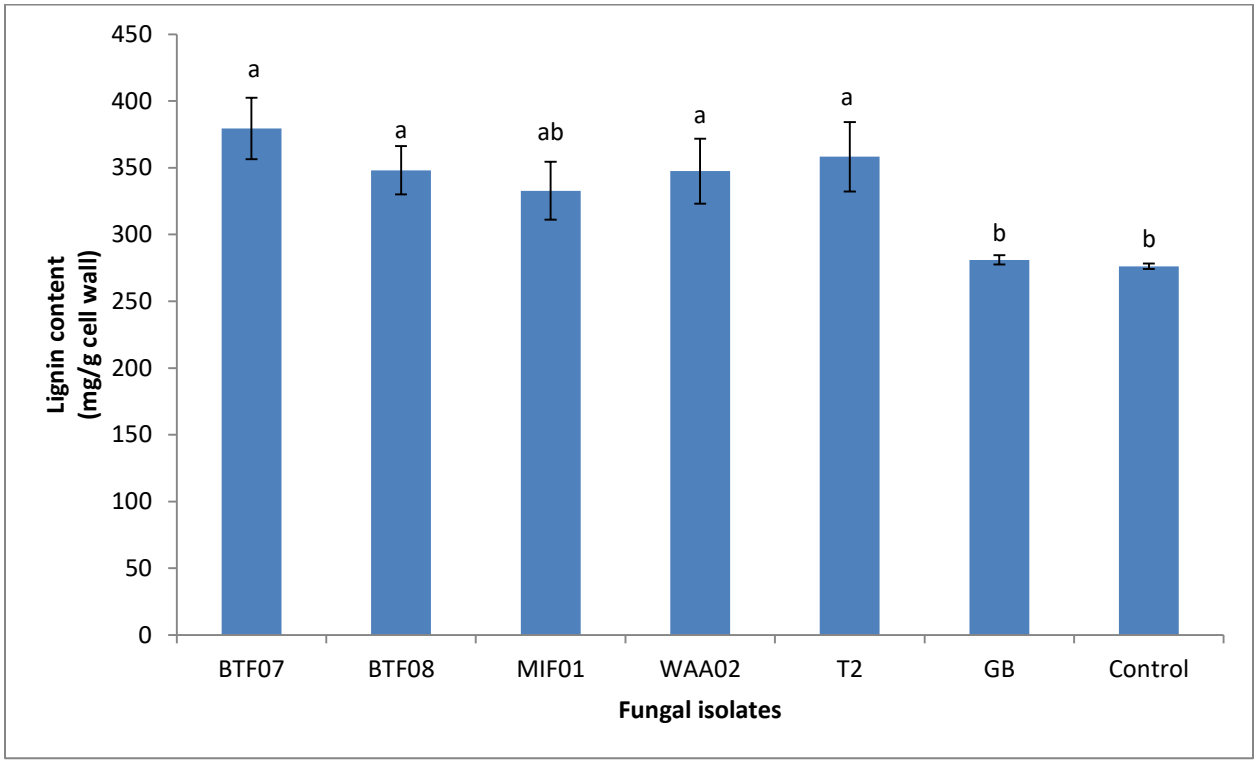
Lignin content (mg/g cell wall)
450
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350
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250
200
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BTF07
BTF08
MIF01
WAA02
T2
GB
Control
Fungal isolates

Figure 1.

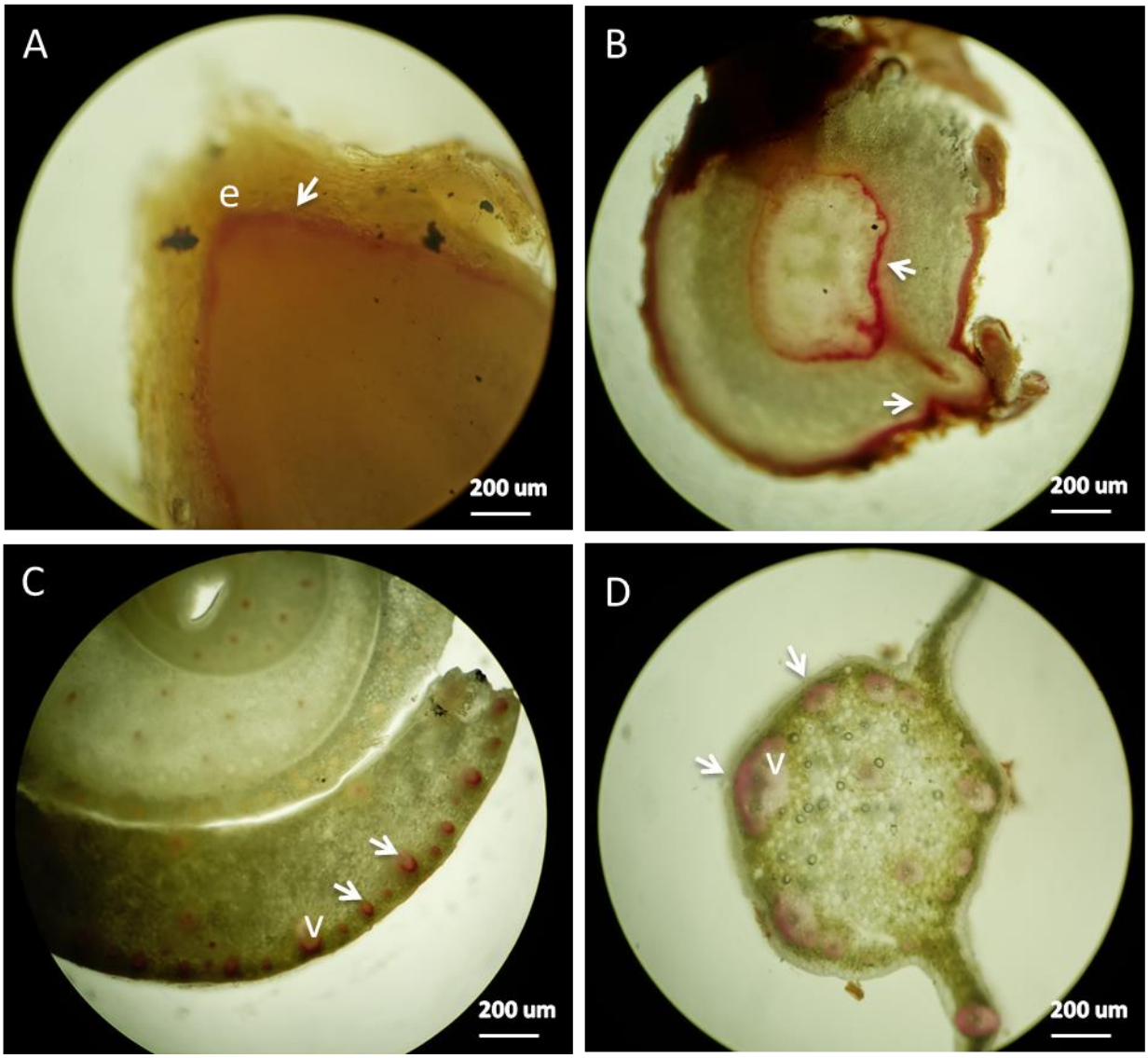

Figure 2.

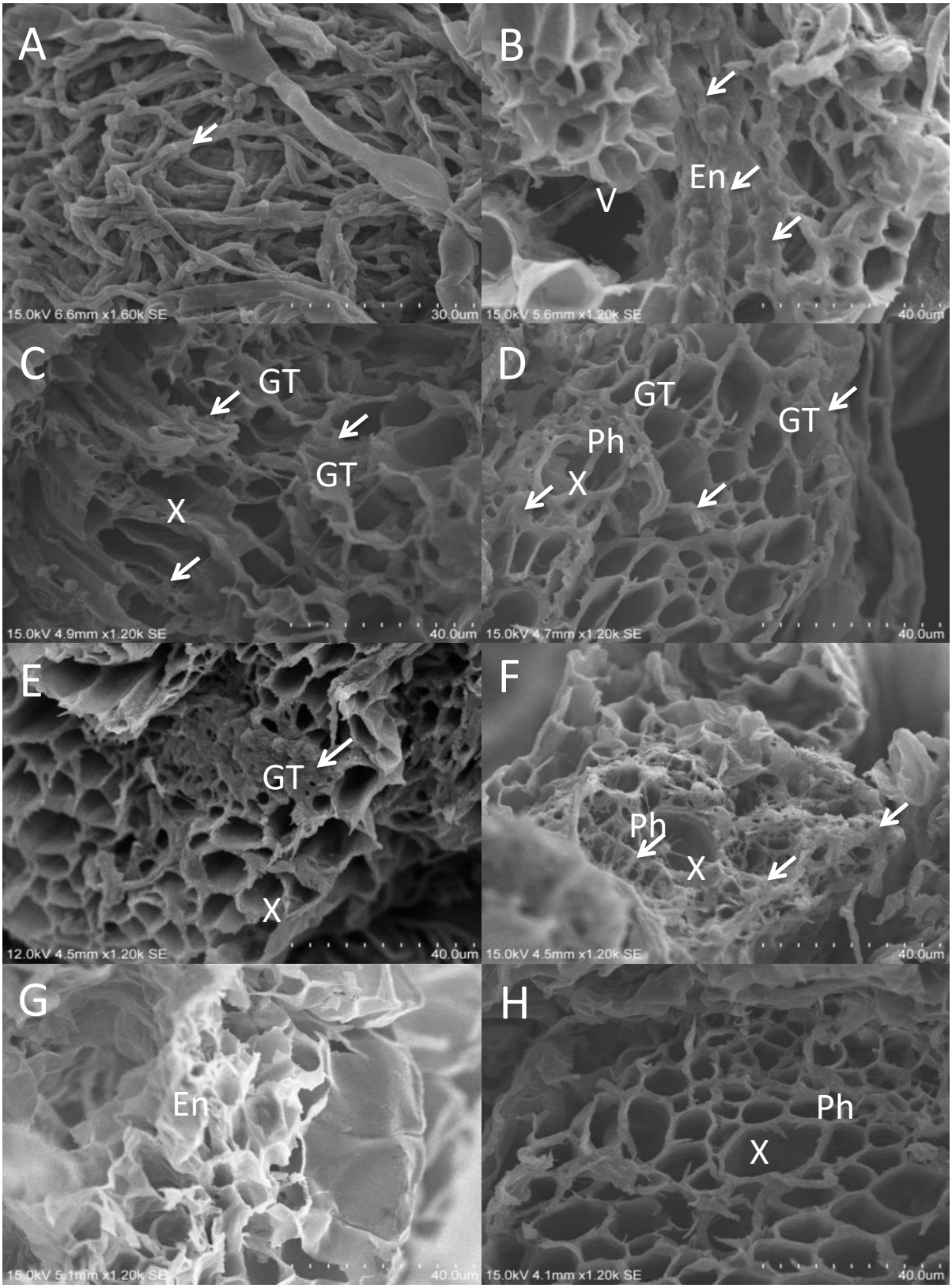
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Figure 3.

Figure captions

Figure 1: Mean lignin content in oil palm ramets. Means with the same letters between isolates are not significantly different at $HSD_{(0.05)}$. Bars indicate standard deviation of means (±SD).

Figure 2. Oil palm tissue stained with Phloroglucinol-HCl showing endophyte inoculated plantlet. Different parts of oil palm ramets showing (A). Root tip, (B). Cross section of primary root, (C). Stem and (D). Leaf. e: epidermis, v: Vascular bundle. Arrows indicating lignin deposition where relevant.

Figure 3: Scanning electron micrograph showing different parts of oil palm affected with fungus. Root surface of endophytic/pathogenic fungi infected ramets (A) while transverse root section endophytic/pathogenic fungi infected ramets can be seen with mycelial projections and wall apposition (B-F). Control sections of roots (G) and stem (H) is shown for comparison. X: xylem; Ph: Phloem; V: vascular cylinder; En: endodermis; GT: ground tissue. Arrows indicating wall apposition where relevant.

Chapter 4

Potential of endophytic fungi in producing volatile compounds with antagonistic activity towards *Ganoderma boninense*

Overview

The endophytic biocontrol agents, similar to other biocontrol agents, are able to produce compounds that are volatile in nature to inhibit pathogen growth. This particular chapter presents the profiles retrieved, as well as the antagonistic effect of volatile metabolites produced by endophytic fungi against *G. boninense*. As such, the solid-phase micro-extraction (SPME) method was applied to determine the volatile compounds generated in the double plate assay at the headspace between the fungal cultures. The Gas Chromatography-Mass spectroscopy (GC-MS) analysis was performed to profile the volatile compounds derived from the monoculture of endophytic fungi and the co-culture with *G. boninense*. Comparisons were made to determine the volatile compounds that were produced by endophytic isolates when co-cultured with the pathogen. The volatile compounds of five endophytic isolates (BTF07, BTF08, MIF01, WAA02, and T2) were examined to detect the antifungal metabolites. Results revealed that isolate T2 (*Trichoderma asperellum*) displayed the highest inhibition rate (68.3%) towards *G. boninense*, followed by WAA02 (56.7%), MIF01 (65.0%), BTF08 (29.1%), and BTF07 (6.7%). In fact, the analysis of volatile compounds revealed that the inhibitory action could be exhibited by single compounds, such as1,2-Benzenediol; 3,5-bis(1,1-dimethylethyl); 3-Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthenecarbox amide; Benzene; 1-methyl-4-(1-methylethyl); Benzothiophene-3-carboxylic acid,4,5,6,7-tetrahydro-2-amino-6-ethyl; ethyl ester, or their interactions in a synergistic manner. This finding strongly suggests the antifungal nature portrayed by these volatile compounds towards *G. boninense*. Therefore, apart from projecting exceptional host compatibility and the capability of inducing lignification, as depicted in Chapters 2 and 3, respectively, the endophytic isolates investigated in this study also produced volatile compounds with excellent antagonistic activities towards *G. boninense.* With that, this

study had proven that the tested endophytic fungi have the potential to function as biocontrol agents in oil palm.

This chapter reflects the manuscript drafted for the Journal of Biocontrol Science and Technology, as follows:

Potential of endophytic fungi in producing volatile compounds with antagonistic activity towards *Ganoderma boninense*

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Abstract
This study identified the antagonistic activities of volatile compounds produced by five fungal endophytes (*Aspergillus calidoustous* BTF07, *Penicillium citrinum* BTF08, *Trichoderma asperellum* T2, *Diaporthe phaseolorum* WAA02, *Diaporthe phaseolorum* MIF01) and profiled the compounds produced. Antagonctic activities were evaluated via double plate test. The volatiles produced by the endophytic fungi-pathogen culture were obtained using the solid-phase micro-extraction (SPME) technique which was then subjected to GC-MS analysis. Various types of volatile compounds were detected from five endophytic fungi isolated from different host species. All isolates produced volatile compounds which are capable of inhibiting growth of the pathogenic fungi *Ganoderma boninense* (GB). Compounds produced by isolate T2 (*Trichoderma asperellum*) showed highest inhibition rate at 68.3% inhibition towards GB. Volatile compounds profiles showed that inhibition action may be contributed by single compounds such as1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl), 3-Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthenecarbox amide, Benzene, 1-methyl-4-(1-methylethyl) and Benzothiophene-3-carboxylic acid,4,5,6,7-tetrahydro-2-amino-6-ethyl , ethyl ester, or their action synergistically. These observations strongly suggest the antifungal nature of these compounds towards GB.

Keyword: Antifungal; endophytic fungi; *G. boninense*; volatile metabolites

4.1 Introduction

The Basal Stem Rot (BSR) disease, which is caused by a white rot fungus called *Ganoderma boninense*, is deadly to *Elaeis guineensis* (oil palm) (Turner, 1981). The BSR disease has been reported to be the primary cause for the massive losses recorded in the Southeast Asia palm oil industry (Breton et al., 2006). The *G. boninense* pathogen infects the basal trunk via enzymatic degradation, hence impeding water and nutrient uptake to leaves and other plant parts, and finally, the inevitable death of oil palm trees (Hama-Ali et al., 2015). The spread of disease occurs via roots (Ariffin et al., 2000). The sources of infection could be found at other infected plant parts, such as palm, stump, or even fragmented tissues from other infected trees (Ariffin et al., 2000). Coventional control measures involved physical and chemical techniques. Trunk surgery is a common physical method, in which the affected tissues are surgically eliminated and mounded to avoid further spread of infection (Cheng Tuck & Hashim, 1997). On the other hand, the chemical approaches are inclusive of fungicides use, for example, triadimefon, carboxin, carbendazim, and methfuroxam (Jollands, 1983). Nevertheless, fungicides, at times, have little effect on mycelial growth (Jollands, 1983). Extensive application of chemicals also leads to resistance towards pathogens and severe pollution of the environment (Harish et al., 2008; De Curtis et al., 2010; Ma et al., 2015; Yang et al., 2015; Gao et al., 2017). Thus, other effective and environment-friendly methods are sought to overcome further spread of the BSR infection, especially in oil palms, as investigated in this study.

Biological control agents are important for biocontrol of BSR disease. Several studies have examined this and preliminary results have been documented (Susanto et al., 2005; Mohd Zainudin & Abdullah, 2008; Ting & Jioe, 2016). Endophytic fungi and their volatile antifungal

metabolites may have antifungal properties to control *G. boninense*. Fungi-generated volatile compounds have been identified as either intermediate and/or end results of some metabolic channels, which could be categorised as sesquiterpenes, alcohols, lactones, esters, ketones or C8 compounds (Schnürer et al., 1999; Korpi et al., 2009). These metabolites can mediate defences against parasites, predators, and diseases in an effective manner, besides increasing its production at competition among species (Sivasithamparam & Ghisalberti, 1998). Volatile metabolite derived from *Trichoderma* was reportedly able to inhibit the growth of plant pathogenic moulds and displayed growth-promoting aspects in treated plant (Vinale et al., 2008). Therefore, exploitation of these fungi-produced volatile metabolites is favourable due to their co-existence with the environment and their ability to decompose naturally (Gao et al., 2017).

As for this particular research, endophytic fungal species (*Aspergillus calidoustous* BTF07, *Penicillium citrinum* BTF08, *Trichoderma asperellum* T2, *Diaporthe phaseolorum* WAA02, and *Diaporthe phaseolorum* MIF01) were investigated to determine if their volatile metabolites possess antifungal properties to inhibit the growth of *G. boninense.* This study carried out the profiling of endophytes-derived volatile organic compounds using the SPME GC-MS analytical method.

4.2 Materials and methods

4.2.1 Culture establishment

The fungal endophytes investigated in this study were obtained from Monash Malaysia Microbiology Laboratory culture collection, *Aspergillus calidoustous* BTF07 (GenBank accession no. KT329189), *Penicillium citrinum* BTF08 (GenBank accession no. KT964566), *Trichoderma asperellum* T2 (GenBank accession no. KT964564) (Ting et al., 2009, 2012; Ting & Jioe, 2016), *Diaporthe phaseolorum* WAA02 (GenBank accession no. KT964567) (Ting et al.,

2009, 2010), and *Diaporthe phaseolorum* MIF01 (GenBank accession no. KT964565) (Ting et al., 2010).The pathogenic fungi, *G. boninense*, had been kindly contributed by Professor Dr Sariah Meon from University Putra Malaysia. Both endophytic and pathogenic fungi had been grown and retained on Potato Dextrose Agar (PDA) (Merck) at room temperature (25 ± 2°C).

4.2.2 Antifungal assay of volatile metabolites of endophytic fungi

The double plate assay was employed to identify antifungal activity by the volatile metabolites produced from various endophytic fungi (Gao et al., 2017). Two petri dishes containing 15 ml of PDA were prepared. Next, a 7-day-old mycelial plug of the endophyte (diameter of 5 mm) was placed at the centre of the PDA plate, while another 7-day-old *G. boninense* agar plug had been placed on a different PDA plate. The PDA plate with *G. boninense* culture was positioned inversely over PDA plate with endophyte culture with both coverlids removed. In addition, both the plates were sealed with parafilm and incubated at room temperature (25 ± 2°C) for a week. This experiment was conducted in triplicate. The percentage of inhibition was recorded by measuring the diameter growth (PIDG) recorded after a week, as depicted in the equation that follows:

PIDG (%) = $\frac{D1-D2}{D1}$ x 100%

Where D1 denotes the diameter growth of *G. boninense* co-inoculated with plain agar plug (control), while R2 refers to the growth diameter of *G. boninense* co-inoculated with fungal endophytes

4.2.3 SPME-GC-MS analysis

The volatiles that were generated by the endophytic fungi-pathogen culture had been gathered by the SPME method, which entrapped the volatile compounds produced at the headspace between

the fungal cultures found in the double plate assay (Strobel et al., 2001; Wan et al., 2008). To collect the volatiles, a small opening was punctured at the side of the Petri dish with a sterile needle. The SPME syringe (Supelco), which was equipped with fibre material (50/30 divinylbenzene/carburen on polydimethylsiloxane on a 65 μm stable flex fibre) (Supelco), was later inserted through the hole to gather the volatiles for a period of 40 min to ensure volatiles were saturated within the fibre material. Following that, the SPME fibre was placed into the Gas Chromatography-Mass Spectrometer (GCMS) (Shimadzu GCMS QP2010) for further analysis. The column temperature was set as: 40°C for initial temperature for 2 min, which was increased to 150°C at 2°C min^{-1}, then further increased to 280°C at 5°C min^{-1}, and finally, retained at 280°C for the next 2 min. In addition, helium was applied as carrier gas at 1 ml min^{-1} flow velocity. On top of that, other unknown compounds were analysed by employing the NIST (National Institute of Standards and Technology) database on the mass spectrometer. This GCMS analysis was repeated once.

4.2.4 Data analysis

All *in vitro* experiments were performed in triplicate. The gathered data were analysed by using the Statistical Package for the Social Sciences (SPSS) version 20.0. After that, the mean values were compared by employing the Tukey-Kramer multiple comparison test (Honestly Significant Difference, HSD, $P<0.05$).

4.3 Results

4.3.1 Inhibitory effect of endophytic fungi towards GB

The double plate test revealed that all the tested isolates were positive for inhibitory effect towards GB. Three of the isolates; T2, WAA02, and MIF01, appeared to exhibit strong inhibitory effect (PIDG %) at 68.3, 56.7, and 65.0%, respectively, which were higher compared

to the other two isolates (BTF08 and BTF07) with 29.1 and 6.7 % of PIDG %, respectively (Figure 1).

4.3.2 Characterization of volatile compounds produced by endophytic fungi

The profiling revealed that all the five endophytic fungi produced between 13 and 74 volatile compounds upon being cultured in a monoculture environment. Isolate WAA02 produced the lowest number of volatiles (13 compounds), whereas isolate MIF01 produced the highest number of volatiles (74 compounds). Interestingly, a total of 27 volatile compounds were derived from the pathogenic GB. This study also found that the amount of volatile compounds generated from endophytic fungi differed significantly after they had been co-cultured in a sealed condition with GB (dual plate assay). In particular, the quantity of volatile compounds escalated after both WAA02 and BTF07 isolates were cultured in dual plate assay with GB. Nevertheless, a decrease in volatile number was noted for MIF01 and BTF08 isolated when cultured in dual plate assay with GB. Thus, the findings showed that the amount of volatiles produced depended on the possible elicitation by the other fungi.

Approximately 44 volatile compounds had been identified as presented in Table 1. Moreover, these selected volatile compounds were derived from monoculture control plates (both endophytes and pathogen). Furthermore, these compounds, for instance, nitrous oxide, cyclotrisiloxane hexamethyl, and ethyl benzene, had been detected in the monoculture control plates, perhaps because of their natural presence in the headspace analysis and may not be due to the isolates (Table 1-6). In another instance, the compound cyclotrisiloxane hexamethyl, which did not exhibit any antifungal property or any inhibitory effect, was also detected naturally in GB.

In addition, the response of every endophytic fungi differed when cultured with GB in dual plate assay, as they were found to produce a unique set of induced volatile metabolites,

while some displaying potential antifungal properties. For example, 1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl), 3-Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthenecarbox amide, 1-methyl-4-(1-methylethyl), Benzene, 1-methyl-4-(1-methylethyl), and Benzothiophene-3-carboxylic acid,4,5,6,7-tetrahydro-2-amino-6-ethyl -, ethyl ester (Table 7). On the other hand, 1, 2-Benzenediol, 3, 5-bis (1, 1-dimethylethyl) had been detected at high levels in isolates T2 (2.82 %) and WAA02 (1.77 %) (Table 7). This finding reflects the presence of significant compounds in inhibiting GB, corresponding with the respective isolates T2 and WAA02 in recording high PIDG values of 68.3 % and 56.7%, respectively.

Specifically in isolate T2, the 3-Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthenecarbox amide had been produced as a significantly major compound with 33% composition, which hinted high inhibitory effect towards GB (Table 7). Therefore, the amount of 3-Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthenecarbox amide compound released, which correlated with its volatile profile, had been found to promote the effect of inhibition towards GB. Other than that, the strong inhibitory effect portrayed by isolate T2 could perhaps originate from compounds 1,2-Dihydroanthra[1,2-d]thiazole-2, 6,11-trione, trans-4-(2-(5-Nitro-2-furyl)vinyl)-2-quinolinamine, 1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl), and Benzoic acid, 5-methyl-2-trimethylsilyloxy-, trimethylsilyl ester as well (Table 7). The relatively high composition of -Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthenecarbox amide displayed in T2 volatile profile, which exceeded 2 %, strongly indicated their antifungal potential towards GB. In fact, all the compounds, excluding 1, 2-Benzenediol, 3, 5-bis (1, 1-dimethylethyl), are indeed exclusive for isolate T2 (Table 7).

As for isolate WAA02, the significant presence of a compound with high antifungal potential (1, 2-Benzenediol, 3, 5-bis (1, 1-dimethylethyl)) may have promoted its capability to

inhibit GB (Table 7). Isolate MIF01, on the other hand, produced benzothiophene-3-carboxylic acid, 4, 5, 6, 7-tetrahydro-2-amino-6-ethyl, ethyl ester as its major compound by 2.67%. Therefore, GB inhibition displayed by isolate MIF01 could be related to the 18 types of volatiles that ranged from peak percentages of 0.07 to 2.67% (Table 7). Nonetheless, the PIDG value at 65% for MIF01 suggested that its capability of inhibition may not necessarily derive from the substantial amount of volatiles produced (low peak percentage), but the synergistic effect obtained from the combination of the volatile compounds generated (Figure 1 and Table 7).

