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Molecular Interaction Between *Ganoderma boninense* On Young Oil Palm

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ABSTRACT

Oil palm is an important crop to the economy of Malaysia and Indonesia. There have been considerable efforts to improve crop quality of crop to meet the growing demands for edible oil in the world. However, the threat of pests and diseases in Malaysian oil palm has increasingly challenged the production of crude palm oil, sometimes to the point of driving plantations to closure and conversion of estate land for commercial development. The most devastating disease in oil palm is basal stem rot (BSR), caused by the root-rot pathogen *Ganoderma boninense*. The disease has been observed in both young and mature oil palm and in both inland and coastal plantations. Epidemiology of BSR was explained through infection by spores and by mycelium from previously infected oil palm and coconut stands, and the presence of *G. boninense* in the basal stem and soil surrounding infected palms. The life cycle of *G. boninense* could extend to years as the fungus can remain as resting structures in palm tissues and as recalcitrant spores spread by wind and rain splash in estates. Shade house trials have been successful in producing artificially infected *Ganoderma*-oil palm BSR symptoms. However, the current method uses oil palm seedlings of three to 12 months and involves inoculation with *G. boninense* that has pre-colonised a rubber wood block for one month. This method requires a minimum of six months to observe BSR-like symptoms, a time consuming effort. The aim of this work was to develop an efficient artificial infection assay that uses clonal oil palm plantlets as hosts for BSR disease through the

inoculation of *G. boninense* isolate GBLS. The experiment was set up with treatments of T1: non-treatment control, T2: wounded plant control and T3: wounded and GBLS-infected plants. During the incubation period of 42 days, T3 plants consistently showed significant stunting (5.18% and 13.41% shorter than T1 and T2, respectively) and loss of weight (57.58% and 61.00% lighter than T1 and T2, respectively). The T3 plants also had significantly thinner leaves (38.70% and 37.71% narrower than T1 and T2, respectively) and lower chlorophyll contents (42.95% and 64.88% lower SPAD readings than T1 and T2, respectively). Disease severity on the T3 plants was 100% by 6 weeks, indicating death of oil palms. The quantity of GBLS DNA present in T3 samples was highest at Day 14, corresponding to the active growth phase of the pathogen, while on Day 42, the quantity of DNA increased to 13.58% of Day 14 readings, indicating continuous growth *in vivo*. The method developed was time-sensitive and reliable for screening oil palm for response during the plant-pathogen interaction. The work examined the hypothesis that *G. boninense* utilizes lignin degrading enzymes (LDEs) such as laccase, lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) to breakdown oil palm lignin, causing primary cell, tissue and stem rot. Therefore, the role of laccase was investigated during the interaction in otherwise symptomless oil palm tissues. A small gene fragment (208 bp) of laccase was isolated from total DNA of *G. boninense* GBLS and sequencing showed it to contain 89% homology to basidiomycete laccase. GBLS reduced the total lignin content of oil palm in T3 plants (48.86%

and 53.18% lower than T1 and T2, respectively). However, neither laccase nor MnP enzymes were produced in significantly higher amounts in T3 as compared to T1 and T2, indicating the need to differentiate the presence of plant and fungal laccases. Transcript abundance for GBLS laccase gene using qPCR indicated that laccase was induced during the interaction, with maximum laccase detected on Day 28. However, this did not place laccase as a virulence factor although the presence of higher amounts of laccase towards the end of the experiment corresponds to loss of lignin and plant death. Therefore, laccase and other LDEs need further investigations to be confirmed as virulence factors.

This work reports a novel infection assay for *G. boninense* interaction with oil palm and was the first study to have investigated the role of *G. boninense* laccases in the devastating BSR disease.

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DEDICATION

This report is dedicated to my daughter, Isabel Ilina Vasavan and my son Jeremy Kiran Vasavan.

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DECLARATION

I hereby declare that the thesis is based on my original work, except for the quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at the University of Nottingham Malaysia Campus or other institutions.