4.4 Discussion

The findings obtained from the GCMS analysis showed that all five endophytic fungi did produced varied volatile compounds, which had been produced either naturally or upon trigger due to the presence of other isolates. Most importantly, this particular research portrayed that having or producing the most number of volatile compounds may not necessarily indicate the isolate is most effective in inhibiting the growth of GB. This may be attributed to the fact that majority of the compounds are not antifungal in nature. Therefore, although 30-70 volatile compounds may be produced by a single endophyte, it is possible that only one compound has significant antimicrobial properties to inhibit a pathogen. This scenario is reflected in the antifungal potential displayed by volatiles of T2 (*T. asperellum*). This study may be the pioneer in reporting such volatile compound with antifungal properties generated by endophytic fungus (T2) to inhibit the GB pathogen, particularly in oil palms. In fact, the method employed to obtain the profiling of volatile compounds produced by endophytic fungi can also be applied to determine other volatiles for further analysis, potential antimicrobial agents. This method can significantly guide one to exclude compounds with non-inhibitory effects, thus enabling one to shortlist only the compounds with inhibitory properties for further use or analysis. This is rather significant because these shortlisted compounds can be tested either on its own or in combination

to investigate the potential of functioning as a biocontrol against pathogens. Research showed volatile compounds of *Trichoderma* inhibited pathogenic growth of *Fusarium oxysporum* (chilli wilt), *Rhizoctonia solani* (sheath blight of rice), *Sclerotium rolfsii* (collar rot of tomato), *Sclerotinia sclerotiorum* (web blight of beans), *Colletotrichum capsici* (anthracnose of chilli fruit), *Helminthosporium oryzae* (brown spot of rice), *Alternaria brassicicola* (Alternaria blight of cabbage) (Amin et al., 2010). Our results showed volatile compounds produced by *T. asperellum* reduced the mycelia growth of *G. boninense* which may indicates disease reduction by reducing the spread by pathogen.

Of all the volatile compounds determined from the isolates tested in this study, only 3-Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthenecarbox amide detected in isolate T2 (*Trichoderma asperellum*) appeared to display antifungal potential against GB. This compound is likely a derivative of metabolic activity and may contain quinazoline as a parent compound. Quinazolines and their by-products are important classes of heterocyclic compounds that promote a wide range of biological activities, mainly due to their acaricidal (Lamberth et al., 2000), weedicide (Khan et al., 2003), antibacterial (Rohini et al., 2009), and antifungal (Liu et al., 2004; Ouyang et al., 2006) properties. In fact, several quinazolines with a broad spectrum of bioactivities are used in both pharmaceutical and agrochemical industries (Liu & Huang, 2011).

For isolate WAA02 (*D. phaseolorum*), the presence of 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl) compound also known as γ-Terpinene at 6.93%, which could have strongly contributed to the antifungal property towards inhibiting pathogen GB. This 1, 4-Cyclohexadiene, 1-methyl-4-(1-methylethyl) compound was only produced exclusively by isolate WAA02. In fact, Tian et al., (2011) asserted that γ-Terpinene, displayed potential antifungal activities against

Aspergillus flavus, *Aspergillus oryzae*, *Aspergillus niger*, and *Alternaria alternata*, which is similar in this case for isolate WAA02.

As for isolate MIF01 (*D. phaseolorum*), it had been found to produce the highest number of volatile compounds in this study, with most compounds at less than 1% of peak percentage. However, Benzothiophene-3-carboxylic acid, 4, 5, 6, 7-tetrahydro-2-amino-6-ethyl, ethyl ester appeared to be a massively produced compound at 2.67% peak percentage. Although the presence of this compound seemed to inhibit the growth of GB, it had been unsure if this particular compound is indeed an antifungal produced naturally or triggered by other elements. It is also possible that synergistic interactions may occur between the minute compounds in inhibiting GB growth. However, this was not further pursued in this study as the focus is to profile the various compounds produced by the endophytes. The remaining volatile compounds identified in control plates (both endophytic and pathogen) were non-active compounds, for example, nitrous oxide, cyclotrisiloxane hexamethyl, and ethyl benzene (Table 1), thus were not accounted as inhibitory volatiles.

The *in vitro* studies, coupled with GCMS profiling of volatile compounds, exemplified the potential of endophytic fungi to function as ideal antifungal agents to inhibit the pathogen. This particular method has channelled the focus of this study to investigate several significant compounds that may function as a defence mechanism in combating pathogens and further inhibit the spread of GB. Hence, more tests are needed to identify the efficacy of several selected compounds as fungicides to hamper the spread of GB. The findings retrieved from this study vividly demonstrated the significance of volatile metabolites in impeding GB growth found in double plate assay. Fungal inhibition noted in double plate assay perhaps could be due to the hydroxyl groups contained in antimicrobial compounds (Juglal et al., 2002). The secondary

metabolites of endophytic fungi exerted greater antifungal activities primarily due to the synergistic effects of the metabolites and active components; suggesting more promising results from mixture of several compounds, in comparison to those from single pure extracts. On top of that, the application of such volatiles via vapour treatment may be ideal in controlling the spread of plant pathogens mainly due to minimal environmental impact. Hence, the endophytic fungi tested in this study displayed significant potential for further development as biocontrol agents, for long term control measure against the GB plant pathogen.

4.5 Conclusion

This study highlighted the effectiveness of the simple profiling technique in determining volatile compounds from potential biocontrol isolates. The volatile profiles discovered corresponded with the strong *in-vitro* antifungal activities against the GB pathogen. The volatile inhibitory compounds with antifungal properties towards GB had been generated by isolates T2, MIF01, and WAA02. The significant compounds identified were 1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl), 3-Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthenecarbox amide, Benzene, 1-methyl-4-(1-methylethyl), and Benzothiophene-3-carboxylic acid,4,5,6,7-tetrahydro-2-amino-6-ethyl, ethyl ester, which inhibited growth of GB effectively. These can be further developed as bioagents for use in managing BSR disease in oil palm.

4.6 References

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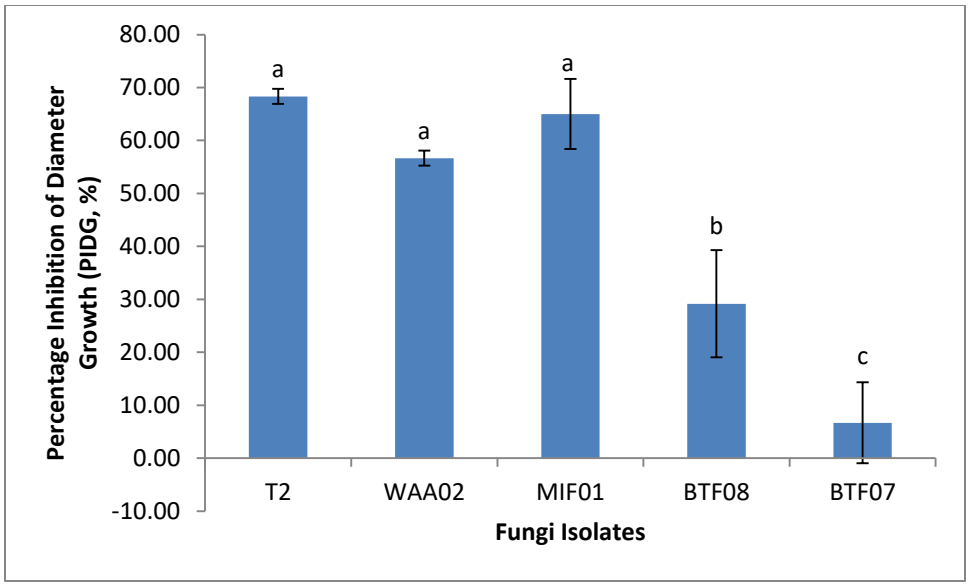
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
-10.00
Percentage Inhibition of Diameter Growth (PIDG, %)
a
a
a
b
c
T2
WAA02
MIF01
BTF08
BTF07
Fungi Isolates

Figure 1.

Figure caption

Figure 1. Inhibition of *G. boninense* by endophytic fungi in dual plate assay. Means with the same letters between isolates are not significantly different at $HSD_{(0.05)}$. Bars indicate standard deviation of means (±SD).

Table 1. Volatile compound produced by *G. boninense*, with a total of 27 compounds produced.

Volatile compounds
GB (control)
Butane, 2-methyl-
Pentane
Cyclotrisiloxane, hexamethyl-
p-Xylene
Styrene
Oxime-, methoxy-phenyl-_
Cyclotetrasiloxane, octamethyl-
D-Limonene
Aminothiazole
Benzo[h]quinoline, 2,4-dimethyl-
Silicic acid, diethyl bis(trimethylsilyl) ester
Acetamide, N-[4-(trimethylsilyl)phenyl]-
5-Methyl-2-trimethylsilyloxy-acetophenone
N-Methyl-1-adamantaneacetamide
2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-
3,6-Bis(N-dimethylamino)-9-ethylcarbazole
2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene
Methyltris(trimethylsiloxy)silane
trans-4-(2-(5-Nitro-2-furyl)vinyl)-2-quinolinamine
2-Methyl-7-phenylindole
4-Methyl-2-trimethylsilyloxy-acetophenone
3,3-Diisopropoxy-1,1,1,5,5,5-hexamethyltrisiloxane
Tetrasiloxane, decamethyl-
Silane, 1,4-phenylenebis[trimethyl
Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane
1,2-Benzisothiazol-3-amine tms
1H-Indole, 1-methyl-2-phenyl-

Table 2: Volatile compound produced by T2 (control) and dual plate culture. Fifteen compounds produced from T2 (control). Fifteen compounds produced in dual plate culture.

Volatile compounds	
T2 (control)	**T2/GB dual plate culture**
Nitrous Oxide	3-Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthenecarbox amide
1,2,5-Oxadiazole	Nitrous Oxide
Ethylbenzene	Silicic acid, diethyl bis(trimethylsilyl) ester
Styrene	3,3-Diisopropoxy-1,1,1,5,5,5-hexamethyltrisiloxane
Silane, 1,4-phenylenebis[trimethyl	Cyclotrisiloxane, hexamethyl-
1,4-Benzenediol, 2,5-bis(1,1-dimethylethyl)-	2-p-Nitrophenyl-oxadiazol-1,3,4-one-5
Cyclotrisiloxane, hexamethyl-	2-(Benzthiazol-2-yl)-6-methoxybenzofuran
1,2-Bis(trimethylsilyl)benzene	Methyltris(trimethylsiloxy)silane
2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-
2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene	Tetrasiloxane, decamethyl-
Methyltris(trimethylsiloxy)silane	1-Methyl-3-phenylindole
Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane	1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl)-
2-Ethylacridine	1,2-Dihydroanthra[1,2-d]thiazole-2, 6,11-trione
1-Methyl-3-phenylindole	trans-4-(2-(5-Nitro-2-furyl)vinyl)-2-quinolinamine
2-Methyl-7-phenylindole	Benzoic acid, 5-methyl-2-trimethylsilyloxy-, trimethylsilyl ester

Table 3. Volatile compound produced by WAA02 (control) and dual plate culture. Thirteen compounds produced from WAA02 (control). Twenty four compounds produced in dual plate culture.

Volatile compounds	
WAA02 (control)	**WAA02/GB dual plate culture**
Nitrous Oxide	Butane, 2-methyl-
1,3,5,7-Cyclooctatetraene	Pentane
1,2-Benzisothiazol-3-amine tbdms	Ethylbenzene
1H-Indole, 1-methyl-2-phenyl-	p-Xylene
1,2-Benzisothiazol-3-amine tms	Styrene
Cyclotrisiloxane, hexamethyl	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-
Methyltris(trimethylsiloxy)silane	Benzene, 1-methyl-4-(1-methylethyl)-
2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	3-Carene
Tetrasiloxane, decamethyl-	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-
2-Methyl-7-phenylindole	Phenol, 4,4'-(1-methylethylidene)bis-
Benzo[h]quinoline, 2,4-dimethyl-	Indole-2-one, 2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimethyl-
2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene	3,3-Diisopropoxy-1,1,1,5,5,5-hexamethyltrisiloxane
3H-3a-Azacyclopenta[a]indene-2-carbonitrile, 3-oxo-1-(piperidin-1-yl)-4,5,6,7-tetrahydro-	Silane, 1,4-phenylenebis[trimethyl
	trans-4-(2-(5-Nitro-2-furyl)vinyl)-2-quinolinamine
	Silicic acid, diethyl bis(trimethylsilyl) ester
	Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane
	1,4-Benzenediol, 2,5-bis(1,1-dimethylethyl)-
	1-Methyl-3-phenylindole
	1,2-Dihydroanthra[1,2-d]thiazole-2,6,11-trione
	2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene
	Cyclotrisiloxane, hexamethyl-
	Benzo[h]quinoline, 2,4-dimethyl-
	1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl)-
	Methyltris(trimethylsiloxy)silane

Table 4. Volatile compound produced by MIF01 (control) and dual plate culture. Seventy four compounds produced from MIF01 (control). Fourty compounds produced in dual plate culture.

Volatile compounds	
MIF01 (control)	**MIF01/GB dual plate culture**
Carbon dioxide	Nitrous Oxide
Ethanol	Ethanol
Acetic acid	Pentane
Toluene	Hexane
Cyclotrisiloxane, hexamethyl-	Ethylbenzene
Benzene, 1-chloro-4-(trifluoromethyl)-	p-Xylene
Ethylbenzene	Styrene
Benzene, 1,1'-(1,2-cyclobutanediyl)bis-, cis-	Bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl-
Oxime-, methoxy-phenyl-_	Benzene, 1-methyl-2-(1-methylethyl)-
Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-
4-Carene, (1S,3S,6R)-(-)-	1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-
Cyclohexene, 4-methylene-1-(1-methylethyl)-	Cyclohexene, 1-methyl-3-(1-methylethenyl)-, (.+/-.)
Cyclotetrasiloxane, octamethyl-	1-(3-Methylbutyl)-2,3,5,6-tetramethylbenzene
1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-
Benzene, 1-methyl-4-(1-methylethyl)-	1H-Indole, 5-methyl-2-phenyl-
Cyclopentene, 3-isopropenyl-5,5-dimethyl-	Silicic acid, diethyl bis(trimethylsilyl) ester
1-Hexanol, 2-ethyl-	Furazano[3,4-b][1,2,4]-triazolo[4,3-d]pirazine, 5-(2,3-dimethylphenylamino)-
1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	Phenol, 4,4'-(1-methylethylidene)bis
Bicyclo[3.1.0]hexan-2-ol, 2-methyl-5-(1-methylethyl)-, (1.alpha.,2.beta.,5.alpha.)-	3-Ethoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy)trisiloxane
Cyclohexene, 1-methyl-4-(1-methylethylidene)-	2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-
2-Ethylacridine	1,2-Dihydroanthra[1,2-d]thiazole-2
Nonanal	Cyclotrisiloxane, hexamethyl-

Table 4 continued

Volatile compounds	
MIF01 (control)	**MIF01/GB dual plate culture**
1H-Trindene, 2,3,4,5,6,7,8,9-octahydro-1,1,4,4,9,9-hexamethyl-	Anthracene, 9-ethyl-9,10-dihydro-10-t-butyl-
m-Hydroxymandelic acid, tris(trimethylsilyl)-	Benzene, 2-[(tert-butyldimethylsilyl)oxy]-1-isopropyl-4-methyl-
3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-	trans-4-(2-(5-Nitro-2-furyl)vinyl)-2-quinolinamine
4H-3,1-Benzoxazine, 6,7-dimethoxy-2-(4-methoxyphenyl)-4-propyl-	Silane, 1,4-phenylenebis[trimethyl
Oxirane, decyl-	Benzo[h]quinoline, 2,4-dimethyl-
Tetracyclo[6.1.0.0(2,4).0(5,7)]nonane,3,3,6,6,9,9-hexamethyl-(1.alpha.,2.alpha.,4.alpha.,5.beta.,7.beta.,8.alpha.)-	1,2-Benzisothiazol-3-amine tms
2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)-	2-p-Nitrophenyl-oxadiazol-1,3,4-one-5
Propan-2-ol, 1-(1-chloronaphthalen-2-yloxy)-3-morpholin-4-yl-	2H-1,3,4-Benzotriazepine-2-thione,5-benzyl-1,3-dihydro-3-methyl-
Benzoic acid, 3-methyl-2-trimethylsilyloxy-, trimethylsilyl ester	1-Methyl-3-phenylindole
Phenol, 2,6-bis(1,1-dimethylethyl)-4-(1-methylpropyl)-	Anthracene, 9,10-dihydro-9,9,10-trimethyl-
2,6-Difluoro-3-methylbenzoic acid,tridecyl ester	Tetrasiloxane, decamethyl-
Mercaptoacetic acid, bis(trimethylsilyl)-	3,3-Diisopropoxy-1,1,1,5,5,5-hexamethyltrisiloxane
Morpholine, 4-octadecyl-	Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane
5,6,7-Trimethoxy-1-indanone	2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene
Benzene, 1,1'-(1,4-dimethyl-2,5-cyclohexadiene-1,4-diyl)bis-	2-Methyl-7-phenylindole
2,6-Di-t-butyl-4-dimethylaminophenol	Benzothiophene-3-carboxylic acid,4,5,6,7-tetrahydro-2-amino-6-ethyl -, ethyl ester
9,10-Anthracenedione, 1-(3-butenyl)-	4-Methyl-2-trimethylsilyloxy-acetophenone
2',4',6'-Triisopropylacetophenone	1,2-Bis(trimethylsilyl)benzene
N-Benzyl-N-ethyl-p-isopropylbenzamide	
3,5-di-tert-Butyl-4-hydroxyacetophenone	
6H-Chromene-4,5-dione, 2-butyl-3-ethyl-7,7-dimethyl-7,8-dihydro-	
Morpholine, 4-octadecyl-	
Benzothieno[2,3-d]azepino[1,2-a]pyrimidin-13(11H)-one, 1,2,3,4,7,8,9,10-octahydro-	

Table 4 continued

Volatile compounds	
MIF01 (control)	**MIF01/GB dual plate culture**
2,6-Bis(1,1-dimethylethyl)-4-(1-oxopropyl)phenol	
1-Ethyl-2-phenylpyrazolium bromide	
Silicic acid, diethyl bis(trimethylsilyl) ester	
3-Isopropoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy)trisiloxane	
Methyl ethyl ketone, N-(3-methylbenzothiazol-2-ydene)hydrazone€	
Indole-2-one, 2,3-dihydro-N-hydrox	
Acrylophenone, 3,3-diphenyl-, semicarbazone	
Methyl ethyl ketone, N-(3-methylbenzothiazol-2-ydene)hydrazone€	
Pyrimidine-2,4-dione, hexahydro-3,6-dimethyl-1-(4-morpholinobutyl)-	
Phenol, 4,4'-(1-methylethylidene)bis-	
3-Ethoxy-1,1,1,5,5,5-hexamethyl-3-	
1H-2-Benzopyran-3-one, 7-ethoxy-4-hydroxy-4-methoxycarbonyl-	
Cyclotrisiloxane, hexamethyl-	
N-Methyl-1-adamantaneacetamide	
2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	
2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene	
2-Ethylacridine	
N,N-Dimethyl-4-nitroso-3-(trimethylsilyl)aniline	
N-Methyl-1-adamantaneacetamide	
Silane, 1,4-phenylenebis[trimethyl	
Benzo[h]quinoline, 2,4-dimethyl-	
Tetrasiloxane, decamethyl-	
4-Methyl-2-trimethylsilyloxy-acetophenone	
Purine-2,6-dione, 8-(3-ethoxypropylamino)-1,3-dimethyl-3,9-dihydro-	

Table 4 continued

Volatile compounds	
MIF01 (control)	**MIF01/GB dual plate culture**
Acetamide, N-[4-(trimethylsilyl)phAcetamide, N-[4-(trimethylsilyl)phenyl]-	
Silicic acid, diethyl bis(trimethylsilyl) ester	
2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	
Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane	
1,2-Bis(trimethylsilyl)benzene	
Trimethyl[4-(1,1,3,3,-tetramethylbutyl)phenoxy]silane	

Table 5. Volatile compound produced by BTF08 (control) and dual plate culture. Eighteen compounds produced from BTF08 (control). Sixteen compounds produced in dual plate culture.

Volatile compounds	
BTF08 (control)	**BTF08/GB dual plate culture**
Nitrous Oxide	Nitrous Oxide
Carbon dioxide	Butane, 2-methyl-
o-Xylene	Pentane
Styrene	Ethylbenzene
Cyclotrisiloxane, hexamethyl-	Bicyclo[4.2.0]octa-1,3,5-triene
Methyltris(trimethylsiloxy)silane	Phenol, 4,4'-(1-methylethylidene)bis-
trans-4-(2-(5-Nitro-2-furyl)vinyl)-2-quinolinamine	trans-4-Dimethylamino-4'-methoxychalcone
Benzoic acid, 5-methyl-2-trimethyl silyloxy-, trimethylsilyl ester	2-Ethylacridine
Anthracene, 9,10-diethyl-9,10-dihydro-	2-Methyl-7-phenylindole
Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane	Cyclotrisiloxane, hexamethyl-
2-Butenenitrile, 2-chloro-3-(4-methoxyphenyl)-	Tetrasiloxane, decamethyl-
Methyltris(trimethylsiloxy)silane	1,4-Benzenediol, 2,5-bis(1,1-dimethylethyl)-
2,4,6-Cycloheptatrien-1-one, 3,5-bis-trimethylsilyl-	Methyltris(trimethylsiloxy)silane
Benzene, 2-[(tert-butyldimethylsilyl)oxy]-1-isopropyl-4-methyl-	Silicic acid, diethyl bis(trimethylsilyl) ester
Benzo[h]quinoline, 2,4-dimethyl-	3,3-Diisopropoxy-1,1,1,5,5,5-hexamethyltrisiloxane
Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane	p-Xylene
4-Methyl-2-trimethylsilyloxy-acetophenone	
Silane, 1,4-phenylenebis[trimethyl	

Table 6. Volatile compound produced by BTF07 (control) and dual plate culture. Fifteen compounds produced from BTF07 (control). Sixteen compounds produced in dual plate culture.

Volatile compounds	
BTF07 (control)	**BTF07/GB dual plate culture**
Nitrous Oxide	Nitrogen
Carbon dioxide	Benzaldehyde, 2-nitro-, diaminomet hylidenhydrazone
Pyrene, 1,2,3,3a,4,5,5a,6,7,8,8a,9 ,10,10a-tetradecahydro-	Ethylbenzene
Ethylene oxide	Acetaldehyde
Disulfide, dimethyl	Butane, 2-methyl-
Silane, trimethyl[5-methyl-2-(1-methylethyl)phenoxy]	Styrene
Styrene	Pentane
2-Methyl-7-phenylindole	p-Xylene
Cyclotrisiloxane, hexamethyl-	Silicic acid, diethyl bis(trimethy lsilyl) ester
1,2-Benzisothiazol-3-amine tbdms	1,2-Dihydroanthra[1,2-d]thiazole-2 ,6,11-trione
2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene	Cyclotrisiloxane, hexamethyl-
Tetrasiloxane, decamethyl-	Benzene, 2-[(tert-butyldimethylsil yl)oxy]-1-isopropyl-4-methyl-
Silane, 1,4-phenylenebis[trimethyl	Benzo[h]quinoline, 2,4-dimethyl-
Silicic acid, diethyl bis(trimethylsilyl) ester	Silicic acid, diethyl bis(trimethy lsilyl) ester
Trimethyl[4-(2-methyl-4-oxo-2-pentyl)phenoxy]silane	3,3-Diisopropoxy-1,1,1,5,5,5-hexam ethyltrisiloxane
	Methyltris(trimethylsiloxy)silane
	trans-4-(2-(5-Nitro-2-furyl)vinyl) -2-quinolinamine

Table 7. Main volatile compounds produced by the endophytic fungi expressed as percentages of peak areas

Volatile compounds	Fungal isolate				
	T2	**WAA02**	**MIF01**	**BTF08**	**BTF07**
3-Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthenecarbox amide	33.13				
2-p-Nitrophenyl-oxadiazol-1,3,4-one-5	1.83				
2-(Benzthiazol-2-yl)-6-methoxybenzofuran	1.34				
1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl)-	2.82	1.77			
1,2-Dihydroanthra[1,2-d]thiazole-2, 6,11-trione	3.88				
trans-4-(2-(5-Nitro-2-furyl)vinyl)-2-quinolinamine	3.81				
Benzoic acid, 5-methyl-2-trimethylsilyloxy-, trimethylsilyl ester	2.63				
1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-		1.56			
Benzene, 1-methyl-4-(1-methylethyl)-		3.91			
3-Carene		1.21			
1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-		6.93			
Phenol, 4,4'-(1-methylethylidene)bis-		0.76		1.07	
1,4-Benzenediol, 2,5-bis(1,1-dimethylethyl)-		1.03		3.16	
1-Methyl-3-phenylindole		0.80			
1,2-Dihydroanthra[1,2-d]thiazole-2,6,11-trione		0.68	0.19		
Hexane			0.34		
Bicyclo[4.1.0]hept-2-ene, 3,7,7-trimethyl-			1.86		
Benzene, 1-methyl-2-(1-methylethyl)-			3.20		
Cyclohexene, 1-methyl-3-(1-methylethenyl)-, (.+/-.)			0.44		

Table 7 continued

Volatile compounds	Fungal isolate				
	T2	**WAA02**	**MIF01**	**BTF08**	**BTF07**
Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-			1.62		
1H-Indole, 5-methyl-2-phenyl-			0.11		
Silicic acid, diethyl bis(trimethylsilyl) ester			0.11	3.87	
Furazano[3,4-b][1,2,4]-triazolo[4,3-d]pirazine, 5-(2,3-dimethylphenylamino)-			0.07		
3-Ethoxy-1,1,1,5,5,5-hexamethyl-3-(trimethylsiloxy)trisiloxane			0.12		
Anthracene, 9-ethyl-9,10-dihydro-10-t-butyl-			0.29		
Benzene, 2-[(tert-butyldimethylsilyl)oxy]-1-isopropyl-4-methyl-			0.16		
2-p-Nitrophenyl-oxadiazol-1,3,4-one-5			0.76		
2H-1,3,4-Benzotriazepine-2-thione,5-benzyl-1,3-dihydro-3-methyl-			0.74		
1-Methyl-3-phenylindole			1.13		
Anthracene, 9,10-dihydro-9,9,10-trimethyl-			0.58		
Benzothiophene-3-carboxylic acid,4,5,6,7-tetrahydro-2-amino-6-ethyl -, ethyl ester			2.67		
Bicyclo[4.2.0]octa-1,3,5-triene				32.20	
trans-4-Dimethylamino-4'-methoxychalcone				0.68	
2-Ethylacridine				1.50	
Nitrogen					14.72
Benzaldehyde, 2-nitro-, diaminomet hylidenhydrazone					1.02
Acetaldehyde					0.76
1,2-Dihydroanthra[1,2-d]thiazole-2 ,6,11-trione					0.32
Benzene, 2-[(tert-butyldimethylsil yl)oxy]-1-isopropyl-4-methyl-					0.21

Table 7 continued

Volatile compounds	Fungal isolate				
	T2	**WAA02**	**MIF01**	**BTF08**	**BTF07**
3,3-Diisopropoxy-1,1,1,5,5,5-hexam ethyltrisiloxane					1.72
trans-4-(2-(5-Nitro-2-furyl)vinyl) -2-quinolinamine					2.00

Chapter 5

Isolation and characterization of non-volatile antifungal metabolites of *Penicillium citrinum* BTF08 towards fungal pathogen *Ganoderma boninense*

Overview

The results from prior experiments suggested BTF08 (*Penicillium citrinum*) as a promising isolate for the biocontrol of *G. boninense*, with growth-promoting properties (Chapter 2), host compatibility (Chapter 2), as well as the ability to induce lignin production as a defence mechanism (Chapter 3) in oil palm. As such, this chapter explores the antagonistic potential among non-volatile metabolites produced by BTF08 against *G. boninense* via broth microdilution method to determine the minimum inhibitory concentration (MIC). The crude extract of BTF08 was tested. The crude extract of BTF08 displayed antifungal activities (100% inhibition) in the broth microdilution assay against *G. boninense*. To obtain the crude extract, the cultures of *P. citrinum* (on agar) were first extracted using methanol as solvent, partitioned with solvents (hexane, dichloromethane, etyl acetate and methanol), followed by column chromatography, and lastly, purified via reversed-phase high-performance liquid chromatography (HPLC) to obtain a single pure compound. This compound was subsequently identified using NMR spectroscopy and was revealed to be citrinin. The MIC of the citrinin was determined at 100 μg/ml. It was concluded that *P. citrinum* citrinin, which has tremendous potential as a natural fungicide towards *G. boninense*. This is the first documentation of citrinin as as effective inhibitors of *G. boninense*. Hence, future studies can further explore the development of citrinin for application in oil palm plantations.

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Isolation and characterization of non-volatile antifungal metabolites of *Penicillium citrinum* BTF08 against fungal pathogen *Ganoderma boninense*

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Abstract

This study aimed to determine the antagonistic potential among non-volatile metabolites produced by BTF08 (*Penicillium citrinum*) against *Ganoderma boninense*. The metabolites were extracted using methanol as solvent. Extracted crude extract were tested via broth microdilution method to determine the antagonistic potential according to its minimum inhibitory concentration (MIC) needed for the control of *G. boninense*. Extracted compounds were isolated via HPLC for the active compound responsible for *P. citrinum* antifungal properties. Identification of compounds was done via NMR analysis. Results showed crude extract of BTF08 displayed antifungal activities (100% inhibition) in the broth microdilution assay against *G. boninense*. The active compound compound was subsequently identified using NMR spectroscopy and was revealed to be citrinin. The MIC of the citrinin was determined at 100 μg/ml. This suggested antagonistic potential of *P. citrinum* was due to the secretion of citrinin. This also suggests the potential of citrinin as a natural fungicide towards *G. boninense*.

Keywords: Biocontrol, Endophyte; *G. boninense*; Metabolites; *P. citrinum*

5.1 Introduction

Endophytes are fungi that cause symptomless infections among plants. Although endophytes are closely related to virulent pathogens, they generally possess limited pathogenic effects. Furthermore, endophytes are found to have plant-protecting properties such as biocontrol agents to their host plants, growth stimulation in plants, increment in nutrient uptake, growth inhibition of pathogens in plants, reduction in disease symptoms in plants, as well as enhanced tolerance in plants towards harsh environments (Carroll, 1988; Rodriguez et al., 2009). The mechanisms of plant protection include a consortium independently trigger plant defense cascades as pathogens of other hosts (via induced systemic resistance), certain fungi to trigger insect resistance (via Jasmonate pathway) as well as antibiosis (Arnold et al., 2003; Aneja et al., 2005). The latter is of immense interest as it involves production of metabolites such as lytic agents, enzymes, volatile compounds, or other toxic substances with inhibitory properties (Fravel, 1988). These compounds are attractive alternatives to chemical (Berg & Hallmann, 2006). Chitinase and β-1, 3-glucanase are considered as hydrolytic enzymes in the lysis of fungal cell walls. These lytic enzymes were known to lyse cell walls of plant pathogen *Fusarium oxysporum*, *Sclerotinia minor*, and *Sclerotinia rolfsii* (Singh et al., 1999; El-Tarabily et al., 2000; El-Tarabily et al., 2001). Volatile compounds are important aspect in biocontrol. Claydon et al. (1987) uses biocontrol fungi *Trichoderma harzianum* for the control of dampig off disease caused by *Rhizoctonia solani*. *Trichoderma harzianum* produced the volatile metabolites 6-n-pentyl-2H-pyran-2-one and 6-n-pentenyl-2H-pyran-2-one. The pentyl analogue which is the major product in the compound demonstrated inhibitory properties against plant pathogenic fungi (*Rhizoctonia solani*) and reduced the rate of damping off disease in lettuce. Endophytic fungus *Phoma* species ZJWCF006 in *Arisaema erubescens* produced α-tetralone derivative, (3S)-3, 6, 7-trihydroxy-α-tetralone which showed growth inhibition against *F. oxysporium* and *R. solani*.

P. citrinum was reported to produce a variety compounds such as compactin (Endo et al., 1976), 4a, 5-dihydrocompactin (Lam et al., 1981), citrinin (Vazquez et al., 2001), tanzawaic acids (El-Neketi et al., 2013), 6-methylcurvulinic acid (El-Neketi et al., 2013), 8-methoxy-3,5-dimethylisoquinolin-6-ol (El-Neketi et al., 2013), 1,2,3,11b-tetrahydroquinolactacide (El-Neketi et al., 2013). Compactin has antifungal properties against *Aspergillus* spp. and *Candida* spp (Larsen et al., 2007). Citrinin has antifungal properties against various clinical pathogenic fungi such as *Saccharomyces cerevisia*, *Rhizopus chinensis*, *Fusarium* sp., and *Aspergillus niveus* (Haraguchi et al., 1989; Devi et al., 2009). Tanzawaic acid was reported to have antimicrobial activity against *Klebsiella pneumonia* and *Staphylococcus aureus* (Cardoso-Martinez et al., 2015). No reports documented the antimicrobial and antifungal properties of 6-methylcurvulinic acid, 8-methoxy-3,5-dimethylisoquinolin-6-ol and 1,2,3,11b-tetrahydroquinolactacide.

P. citrinum was documented to have antifungal activities as antagonistic fungi towards plant pathogen *G. boninense*, *Fusarium oxysporum* and *Botrytis cinerea* (Ting et al., 2012; Sreevidya et al., 2015; Ting and Jioe, 2016). However, there has not been any report on the isolation of bioactive compounds of *P. citrinum* and the susceptibility of *G. boninense* towards the bioactive compounds of *P. citrinum*. In addition, there were only few reports on bioactive compounds isolated from endophytic fungi for the control of *G. boninense*. Report showed *Trichoderma harzanum* produced diffusible toxic substance which suppresses the growth of *G. boninense*; however, there were no identification on the bioactive substance responsible for the growth suppression of *G. boninense* (Siddiquee et al., 2009). An antifungal compound known as phenylethyl alcohol was isolated from *Trichoderma virens* inhibits the growth of *G. boninense* (Fiedler et al., 2001).

Several steps were taken before the isolation and characterization of bioactive compounds from fungal origin. Initially, the antifungal properties of the selected fungal

strains are tested in *in-vitro*. Once the fungal strain has potent bioactivity (antifungal), it will then be subjected to mass cultivation and extraction. Cultivation of fungal strain was done on fungal culture medium such as potato dextrose agar or potato dextrose broth. Fungi are known to secrete metabolites into the culture medium. Thus, the culture medium and the fungi biomass were subjected to solvent extraction. Methanol is generally used as solvent for extraction purpose due to its amphiphilic nature; which is a compound consisting of molecules is having a polar water-soluble group attached to a water-insoluble hydrocarbon chain (Patersen and Voth, 2006). This is an ideal solvent as methanol can dissolve polar molecules and also non-polar molecules. The extract obtain from methonal consist of compounds of polar and non-polar molecule. These mixtures of molecules were then further separated based on their polarity via fractionation using solvents of different polarity (hexane, dichloromethane, ethyl acetate and methanol). Bioactivity test were carried out using these 4 fractions (hexane, dichloromethane, ethyl acetate and methanol). Once the active fraction were identified, the active fraction were further purified using HPLC and to obtain a pure compound. The identification of the pure compounds was done via NMR identification to illucidate the structure of the compound.

Therefore, this study evaluates the potentials of endophytic *P. citrinum* as a biocontrol agent against basal stem rot disease in oil palm. *P. citrinum* was selected due to its inhibitory potential against *G. boninense*. However, the mode of action for this antagonism remained unclear. Antagonism by biocontrol agent is attributed to physical, chemical, and biochemical mechanisms (Knudsen and Dandurand, 2014). The present work attempts to identify the antagonistic mechanisms that may be responsible in this pathogen and endophytic biocontrol agent interaction. The antifungal properties of non-volatile crude extracts of *P. citrinum* (BTF08) were first tested in *in vitro*, followed by bioassay-guided isolation of the antifungal

compound, phytochemical screening, structural elucidation, and lastly, identification of the antifungal secondary metabolite.

5.2 Materials and methods

5.2.1 Culture establishment

Isolate *P. citrinum* (BTF08) (GenBank accession no. KT964566) from Monash Malaysia Microbiology Laboratory culture collection was cultured on Potato Dextrose Agar (PDA) (Merck) for 14 days at room temperature (25 ± 2°C) (Ting et al., 2009, 2012; Ting & Jioe, 2016). The pathogen, *G. boninense* was obtained from Professor Dr Sariah Meon from Universiti Putra Malaysia, as pure cultures grown on Potato Dextrose Agar (PDA) (Merck) for 14 days at room temperature (25 ± 2°C).

5.2.2 Extraction and isolation of non-volatile antifungal compound from BTF08

In order to extract the metabolites from solid culture, agar plate cultures containing fungal mycelia and agar were first separated from the petri dish and soaked in 4 L methanol (MeOH) in conical flask overnight. Next, the sample was sonicated for 10 min in the Ultrasonic Sonicator Bath, followed by filtration (using Whatman qualitative filter paper, Grade 1(Sigma –Aldrich)). The extraction procedure was repeated thrice. The extract retrieved was evaporated using the ROTAVAPOR R-210 (BUCHI) to obtain the crude extract residue (weighing approximately 12.0 g). This residue was then subjected to solvent partitioning in different polarity using n-hexane, dichloromethane (CH_2Cl_2) and ethyl acetate (EtOAc) singly. After that, column chromatography was performed for CH_2Cl_2 fraction using RP-18 to yield 2 fractions (MeOH-H_2O, 1:1 and MeOH: H_2O, 1:0). The sub-fraction (2.4 g) was eluted with MeOH: H_2O, 1:1 was further purified by preparative reversed-phase high-performance liquid chromatography (HPLC) (Zorbax SB 5-µm-particle-size C_{18} column; 40 to 60% MeOH in H_2O for 10 min) to yield a pure compound (8.4 mg). Later, this pure compound was subjected to NMR analysis (^{13}C NMR, ^{1}H NMR, DEPT, HMBC, COSY). Finally, the NMR

spectrum was analysed to determine its resulting structure, which was then compared with the results reported in the literature.

5.2.3 Preliminary phytochemical studies of extract from BTF08

The crude extract from BTF08 was subjected to a number of phytochemical tests to determine the active constituents present in the extracts of crude methanol, as well as the active dichloromethane fraction. This was done to compare the groups of compound presences in both crude extract and the active dichloromethane fraction. The phytochemical screening was performed based on methods by Okerulu and Ani (2001), Veerachari and Bopaiah (2012), Sofawara (1993), Trease (1989), Harborne (1973), and Edeoga et al., (2005) as presented as follow:

Phytochemical	**Methods**
Alkaloids	2ml of Drangendroff's reagent was added to 1ml of extract. Formation of cloudy opaque orange coloration indicated alkaloids.
Tannins	2ml of Ferric chloride solution was added to 1ml extract. Dark green coloration showed positive for tannins.
Saponins	2ml distilled water was added to 1ml of extract and shaken vigorously before allowing to stand for 10 minutes. Formation of foam above the suspension, lasting for 10 minutes would indicate presence of saponins
Anthraquinones	Mixture of 1ml extract and 10ml benzene was filtered followed by addition of 5ml of 10% (v/v) ammonia. The mixture was shaken well. Formation of pinkish colored solution indicated positive result.
Anthocyanides	5ml of dilute HCl was added directly to the extract. Formation of pinkish colour solution indicates presence of anthocyanides.
Phenolic flavonoids	10% lead acetate was added to 1ml of extract. Brown precipitate showed presence of phenolic flavonoids.
Flavonoids	Diluted NaOH was added to 1ml of extract. Formation of golden yellow coloration showed flavonoids.

Steroids	Equal amounts of chloroform and H_2SO_4 was added slowly along the sides of the test tube containing the extract. Positive result in inducated by the presence of dual layer, where top layer was red in color, while the sulphuric acid layer turned yellow with green fluorescence
Terpenoids	$CHCl_3$ was added to the sample, followed by a few drops of concentrated H_2SO_4 to the extract. Reddish-brown interface formation indicated positive result.

5.2.4 Antifungal bioassay of crude extract of *P. citrinum* BTF08

The citrinin extracted from BTF08 was first prepared as a stock solution by dissolving each extracted compound in methanol to a concentration of 800 µg/ml. The broth microdilution assay was carried out using the sterile multi-well plate (96-well plate, Thermo Fisher Scientific, MA, USA). Later, each compound was diluted 2-fold with methanol and tested in triplicates. The homogenized *G. boninense* was adjusted approximately to 10^4 cfu/ml in potato dextrose broth, from which 10 µl was dispensed into each well to achieve a concentration of 0.39 - 800.00 µg/ml for each compound examined. The total final volume in each well was made up to 200 µl with approximately 90 µl of potato dextrose broth. The multi-well plates were incubated for 5 days to assess the viability of the pathogen upon exposure to the various concetrations of the citrinin extracted from BTF08. On the 5^{th} day, the mixture was transferred to PDA via spread plate method and incubated for a week at room temperature (25 ± 2°C). Growth of *G. boninense* on PDA indicated citrinin slows down or prevents the growth of the pathogen, while the absence of *G. boninense* growth on PDA suggests citrinin inhibits fungal growth by killing off the fungi. The inhibition was recorded as minimum inhibitory concentrations (MIC) of the compound with antifungal efficacy in microdilution in broth, whereby the least concentration of compound needed to obtain total growth inhibition of *G. boninense*.

5.2.5 NMR Analysis of pure compound obtained from BTF08

The pure compound (8.4 mg), purified using preparative reversed-phase high-performance liquid chromatography (HPLC) method, was subjected to analsyis using the NMR (13C NMR, 1H NMR, DEPT, HMBC, COSY). In 1H NMR information can be obtained are integration, multiplicity and coupling. DEPT type 13C NMR experiments identified different carbon environments. While 2D NMR experiments such as HMBC can be important for fully characterising more complex or new compounds. They bring together both 1H and 13C NMR and show correlations between proton and carbon atoms. HMBC shows multiple bond interactions between carbon atoms and protons (eg. C-C-H). NMR information was recorded on a Varian Unity Bruker Ascend 700 MHz NMR. The analysis was carried out at room temperature in $CDCl_3$ (98% D) with TMS as reference, in which the ^{13}C NMR data had been compared with the data reported in the literature (Barber et al., 1981; Sankawa et al., 1983).

5.3 Results

5.3.1 Phytochemical screening of crude extract of BTF08

The extraction process using various solvents (i.e. methanol, hexane, dichloromethane, and ethyl acetate) produced various fractions of the crude extracts. It was observed that methanol and dichloromethane crude extracts demonstrated positive antifungal activities towards *G. boninense.* Hexane and ethyl acetate fraction did not demonsted antifungal potential. As such, the phytochemical analysis was conducted for only methanol and dichloromethane crude extracts. The phytochemical analysis revealed that methanol-derived crude extracts of BTF08 comprised of alkaloids, tannin, saponin, anthraquinone, anthocyanosides, phenolic flavonoids, and terpenoids. These tests were positive as the entire test yielded positive results (Table 2). On the other hand, the dichloromethane fraction has a similar profile except that anthocyanosides, flavonoids, and steroids were absent (Table 2).

5.3.2 Bioassay for antifungal activity

The methanol fractioned crude extracts demonstrated potent antifungal activities in broth microdilution assay against *G. boninense*. Methanol crude extract of BTF08 consist of compounds of polar and non-polar molecule. Solvent fractionation of the crude extract yielded 4 fractions (hexane, ethyl acetate, dichloromethane, methanol). These 4 fraction were tested for its antifungal properties. Positive antifungal activities had been identified in the dichloromethane (CH_2Cl_2) fraction with total inhibition of *G. boninense* growth. This proves that the active compound with antifungal properties was only deposited in the dichloromethane fraction and the compound is moderately polar. The antifungal activity of the dichloromethane fractions were validated with a MIC value of 100 μg/ml. No fungal growth was detected on PDA plates inoculated with *G. boninense*.

5.3.3 NMR analysis of BTF08 pure compound

The active compound obtained from the dichloromethane fraction appeared as yellow amorphous solid particles. The NMR analysis revealed this antifungal compound as citrinin. The profile of the compound as citrinin was based on comparison of the spectrum of ^{13}C NMR with those reported in the literature: δ 183.83 (C-6, lit. 183.7), 177.22 (C-8, lit. 177.2, 162.69 (C-1, lit. 162.9), 174.53 (C-12, lit. 174.1), 139.01 (C-4a, lit. 139.2), 123.13 (C-5, lit. 122.6), 107.43 (C-8a, lit. 107.1), 100.35 (C-7, lit. 100.0), 81.65 (C-3, lit. 81.8), 34.61 (C-4, lit. 34.5), 18.52 (C-11, lit. 18.4), 18.26 (C-9, lit. 18.2), and 9.46 (C10, lit. 9.4). In addition, the spectrum of ^{1}H NMR were also in agreement with reports in the literature: δ 1.25 (11-H_3, lit. 1.23), 1.37 (9-H_3, lit. 1.35, 2.04 (10-H_3, lit. 2.02), 3.00 (4-H, lit. 2.98), 4.79 (3-H, lit. 4.78), 8.25 (1-H, lit. 8.24), 15.13 (8-OH, lit. 15.09), and 15.88 (CO_2H, lit. 15.88) (Figure 1). Additionally, it was found that this particular compound was soluble in ethyl acetate and methanol, but insoluble in hexane and water, which are typical attributes of citrinin.

5.4 Discussion

This study showed that the endophytic isolate *P. citrinum* (BTF08) exhibited antifungal activities towards the oil palm pathogen *G. boninense* via production of non-volatile inhibitory compounds. In fact, similar observations had been reported by Khamthong et al., (2012) and Wakana et al., (2006) on the antifungal potential displayed by *P. citrinum*, but these reports did not further investigate the compounds responsible for the antifungal activity. The phytochemical screening of the crude extracts showed that *P. citrinum* produces a variety of alkaloids, tannin, saponin, anthraquinone, anthocyanosides, phenolic flavonoids, and terpenoids. This was the first attempt in performing phytochemical test on the extracted metabolites of *P. citrinum*. Fungi are known to produce tannin, flavonoids, tepenoids, phenol and saponins (Govindappa et al., 2011; Devi et al., 2012). These compounds are typical in fungi. The results of phytochemicals analysis showed that *P. citrinum* produces more groups of compounds such as alkaloids, anthraquinone and anthocyanosides, which was not reported in previous studies. Both crude extract and dichloromethane fractions yielded similar compounds, with the exception that crude extract consist of alkaloids, tannin, saponin, anthraquinone, anthocyanosides, phenolic flavonoids, and terpenoids. On the contrary, the dichloromethane extract was only comprised of alkaloids, tannin, saponin, anthraquinone, phenolic flavonoids, and terpenoids. This shows that anthicyanosides are not responsible for the bioactivity of BTF08. The various compounds may be responsible for the antifungal activities of *P. citrinum* towards *G. boninense*. Both phenol and phenolic compounds have been proven to be effective as fungicide against *Aspergillus niger*, *Aureobasidium pullulans*, *Chaetomium* sp., *Cladosporium* sp., *Fusarium* sp., *Paecilomyces lilacinus* and *Penicillium* sp. (Haines and Stuart, 1986; Doherty et al., 2010). The antimicrobial nature of phenolic compounds is attributed to their role as electron donors, which displayed easy oxidation to form phenolate ion, an electron acceptor (Doherty et al., 2010). Besides that, phenols being

lipophilic in nature are able to to inhibit the activityof ATP-binding cassette transporter in fungal pathogen, thus making the fungal pathogen more susceptible to antifungal compounds (Wink and Schimmer, 2010). Alkaloids were isolated from endophytic fungi as they projected pathogen growth inhibition properties against phytopatogenic fungi (Fu et al., 2011; Lu et al., 2000). Several studies on saponins hinted antifungal activities (Grayer & Harborne, 1994). Anthraquinones and their derivatives had been reported to be active against human pathogenic fungi, such as *Cladosporium cucumerinum* and *Candida albicana* (Rath et al., 1995). Terpenoids have been proven to exert antifungal activities against a wide range of pathogens (Pare et al., 1993; Rao et al., 2010). This study demonstrated that the endophytic *P. citrinum* is indeed capable of generating a wide array of bioacitve compounds, along with the potential to exert antifungal activities, as reported in other studies.

A substantially growing number of studies have begun looking into *in vitro* assay of antifungal potential among non-volatile metabolites, especially those produced by endophytic fungi. This is because; researchers have observed the positive correlations between assays *in-vitro* and *in-vivo* biocontrol. For instance, the non-volatile antibiotic chetomin produced by *Chaetomium globosum in-vitro* had been found to be positively correlated with antagonism towards *Venturia inequalis* on apple plants in a nursery (Cullen & Andrews, 1984). On top of that, researchers have started using cell-free culture filtrates or extracts of these filtrates to portray the probable role of antibiosis in biocontrol (Ait-Lahsen et al., 2001; Fravel, 1988). With that, the method of antibiosis has often been applied for fungal competition and/or parasitism. The use of *in-vitro* assays for assessment of antibiosis is indeed crucial, while the utility of these assays may be associated to one's level of comprehending the compounds involved. This study had conducted an *in-vitro* assay upon the non-volatile secondary metabolite of BTF08 (*P. citrinum*) in determining its antifungal potential against *G.*

boninense, which appears to be the causal agent of the BSR disease in oil palm (Spaincer, 2000).

The NMR analysis identified the non-volatile antifungal metabolite produced by *P. citrinum* as citrinin. Citrinin is known to be a mycotoxin, and several other studies have reported that this compound can be produced by several fungal species. This include *Aspergillus terreus* (Sankawa et al., 1983), *Penicillium expansum* (Ciegler et al., 1977), *Penicillium verrucosum* (Schmidt-Heydt et al., 2015), *Penicillium chrysogenum* (Devi et al., 2009), and *Penicillium citrinum* (Barber et al., 1981), include the species here and mention their citations (Haraguchi et al., 1989; He et al., 2004). Apart from that, citrinin has also been reported as having strong antibacterial activities (Ambrose & Deeds, 1946; Subramani et al., 2013). The antifungal nature of citrinin has also been reported, mainly towards *Rhizopus chinensis* (Haraguchi et al., 1989; He et al., 2004). This however, is one of the few reports on citrinin towards *G. boninense*. Furthermore, citrinin has been proven by many researchers to inhibit both respiration and macromolecular syntheses, hence serving primarily in the mitochondrial electron transport system within the targeted fungi (Haraguchi et al., 1987). Although citrinin has been successfully determined to be a metabolite that contributes to the antifungal activities towards *G. boninense*, the application of endophytic isolate, instead of citrinin, in the oil palm plantation has been deemed as more beneficial. This is due to the fact that *P. citrinum* is not only capable of producing citrinin, but it can also elicit a defensive mechanism via lignification in oil palm. As such, endophytic fungi can absolutely function as metabolite producers with phytotoxic and growth-regulating properties (Hussain et al., 2007). In fact, endophytes possess the capability to compete with pathogens for growing niche by producing secondary metabolites that exhibit antibiotic properties (Gunatilaka, 2006; Hallmann & Sikora, 1996). Researcher found that most of the secondary metabolites generated by endophytes did display good antibiotic activities against microorganisms

(Kusari et al., 2012; Tan & Zou, 2001). Therefore, endophyte *P. citrinum* and citrinin may be apply for the control of *G. boninense*, whereby *P. citrinum* presents as a competition for both space and nutrient with *G. boninense* and citrinin inhibit both respiration and macromolecular syntheses of *G. boninense*.

5.5 Conclusion

P. citrinum, an endophyte that grows in oil palm, could become a valuable source of natural fungicide. It is discovered to produce citrinin, a widely reported mycotoxin. Citrinin may be useful for the control of pathogenic fungi. *P. citrinum* could be recommended as an organism of agricultural importance. However, further studies will be needed to ascertain fully its effect on the ecosystem and agricultural products.

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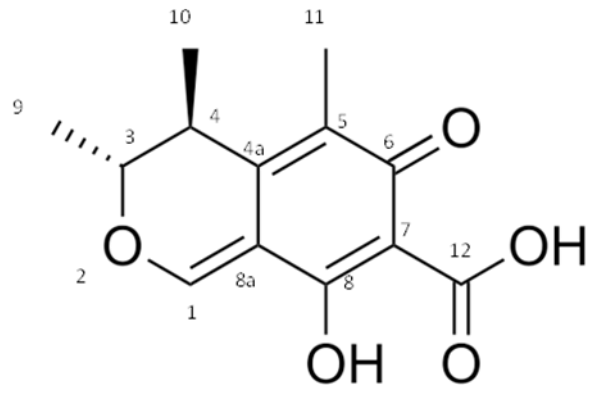

Figure 1.

Figure caption

Figure 1. Chemical structure of citrinin elucidated from NMR analysis with position of carbon atoms labeled accordingly.

Table 1: Biochemical tests for identification of phytochemical constituents in extract from BTF08

Phytochemical	**Methods**
Alkaloids	2ml of Drangendroff's reagent was added to 1ml of filtrate. Formation of cloudy opaque orange coloration indicated alkaloids.
Tannins	2ml of Ferric chloride solution was added to 1ml filtrate. Dark green coloration showed positive for tannins.
Saponins	2ml distilled water was added to 1ml of filtrate and shaken vigorously before allowing to stand for 10 minutes. Formation of foam above the suspension, lasting for 10 minutes would indicate presence of saponins
Anthraquinones	Mixture of 1ml filtrate and 10ml benzene was filtered followed by addition of 5ml of 10% (v/v) ammonia. The mixture was shaken well. Formation of pinkish colored solution indicated positive result.
Anthocyanides	5ml of dilute HCl was added directly to the filtrate. Formation of pinkish colour solution indicates presence of anthocyanides.
Phenolic flavonoids	10% lead acetate was added to 1ml of filtrate. Brown precipitate showed presence of phenolic flavonoids.
Flavonoids	Diluted NaOH was added to 1ml of filtrate. Formation of golden yellow coloration showed flavonoids.
Steroids	Equal amounts of chloroform and H_2SO_4 was added slowly along the sides of the test tube. Positive result in inducated by the presence of dual layer, where top layer was red in color, while the sulphuric acid layer turned yellow with green fluorescence
Terpenoids	$CHCl_3$ was added to the sample, followed by a few drops of concentrated H_2SO_4 to the filtrate. Reddish-brown interface formation indicated positive result.