_____

CHRISTINA SUPRAMANIAM

Date:

ABBREVIATIONS

%	percentage
°C	degree Celcius
2XYT	Two times of Yeast-Tryptone media
3’	3’ prime-end of a DNA strand
5’	5’ prime-end of a DNA strand
µm	micro meter
µg	microgram
µL	microliter
µM	micromolar
AARSB	Applied Agriecological Research Sdn Bhd
BLAST	Basic local alignment search tool
Boud	Boudier (taxonomy)
bp	base pair
BSR	Basal stem rot disease
$CaCl_2$	Calcium chloride
CAGE	cellulose acetate gel electrophoresis
CCD	charged-coupled device
CDA	Czapek dox agar
cDNA	complementary DNA
cm	centimetre
CPO	crude palm oil
CTAB	Cetyl trimethylammonium bromide
Cu	copper
DI	disease incidence
DNA	deoxyribonucleic acid

dNTP	deoxynucleotide triphosphate
DS	disease severity
DXP	Dura x Pisifera oil palm breed
EDTA	Ethylenediaminetetraacetic acid
et. al.	Collective authors
FFB	fresh fruit bunch
Foe	*Fusarium oxysporum* f.sp. *elaeidis*
Fr	Fries (taxonomy)
g	gram
GbLS	*Ganoderma boninense* isolate LS
GSM	*Ganoderma* Selective Medium
HCl	hydrochloric acid
Imp	Improved
IPM	Integrated Pest Management
IPTG	Isopropyl-ß-D-thiogalactopyranoside
ITS	internal transcribed spacer region
Jacq	Jacquard (taxonomy)
L	litre
Lac	laccase
LDE	lignin degrading enzyme
LiP	lignin peroxidase
m	meter
M	molar
mg	milligram
$MgCl_2$	magnesium chloride
ml	millilitre

mm	millimetre
mM	millimolar
MnP	mangan peroxidase
MPOB	Malaysian Palm Oil Board
MPOC	Malaysian Palm Oil Council
mtDNA	mitochondrial DNA
NaCl	sodium chloride
NC	No change
ng	nanogram
nm	nanometre
NaOH	sodium hydroxide
Pat	Patouillard (taxonomy)
PAL	phenylalanine ammonia lyase
PCR	polymerase chain reaction
PDA	Potato dextrose agar
Pers	Persoon (taxonomy)
Pers comm	personal communication
pH	acid/base value
pmol	picomol
PO	phenol oxidase
PPO	polyphenol oxidase
PVP	polyvinyl pyrrolidone
qPCR	quantitative real-time polymerase chain reaction
RBBR	Remazol Brilliant Blue R dye
RNA	ribonucleic acid
rpm	revolution per minute

RWB	rubber wood block
Ryv	Ryvarden
Sdn Bhd	Private limited
SDS	Sodium dodecyl sulphate
Sg	Sungai (address)
SGP	small grain and erect panicle
SOD	Sudden Oak Death
spp	species
Taq	*Thermus aquaticus* bacterium polymerase
TBE	Tris borate buffer
Tm	melting temperature value
Tris	tris(hydroxymethyl)aminomethane
UK	United Kingdom
US	United States of America
USR	Upper stem rot
UV/Vis	UV and Visible light spectrum
v/v	volume/volume
Willd	Willdenow (taxonomy)
Wors	Worsen
WRF	white rot fungus
w/v	weight/volume
X-Gal	5-bromo-4-chloro-indolyl-galactopyranoside