Table 2: Phytochemical detected in extracts of BTF08

Phytochemical test	**Methanol crude extract**	**Dichloromethane crude extract**
Alkaloids	+	+
Tannin	+	+
Saponin	+	+
Anthraquinone	+	+
Anthocyanosides	+	-
Phenolic flavonoids	+	+
Flavonoids	-	-
Steroids	-	-
Terpenoids	+	+

Key: + indicates present; - indicates absent

Table 3: Assignment for ^{13}C NMR of citrinin

Position	Citrinin from BTF08, δ (ppm)	Reference , δ (ppm), (Sankawa et al., 1983)
	^{13}C (700 MHz)	^{13}C (300 MHz)
1	162.69	162.9
3	81.65	81.8
4	34.61	34.5
4a	139.01	139.2
5	123.13	122.6
6	183.83	183.7
7	100.35	100
8	177.22	177.2
8a	107.43	107.1
9	18.26	18.2
10	9.46	9.4
11	18.52	18.4
12	174.53	174.1

Table 4: Assignment for ^{1}H NMR of citrinin

Position	Citrinin fromBTF08, δ (ppm), J in Hz	Reference, δ (ppm), (Barber et al., 1981)
	^{1}H (700 MHz)	^{1}H (300 MHz)
11-H_3	1.25d, 7	1.23d
9-H_3	1.37d, 7	1.35d
10-H_3	2.04s	2.02s
4-H	3.00q, 7	2.98q
3-H	4.79q, 7	4.78q
1-H	8.25s	8.24s
8-OH	15.13s	15.09s
CO_2H	15.88s	15.88s

Chapter 6

Efficacy of single and mixed treatment of endophytic fungi for the biocontrol of *Ganoderma boninense* in oil palm seedlings

Overview

This chapter presents the last part of the study, in which the biocontrol efficacy of selected endophytes was evaluated for the control of *G. boninense* in oil palm seedlings. The endophytes selected were T2 (*T. asperellum*) and BTF08 (*P. citrinum*), and these two isolates were applied as single or mixed treatments. The selection of endophytes was carried out based on the results from Chapters 2, 3, 4, and 5 whereby T2 and BTF08 generally showed antagonisntic potential against *G. boninense* with the production of antifungal volatile, production of antifungal citrinin, mycoparasitism, induced host defense via lignification, and plant growth promoting potential. This study has discovered the potential of endophytic fungi in producing volatile (Chapter 4) and non-volatile (Chapter 5) antifungal metabolites. Isolates T2 (*T. asperellum*) and BTF08 (*P. citrinum*) had been revealed to generate both volatile and non-volatile metabolites, respectively, along with antagonistic activities that are potent against *G. boninense*. Besides, isolate T2 displayed antifungal properties via mycoparasitism as space and nutrient competition took place with the pathogen; *G. boninense*. Isolate BTF08 yielded citrinin (Chapter 5), which refers to a strong and powerful antifungal compound against *G. boninense*. Both of these isolates had been determined to trigger the induced defence response of oil palm so as to hike the production of lignin (Chapter 3), which serves as a defence mechanism against the spread of fungal pathogenic infection by *G. boninense.* Isolate BTF08 also exerted plant growth promoting potential towards oil palm (Chapter 2). As such, this particular chapter investigated the potential of these two isolates (T2 or BTF08) to exert antifungal activities upon combined treatment and single endophyte treatment on oil palm seedlings infected with *G. boninense* in a greenhouse study. To initiate this experiment, several healthy oil palm seedlings were infected with *G. boninense* and later, were treated with various treatments of endophytic fungi; either as single (T2 or BTF08) or combined treatments (BTF08+T2). The seedlings were inoculated with endophytes via soil drenching

before *G. boninense* infection. After a week seedling were then infected with *G. boninense* for a period of 7 weeks and treated with endohytic fungi immediately. The following parameters were assessed; plant height, stem diameter, root count and root mass. The first symptoms of the disease appeared at 3rd week post-infection with *G. boninense*. At this stage, the Disease Index (DI) of 40% had been recorded for non treated seedlings. All the seedlings that were treated with endophytic fungi were asymtomless (DI percentage 0%). During the 4thweek, the DI for non treated seedlngs escalate up to 60%, where those samples treated with the combination of T2+BTF08 exhibited an initial DI percentage of 20%. After the 7th week of infection, 100% DI was recorded for non treated seedlings, indicating severe infection. Samples that were given treatment with endophytic fungi portrayed a lower DI percentage of 60% in the combined isolates; T2+BTF08, whereas T2 (40%) and BTF08 (20%) for single isolate treatments.

As for vegetative growth, the samples treated with endophytic fungi displayed increment in the height of the plant. At the 7th experimental week, increment in plant height was noted to be significant for BTF08 and T2 single strain treatment, in comparison to those untreated samples infected with pathogenic *G. boninense*. The BTF08 treatment was found to enhance the growth of stem. The quantity and mass of root also increased for samples treated with endophytic BTF08 and T2, hence indicating enhance growth. The average number of root found in endophytic-treated seedlings had been significantly higher, in comparison to samples that were untreated.

As such, this present study showcased that both T2 and BTF08 have potential as biocontrol agents, whereby the use of these endophytes displayed a significant reduction in the spread of the BSR disease. The application of endophytes singly (either T2 or BTF08) enhanced disease suppression, in comparison to the mixed treatment (T2+BTF08). As a conclusion, both T2 and BTF08 endophytic fungi do possess the potential to serve as

biocontrol agents to limit the spread of BSR disease in oil palm, in which it was noted that the application of T2 and BTF08 single strain inoculation had successfully decreased the spread of infection among infected oil palm seedlings.

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Efficacy of single and mixed treatment of endophytic fungi as biocontrol agents of *Ganoderma boninense* in oil palm seedlings

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Abstract

This study aimed to investigate the potential of these two isolates (T2 or BTF08) to exert antifungal activities upon combined treatment and single endophyte treatment on oil palm seedlings infected with *G. boninense* in a greenhouse study. Single and mixed endophytic treatment comprising *Penicillium citrinum* BTF08 and *Trichoderma asperellum* T2 were tested as biocontrol agents for basal stem rot in oil palm seedlings which was pre-infected with *G. boninense* through artificial infection method via rubber wood block. The treatment was performed by pre-inoculating the seedling with endophytic fungi and application of endophytic fungi soil drench made from homogenized fungi mycelia. A disease severity index ranging from 1 to 5 was utilized to assess the disease severity after endophytic treatment. A single strain application of *T. asperellum* T2 gave good disease suppression with the low DSI of 10 compared to infected but non-treated control plant (DSI value of 50). Single application of *P. citrinum* was effective in suppressing symptom of *G. boninense* and promotes growth of the plant with DSI value of 5. However, a mixed treatment of both fungi did not perform better than a single strain inoculation treatment, with DSI 15. The biological control property of both fungi was shown to be an effective control measure against *G. boninense*. Single isolate application of either T2 or BTF08 displayed better growth of oil palm seedlings, when compared to mixed treatment of T2+BTF08

Keywords: Biocontrol; Disease severity; Endophyte; *G.boninense*; pathogen

6.1 Introduction

The pathogen *Ganoderma boninense* has been found to be the causal agent of the Basal Stem Rot (BSR) disease in oil palm (Ho & Nawawi, 1985; Abdullah et al., 2009). As such, this particular disease has been deemed fatal among oil palm trees and emerged as a significant threat to the oil palm industry (Liaghat et al., 2014). Several control measures have been introduced to control the BSR disease, but none have promised to be prominent, effective and feasible for sustainable application (Hushiarian et al., 2013). Cultural approaches were discovered to be inefective; chemical mesures were trunk injection with fungicides and foliar spray with chemical fungicides. Trunk injections are rather impractical due to the high cost incurred and the intensive labour requirement. This method only decreases the rate of disease spread. The application of chemical fungicides has displayed some positive effects upon inhibition of *G. boninense*, but again, fail in eradicating the disease. This is because chemical application had been found to degrade in soil before it could even reach the infected areas (Susanto et al., 2005). Chemical application on oil palm negatively affects the environment through the use of fungicides, which is witnessing a reduction due to awareness of being environmental-friendly. Hence, biocontrol agents have appeared to be a more promising alternative for sustainable and long-term control measure that impedes the spread of plant pathogens (Park et al., 2014; Sapak et al., 2008a).

The use of endophytes as bicontrol agents are investigated as endophytes can colonize the internal plant tissues without causing any vivid symptoms to their host (Wilson, 1995). Several studies have found endophytes demonstrating inhibitory effect towards pathogens of banana (*Fusarium oxysporum*) (Zacky & Ting, 2015), barley (*Pochonia chlamydosporia*) (Macia et al., 2009), and cocoa (*Moniliophthora roreri*) (Mejía et al., 2008). In addition, Susanto et al (2005) and Ting and Jioe (2016) have carried out preliminary studies on

endophytes and their biocontrol activities towards *G. boninense* and found endophytic fungi are capable of inhibiting the growth of *G. boninense in vitro*.

In many studies, the biocontrol agents are often evaluated when applied as single combinations. A single isolate of *Trichoderma* was applied via soil drenching to suppress basal stem rot in oil palm (Sundram, 2013). Only a few studies examined the biocontrol efficacy for treatments using mixed (combined) treatments. It is important to evaluate the feasibility of mixed culture as it has been shown to have better control ability in certain biocontrol studies. Mixed treatments are important as it determines whether the co-existence of more than one biocontrol agent may lead to synergistic, antagonistic or mutualistic relationship with one another. Etebarian et al. (2000) combined *T. harzianum* with *T. virens* as a mixed treatment to decrease the severity of disease in shoots and roots of potatoes infected with *Phytophthora erythroseptica* (Etebarian et al., 2000). Another study by Tarabily et al. (2009) used a combination of three biocontrol isolates for the control of *Phythium aphanidermatum*. The findings revealed that the combination treatment (*Actinoplanes campanulatus*, *Micromonospora chalcea Streptomyces spiralis*) had successfully and significantly suppressed the spread of disease, when compared to individual strains (Tarabily et al., 2009). Sundaramoorthy et al. (2012) employed a combination of bacterial strains (*Bacillus subtilis*; EPCO16, and EPC5) and rhizobacterial strain (*Pseudomonas fluorescens*; Pf1) to control the spread of chili wilt disease. The results pointed out that the combination of antagonistic bacterial strains had managed to significantly reduce the progression of disease, and that the control of wilt disease was mainly due to the synergistic effect by the biocontrol agent in the combined treatment.

In this study, two endophytic isolates were slected for evaluation. The isolates were *P. citrinum* (BTF08) and *T. asperellum* (T2), which has shown antagonistic properties against *G. boninense* and displayed high compatibility with oil palm (Cheong et al., 2017). The efficacy

of both single and mixed treatments of the selected endophytic fungi (BTF08 and T2) against the spread of *G. boninense* was conducted in a greenhouse trial. The criteria assessed include BSR disease incidence (DI) of oil palm seedling, disease severity (calculated by employing the AUDPC (area under the disease progress curve)) (Simko & Piepho, 2012) and the vegetative growth of seedling (height, stem diameter, root count and root mass). Disease incidence was measured by counting the number of plants that were infected (disease incidence). Disease severity was assessed by estimating the proportion of total photosynthetic area that is diseased. Observing the proportion of diseased leaf by eye is unreliable, disease assessment keys, showing different disease severities as blackened areas or tissue rottings have been devised for oil palm seedlings. The disease assessment keys were generated by Kok et al., (2013), whereby the estimation adheres to a scale of 0 to 4, by observing the tissues of oil palm seedlings for symptoms (rotting of tissues). AUDPC is an epidemiological indicator of the sum of accumulated disease over time. Treatment showing lowest AUDPC was considered as promising in tolerance to disease.

6.2 Materials and methods

6.2.1 Fungal culture establishment

Fungal endophytes; *P. citrinum* (BTF08) (GenBank accession no. KT964566) and *Trichoderma asperellum* (T2) (GenBank accession no. KT964564) obtained from Monash Malaysia Microbiology Laboratory culture collection, were cultured on Potato Dextrose Agar (PDA) (Merck). The pathogenic *G. boninense*, was obtained from Prof. Sariah Meon from Universiti Putra Malaysia, had been employed as pathogen in this study. The fungal inoculum was prepared by cultivating BTF08 and T2 in Potato Dextrose Broth (PDB) (Merck) by inoculating 5 mycelial disc into 500 ml of PDB. The mycelial in PDB was incubated for 14 days at room temperature (25 ± 2°C). The homogenized fungal mycelial (BTF08 and T2) had been adjusted to concentrations of 2×10^7 colony forming units per ml (cfu ml^{-1}), respectively,

based on the standard growth curves (Appendix 1, 2 and 5). Meanwhile, the treatment that employed the mixture of T2 and BTF08 was prepared by mixing the suspension in ratio of 1:1 (v/v). Mycelial solutions (150 ml) were applied to oil palm seedlings via soil drenching. Treatments include the combination of endophytes (T2 and BTF08), single strain treatment (BTF08) and single strain treatment (T2). Positive control was seedlings infected with *G. boninense*.

6.2.2 Preparation of *G. boninense* inoculum on rubber wood block

Rubber wood blocks (12 cm x 6 cm x 6 cm dimension, approximately 450 – 500 g) obtained from a local sawmill (Impressive Trasforms Plt Ltd) in Malacca, Malaysia. Rubber wood blocks were thoroughly washed with distilled water to rehydrate the wood blocks prior to inoculation. The rubber wood blocks were then placed into individual polypropylene bags (14cm x 7cm x 7cm), and sterilized by autoclaving (121°C, 15 p.s.i., 20 min) twice. After cooling to ambient temperature, 10 ml of molten PDA was added into each bag using hand pipetteas as starting nutrient source for *G. boninense*. The PDA gradual solidies and forms a thin layer on the wooden block. Inoculation was performed by inoculating the wood block with 20 mycelial disc (1 cm). The wooden blocks were then incubated for a month at room temperature (25°C ± 2) until use (Sapak et al., 2008b).

6.2.3 Establishment and inoculation of oil palm seedlings

Oil palm seedlings (Dura x Pisifera) were obtained from Applied Agricultural Resources Pte Ltd as 4-leaf stage seedlings grown in coco peat. The endophytes were first inoculated to the seedlings via soil drenching technique (150 ml of inoculum suspension, 2 x 10^7 cfu/ ml). For control treatments, the seedlings were drenched with 150 ml sterile distilled water (SDW). After 2 weeks, the seedlings were uprooted and transferred to larger pots containing 3 kg of soil mixture (3:2:1 v/v/v top soil: peat: sand) and *G. boninense* infected rubber wood block.

In order to esthablish successful infection by *G. boninense* in oil palm, *G. boninense* colonized rubber wood blocks were places in direct contact with the primary root of oil palm. Pots were placed on benches in the greenhouse and watered twice daily.

6.2.4 Biocontrol efficacy of endophytic fungi against *G. boninense* in oil palm seedlings

The quantitative assessment of disease development was performed using the Disease Incidence (DI) percentage at monthly intervals (as formula below. DI denotes the number of seedlings that visually portrayed the features of the disease (chlorosis & necrosis of leaves, with or without sporophore), as reported by Campbell and Madden (1990) and Ili Nadhraan et al., (2015).

$$DI = \frac{number\ of\ seedlings\ infected}{total\ number\ od\ seedlings\ in\ study} \times 100$$

A decrease in DI (Disease incidence), when compared to control, indicates the efficacy of the treatment in controlling the spread of disease. This is performed by gathering and plotting the data regularly, along with the disease progress curve, by employing the AUDPC (Area under the Disease Progress Curve) method, where R refers to the DI value, n denotes the number of evaluation, and ($t_{i+1} - t_i$) indicates the time interval between each time point. After that, the values obtained from AUDPC, which had been based on DSI, were computed using the formula suggested by Shaner and Finner (1977),

$$AUDPC = \sum_{i=1}^{n} [R_{i+1} + R_i][(t_{i+1} - t_i)]$$

This estimation adheres to a scale where 0 = healthy: no internal rot, 1 = 20% rotting of tissues, 2 = 20 to 50% rotting of tissues, 3 = > 50% rotting of tissues, and lastly, 4 = > 90% rotting of tissues. In addition, Disease Severity (DS) for internal symptoms had been

determined based on the number of vivid and observable symptoms derived from "disease rating" of infected seedlings converted to the range of 1 – 4.

$$DS = \frac{\sum(x + y)}{\sum Y + 4} \times 100$$

Where: X refers to the various disease range (0 - 4); Y denotes the number of seedlings that displayed a particular disease range, in which number 4 represents the highest value of a particular disease range. Furthermore, the classes of the disease applied for DSI calculation had been adopted from those suggested by Kok et al., (2013).

6.2.5 Effect of endophytic fungi on plant vigor

The evaluation of endophytic infection (single or mixture) on plant vigour was observed. The vegetative growth was estimated based on plant height, stem diameter, as well as root count and mass. Plant height (cm) and stem diameter (mm) was measured 1 cm above the soil level until the tip of the most mature leaves (Baset, 2010). For stem diameter, this was measured 1 cm above the ground level. At the end of the experiment, the seedlings were uprooted and root count and mass (g) was recorded for each oil palm seedling (López, 2007).

6.2.6 Statistical analysis

The percentages of both DI and DS were analysed by using ANOVA, while the mean values obtained were compared using the Tukey-Kramer multiple comparison test (Honestly Significant Difference, HSD, $P<0.05$). On top of that, the parameters of the vegetative growth for the samples pre-inoculated with endophytes and challenged with and without *G. boninense* had been compared by using the mean values obtained via ANOVA and Tukey-Kramer multiple comparison test (Honestly Significant Difference, HSD, $P<0.05$).

6.3 Results

6.3.1 Biocontrol efficacy of selected endophytic isolates

Disease Incidence (DI) had been examined based on several vivid symptoms, for example, chlorosis and necrosis of leaves. By monitoring oil palm seedlings with disease symptoms, this allows the comparison of epidemic progress of Basal Stem Rot under different treatment of endophytic fungi (single strain and combination treatment). This also allows the prediction of crop loss for *G. boninense* infection after endophytic treatment. Results showed endophytic treated seedlings (single or combination treatment) managed to reduce disease incidence when infected by *G. boninense*. Single endophytic treatment by BTF08 and T2 showed better biocontrol potential when compared to combination treatment (T2+BTF08), with lower disease incidence during the experimental period. As such, the symptoms of the disease were initially recorded in the non-treated samples 3 weeks after they were infected with *G. boninense*, which displayed as DI percentage of 40% (Figures 1 and 3). The other treated samples (BTF08, T2, and BTF08 + T2) did not portray any disease symptoms during the 3rd week. During the 4th weeks of inoculation, the DI for non-treated samples infected with *G. boninense* began to escalate until 60% (Figures 1 and 3). Samples treated with the T2 and BTF08 combination began exhibiting a DI of 20% (Figure 1). The initial disease symptoms were only observed on the 5th week for sample treated with T2; a DI of 20%, whereas that treated with BTF08 displayed disease symptoms in week 6 with a DI of 20% (Figures 1 and 3).

At the end of the experiment (7 weeks after inoculation), the non-treated samples portrayed the highest DI percentage, which was 100% (Figures 1 and 3). On the other hand, All the treated samples exhibited lower DI percentages; combination of T2 + BTF08 (60%), T2 (40%), and BTF08 (20%) (Figures 1 and 3). Furthermore, the symptoms of the infection reflected yellowing of fronds and necrosis. Meanwhile, desiccation of leaves was observed

beginning from the matured leaves and progressed towards younger leaves. White mycelia had been noted at the roots of the infected plants, along with some white fungal mass. Retarded growth was also observed for the non-treated seedlings. Nonetheless, the growth of seedlings treated with endophytic fungi had not been hindered that suggests better suppression of disease spread with endophytic fungi treatment.

Results showed low disease severity discovered in samples treated with endophytic fungi with slow progression of *G. boninense* infection in oil palm. The treatment of endophytic fungi (either single or combination treatment) generated improved effect upon BSR severity (lower DSI value), when compared to untreated seedlings infected with *G. boninense*. Results showed simgle endophytic treatment (T2 and BTF08) have better biocontrol potential when compared to combination treatment (T2+BTF08). At the initial stage of the experiment, those untreated and infected samples (positive control) recorded the highest DSI value of 50 (Figure 2). Samples treated with endophytic fungi (either single or combined treatment) exhibited lower DSI, where the T2+BTF08 combination had the highest DSI value among all other treatments at 15, followed by T2 and BTF08 treatments at values 10 and 5, respectively (Figure 2). This study projected that single strain treatment with BTF08 and T2 displayed better efficacy, in comparison to the mixed treatment of T2+BTF08 to reduce the development of disease severity in oil palm seedlings.

6.3.2 Area under the Disease Progress Curve (AUDPC)

Results showed endophytic treated seedling (single strain or combination treatment) showed promising tolerance to *G. boninense* infection with reduced disease symptoms. Single strain treated (T2 or BTF08) seedling showed better tolerance to *G. boninense* when compared to combination treatment (T2+BTF08). At the end of the 7^{th} week of experimental period, samples infected with *G. boninense* (without endophytic inoculation or control sample) displayed the highest AUDPC of 95 units^2. While, samples treated with endophytic fungi

exhibited lower AUDPC values. In precise, the inoculation of single strains (BTF08 or T2) had been proven to be better at decreasing the severity of the BSR disease caused by *G. boninense*, in comparison to treatment with mixed endophytic fungi (T2+BTF08), which exhibited an AUDPC value of 15 units^2 (Table 1). However, samples that were treated with BTF08 displayed lower AUDPC value, when compared to that of T2, which had been 7.5 and 15 units^2, respectively (Table 1). Generally, lower AUDPC value points out promising effect of endophytic treatment to serve as a biocontrol agent in suppressing the symptoms of the disease due to infection of *G. boninense*. Thus, samples treated with BTF08 emerged as the most effective treatment that inhibited the development of disease caused by the pathogenic *G. boninense*.

6.3.3 Vegetative growth of oil palm seedlings

Results showed samples treated with endophytic fungi displayed increment in the height of the plant. Single strain (T2 or BTF08) treated seedling performed better when compared to combination treatment (T2+BTF08) in terms of vegetative growth (stem diameter, leaf heigt, root count and root mass). At the 7th experimental week, increment in plant height was noted to be significant for BTF08 and T2 single strain treatment, in comparison to those untreated samples infected with pathogenic *G. boninense*. No variation was found for samples that were given mixed endophytic (T2+BTF08) treatment for 7 weeks, when compared to those uninfected control (Figure 3). Observation of stem diameter, which also projects the growth of a plant, had been made in this study so as to compare between samples treated with endophytic fungi and those untreated. The BTF08 treatment was found to enhance the growth of stem in the sample at the diameter of the stem was recorded at 3.68 cm (Figure 4). This was followed by the single strain treatment of T2 and mixed treatment of T2+BTF08, with stem diameters at 3.48 cm and 3.26 cm, respectively. In addition, the stem growth of endophytic-treated seedling differed significantly from that untreated and uninfected control

(2.80 cm), as well as untreated but infected control (2.42 cm) (Figure 4). This showed that apart from possessing biocontrol potential, the inoculation of either single or mix-treatment of endophytic fungi displayed stem-promoting attribute in oil palm seedlings.

The quantity of root hair also increased for samples treated with endophytic BTF08 and T2, hence indicating enhance growth. It was noted that the average number of root hair found in endophytic-treated seedlings had been significantly higher, in comparison to samples that were untreated (114 root hair strands), as well as untreated and uninfected seedlings (149 root hair strands), whereas BTF08 and T2 recorded an average root hair count of 191 and 188 strands, respectively (Figure 5). Results showed mixed treatment of T2+ BTF08 failed in stimulating root growth as the average root hair count was 138 strands (Figure 5). Furthermore, the highest root mass was recorded in samples inoculated with single T2 and BTF08 isolates, with mean weights of 0.592 g and 0.600 g, respectively, after 7 weeks of incubation period (Figure 6). Untreated samples infected with *G. boninense* displayed rather slow progress for root growth with root mass of 0.498 g (Figure 6). Meanwhile, the combined treatment of isolates T2 and BTF08 (0.53g) exhibited insignificant variation in root mass, in comparison to control (0.53 g) (Figure 6).

6.4 Discussion

This study showed single strain treatment (T2 or BTF08) have better biocontrol potential (lowering disease incidence and promoting growth of seedlings) when compared to combination treatment (T2+BTF08). This may be due to competition between isolate T2 and BTF08. These 2 isolate may exert antagonistic action against each other, whereby T2 has mycoparasitic properties and BTF08 produces antifungal metabolites. When both isolate (T2+BTF08) were inoculated into oil palm seedlings, this may also shift the balance of nutrient supply of host plants hence hindering plant growth. Research was done by Chaves et al. (2009) on combination treatment using biocontrol agent of different species with varied

biocontrol mechanism for the control of plant pathogen *Radopholus similis* in banana plants. Some combinations of biocontrol agent provided no increased or decreased plant growth, and did not reduced disease severity caused by plant pathogen *R. similis*. Certain treatments increased root mass but have no biocontrol effect against *R. similis*. For example, combined inoculations of biocontrol agents, which is a non-pathogenic *F. oxysporum* and *Pseudomonas* increased root mass but exerted low suppression of *R. similis*. Effective combination of biocontrol agent (*Trichoderma atroviride* and *Pseudomonas*) provides high biocontrol potential in controlling *R. similis*. Several researches have recorded the effect of combining biocontrol agents against plant pathogens (Zum et al., 2006). Although some combinations may have negative effect on host plant (Mayer and Roberts, 1999; Chaves et al., 2009), many have resulted in increased biocontrol potential (Mejía at al., 2008; Martinuz et al., 2012

The results retrieved for combined treatment of both T2 and BTF08 endophytic isolates failed to enhance the growth of plant, especially when compared to single endophytic treatment. This result could be due to the competition and the inhibition that took place between both endophytic fungi of varied genus. The results obtained in this study contradicted with those reported in a prior research work that looked into combined fungal endophyte treatment, which consisted of *T. harzianum* and *T. Atroviride* isolates (Vinale et al., 2004). Moreover, their evaluation demonstrated that the combined treatment suppressed the disease symptoms in tomato caused by pathogenic *Fusarium*, besides promoting the growth of the host plant (Vinale et al., 2004). The success of inhibiting the disease that affected tomato could be due to the strains that derived from identical genus (*Trichoderma*), which minimised both competition and inhibition. The findings portrayed that the endophytic fungi could have promoted the inhibition of pathogen growth, hence impeding *G. boninense* penetration into the vascular system. Varied inhibitory mechanisms of endophytic fungi had been noted from the investigation in this study. For example, isolate T2 (*T. asperellum*)

displayed competitive exclusion for nutrients and managed to overgrow *G. boninense* when cultured together (Ting & Jioe, 2016). Isolate BTF08, which is also known as *P. citrinum*, also had been found to suppress the growth of *G. boninense* by generating several inhibitory compounds, such as citrinin (Wakana et al., 2006). In this experiement, although both T2 and BTF08 are recognized as biocontrol agents, more research is needed to understand their mutual interactions between endophytes and their interactions with oil palm.