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1. Introduction

1.1 Oil palm

1.1.1 The oil palm tree

Oil palm is a monocotyledon that belongs to the family Arecaceae, order Cocoeae and genus *Elaies* (Corley and Tinker, 2003). Documented records show that oil palm grows well in the tropical belt, parallel to the Atlantic coast of West Africa, stretching towards Cape Verde in Angola, Africa (Gerritsma *et al*., 1977). It was hypothesized that before the formation of continents, oil palm originated from the African end of the land mass to South America with the earliest fossil of *Elaies* pollen found in the Niger delta (Zeven, 1972). Therefore, the species *Elaies guineensis* was identified as the African oil palm whilst the species found in Central and South America was described as *Elaies oleifera* (Zeven, 1972). Africans use the mesocarp-oil and kernel-oil for food, soap and candle making (Zeven, 1972). Oil palm grew as a wild tree crop, growing in low-land rainforest in Africa before it was brought into South East Asia in the 19th century (Gerritsma *et al*., 1977). Although, it was first cultivated as an ornamental palm in Medan, Northern Sumatra the commercial potential of palm oil was realized when it was cultivated on a large scale in plantations of Sumatra and Malaysia (Gerritsma *et al*., 1977).

The transfer of oil palm to South East Asia had proven favourable as the climatic conditions are suitable for the cultivation of this crop as compared to the African climate (Gerritsma *et al*., 1977). A monoecious plant, oil palm bears both male and female characteristics and each tree

can produce fruit bunches weighing 25 to 40 kilograms. Each fruit bunch can carry approximately 1000 to 3000 fruitlets that contain oil-rich mesocarps. (www.mpoc.org.my). First seeds were selected at the Elmina Estate in Malaysia, while fourth generation seeds have been maintained at the Banting Oil Palm Research Station, Malaysia (Gerritsma *et al*., 1977). The variety of oil palm grown in Malaysia is *tenera*, a hybrid between the thick shelled Deli *dura* and the high yielding female palm, *pisifera*. The fully domesticated oil palm was first achieved in Malaysia, where the palms depended entirely on humans for nutrition and space (Gerritsma *et al*., 1977). Oil palm trees survive up to a hundred years but the yield of good quality oil is limited to ages five to 30 years (www.mpoc.gov.my) (Fig. 1). A common practice in Malaysian plantations has been the replacement of 25 to 30 years old 'mature' palms with the younger generation for continuous cropping (www.mpoc.org.my). The trunk of the oil palm is wrapped with layers of palm leaves or fronds. An average mature oil palm can reach a height of 50-60 feet. Oil palm is propagated from seeds and it takes about five years to achieve maturity and to bear fruits (www.oilpalmtree.net). Most soil types in Malaysia are suitable for oil palm cultivation but oil palm was first grown to replace coconut on the coastal lands and rubber plantations in the inland areas (www.oilpalmtree.net).

Figure 1: An oil palm tree (17 years old) growing at The University of Nottingham Malaysia campus. This photo was taken on 22 April 2011.

1.1.2 Oil palm and the economy

Since the planting of oil palm in Malaysia and Indonesia, new land areas have been cleared for oil palm cultivation. These include land previously planted with coconut and rubber. In a report in 2000, the total land use for oil palm in Malaysia was recorded as 3.31 million hectares (Teoh, 2000). It was reported that 2 million hectares or 62% of this land was situated in Peninsular Malaysia, while 940,000 hectares (28%) and 320,000 hectares (10%) was in Sabah and Sarawak, respectively (Teoh, 2000). In 2010, the Malaysian Palm Oil Board reported plans to replant 365,000 hectares of mature oil palm plantation with younger palms to boost yield. This move was prompted when Malaysia lost the position of

top palm oil producer in the world to Indonesia (Reuters, 2010). In 2009, Malaysia exported about 90% of its palm oil output and this contributed to RM37 billion in crude palm oil (CPO) export and a further RM 13 million in refined products and oleo chemicals (Reuters, 2010).