This study also revealed that the values of DI, DSI, and AUDPC of endophyte-treated seedlings had been lower than untreated and *G. boninense* infected seedling. Hence, both endophytic fungi of BTF08 and T2 exhibited exceptional potential to suppress both the development of BSR disease and the growth of pathogenic *G. boninense*. This is reflected from the decrease in DI% and DSI among samples treated with the selected endophytic isolates after they were inoculated with *G. boninense* for as long as 7 weeks. In fact, the untreated samples infected with *G. boninense* exemplified severe symptoms of the disease, in which the DSI was recorded at 50 after 7 weeks of inoculation, indicating a fast infection rate within just 7 weeks, along with severe symptoms observed, for instance, necrosis. Nevertheless, samples that were treated with T2 and BTF08 endophytic fungi displayed lower DSI that ranged only between 5 and 15. As such, the findings showed that endophytic fungi are indeed effective disease suppressor and excellent to serve as biocontrol agents against the pathogenic *G. boninense* in oil palms.

The vegetative responses of oil palm seedlings towards endophytic fungi inoculants that consisted of a variety of isolates species had been investigated in this study for both single and mixed treatments. Study showed single strain inoculation with endophyte T2 or BTF08 have better biocontrol potential. This study also illustrated that endophytic fungi could actually significantly enhance the vegetative growth of oil palm seedlings in terms of plant height, stem diameter, root mass and root hair count. In fact, particular endophytic fungi

were discovered to boost plant growth, trigger plant defence mechanisms, and induce tolerance towards fungal pathogen that caused physical damage (Arnold et al., 2003; Varma et al., 1999; Waller et al., 2005; Waqas et al., 2012). T2 proved to have some plant growth-promoting compounds, for instance, phosphate solubilisation, 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, auxin, and siderophore (Qi & Zhao, 2013). Other than that, *P. citrinum* had been found to possess phosphate solubilizing potential, besides producing plant hormones (indole acetic acid and gibberellins), thus enhancing soil fertility and plant growth (Khan et al., 2008; Yadav et al., 2011). As such, it had been postulated that the single strain inoculations of T2 and BTF08 endophytic fungi may promote enhanced interaction with oil palm seedlings, which conjure positive outcomes for both parties.

6.5 Conclusion

This study had successfully proven that T2 and BTF08 endophytic fungi do possess the potential to serve as biocontrol agents against BSR disease in oil palm, primarily to decrease the symptoms of disease among infected oil palm seedlings. In addition, single isolate application of either T2 or BTF08 displayed better growth of oil palm seedlings, when compared to mixed treatment of T2+BTF08. As such, T2 and BTF08 isolates could be further developed to function as biofungicides. With that, a field study could be conducted so as to ascertain their efficacy *in vivo*, as well as to determine the effective dosage for optimum biocontrol activities and growth-promoting effects.

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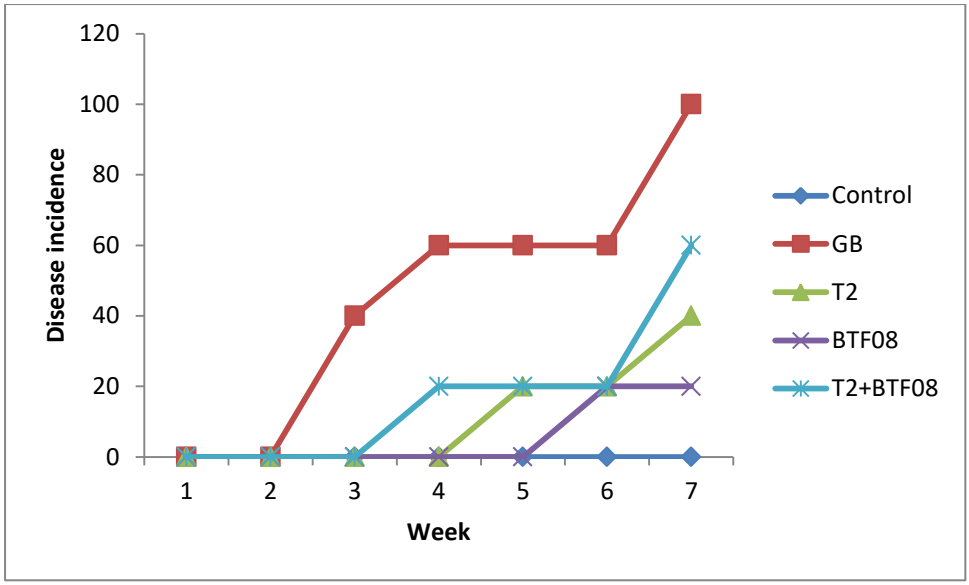
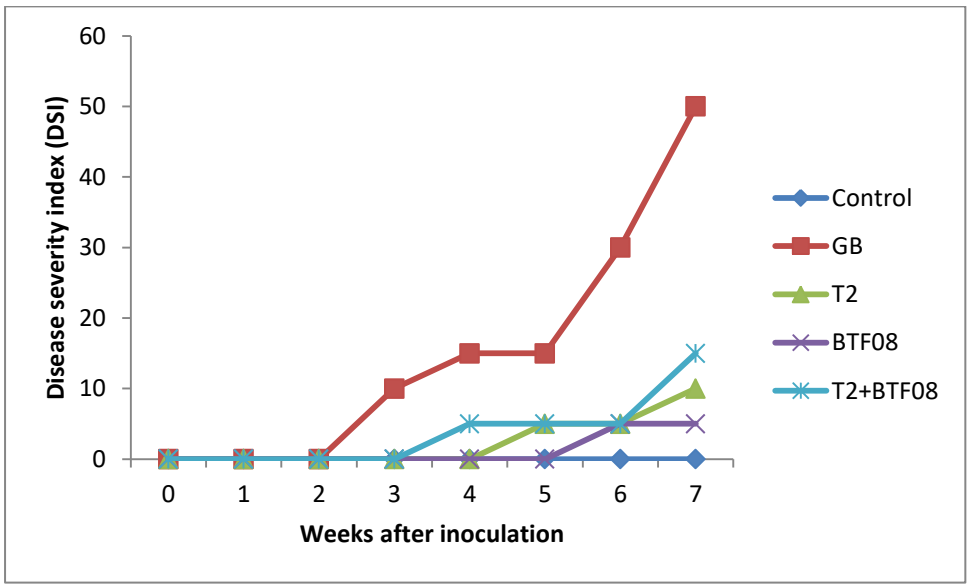


Figure 1.



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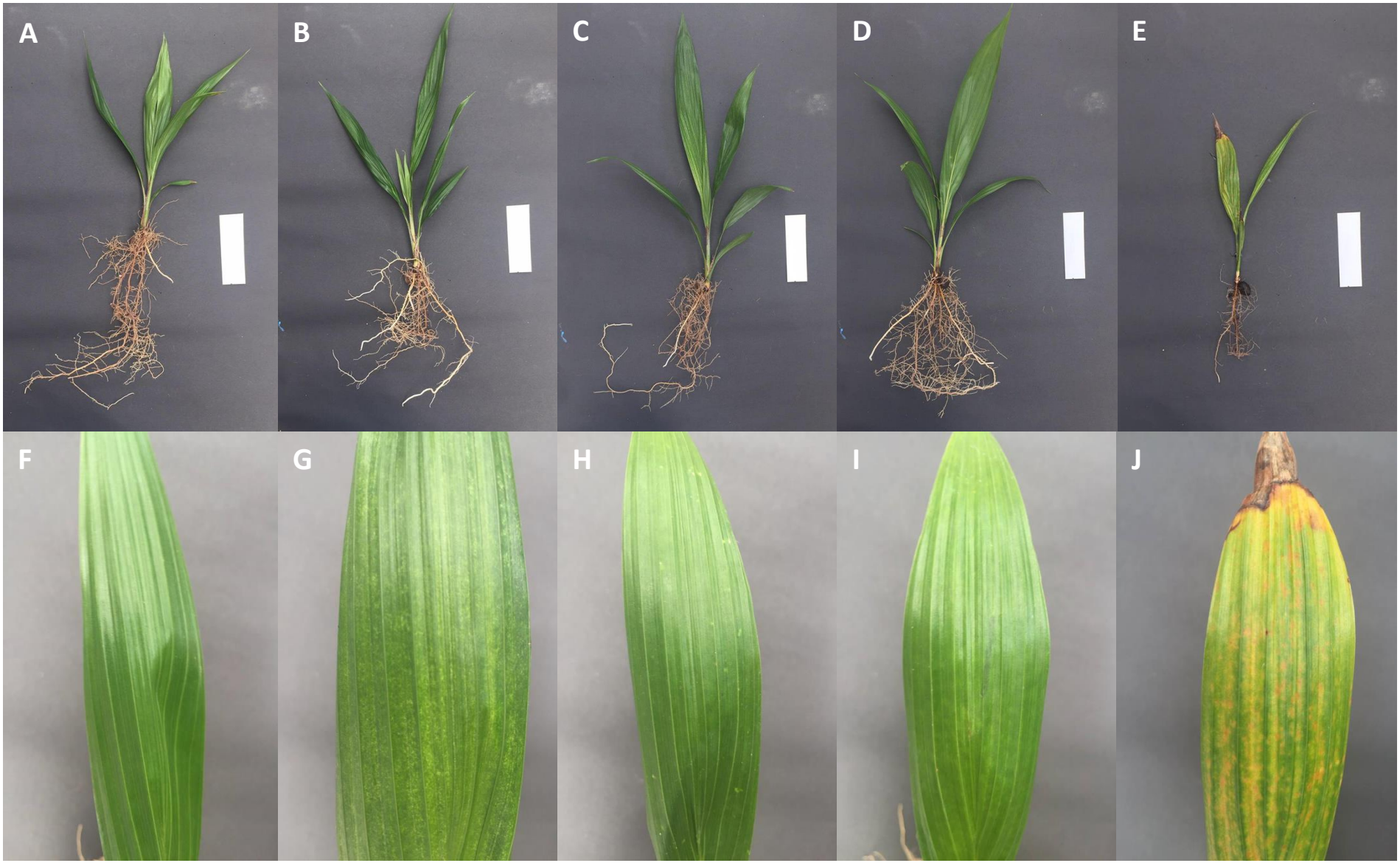

Figure 3.

Figure 4.

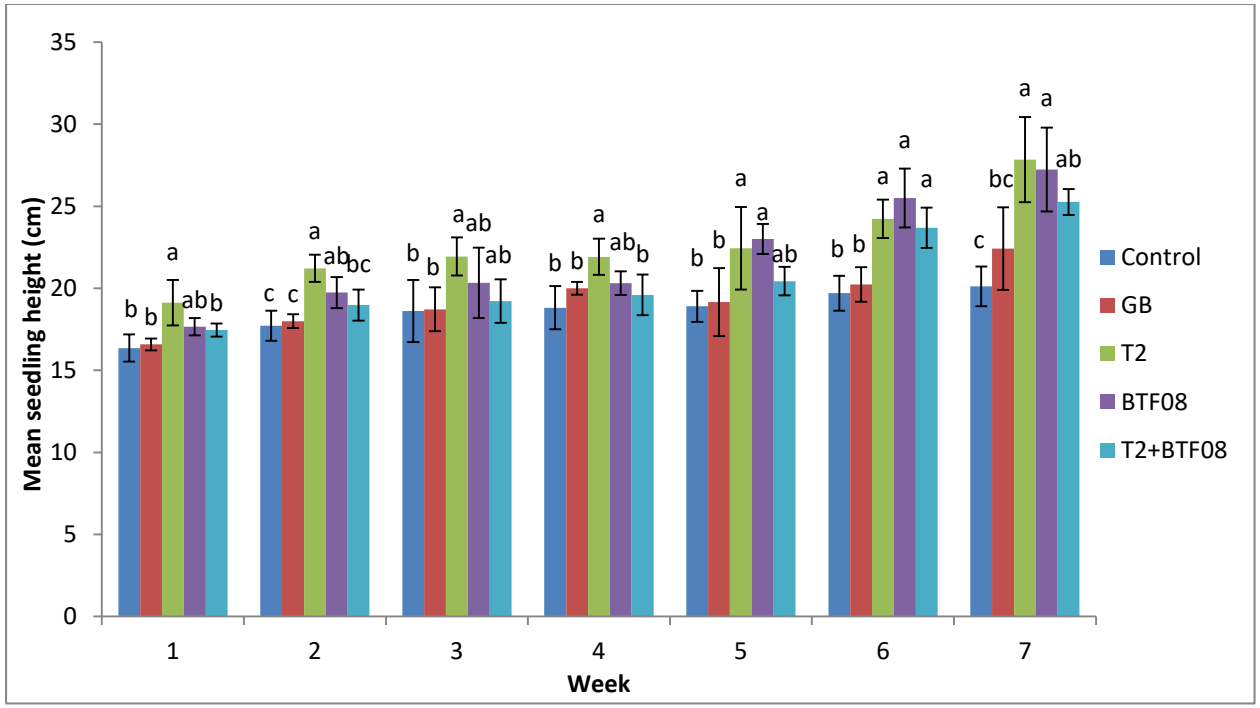
Mean seedling height (cm)
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
Week
Control
GB
T2
BTF08
T2+BTF08

Figure 5.

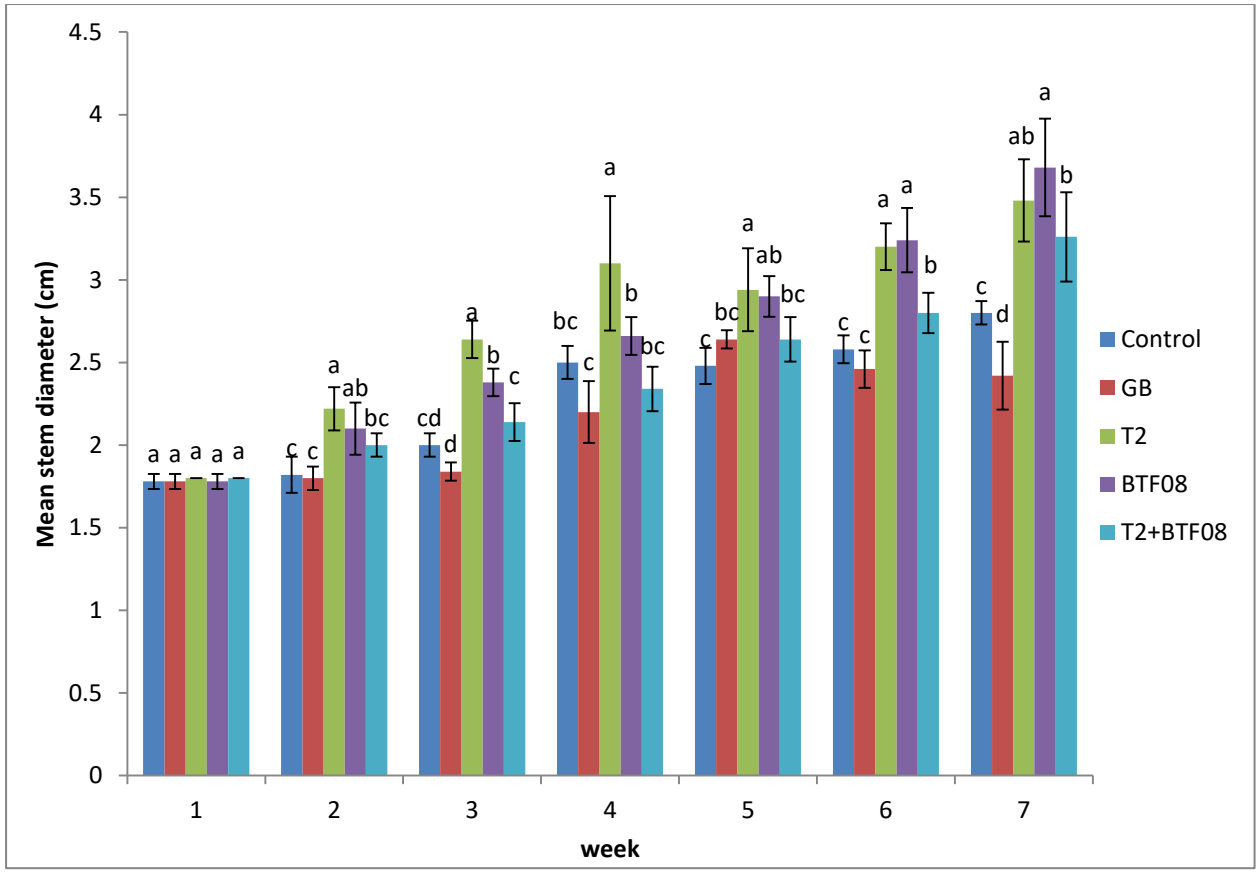
Mean stem diameter (cm)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
week
a a a a a
c c a ab bc
cd d a b c
bc c a b bc
c bc a ab bc
c c a a b
c d a ab b
Control
GB
T2
BTF08
T2+BTF08

Figure 6.

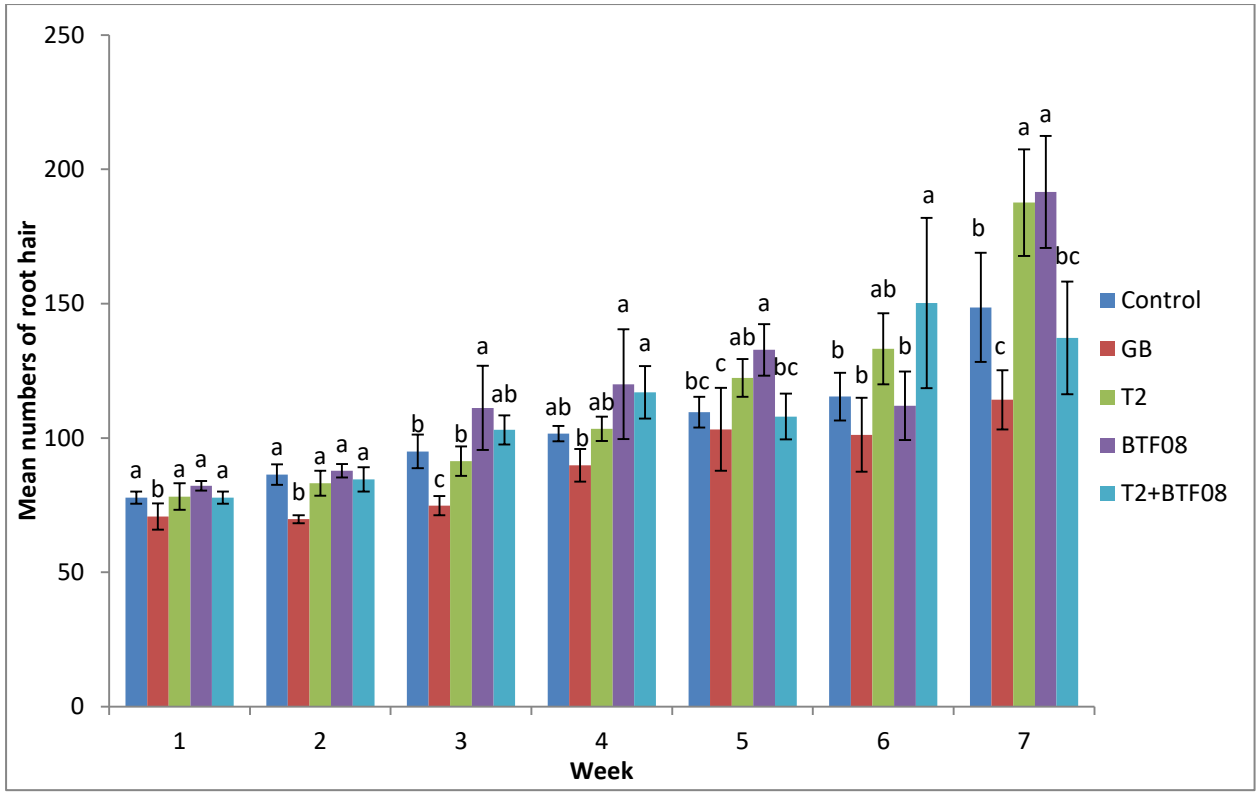
Mean numbers of root hair
250
200
150
100
50
0
1
2
3
4
5
6
7
Week
Control
GB
T2
BTF08
T2+BTF08

Figure 7.

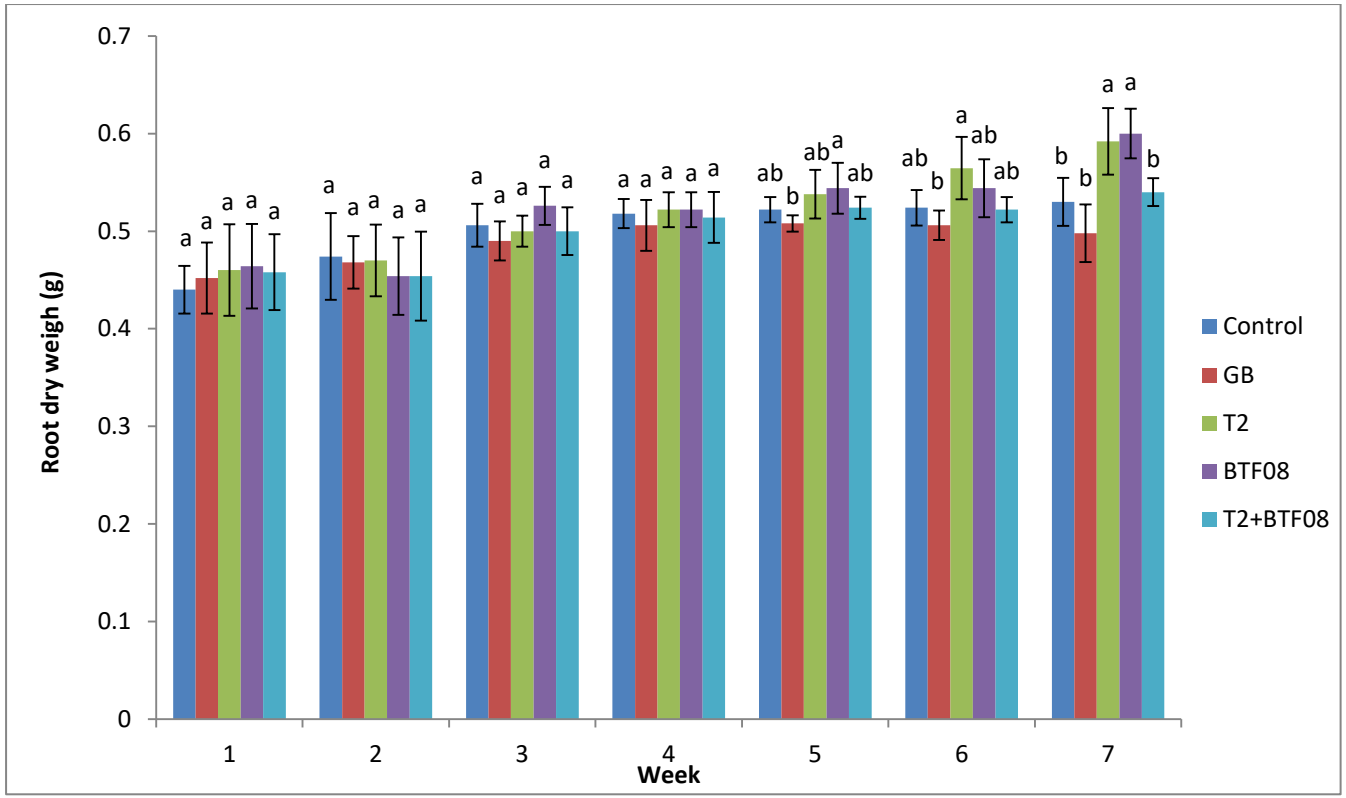
Root dry weigh (g)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6
7
Week
Control
GB
T2
BTF08
T2+BTF08

Figure 8.

Figure caption

Figure 1: Disease Incidence of oil palm seedling after *G.boninense* artificial inoculation

Figure 2: Disease Severity Index of oil palm seedling after *G.boninense* artificial inoculation.

Figure 3: **The effect of biocontrol agents' formulation in controlling Basal Stem Rot disease incidence in oil palm seedlings**. Each photo (A-E) shows different conditions of oil palm seedlings with their corresponding leaf conditions (F-J) in the presence of different types of endophyte. (A and F) are T2 treated seedlings. (B and G) are BTF08 treated seedlings. (C and H) reflect T2+BTF08 treated seedlings. (D and I) are control seedlings. (E and J) are untreated seedlings. The white bar represents 10cm.

Figure 4: **Growth of G. boninense on oil palm seedling and its effect towards the roots**. (A) shows degradation of root by G. boninense. (B) shows G. boninense mycelial masses growing on root. (C) shows G. boninense fruiting body emerging from the rubber wood block

Figure 5: **Vegetative growth of oil palm seedlings by height.** Means with the same letters between oil palm seedlings treated with endophytic isolates are not significantly different at $HSD_{(0.05)}$. Bars indicate standard deviation of means (±SD).

Figure 6: **Vegetative growth of oil palm seedlings by stem diameter.** Means with the same letters between oil palm seedlings treated with endophytic isolates are not significantly different at $HSD_{(0.05)}$. Bars indicate standard deviation of means (±SD).

Figure 7: **Vegetative growth of oil palm seedlings by amount of root.** Means with the same letters between oil palm seedlings treated with endophytic isolates are not significantly different at $HSD_{(0.05)}$. Bars indicate standard deviation of means (±SD).

Figure 8: **Vegetative growth of oil palm seedlings by mass of root.** Means with the same letters between oil palm seedlings treated with endophytic isolates are not significantly different at $HSD_{(0.05)}$. Bars indicate standard deviation of means (±SD).

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Table 1: Effect of endophytes on BSR development onto oil palm seedlings after 7 weeks

Treatment	control	GB	T2	BTF08	T2+BTF08
AUDPC (unit^2)	0	95	15	7.5	22.5

AUCPC: Area under disease progressive curve (based on figure 2)

Chapter 7

General discussion and conclusion

This study has proven that endophytic fungi could serve as potential biocontrol agents against *G. boninense* in oil palm. The selected endophytic isolates tested (BTF07, BTF08, MIF01, WAA02, and T2) exerted positive antagonistic activities against *G. boninense* with isolate BTF07 appearing to possess good antifungal activities towards *G. boninense* (PIRG value of 49.55%), followed by T2, WAA02, MIF01, and BTF08 with 47.75, 39.64, 36.04, and 13.51%, respectively. A host compatibility test had been carried out, which displayed all the tested endophytic isolates did portray exceptional compatibility with oil palm without harming its host. Isolate BTF08 was found to exert the best growth-promoting effect towards the oil palm host. Endophytes BTF07, T2, WAA02 and MIF01 are potential candidates to be developed as biocontrol agents as they showed high inhibitory activity against *G. boninense*. Previous study by Chow et al. (2016) proved the presence of endophytes (T2, WAA02 and BTF08) via plating assay within 7 days after inoculation. Detection of fungal DNA was done via Polymerase Chain Reaction (PCR) to identify the isolates from the plant tissues in root, stem and leaf tissues. An ergosterol assay was also performed to assess proliferation rate of these endophytes (T2, Waa02 and BTF08). The endophytes were found to be fast plant (oil palm) colonizer, but the minor changes in ergosterol level suggested the endophytes T2, WAA02 and BTF08 colonized the oil palm upon inoculation with a slower rate of proliferation. Endophytic isolates (WAA02, T2, BT0F8) were found to have similar colonization potential with *G. boninense*, with the ability to colonize roots to leaves within 7 days after inoculation which may suggest endophytes could be introduced prior to contact with *G. boninense*, and *G. boninense* infection may be halted via competitive exclusion for space and nutrients

Lignification in palm reflects a significant mechanism for plant defence to inhibit harm caused by pathogens. The selected endophytic isolates (BTF07, BTF08, MIF01, WAA02, and T2) had successfully induced higher production of lignin. The pathogenic *G.*

boninense failed in inducing higher lignin content among the infected ramets. It is a fact that increment in lignification level functions as a barrier against further pathogenic infection, which could lessen or even halt further infections. Eventually, this signifies a closer bond between endophyte and its host, verifying the mutual correlation between endophytes and host.

It was revealed that the endophytic fungi produced volatile compounds, which possessed biocontrol potential against pathogenic *G. boninense.* Double plate test displayed that the volatile compounds produced by endophytic fungi (BTF07, BTF08, MIF01, WAA02, and T2) exerted antagonistic activities towards *G. boninense*. The GC-MS analysis exhibited a variety of compounds relevant in double plate test generated by both *G. boninense* and endophytes. These compounds exhibited various antagonistic effects towards the pathogen. For instance, the compound 3-Chloro-N-[2-methyl-4(3H)-oxo-3-quinazolinyl]-2-thianaphthene carboxamide or a quinazoline derivative had been produced in large quantities in isolate T2, in which quinazoline may serve as a fungicide. Meanwhile, 1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl), and 1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl) had been produced in abundance by isolate WAA02, which had been classified as terpenes. Terpene is produced by fungi when they are starved from nutrients, which is commonly noted at the later growth stage of fungi (Schmidt et al., 2016). In this study, the terpenes produced had been triggered to eradicate potential pathogens, such as *G. boninense*. Additionally, fungal terpenes have the capability to generate a defence system to inhibit fungal pathogens, besides acting as a signalling molecule (Buśko et al., 2014; Martins et al., 2007). Terpenes may also decrease the development of fungal pathogens, especially when the host plant is infiltrated (Schmidt et al., 2016). Thus, volatile compounds generated by the selected endophytic fungi in this study have exerted good potential as antifungal agents against *G. boninense*. T2 emerged as exceptional in generating non-volatile compounds, such as quinazoline derivative,

which could serve as antifungal agents towards *G. boninense*. T2 could function as a biocontrol agent against pathogen by producing antifungal non-volatile compounds.

Endophytic fungi also generated non-volatile antifungal metabolites against *G. boninense*. Apart from displaying excellent growth-promoting attribute in oil palm, BTF08 (*P. citrinum*) also generated non-volatile metabolite with antagonistic properties against *G. boninense*. The active non-volatile compound produced by BTF08 is known as citrinin. Citrinin refers to a potent antifungal compound without any effect upon cell permeability, but only targets cellular respiration via mitochondria by hampering succinate oxidase and NADH oxidase (Haraguchi et al., 1987). As BTF08 isolate had displayed the ability to enhance plant growth and to generate potent inhibitory compound against *G. boninense*, it is definitely beneficial to inoculate this isolate into the plantation as biocontrol agent for its pathogen-inhibiting activities. As T2 and BTF08 endophytic fungi had exhibited the capability of generating both volatile and non-volatile compounds, respectively, against *G. boninense*, the effect of combining these two isolates had been investigated in treating oil palm ramets infected with *G. boninense*. As a result, it was discovered that the usage of a single species of endophytic fungi displayed better biocontrol ability against *G. boninense*, in comparison to the combination of T2 and BTF08 isolates. The findings showed that the single strain treatment of isolates BTF08 and T2 had been more effective in promoting growth, when compared to the T2+BTF08 combination treatment. BTF08 had exerted better plant-protecting potential, in comparison to isolate T2, in terms of disease inhibition, which had been measured by employing the AUDPC. Samples treated with single isolate of either T2 or BTF08 demonstrated better vegetative growth, evidenced by the increment in root hair strands, root mass, bigger stem diameter, and lengthier seedling height. One possible justification of such scenario is the intraspecific interaction that might have taken place, such as the production of mycotoxin among fungal species and competition. The investigation

performed on citrinin (a mycotoxin) showed antagonistic effect against other fungi (Speijers & Speijers, 2004). In fact, mycotoxins can gather and offer either synergistic, additive or antagonistic effect upon each other and lead to precipitation of numerous symptoms towards its host (Speijers & Speijers, 2004). The variation in the effect of the combined treatment could also be due to the competition that took place between two endophytes found within the same host. In comparison to oil palm seedling infected by only one endophyte, the combination treatment required the two endophytes to share a common host, thus suggesting competition between the two endophytes for both nutrients and space. However, the analysis on mutualism between the endophytes (T2 and BTF08 isolates) has yet to be performed. Hence, one can presume that these endophytes possess an antagonistic correlation. The competition between these two endophytes had been further strained due to the existence of *G. boninense*, which forced the host to provide balanced nutrients for the endophytes while exerting a defence barrier against *G. boninense*. Thus, the single strain application of endophytic fungi is more appropriate for application onto oil palm to serve as biocontrol agents against *G. boninense*.

This study suggests that some mechanisms had been exerted by the endophytic fungi so as to control the pathogenic *G. boninense* in oil palm. In fact, the biocontrol of *G. boninense* via antibiosis through secretion of both volatile and non-volatile antifungal compounds appear to be the major mechanism, mainly because the selected isolates (T2 and BTF08) displayed biocontrol activities in soil condition. These non-specific metabolites derived from endophytic fungi might have consisted of lytic agent or enzymes. Therefore, more investigations should be conducted to look into the nature of biocontrol within these isolates (T2 and BTF08). Application of endophytic fungi through the technique of soil drenching resulted in the highest rate of healthy seedlings. T2 and BTF08 demonstrated their endophytic attributes in oil palm, in which the oil palm tissues were colonized without

harming the host. This endophytic nature has emerged as effective biocontrol measure against *G. boninense*, primarily due to their capability of colonizing plant tissues and exerting antifungal properties. The combined mode of action (antibiosis and competition for niche and nutrient) employed by these endophytic fungi are highly adequate to inhibit the pathogenic *G. boninese*. Besides, endophytic fungi have been proven to significantly improve vegetative growth of oil palm seedlings in terms of plant height, stem diameter root mass and root count. Such enhancement of growth by T2 and BTF08 fungi endophytes could potentially contribute to plant protection against the disease spread caused by *G. boninense*.

This study has demonstrated the potential of endophytic fungi to function as biocontrol agents against the *G. boninense* pathogen in oil palm. BTF08 isolate had been chosen as the most suitable endophyte in hampering the BSR caused by *G. boninense*, with properties such as plant growth promotion, induced lignification, production of citrinin, good biocontrol potential (with low AUDPC). Further studies have to be conducted in order to investigate the primary function of secondary metabolites found in other endophytes in infected oil palms. This, eventually, may provide fresh opportunities for new biocontrol agent that may not only impede the spread of BSR disease, but also to provide effective growth-promoting factors to infected oil palms. The application of isolated secondary metabolites may also be introduced onto field work upon determining and verifying the precise and exact role of each metabolite used.

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Appendix

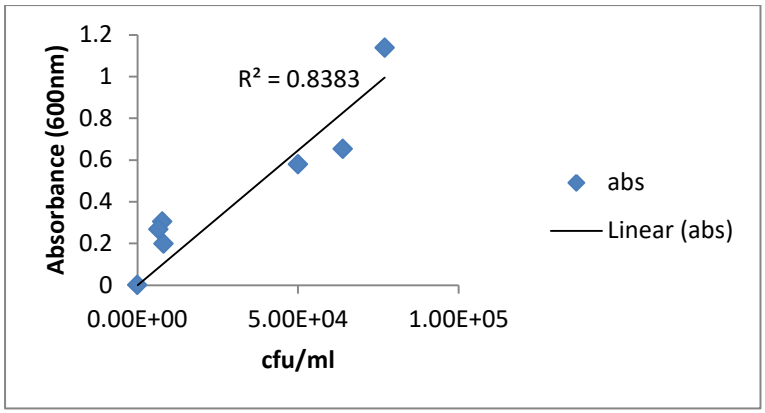
Appendix I

Characterizing antagonistic activities and host compatibility (via simple endophyte-calli test) of endophytes as biocontrol agents of Ganoderma boninense

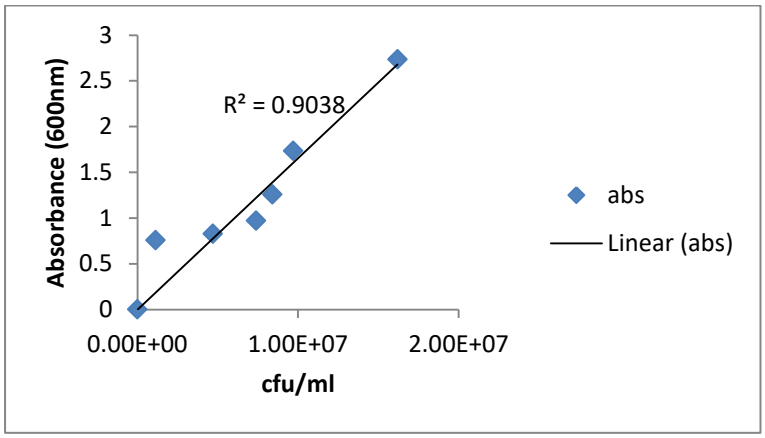
The following appendix contains:

1. Standard curve for endophytic and pathogen isolate
2. Standard curve for lignin
3. Results of One-way ANOVA with Tukey's post hoc test for dual culture assay
4. Results of T-test comparing endophytic dual culture with calli and monoculture control
5. Result of One-way ANOVA with Tukey's post hoc test for calli growth for all treatments

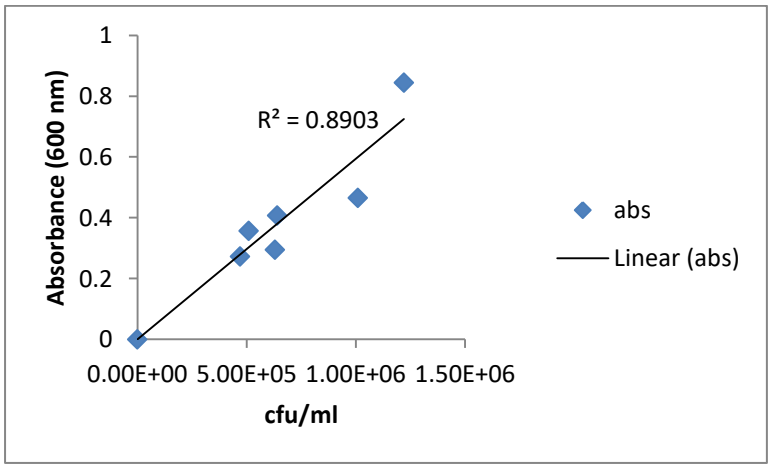
Appendix 1. Standard curve for *G. boninense*



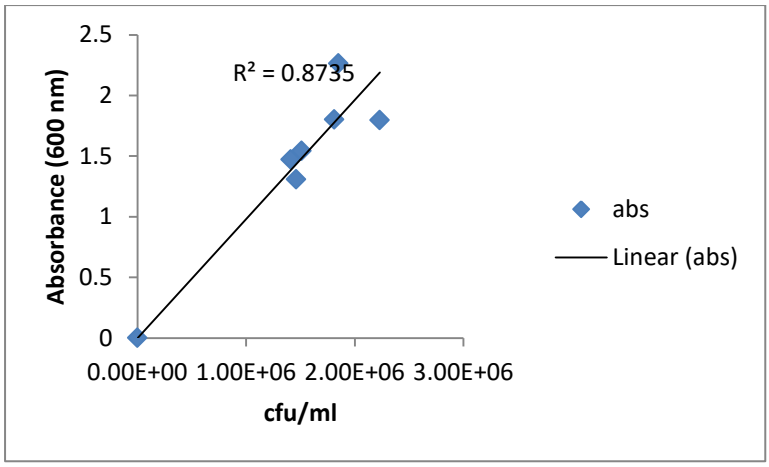
Appendix 2. Standard curve for T2



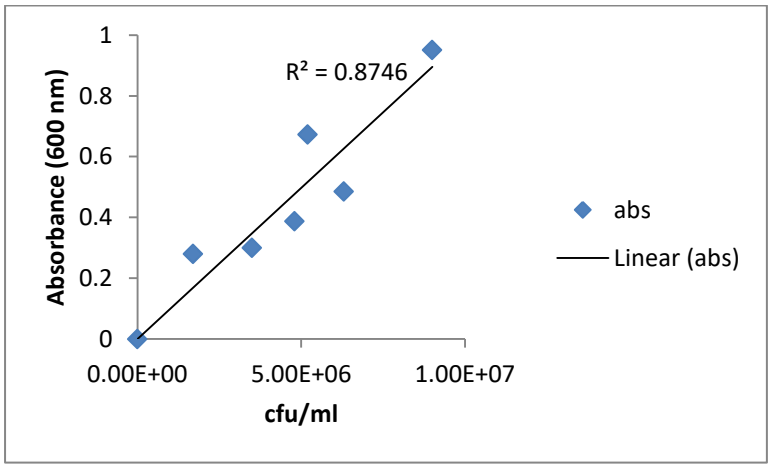
Appendix 3. Standard curve for WAA02



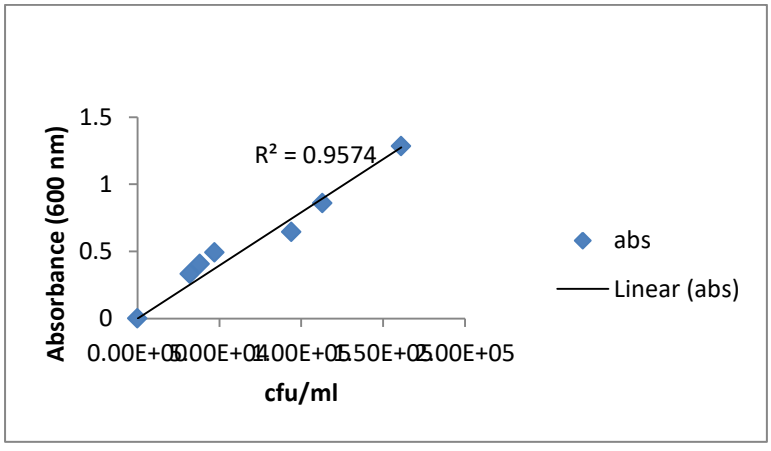
Appendix 4. Standard curve for MIF01



Appendix 5. Standard curve for BTF08



Appendix 6. Standard curve for BTF07



Appendix 7. One-way ANOVA for dual culture assay

Descriptives

PIRG (Percentage Inhibition of Radial Growth)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
T2	3	47.7487	1.56118	.90135	43.8705	51.6268	45.95	48.65
WAA02	3	39.6433	5.62610	3.24823	25.6673	53.6193	35.14	45.95
MIF01	3	36.0367	4.12868	2.38370	25.7804	46.2929	32.43	40.54
BTF08	3	13.5133	5.40500	3.12058	.0866	26.9401	8.11	18.92
BTF07	3	49.5467	10.92718	6.30881	22.4021	76.6913	37.83	59.46
Total	15	37.2977	14.38166	3.71333	29.3334	45.2620	8.11	59.46

ANOVA

PIRG (Percentage Inhibition of Radial Growth)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2496.144	4	624.036	15.620	.000
Within Groups	399.507	10	39.951		
Total	2895.651	14			

PIRG (Percentage Inhibition of Radial Growth)

Tukey HSD

Fungi Isolates	N	Subset for alpha = 0.05	
		1	2
BTF08	3	13.5133	
MIF01	3		36.0367
WAA02	3		39.6433
T2	3		47.7487
BTF07	3		49.5467
Sig.		1.000	.140

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 8. T-test comparing BTF08 dual culture with calli and monoculture control

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Treatment	2.3633	3	.23094	.13333
	Control	2.1000	3	.17321	.10000

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Treatment & Control	3	1.000	.000

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Treatment - Control	.26333	.05774	.03333	.11991	.40676	7.900	2	.016

Appendix 9. T-test comparing BTF07 dual culture with calli and monoculture control

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Treatment	2.4750	3	.13919	.08036
	Control	2.0417	3	.18764	.10833

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Treatment & Control	3	-.993	.075

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Treatment - Control	.43333	.32628	.18838	-.37719	1.24386	2.300	2	.148

Appendix 10. T-test comparing WAA02 dual culture with calli and monoculture control

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Treatment	2.5500	3	.08660	.05000
	Control	2.4333	3	.16073	.09280

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Treatment & Control	3	.359	.766

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Treatment - Control	.11667	.15275	.08819	-.26279	.49612	1.323	2	.317

Appendix 11. T-test comparing T2 dual culture with calli and monoculture control

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Treatment	2.3750	3	.18875	.10897
	Control	2.2750	3	.10897	.06292

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Treatment & Control	3	.866	.333

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Treatment - Control	.10000	.10897	.06292	-.17070	.37070	1.589	2	.253

Appendix 12. T-test comparing MIF01 dual culture with calli and monoculture control

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Treatment	2.4833	3	.06292	.03632
	Control	2.4667	3	.05204	.03005

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Treatment & Control	3	.795	.415

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Treatment - Control	.01667	.03819	.02205	-.07820	.11153	.756	2	.529

Appendix 13. T-test comparing GB dual culture with calli and monoculture control

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	Treatment	2.3167	3	.15069	.08700
	Control	2.1500	3	.13919	.08036

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	Treatment & Control	3	.968	.160

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Treatment - Control	.16667	.03819	.02205	.07180	.26153	7.559	2	.017

Appendix 14. One-way ANOVA for calli growth for all treatments

Descriptives

Weight

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
BTF07	3	1007.0000	3.00000	1.73205	999.5476	1014.4524	1004.00	1010.00
BTF08	3	1011.6667	1.15470	.66667	1008.7982	1014.5351	1011.00	1013.00
WAA02	3	1003.6667	.57735	.33333	1002.2324	1005.1009	1003.00	1004.00
MIF01	3	1000.6667	.57735	.33333	999.2324	1002.1009	1000.00	1001.00
T2	3	1000.0000	.00000	.00000	1000.0000	1000.0000	1000.00	1000.00
GB	3	1007.6667	4.04145	2.33333	997.6271	1017.7062	1004.00	1012.00
Control	3	1007.3333	2.08167	1.20185	1002.1622	1012.5045	1005.00	1009.00
Total	21	1005.4286	4.35398	.95012	1003.4467	1007.4105	1000.00	1013.00

ANOVA

Weight

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	315.810	6	52.635	11.635	.000
Within Groups	63.333	14	4.524		
Total	379.143	20			

Weight of calli

	Treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	T2	3	1000.0000		
	MIF01	3	1000.6667		
	WAA02	3	1003.6667	1003.6667	
	BTF07	3		1007.0000	1007.0000
	Control	3		1007.3333	1007.3333
	GB	3		1007.6667	1007.6667
	BTF08	3			1011.6667
	Sig.		.397	.307	.172

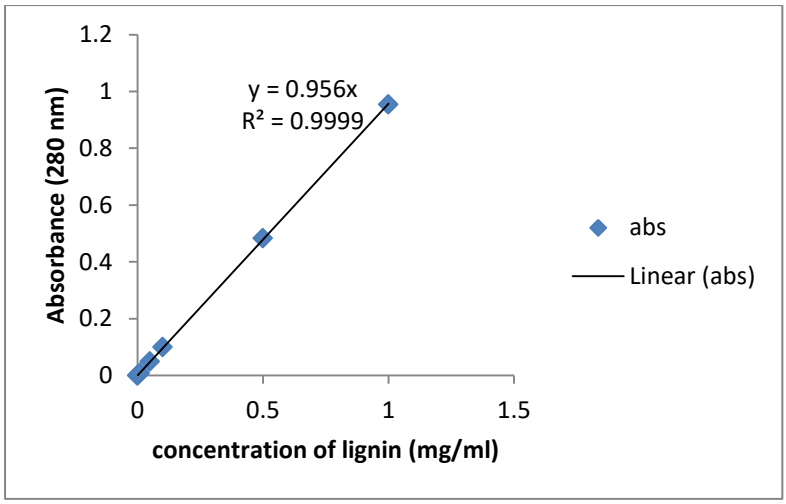
Appendix II

Comparing lignification in oil palm ramets elucidated by endophytic and pathogenic infection

The following appendix contains:

1. The standard cure for lignin content
2. The results of One-way ANOVA with Tukey's post hoc test comparing lignin content in oil palm after different treatment of endophytic fungi

Appendix 15. Standard curve for lignin content



Appendix 16. One-way ANOVA lignin content in oil palm for all treatments

Descriptives

Lignin

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
BTF07	3	379.4748	23.06847	13.31859	322.1695	436.7800	356.75	402.87
BTF08	3	348.0612	18.12948	10.46706	303.0251	393.0973	331.01	367.11
WAA02	3	347.4655	24.35327	14.06037	286.9686	407.9624	322.63	371.30
MIF01	3	332.7063	21.66401	12.50772	278.8899	386.5227	312.30	355.44
T2	3	358.2282	26.02200	15.02381	293.5859	422.8704	332.37	384.42
GB	3	280.9886	3.48081	2.00965	272.3418	289.6354	278.66	284.99
Control	3	276.2237	2.13341	1.23173	270.9240	281.5234	274.28	278.50
Total	21	331.8783	40.42988	8.82252	313.4749	350.2818	274.28	402.87

ANOVA

Lignin

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	27457.393	6	4576.232	12.240	.000
Within Groups	5234.110	14	373.865		
Total	32691.503	20			

Lignin

	Treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	Control	3	276.2237		
	GB	3	280.9886	280.9886	
	MIF01	3		332.7063	332.7063
	WAA02	3			347.4655
	BTF08	3			348.0612
	T2	3			358.2282
	BTF07	3			379.4748
	Sig.		1.000	.064	.110

Means for groups in homogeneous subsets are displayed.

Appendix III

Profiling volatile compounds produced by endophytes to inhibit *Ganoderma boninense*

The following appendix contains:

1. The results of One-way ANOVA with Tukey's post hoc test for double plate test

Appendix 17. One-way ANOVA for double plate test

Descriptives

PIDG (Percentage Inhibition of Diameter Growth)

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
T2	3	68.3333	1.44338	.83333	64.7478	71.9189	67.50	70.00
WAA02	3	56.6667	1.44338	.83333	53.0811	60.2522	55.00	57.50
MIF01	3	65.0000	6.61438	3.81881	48.5690	81.4310	57.50	70.00
BTF08	3	29.1667	10.10363	5.83333	4.0679	54.2655	17.50	35.00
BTF07	3	6.6667	7.63763	4.40959	-12.3062	25.6396	.00	15.00
Total	15	45.1667	25.09743	6.48013	31.2682	59.0652	.00	70.00

ANOVA

PIDG (Percentage Inhibition of Diameter Growth)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8401.667	4	2100.417	50.410	.000
Within Groups	416.667	10	41.667		
Total	8818.333	14			

PIDG (Percentage Inhibition of Diameter Growth)

Tukey HSD

Fungi Isolates	N	Subset for alpha = 0.05		
		1	2	3
BTF07	3	6.6667		
BTF08	3		29.1667	
WAA02	3			56.6667
MIF01	3			65.0000
T2	3			68.3333
Sig.		1.000	1.000	.250

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

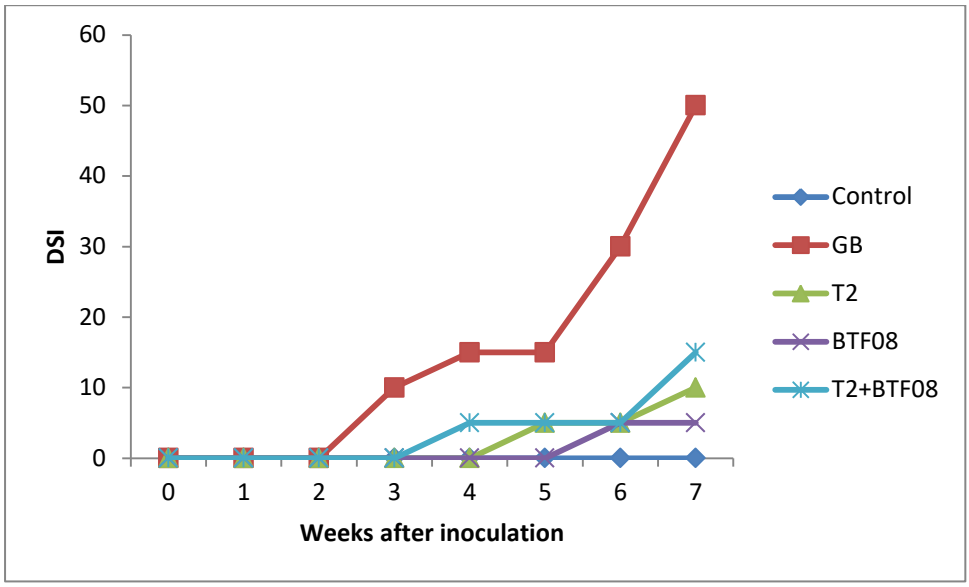
Appendix IV

Exploring the efficacy of single and mixed treatment of different species of endophytic fungi as a potential biocontrol agent against *Ganoderma boninense* basal stem rot in oil palm

The following appendix contains:

1. Disease progress curve
2. The results of One-way ANOVA with Tukey's post hoc test for Disease Severity Index of oil palm seedling after *G.boninense* artificial inoculation
3. The results of One-way ANOVA with Tukey's post hoc test for means of seedling height after *G.boninense* artificial inoculation
4. The results of One-way ANOVA with Tukey's post hoc test for means of seedling stem growth after *G.boninense* artificial inoculation
5. The results of One-way ANOVA with Tukey's post hoc test for means of seedling root growth after *G.boninense* artificial inoculation

Appendix 18. Disease progress curve



Appendix 19. One-way ANOVA for Disease Severity Index of oil palm seedling after *G. boninense* artificial inoculation (Week 3)