The main product of the oil palm tree is crude palm oil (CPO) from the pressing of the fruit mesocarp. CPO is traded as a commodity and is highly valuable for the edible oil market. This market is crucial to Malaysia as oil palm is the highest oil-producing crop in the world, next to 17 different oil crops such as soybean, rapeseed and sunflower. In 1999, Malaysia produced 54% of the total palm oil in the world and in 2015, 37% (Fig. 2). It is projected that by 2016, palm oil will be the leading edible oil (Teoh, 2000). However, the importance of an oil-producing crop such as oil palm goes beyond the edible oil and oleo chemical industries. In recent reports, green technology utilizing biofuels has created a new avenue for the use of palm oil. However, the issues surrounding sustainable palm oil production have prompted an active group called Roundtable Sustainable Palm Oil (RSPO). RSPO is responsible for overseeing good practices for conservation of the environment and by 2016, the group requested the following compliance: (1) Conserve high carbon stock area, (2) Avoid plantings in peatlands, regardless of the depth of peat, (3) reporting of greenhouse gas emission and reduced targets and (4) ensure transparency and traceability back to individual estates (Burger, 2015). Peat is soil area that has decomposed plant biomass and creates wet coastal land where the rate of biomass

accumulation is greater than decomposition. Due to high water content, the aerobic decomposition is limited (Mutert *et al.*, 1999). Due to the spongy character of peat, there is a shringkage following drainage at the beginning of palm oil cultivation, creating more land below sea level and a reduction of biodiversity that peatland has (Mutert *et al.,* 1999).

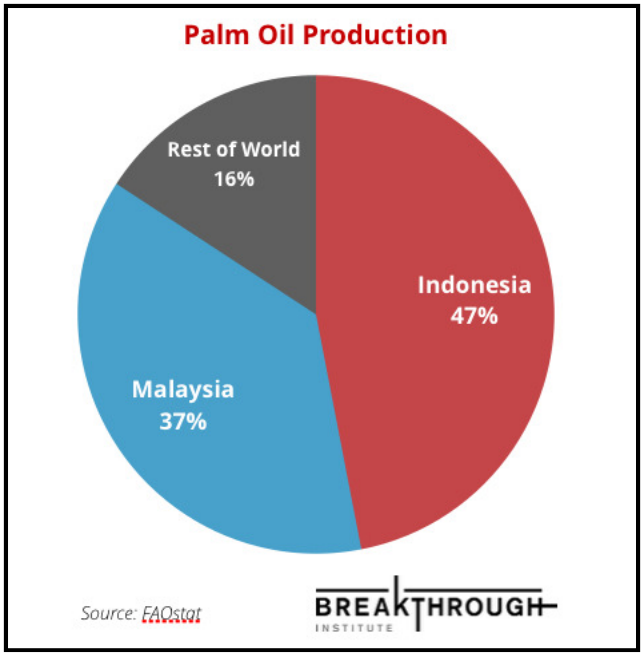


Figure 2: Distribution of the production palm oil according to countries, FAOstat (Burger, 2015).

1.1.3 Oil palm: pests and diseases

The main threats towards oil palm production in Malaysia are plant health disorders and natural pests and diseases resulting in abiotic and biotic stresses in the field. The major contributing factor in health of oil palm is limited genetic diversity; nutrient stress also plays a role. These are manageable threats and can be treated with the use of elite oil palm clones and a strict application of fertiliser regimes (Corley and Tinker,

2003). Early research and development into oil palm production in Malaysia involved plant breeding and selection of superior clonal material. Over the years, agronomists addressed the issue of efficient nutrient delivery and uptake for high yields of fresh fruit bunches (FFB) and crude palm oil (CPO) (Corley and Tinker, 2003). The threat of pests and diseases in oil palm is a continuous struggle in plantations. Major pests in oil palm estates are insects, birds, rats and other mammals (Corley and Tinker, 2003). Some of the insects that cause damage to oil palm are bagworm (*Metisa plana*), rhinoceros beetle (*Oryctes rhinoceros*) and leaf miner (*Coelaenomenodera lameensis*). While rats and snakes have been successfully controlled using bait techniques and owls (*Tyto alba*), insect pests are only manageable with the use of integrated pest management (IPM) which, in some cases, includes a biological control agent (Corley and Tinker, 2003).

Common diseases in oil palm differ according to the geographic location and the growth stage of an oil palm. Most diseases were first recorded in the 1950s (Corley and Tinker, 2003). Diseases that affect young oil palm seedlings are often different from diseases that are prevalent in mature palms. Some of the diseases that affect oil palm seedlings are brown germ caused by *Aspergillus niger* and *Penicillum* spp., *Cercospora* leaf spot caused by *Cercospora elaeidis* and seedling spear and bud rot caused by *Phytophthora* spp. and *Potyvirus* spp. (Corley and Tinker, 2003). Mature palms commonly suffer from leaf wilt and trunk rot diseases. *Cercospora elaeidis* causes brown spots and

orange halos on fronds in Africa, while Orange spot disease on fronds is associated with a viroid (Corley and Tinker, 2003). Root and stem diseases have been related to damage and drying of opened fronds, followed by drying or decay of younger spear fronds (Corley and Tinker, 2003). In Africa, dry basal stem rots are caused by the ascomycete *Ceratocystis paradoxa* or *Thielaviopsis paradoxa* which caused an epidemic in Nigeria in 1967 (Corley and Tinker, 2003). Vascular wilt caused by *Fusarium oxysporum* f.sp*. elaeidis* (*Foe*) is a problem in West Africa and Brazil but less prevalent in Malaysia. The symptoms are differentiated from those of trunk/stem rots by the abscission of the rachis/frond at the base and hanging downwards (Corley and Tinker, 2003). *Foe* is a virulent pathogen that out compete other fungi in the soil environment (Cooper, 2011). The disease is established in the roots and spreads through root to root contact, while spores are carried on male inflorescences (Cooper, 2011). There are also indications that *Foe* spreads via seeds by producing a germ tube on the surface of the fruitlets (Cooper, 2011). The most promising control is the use of resistant varieties, although screening for *Foe* resistance in oil palm is in the initial stages (Rusli *et al*., 2015). Sustainable breeding programmes have markedly reduced disease incidence in the Ivory Coast (Corley and Tinker, 2003). It has also been suggested that the prevention of spread to non-*Foe* nations through good quarantine practice is the best way to avoid this disease (Cooper, 2011; Rusli *et al*., 2015).

In the 2009 International Workshop on Awareness, Detection and Control of Oil Palm Devastating Diseases in Kuala Lumpur, Malaysia, it was reported that the most significant disease of oil palm is basal stem rot (BSR) caused by the hymenomycete fungus, *Ganoderma boninense* (Idris, 2009). Although, for many decades, BSR was limited to old, mature palms, the epidemiology changed in the 1950s to younger palms and in former coconut plantations (Corley and Tinker, 2003). The early symptoms include 'malnutrition'-like wilting of mature fronds, desiccation of fronds and toppling of the oil palm crown. Although the symptoms appear very similar to *Fusarium* wilt, the disease is usually identified by the presence of *Ganoderma* sporophores on the basal stem and roots (Chung, 2011). Upper stem rot (USR) is postulated to be associated with *G. boninense* but also with other fungal species such as *Ganoderma zonatum* and *Phellinus noxious* (Corley and Tinker, 2003); however, recent findings indicate that *G. boninense* is the most prevalent pathogen found in USRs (Hasan *et al*., 2005; Idris, 2009; Rees *et al*., 2012). USR prevalence compared to BSR ranges between 1:10 to 1:1 in some areas (Hasan *et al*., 2005). Since the occurrence of USR is prevalent in oil palm grown in peat soils, roles for basidiospores were proposed (Susanto, 2009). However, a field trial of artificial inoculation using basidiospores did not yield infection and disease in frond bases, possibly due to insufficient inoculum potential (Hasan *et al*., 2005). In a recent study, basidiospores were applied on cut frond surfaces and peduncles of oil palm (Rees *et al*., 2012). The work included scanning electron microscopy

to detect the growth and infectivity of the spores on cut surfaces. The results were similar those of Hasan *et al*. (2005), whereby the spores germinated and exhibited growth on the surface but were unable to gain entry into the tissue. The identification of *Ganoderma* spp. causing USR by DNA analysis demonstrated several infections within the same palm and on adjacent palms. The researchers used ITS fragment of DNA is an RFLP by comparing banding patterns to determine the different genets of the same pathogen. Although the DNA profile showed identical banding patterns for infections within the same tree, there were clear differences between isolates in adjacent trees, further adding to the complexity of *Ganoderma* spp. taxonomy (Rees *et al*., 2012).