Descriptives

DSI

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	.0000	.00000	.00000	.0000	.0000	.00	.00
GB	5	10.0000	13.69306	6.12372	-7.0022	27.0022	.00	25.00
T2	5	.0000	.00000	.00000	.0000	.0000	.00	.00
BTF08	5	.0000	.00000	.00000	.0000	.0000	.00	.00
T2+BTF08	5	.0000	.00000	.00000	.0000	.0000	.00	.00
Total	25	2.0000	6.92219	1.38444	-.8573	4.8573	.00	25.00

ANOVA

DSI

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	400.000	4	100.000	2.667	.062
Within Groups	750.000	20	37.500		
Total	1150.000	24			

DSI

	Treatment	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	control	5	.0000
	T2	5	.0000
	BTF08	5	.0000
	T2+BTF08	5	.0000
	GB	5	10.0000
	Sig.		.112

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 20. One-way ANOVA for Disease Severity Index of oil palm seedling after *G. boninense* artificial inoculation (Week 4)

Descriptives

DSI

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	.0000	.00000	.00000	.0000	.0000	.00	.00
GB	5	15.0000	13.69306	6.12372	-2.0022	32.0022	.00	25.00
T2	5	.0000	.00000	.00000	.0000	.0000	.00	.00
BTF08	5	.0000	.00000	.00000	.0000	.0000	.00	.00
T2+BTF08	5	5.0000	11.18034	5.00000	-8.8822	18.8822	.00	25.00
Total	25	4.0000	9.35414	1.87083	.1388	7.8612	.00	25.00

ANOVA

DSI

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	850.000	4	212.500	3.400	.028
Within Groups	1250.000	20	62.500		
Total	2100.000	24			

DSI

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	control	5	.0000	
	T2	5	.0000	
	BTF08	5	.0000	
	T2+BTF08	5	5.0000	5.0000
	GB	5		15.0000
	Sig.		.852	.302

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 21. One-way ANOVA for Disease Severity Index of oil palm seedling after *G. boninense* artificial inoculation (Week 5)

Descriptives

DSI

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	.0000	.00000	.00000	.0000	.0000	.00	.00
GB	5	15.0000	13.69306	6.12372	-2.0022	32.0022	.00	25.00
T2	5	5.0000	11.18034	5.00000	-8.8822	18.8822	.00	25.00
BTF08	5	.0000	.00000	.00000	.0000	.0000	.00	.00
T2+BTF08	5	5.0000	11.18034	5.00000	-8.8822	18.8822	.00	25.00
Total	25	5.0000	10.20621	2.04124	.7871	9.2129	.00	25.00

ANOVA

DSI

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	750.000	4	187.500	2.143	.113
Within Groups	1750.000	20	87.500		
Total	2500.000	24			

DSI

	Treatment	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	control	5	.0000
	BTF08	5	.0000
	T2	5	5.0000
	T2+BTF08	5	5.0000
	GB	5	15.0000
	Sig.		.122

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 22. One-way ANOVA for Disease Severity Index of oil palm seedling after *G. boninense* artificial inoculation (Week 6)

Descriptives

DSI

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	.0000	.00000	.00000	.0000	.0000	.00	.00
GB	5	30.0000	27.38613	12.24745	-4.0044	64.0044	.00	50.00
T2	5	5.0000	11.18034	5.00000	-8.8822	18.8822	.00	25.00
BTF08	5	5.0000	11.18034	5.00000	-8.8822	18.8822	.00	25.00
T2+BTF08	5	5.0000	11.18034	5.00000	-8.8822	18.8822	.00	25.00
Total	25	9.0000	17.50000	3.50000	1.7764	16.2236	.00	50.00

ANOVA

DSI

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2850.000	4	712.500	3.167	.036
Within Groups	4500.000	20	225.000		
Total	7350.000	24			

DSI

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	control	5	.0000	
	T2	5	5.0000	5.0000
	BTF08	5	5.0000	5.0000
	T2+BTF08	5	5.0000	5.0000
	GB	5		30.0000
	Sig.		.983	.101

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 23. One-way ANOVA for Disease Severity Index of oil palm seedling after *G. boninense* artificial inoculation (Week 7)

Descriptives

DSI

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	.0000	.00000	.00000	.0000	.0000	.00	.00
GB	5	50.0000	.00000	.00000	50.0000	50.0000	50.00	50.00
T2	5	10.0000	13.69306	6.12372	-7.0022	27.0022	.00	25.00
BTF08	5	5.0000	11.18034	5.00000	-8.8822	18.8822	.00	25.00
T2+BTF08	5	15.0000	13.69306	6.12372	-2.0022	32.0022	.00	25.00
Total	25	16.0000	20.25874	4.05175	7.6376	24.3624	.00	50.00

ANOVA

DSI

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7850.000	4	1962.500	19.625	.000
Within Groups	2000.000	20	100.000		
Total	9850.000	24			

DSI

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	control	5	.0000	
	BTF08	5	5.0000	
	T2	5	10.0000	
	T2+BTF08	5	15.0000	
	GB	5		50.0000
	Sig.		.164	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 24. One-way ANOVA with Tukey's post hoc test for means of seedling height after *G. boninense* artificial inoculation (week 1)

Descriptives

Height

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	16.3600	.82946	.37094	15.3301	17.3899	15.50	17.50
GB	5	16.5800	.36332	.16248	16.1289	17.0311	16.20	17.00
T2	5	19.1200	1.38816	.62081	17.3964	20.8436	17.60	21.00
BTF08	5	17.6600	.52726	.23580	17.0053	18.3147	16.80	18.20
T2+BTF08	5	17.4600	.40373	.18055	16.9587	17.9613	17.10	18.10
Total	25	17.4360	1.23554	.24711	16.9260	17.9460	15.50	21.00

ANOVA

Height

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23.886	4	5.971	9.365	.000
Within Groups	12.752	20	.638		
Total	36.638	24			

Height

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	control	5	16.3600	
	GB	5	16.5800	
	T2+BTF08	5	17.4600	
	BTF08	5	17.6600	17.6600
	T2	5		19.1200
	Sig.		.114	.061

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 25. One-way ANOVA with Tukey's post hoc test for means of seedling height after *G. boninense* artificial inoculation (week 2)

Descriptives

Height

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	17.7200	.91488	.40915	16.5840	18.8560	17.00	19.20
GB	5	18.0000	.41833	.18708	17.4806	18.5194	17.50	18.40
T2	5	21.2200	.82280	.36797	20.1984	22.2416	19.90	22.00
BTF08	5	19.7400	.94499	.42261	18.5666	20.9134	18.70	21.00
T2+BTF08	5	18.9800	.95237	.42591	17.7975	20.1625	17.50	20.00
Total	25	19.1320	1.50131	.30026	18.5123	19.7517	17.00	22.00

ANOVA

Height

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	40.138	4	10.035	14.380	.000
Within Groups	13.956	20	.698		
Total	54.094	24			

Height

	Treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	control	5	17.7200		
	GB	5	18.0000		
	T2+BTF08	5	18.9800	18.9800	
	BTF08	5		19.7400	19.7400
	T2	5			21.2200
	Sig.		.160	.611	.073

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 26. One-way ANOVA with Tukey's post hoc test for means of seedling height after *G. boninense* artificial inoculation (week 3)

Descriptives

Height

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	18.6200	1.88733	.84404	16.2766	20.9634	17.20	21.60
GB	5	18.7200	1.33116	.59532	17.0671	20.3729	17.60	21.00
T2	5	21.9400	1.16533	.52115	20.4930	23.3870	20.40	23.00
BTF08	5	20.3400	2.14429	.95896	17.6775	23.0025	17.80	22.00
T2+BTF08	5	19.2200	1.32740	.59363	17.5718	20.8682	17.80	21.00
Total	25	19.7680	1.94737	.38947	18.9642	20.5718	17.20	23.00

ANOVA

Height

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	38.806	4	9.702	3.717	.020
Within Groups	52.208	20	2.610		
Total	91.014	24			

Height

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	control	5	18.6200	
	GB	5	18.7200	
	T2+BTF08	5	19.2200	19.2200
	BTF08	5	20.3400	20.3400
	T2	5		21.9400
	Sig.		.466	.096

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 27. One-way ANOVA with Tukey's post hoc test for means of seedling height after *G. boninense* artificial inoculation (week 4)

Descriptives

Height

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	18.8200	1.31415	.58771	17.1883	20.4517	17.70	21.00
GB	5	20.0000	.39370	.17607	19.5112	20.4888	19.70	20.60
T2	5	21.9200	1.10770	.49538	20.5446	23.2954	20.40	23.40
BTF08	5	20.3200	.72250	.32311	19.4229	21.2171	19.50	21.10
T2+BTF08	5	19.6000	1.23085	.55045	18.0717	21.1283	18.40	21.10
Total	25	20.1320	1.39724	.27945	19.5552	20.7088	17.70	23.40

ANOVA

Height

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	26.270	4	6.568	6.381	.002
Within Groups	20.584	20	1.029		
Total	46.854	24			

Height

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	control	5	18.8200	
	T2+BTF08	5	19.6000	
	GB	5	20.0000	
	BTF08	5	20.3200	20.3200
	T2	5		21.9200
	Sig.		.174	.132

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 28. One-way ANOVA with Tukey's post hoc test for means of seedling height after *G. boninense* artificial inoculation (week 5)

Descriptives

Height

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	18.9000	.94604	.42308	17.7253	20.0747	17.60	20.10
GB	5	19.1600	2.06954	.92553	16.5903	21.7297	17.00	21.40
T2	5	22.4400	2.52052	1.12721	19.3104	25.5696	18.60	25.10
BTF08	5	23.0000	.91378	.40866	21.8654	24.1346	21.50	23.80
T2+BTF08	5	20.4400	.87350	.39064	19.3554	21.5246	19.40	21.80
Total	25	20.7880	2.25745	.45149	19.8562	21.7198	17.00	25.10

ANOVA

Height

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	69.790	4	17.448	6.645	.001
Within Groups	52.516	20	2.626		
Total	122.306	24			

Height

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	control	5	18.9000	
	GB	5	19.1600	
	T2+BTF08	5	20.4400	20.4400
	T2	5		22.4400
	BTF08	5		23.0000
	Sig.		.573	.131

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 29 One-way ANOVA with Tukey's post hoc test for means of seedling height after *G. boninense* artificial inoculation (week 6)

Descriptives

Height

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	19.7000	1.05594	.47223	18.3889	21.0111	18.50	20.80
GB	5	20.2400	1.05262	.47074	18.9330	21.5470	19.00	21.60
T2	5	24.2400	1.16962	.52307	22.7877	25.6923	22.70	25.50
BTF08	5	25.5000	1.79861	.80436	23.2667	27.7333	23.50	27.60
T2+BTF08	5	23.6800	1.22963	.54991	22.1532	25.2068	22.20	25.50
Total	25	22.6720	2.61765	.52353	21.5915	23.7525	18.50	27.60

ANOVA

Height

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	131.098	4	32.775	19.654	.000
Within Groups	33.352	20	1.668		
Total	164.450	24			

Height

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	control	5	19.7000	
	GB	5	20.2400	
	T2+BTF08	5		23.6800
	T2	5		24.2400
	BTF08	5		25.5000
	Sig.		.962	.210

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 30. One-way ANOVA with Tukey's post hoc test for means of seedling height after *G. boninense* artificial inoculation (week 7)

Descriptives

Height

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	20.1200	1.20706	.53981	18.6212	21.6188	18.30	21.20
GB	5	22.4200	2.52329	1.12845	19.2869	25.5531	19.30	25.40
T2	5	27.8400	2.59769	1.16172	24.6145	31.0655	23.50	30.20
BTF08	5	27.2400	2.55010	1.14044	24.0736	30.4064	24.60	30.00
T2+BTF08	5	25.2600	.79246	.35440	24.2760	26.2440	24.20	26.40
Total	25	24.5760	3.53804	.70761	23.1156	26.0364	18.30	30.20

ANOVA

Height

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	213.614	4	53.403	12.303	.000
Within Groups	86.812	20	4.341		
Total	300.426	24			

Height

	Treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	control	5	20.1200		
	GB	5	22.4200	22.4200	
	T2+BTF08	5		25.2600	25.2600
	BTF08	5			27.2400
	T2	5			27.8400
	Sig.		.431	.237	.321

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 31. One-way ANOVA with Tukey's post hoc test for means of seedling stem diameter after *G. boninense* artificial inoculation (week 1)

Descriptives

Diameter

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	1.7800	.04472	.02000	1.7245	1.8355	1.70	1.80
GB	5	1.7800	.04472	.02000	1.7245	1.8355	1.70	1.80
T2	5	1.8000	.00000	.00000	1.8000	1.8000	1.80	1.80
BTF08	5	1.7800	.04472	.02000	1.7245	1.8355	1.70	1.80
T2+BTF08	5	1.8000	.00000	.00000	1.8000	1.8000	1.80	1.80
Total	25	1.7880	.03317	.00663	1.7743	1.8017	1.70	1.80

ANOVA

Diameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.002	4	.001	.500	.736
Within Groups	.024	20	.001		
Total	.026	24			

Diameter

	Treatment	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	control	5	1.7800
	GB	5	1.7800
	BTF08	5	1.7800
	T2	5	1.8000
	T2+BTF08	5	1.8000
	Sig.		.889

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 32. One-way ANOVA with Tukey's post hoc test for means of seedling stem diameter after *G. boninense* artificial inoculation (week 2)

Descriptives

Diameter

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	1.8200	.10954	.04899	1.6840	1.9560	1.70	2.00
GB	5	1.8000	.07071	.03162	1.7122	1.8878	1.70	1.90
T2	5	2.2200	.13038	.05831	2.0581	2.3819	2.00	2.30
BTF08	5	2.1000	.15811	.07071	1.9037	2.2963	1.90	2.30
T2+BTF08	5	2.0000	.07071	.03162	1.9122	2.0878	1.90	2.10
Total	25	1.9880	.19434	.03887	1.9078	2.0682	1.70	2.30

ANOVA

Diameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.650	4	.163	12.703	.000
Within Groups	.256	20	.013		
Total	.906	24			

Diameter

	Treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	GB	5	1.8000		
	control	5	1.8200		
	T2+BTF08	5	2.0000	2.0000	
	BTF08	5		2.1000	2.1000
	T2	5			2.2200
	Sig.		.074	.636	.469

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 33. One-way ANOVA with Tukey's post hoc test for means of seedling stem diameter after *G. boninense* artificial inoculation (week 3)

Descriptives

Diameter

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	2.0000	.07071	.03162	1.9122	2.0878	1.90	2.10
GB	5	1.8400	.05477	.02449	1.7720	1.9080	1.80	1.90
T2	5	2.6400	.11402	.05099	2.4984	2.7816	2.50	2.80
BTF08	5	2.3800	.08367	.03742	2.2761	2.4839	2.30	2.50
T2+BTF08	5	2.1400	.11402	.05099	1.9984	2.2816	2.00	2.30
Total	25	2.2000	.30000	.06000	2.0762	2.3238	1.80	2.80

ANOVA

Diameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.996	4	.499	60.854	.000
Within Groups	.164	20	.008		
Total	2.160	24			

Diameter

	Treatment	N	Subset for alpha = 0.05			
			1	2	3	4
Tukey HSD[a]	GB	5	1.8400			
	control	5	2.0000	2.0000		
	T2+BTF08	5		2.1400		
	BTF08	5			2.3800	
	T2	5				2.6400
	Sig.		.075	.144	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 34. One-way ANOVA with Tukey's post hoc test for means of seedling stem diameter after *G. boninense* artificial inoculation (week 4)

Descriptives

Diameter

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	2.5000	.10000	.04472	2.3758	2.6242	2.40	2.60
GB	5	2.2000	.18708	.08367	1.9677	2.4323	2.00	2.50
T2	5	3.1000	.40620	.18166	2.5956	3.6044	2.80	3.80
BTF08	5	2.6600	.11402	.05099	2.5184	2.8016	2.50	2.80
T2+BTF08	5	2.3400	.13416	.06000	2.1734	2.5066	2.20	2.50
Total	25	2.5600	.37528	.07506	2.4051	2.7149	2.00	3.80

ANOVA

Diameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.416	4	.604	12.531	.000
Within Groups	.964	20	.048		
Total	3.380	24			

Diameter

	Treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	GB	5	2.2000		
	T2+BTF08	5	2.3400	2.3400	
	control	5	2.5000	2.5000	
	BTF08	5		2.6600	
	T2	5			3.1000
	Sig.		.235	.184	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 35. One-way ANOVA with Tukey's post hoc test for means of seedling stem diameter after *G. boninense* artificial inoculation (week 5)

Descriptives

Diameter

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	2.4800	.10954	.04899	2.3440	2.6160	2.40	2.60
GB	5	2.6400	.05477	.02449	2.5720	2.7080	2.60	2.70
T2	5	2.9400	.25100	.11225	2.6283	3.2517	2.50	3.10
BTF08	5	2.9000	.12247	.05477	2.7479	3.0521	2.70	3.00
T2+BTF08	5	2.6400	.13416	.06000	2.4734	2.8066	2.50	2.80
Total	25	2.7200	.22361	.04472	2.6277	2.8123	2.40	3.10

ANOVA

Diameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.756	4	.189	8.514	.000
Within Groups	.444	20	.022		
Total	1.200	24			

Diameter

	Treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	control	5	2.4800		
	T2+BTF08	5	2.6400	2.6400	
	GB	5	2.6400	2.6400	
	BTF08	5		2.9000	2.9000
	T2	5			2.9400
	Sig.		.457	.080	.993

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 36. One-way ANOVA with Tukey's post hoc test for means of seedling stem diameter after *G. boninense* artificial inoculation (week 6)

Descriptives

Diameter

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	2.5800	.08367	.03742	2.4761	2.6839	2.50	2.70
GB	5	2.4600	.11402	.05099	2.3184	2.6016	2.30	2.60
T2	5	3.2000	.14142	.06325	3.0244	3.3756	3.00	3.40
BTF08	5	3.2400	.19494	.08718	2.9980	3.4820	3.10	3.50
T2+BTF08	5	2.8000	.12247	.05477	2.6479	2.9521	2.60	2.90
Total	25	2.8560	.34651	.06930	2.7130	2.9990	2.30	3.50

ANOVA

Diameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2.510	4	.627	33.731	.000
Within Groups	.372	20	.019		
Total	2.882	24			

Diameter

	Treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	GB	5	2.4600		
	control	5	2.5800	2.5800	
	T2+BTF08	5		2.8000	
	T2	5			3.2000
	BTF08	5			3.2400
	Sig.		.640	.119	.990

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 37. One-way ANOVA with Tukey's post hoc test for means of seedling stem diameter after *G. boninense* artificial inoculation (week 7)

Descriptives

Diameter

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	2.8000	.07071	.03162	2.7122	2.8878	2.70	2.90
GB	5	2.4200	.20494	.09165	2.1655	2.6745	2.10	2.60
T2	5	3.4800	.24900	.11136	3.1708	3.7892	3.10	3.70
BTF08	5	3.6800	.29496	.13191	3.3138	4.0462	3.20	4.00
T2+BTF08	5	3.2600	.27019	.12083	2.9245	3.5955	3.00	3.60
Total	25	3.1280	.51439	.10288	2.9157	3.3403	2.10	4.00

ANOVA

Diameter

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.274	4	1.319	24.509	.000
Within Groups	1.076	20	.054		
Total	6.350	24			

Diameter

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	GB	5	2.4200	
	control	5	2.8000	
	T2+BTF08	5		3.2600
	T2	5		3.4800
	BTF08	5		3.6800
	Sig.		.110	.065

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 38. One-way ANOVA with Tukey's post hoc test for means number of roots after *G. boninense* artificial inoculation (week 1)

Descriptives

Roots

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	77.8000	2.28035	1.01980	74.9686	80.6314	75.00	80.00
GB	5	70.8000	4.86826	2.17715	64.7553	76.8447	65.00	78.00
T2	5	78.2000	4.96991	2.22261	72.0290	84.3710	72.00	85.00
BTF08	5	82.2000	1.78885	.80000	79.9788	84.4212	80.00	84.00
T2+BTF08	5	77.8000	2.28035	1.01980	74.9686	80.6314	75.00	80.00
Total	25	77.3600	4.94031	.98806	75.3207	79.3993	65.00	85.00

ANOVA

Roots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	337.760	4	84.440	6.810	.001
Within Groups	248.000	20	12.400		
Total	585.760	24			

Roots

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	GB	5	70.8000	
	control	5		77.8000
	T2+BTF08	5		77.8000
	T2	5		78.2000
	BTF08	5		82.2000
	Sig.		1.000	.313

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 39. One-way ANOVA with Tukey's post hoc test for means number of roots after *G. boninense* artificial inoculation (week 2)

Descriptives

Roots

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	86.4000	3.78153	1.69115	81.7046	91.0954	81.00	90.00
GB	5	69.8000	1.48324	.66332	67.9583	71.6417	68.00	72.00
T2	5	83.2000	4.65833	2.08327	77.4159	88.9841	78.00	89.00
BTF08	5	87.8000	2.48998	1.11355	84.7083	90.8917	86.00	92.00
T2+BTF08	5	84.6000	4.50555	2.01494	79.0056	90.1944	81.00	90.00
Total	25	82.3600	7.37609	1.47522	79.3153	85.4047	68.00	92.00

ANOVA

Roots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1046.960	4	261.740	20.227	.000
Within Groups	258.800	20	12.940		
Total	1305.760	24			

Roots

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	GB	5	69.8000	
	T2	5		83.2000
	T2+BTF08	5		84.6000
	control	5		86.4000
	BTF08	5		87.8000
	Sig.		1.000	.292

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 40. One-way ANOVA with Tukey's post hoc test for means number of roots after *G. boninense* artificial inoculation (week 3)

Descriptives

Roots

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	95.0000	6.24500	2.79285	87.2458	102.7542	88.00	103.00
GB	5	74.8000	3.56371	1.59374	70.3751	79.2249	71.00	80.00
T2	5	91.4000	5.50454	2.46171	84.5652	98.2348	84.00	96.00
BTF08	5	111.2000	15.67482	7.00999	91.7371	130.6629	98.00	134.00
T2+BTF08	5	103.0000	5.38516	2.40832	96.3134	109.6866	98.00	112.00
Total	25	95.0800	14.66833	2.93367	89.0252	101.1348	71.00	134.00

ANOVA

Roots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3737.040	4	934.260	13.096	.000
Within Groups	1426.800	20	71.340		
Total	5163.840	24			

Roots

	Treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	GB	5	74.8000		
	T2	5		91.4000	
	control	5		95.0000	
	T2+BTF08	5		103.0000	103.0000
	BTF08	5			111.2000
	Sig.		1.000	.231	.553

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 41. One-way ANOVA with Tukey's post hoc test for means number of roots after *G. boninense* artificial inoculation (week 4)

Descriptives

Roots

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	101.6000	2.88097	1.28841	98.0228	105.1772	99.00	106.00
GB	5	89.8000	6.05805	2.70924	82.2779	97.3221	84.00	99.00
T2	5	103.4000	4.56070	2.03961	97.7371	109.0629	96.00	107.00
BTF08	5	120.0000	20.45727	9.14877	94.5989	145.4011	100.00	150.00
T2+BTF08	5	117.0000	9.77241	4.37035	104.8660	129.1340	107.00	132.00
Total	25	106.3600	14.92168	2.98434	100.2006	112.5194	84.00	150.00

ANOVA

Roots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3024.560	4	756.140	6.521	.002
Within Groups	2319.200	20	115.960		
Total	5343.760	24			

Roots

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	GB	5	89.8000	
	control	5	101.6000	101.6000
	T2	5	103.4000	103.4000
	T2+BTF08	5		117.0000
	BTF08	5		120.0000
	Sig.		.303	.089

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 42. One-way ANOVA with Tukey's post hoc test for means number of roots after *G. boninense* artificial inoculation (week 5)

Descriptives

Roots

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	109.6000	5.72713	2.56125	102.4888	116.7112	105.00	119.00
GB	5	103.2000	15.41752	6.89493	84.0566	122.3434	84.00	124.00
T2	5	122.4000	7.02140	3.14006	113.6818	131.1182	116.00	132.00
BTF08	5	132.8000	9.62808	4.30581	120.8452	144.7548	119.00	142.00
T2+BTF08	5	108.0000	8.57321	3.83406	97.3549	118.6451	93.00	114.00
Total	25	115.2000	14.27118	2.85424	109.3091	121.0909	84.00	142.00

ANOVA

Roots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2944.000	4	736.000	7.572	.001
Within Groups	1944.000	20	97.200		
Total	4888.000	24			

Roots

	Treatment	N	Subset for alpha = 0.05		
			1	2	3
Tukey HSD[a]	GB	5	103.2000		
	T2+BTF08	5	108.0000	108.0000	
	control	5	109.6000	109.6000	
	T2	5		122.4000	122.4000
	BTF08	5			132.8000
	Sig.		.840	.183	.475

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 43. One-way ANOVA with Tukey's post hoc test for means number of roots after *G. boninense* artificial inoculation (week 6)

Descriptives

Roots

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	115.4000	8.84873	3.95727	104.4129	126.3871	110.00	131.00
GB	5	101.2000	13.80942	6.17576	84.0533	118.3467	80.00	114.00
T2	5	133.2000	13.21741	5.91101	116.7884	149.6116	115.00	148.00
BTF08	5	112.0000	12.74755	5.70088	96.1718	127.8282	99.00	126.00
T2+BTF08	5	150.2000	31.71277	14.18238	110.8234	189.5766	117.00	189.00
Total	25	122.4000	24.09011	4.81802	112.4561	132.3439	80.00	189.00

ANOVA

Roots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7480.400	4	1870.100	5.801	.003
Within Groups	6447.600	20	322.380		
Total	13928.000	24			

Roots

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	GB	5	101.2000	
	BTF08	5	112.0000	
	control	5	115.4000	
	T2	5	133.2000	133.2000
	T2+BTF08	5		150.2000
	Sig.		.071	.576

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 44. One-way ANOVA with Tukey's post hoc test for means number of roots after *G. boninense* artificial inoculation (week 7)

Descriptives

Roots

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	5	148.6000	20.32978	9.09175	123.3572	173.8428	121.00	172.00
GB	5	114.2000	11.03177	4.93356	100.5022	127.8978	101.00	128.00
T2	5	187.6000	19.84439	8.87468	162.9599	212.2401	153.00	201.00
BTF08	5	191.6000	20.88780	9.34131	165.6644	217.5356	156.00	208.00
T2+BTF08	5	137.2000	20.96902	9.37763	111.1635	163.2365	112.00	163.00
Total	25	155.8400	34.95554	6.99111	141.4111	170.2689	101.00	208.00

ANOVA

Roots

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	22106.160	4	5526.540	15.311	.000
Within Groups	7219.200	20	360.960		
Total	29325.360	24			

Roots

	Treatment	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	GB	5	114.2000	
	T2+BTF08	5	137.2000	
	control	5	148.6000	
	T2	5		187.6000
	BTF08	5		191.6000
	Sig.		.065	.997