1.2 Basal Stem Rot (BSR) Disease

1.2.1 Pathogen biology

1.2.1.1 *Ganoderma* spp.

Ganodermataceae belong to the kingdom Fungi and the phylum Basidiomycota. The genus *Ganoderma* was first described by Peter Adolf Karsten in 1881 and commonly is known as a 'bracket fungus' (Seo and Kirk, 2000). At present, the genus contains 80 species that are mainly identified by morphological traits (Seo and Kirk, 2000). The recognition of a *Ganoderma* bracket is through their large, hard/soft and leathery sporophore also known as basidiocarps. They grow and thrive in stems of living or dead trees by digesting lignin. The life cycle of *Ganoderma* spp. includes sporophore structures that store nutrients as well as producing

basidiospores. (Fig. 3) (Seo and Kirk, 2000). *Ganoderma* basidiospores (Fig. 4) have double cell walls and are ovoid, ellipsoid-ovoid and occasionally cylindrical-ovoid but usually have a truncated end of the apex (Seo and Kirk, 2000). Identification tools have been employed to distinguish between the species and subspecies, such as somatic incompatibility (*Ganoderma* has outcrossing mating characteristics) (Pilotti, 2002), isoenzyme classification (Smith and Sivasithamparam, 2000) and DNA fingerprinting (Sun *et al*., 2006; Moncalvo *et al*., 2008). *Ganoderma* has a complex taxonomy because traditional and morphological identification does not differentiate some species (Jean-Marc Moncalvo, Senior Curator of Mycology in the Department of Natural History at the Royal Ontario Museum, and a cross-appointed Professor in the Departments of Ecology and Evolutionary Biology and Cell and Systems Biology at the University of Toronto, personal comm, 11 August 2007). A molecular fingerprinting approach using genetic markers to map phylogenetic relationships was used to rearrange the relationships between *Ganoderma* species, causing some to be moved out of the genus *Ganoderma* such as species of *Amauroderma* (Seo and Kirk, 2000). Other than genetic fingerprinting, vegetative incompatibility remains a key test to select a variety of *Ganoderma* mating types of the same species (Seo and Kirk, 2000).