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 45. One-way ANOVA with Tukey's post hoc test for means root weigh (dry weigh) after *G. boninense* artificial inoculation (week 1)

Descriptives

Weigh

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	.4400	.02449	.01095	.4096	.4704	.42	.48
GB	5	.4520	.03633	.01625	.4069	.4971	.42	.50
T2	5	.4600	.04690	.02098	.4018	.5182	.42	.52
BTF08	5	.4640	.04336	.01939	.4102	.5178	.42	.52
T2+BTF08	5	.4580	.03899	.01744	.4096	.5064	.42	.52
Total	25	.4548	.03641	.00728	.4398	.4698	.42	.52

ANOVA

Weigh

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.002	4	.000	.290	.881
Within Groups	.030	20	.002		
Total	.032	24			

Weigh

	Endophyte	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	Control	5	.4400
	GB	5	.4520
	T2+BTF08	5	.4580
	T2	5	.4600
	BTF08	5	.4640
	Sig.		.862

Appendix 46. One-way ANOVA with Tukey's post hoc test for means root weigh (dry weigh) after *G. boninense* artificial inoculation (week 2)

Descriptives

Weigh

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	.4740	.04450	.01990	.4187	.5293	.42	.53
GB	5	.4680	.02683	.01200	.4347	.5013	.44	.50
T2	5	.4700	.03674	.01643	.4244	.5156	.42	.51
BTF08	5	.4540	.03975	.01778	.4046	.5034	.42	.51
T2+BTF08	5	.4540	.04561	.02040	.3974	.5106	.41	.52
Total	25	.4640	.03686	.00737	.4488	.4792	.41	.53

ANOVA

Weigh

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.002	4	.000	.285	.884
Within Groups	.031	20	.002		
Total	.033	24			

Weigh

	Endophyte	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	BTF08	5	.4540
	T2+BTF08	5	.4540
	GB	5	.4680
	T2	5	.4700
	Control	5	.4740
	Sig.		.926

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 47. One-way ANOVA with Tukey's post hoc test for means root weigh (dry weigh) after *G. boninense* artificial inoculation (week 3)

Descriptives

Weigh

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	.5060	.02191	.00980	.4788	.5332	.48	.54
GB	5	.4900	.02000	.00894	.4652	.5148	.47	.52
T2	5	.5000	.01581	.00707	.4804	.5196	.48	.52
BTF08	5	.5260	.01949	.00872	.5018	.5502	.50	.54
T2+BTF08	5	.5000	.02449	.01095	.4696	.5304	.48	.54
Total	25	.5044	.02238	.00448	.4952	.5136	.47	.54

ANOVA

Weigh

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.004	4	.001	2.118	.116
Within Groups	.008	20	.000		
Total	.012	24			

Weigh

	Endophyte	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	GB	5	.4900
	T2	5	.5000
	T2+BTF08	5	.5000
	Control	5	.5060
	BTF08	5	.5260
	Sig.		.078

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 48. One-way ANOVA with Tukey's post hoc test for means root weigh (dry weigh) after *G. boninense* artificial inoculation (week 4)

Descriptives

Weigh

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	.5180	.01483	.00663	.4996	.5364	.50	.54
GB	5	.5060	.02608	.01166	.4736	.5384	.48	.54
T2	5	.5220	.01789	.00800	.4998	.5442	.50	.54
BTF08	5	.5220	.01789	.00800	.4998	.5442	.50	.54
T2+BTF08	5	.5140	.02608	.01166	.4816	.5464	.48	.54
Total	25	.5164	.02018	.00404	.5081	.5247	.48	.54

ANOVA

Weigh

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	4	.000	.505	.733
Within Groups	.009	20	.000		
Total	.010	24			

Weigh

	Endophyte	N	Subset for alpha = 0.05
			1
Tukey HSD[a]	GB	5	.5060
	T2+BTF08	5	.5140
	Control	5	.5180
	T2	5	.5220
	BTF08	5	.5220
	Sig.		.751

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 49. One-way ANOVA with Tukey's post hoc test for means root weigh (dry weigh) after *G. boninense* artificial inoculation (week 5)

Descriptives

Weigh

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	.5220	.01304	.00583	.5058	.5382	.51	.54
GB	5	.5080	.00837	.00374	.4976	.5184	.50	.52
T2	5	.5380	.02490	.01114	.5071	.5689	.50	.57
BTF08	5	.5440	.02608	.01166	.5116	.5764	.52	.58
T2+BTF08	5	.5240	.01140	.00510	.5098	.5382	.51	.54
Total	25	.5272	.02112	.00422	.5185	.5359	.50	.58

ANOVA

Weigh

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.004	4	.001	3.012	.043
Within Groups	.007	20	.000		
Total	.011	24			

Weigh

	Endophyte	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	GB	5	.5080	
	Control	5	.5220	.5220
	T2+BTF08	5	.5240	.5240
	T2	5	.5380	.5380
	BTF08	5		.5440
	Sig.		.109	.347

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 50. One-way ANOVA with Tukey's post hoc test for means root weigh (dry weigh) after *G. boninense* artificial inoculation (week 6)

Descriptives

Weigh

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	.5240	.01817	.00812	.5014	.5466	.50	.54
GB	5	.5060	.01517	.00678	.4872	.5248	.49	.52
T2	5	.5646	.03187	.01425	.5250	.6042	.52	.60
BTF08	5	.5440	.02966	.01327	.5072	.5808	.51	.58
T2+BTF08	5	.5220	.01304	.00583	.5058	.5382	.51	.54
Total	25	.5321	.02939	.00588	.5200	.5443	.49	.60

ANOVA

Weigh

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.010	4	.003	4.872	.007
Within Groups	.011	20	.001		
Total	.021	24			

Weigh

	Endophyte	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	GB	5	.5060	
	T2+BTF08	5	.5220	.5220
	Control	5	.5240	.5240
	BTF08	5	.5440	.5440
	T2	5		.5646
	Sig.		.104	.056

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix 51. One-way ANOVA with Tukey's post hoc test for means root weigh (dry weigh) after *G. boninense* artificial inoculation (week 7)

Descriptives

Weigh

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	5	.5300	.02449	.01095	.4996	.5604	.50	.56
GB	5	.4980	.02950	.01319	.4614	.5346	.47	.54
T2	5	.5920	.03421	.01530	.5495	.6345	.54	.63
BTF08	5	.6000	.02550	.01140	.5683	.6317	.56	.63
T2+BTF08	5	.5400	.01414	.00632	.5224	.5576	.52	.56
Total	25	.5520	.04619	.00924	.5329	.5711	.47	.63

ANOVA

Weigh

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.037	4	.009	13.338	.000
Within Groups	.014	20	.001		
Total	.051	24			

Weigh

	Endophyte	N	Subset for alpha = 0.05	
			1	2
Tukey HSD[a]	GB	5	.4980	
	Control	5	.5300	
	T2+BTF08	5	.5400	
	T2	5		.5920
	BTF08	5		.6000
	Sig.		.127	.988

Means for groups in homogeneous subsets are displayed.

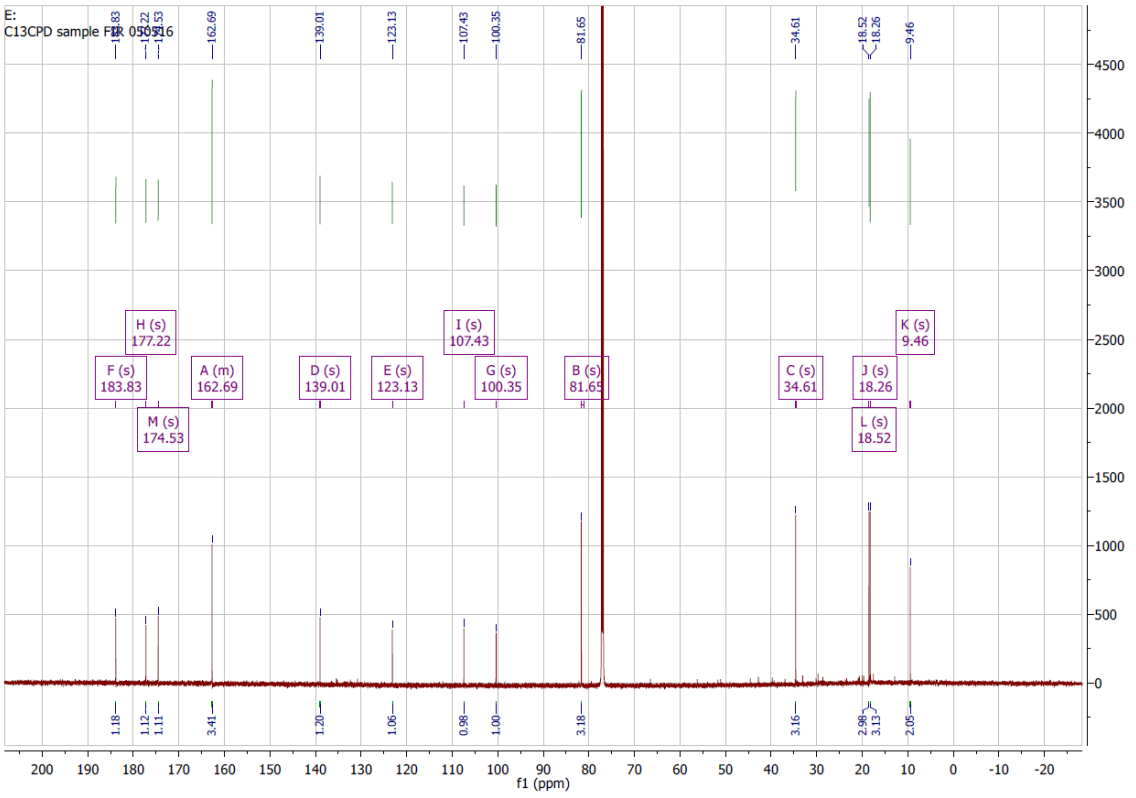
a. Uses Harmonic Mean Sample Size = 5.000.

Appendix V

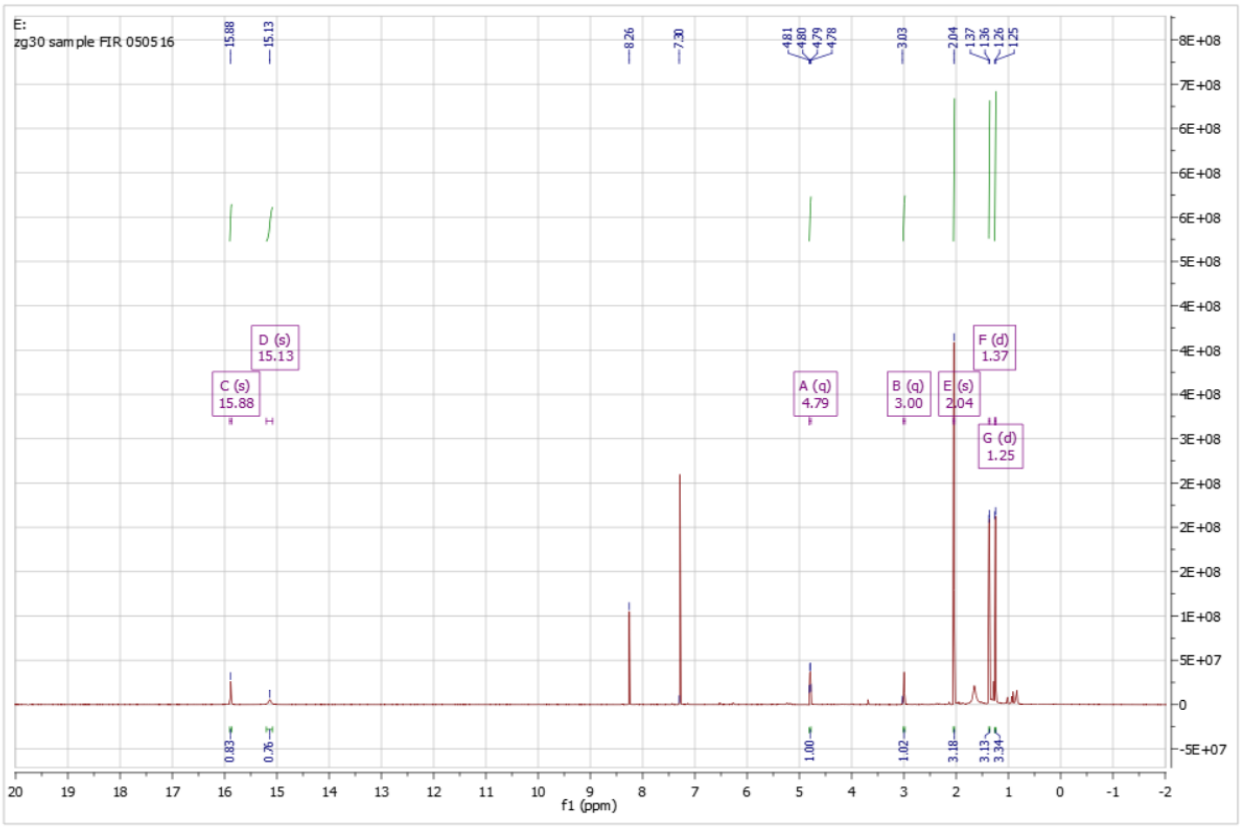
Isolation and characterization of antifungal metabolites of *Penicillium citrinum* against fungal pathogen *Ganoderma boninense*

The following appendix contains Nuclear magnetic resonance spectra of BTF08 extract (citrinin)

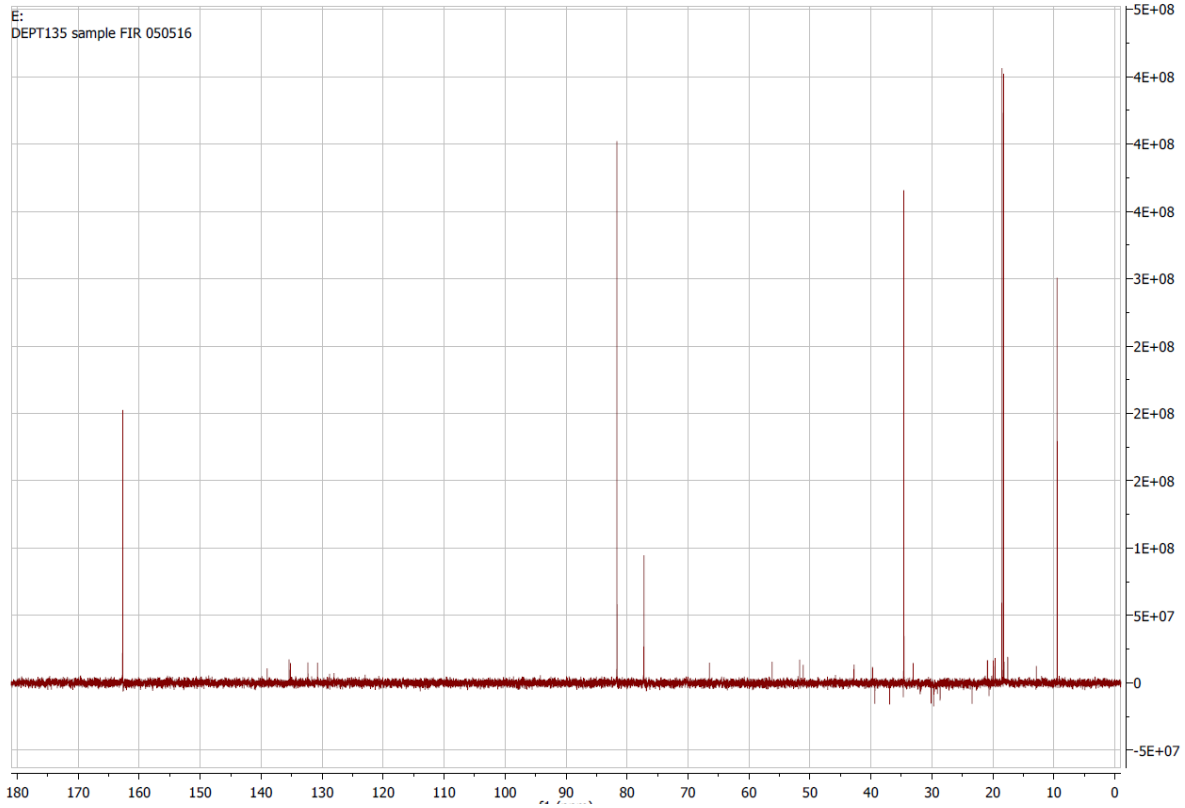
Appendix 52. ^{13}C NMR of citrinin



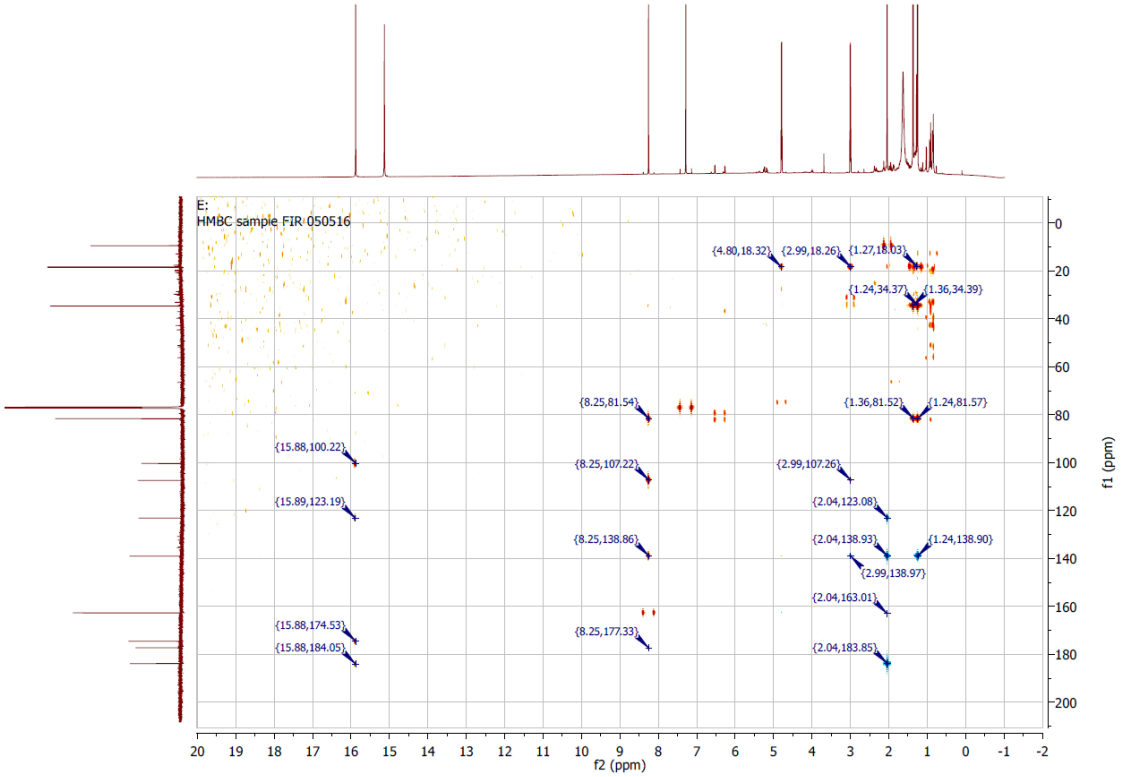
Appendix 53. ^{1}H NMR of citrinin



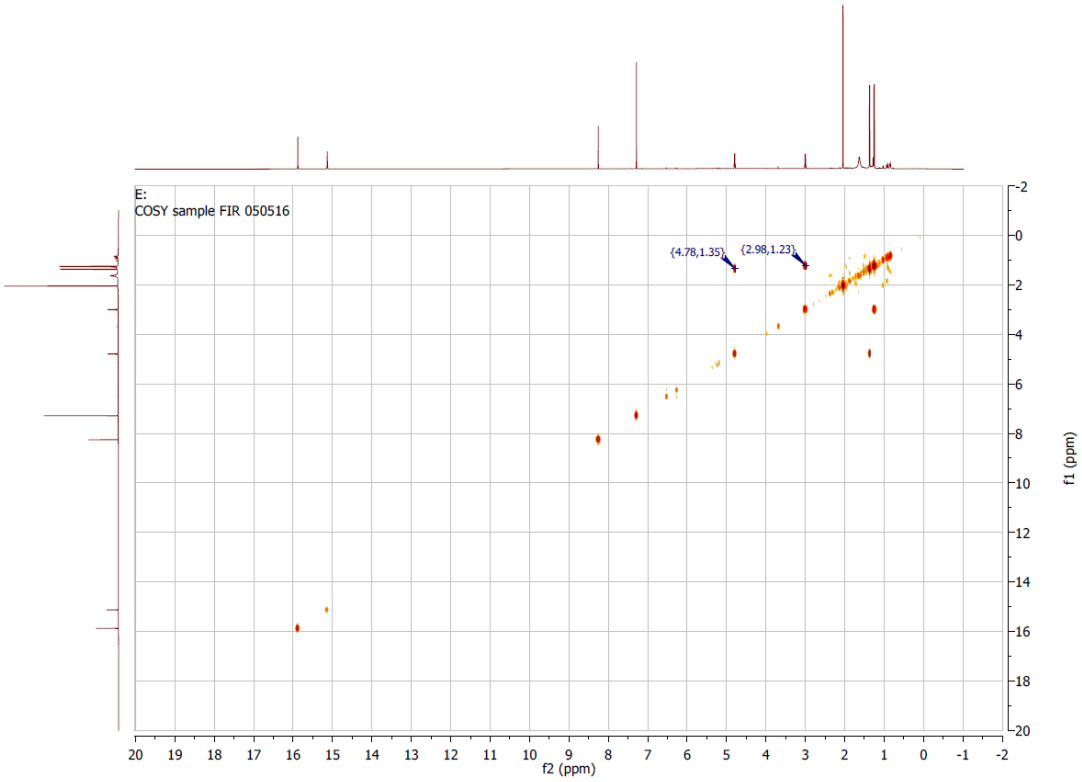
Appendix 54. DEPT spectra of citrinin



Appendix 55. HMBC spectra of citrinin



Appendix 56. COSY spectra of citrinin



Appendix VI

Conference output and awards

List of conference:

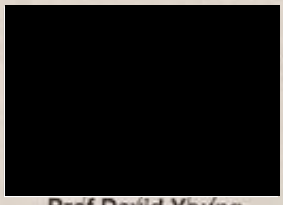
1. Three Minute Thesis 3MT, organised by Monash University Malaysia (2014)
2. Monash Science Symposium 2014, Malaysia
3. 32nd Symposium of the Malaysan Society for Microbiology (MSM2014)
4. Asian Mycology Congress 2015, Goa, India.
5. International Posgraduate Research Awards Seminar (InPRAS2016)
6. Malaysian Society for Microbiology Postgraduate Seminar (MSMPS2016)
7. International Conference on Beneficial Microbes 2016, Phuket, Thailand.

CERTIFICATE OF AWARD

This is to certify that

CHEONG SIEW LOON

has been awarded the 2nd prize in the
Three Minute Thesis 3MT™ 2014 School Level Competition
organised by School of Science,
Monash University Malaysia
on 4 June 2014



Prof David Young
Head of School
School of Science
Monash University Malaysia

MONASH University

School of Science

Certificate of Participation

awarded to

Cheong Siew Loon

for presenting (poster presentation) in Monash Science Symposium 2014 held at Monash University Malaysia, June 18th and 19th, 2014.

Professor David Young,
Head, School of Science
Monash University Malaysia

MONASH University

MALAYSIAN SOCIETY FOR MICROBIOLOGY

Certificate of Appreciation

This certificate is presented to

Mr. Cheong Siew Loon

as the **POSTER PRESENTER** in the

32nd Symposium of the Malaysian Society for Microbiology (MSM2014)

6 – 8 December 2014

at the

Terengganu Equestrian Resort, Terengganu

MALAYSIA

[redacted]

ASSOC. PROF. DR. FARAH DIBA ABU BAKAR
President
Malaysian Society for Microbiology

[redacted]

DR. MOHD NIZAM LANI
Chairman
32nd Symposium of the Malaysian Society for Microbiology (MSM2014)

Mycological Society of India (MSI)

Asian Mycological Association

DEPARTMENT OF BOTANY, GOA UNIVERSITY
ASIAN MYCOLOGICAL ASSOCIATION (AMA)
& MYCOLOGICAL SOCIETY OF INDIA (MSI)

Participation Certificate

This is to certify that Prof./Dr./Mr./Ms Siew Loon Che[illegible],

Monash University Malaysia, has participated in the

Asian Mycological Congress 2015 with ~~*Invited/Oral/Poster*~~ presentation.

Place: Goa University, Goa India.
Date: 7-10 October, 2015

[redacted]

Dr. Chandralata Raghukumar
Convener, AMC 2015

[redacted]

Prof. B. F. Rodrigues
Organizing Secretary 2015

UNIVERSITY OF MALAYA
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MSMBB MALAYSIAN SOCIETY FOR MOLECULAR BIOLOGY AND BIOTECHNOLOGY
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INTERNATIONAL POSTGRADUATE

Research Awards Seminar

7TH-8TH March 2016 • University of Malaya • Kuala Lumpur, Malaysia

FIRST PRIZE (BIOTECHNOLOGY CATEGORY)

This certificate is awarded to

CHEONG SIEW LOON

(Ph.D Candidate)

for best oral presentation at the

International Postgraduate Research Awards Seminar

(InPRAS) 2016

[redacted]

A/P Dr. Kanthimathi A/P M S Subramaniam
Organising Chairperson InPRAS 2016
&
President, Malaysian Society for Molecular Biology and Biotechnology (MSMBB)

Universiti Kebangsaan Malaysia
National University of Malaysia

Malaysian Society for Microbiology

American Society for Microbiology

Certificate of Appreciation

Presented to

CHEONG SIEW LOON

for contribution as

POSTER PRESENTER

at the

40 MSM YEARS

Malaysian Society for Microbiology
Postgraduate Seminar
(MSMPS2016)

Stimulating Excellence in Microbiology Through Human Resource Development

24 August 2016
Kompleks Kedekanan
Faculty of Science and Technology
Universiti Kebangsaan Malaysia

[redacted]

PROF. DR. KALAIVANI NADARAJAH
Chairman
MSMPS2016 Organising Committee

[redacted]

ASSOC. PROF. DR. FARAH DIBA ABU BAKAR
President
Malaysian Society for Microbiology

Certificate of Participation

This is to certify that

Mr. Cheong Siew Loon

participated in the

International Conference on Beneficial Microbes 2016

Phuket, Thailand

31st May - 2nd June 2016

[redacted]

Prof. Dr. Liong Min Tze
ICOBM 2016 Chairman
University Sains Malaysia, Penang, Malaysia

[redacted]

Prof. Dr. Wibool Piyawattanametha
ICOBM 2016 Co-Chairman
King Mongkut's Institute of Technology, Ladkrabang, Thailand