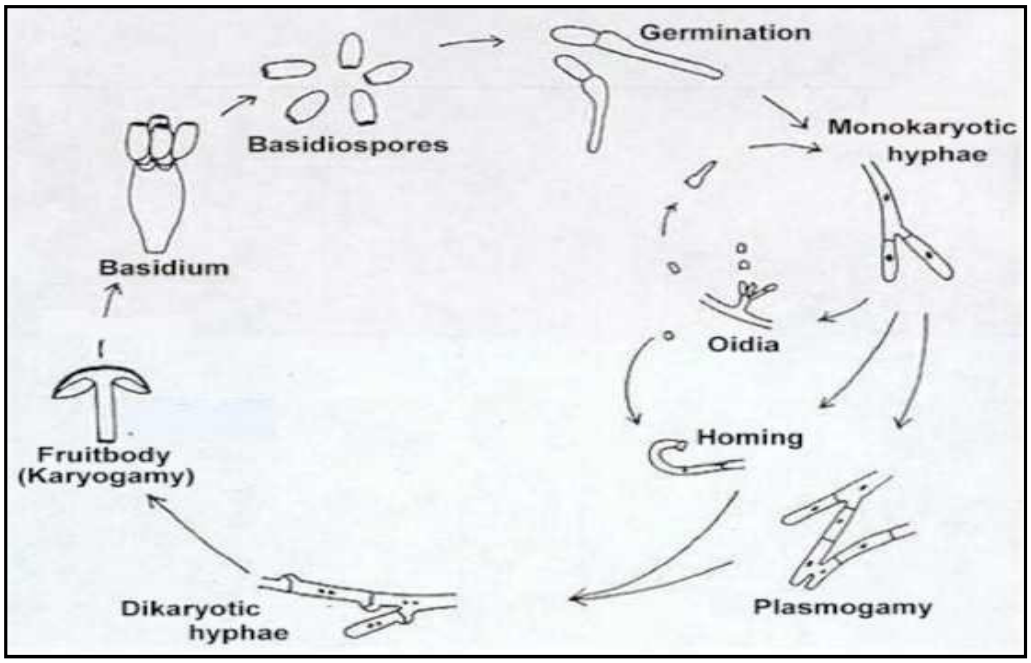


Figure 3: Life cycle of Basidomycota fungi (Deacon, 2006b).

1.2.1.2 *Ganoderma boninense* Pat.

The physical structure of the Ganoderma basidiocarp, (basidiomata, sporophore) includes pores on the underside to disseminate spores, hence the term polypores. The colour of the sporophore, pileus and hymenophore is influenced by environmental factors; for example, the surface of *G. lucidum* appears as shades of red and brown (Volk, 2000). The number of concentric rings on the surface of the basidiocarp also varies from 1-3 to 4-7 (Seo and Kirk, 2000). Basidiospores are usually ellipsoid and brown in colour with an average diameter of 10 µm x 7 µm with the mean spore index (ratio of spore length to width) of 1.6 (Fig. 4) (Seo and Kirk, 2000; Pilloti *et al*., 2004).

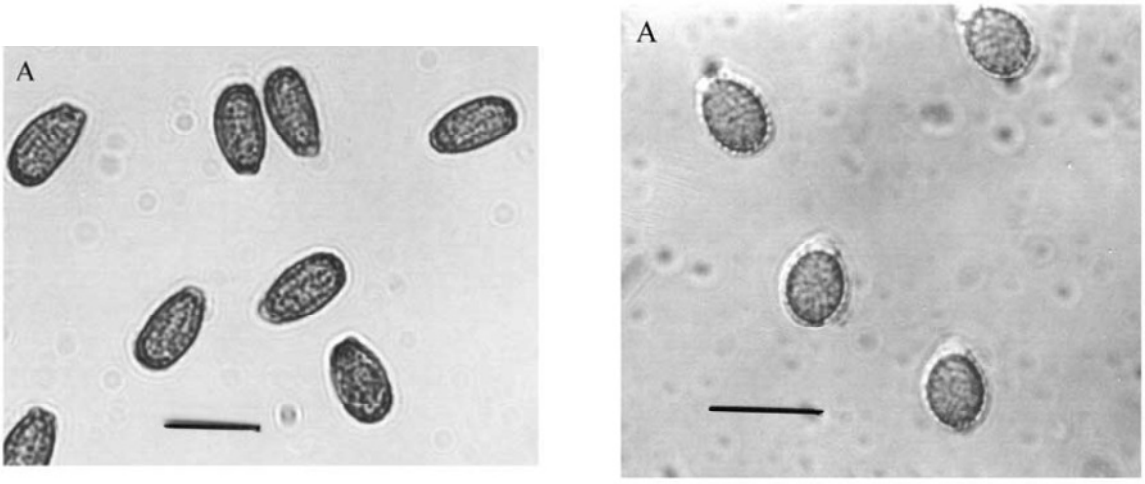


Figure 4: The morphology of *Ganoderma* spp. spores. Above: *G. boninense*; Below: *G. tornatum* (Pilloti *et al*., 2004).

Ganoderma boninense is a necrotrophic fungus that rots and decays oil palm, causing basal stem rot disease. Previously, taxonomic confusion often labelled *G. boninense* Pat. as *G. lucidum* although the basidiocarps are clearly look different, with *G. lucidum* basidiocarps being shades of red whilst *G. boninense* are dark brown (Thompson, 1931). Researchers have argued over the accuracy of the nomenclature because morphology is influenced by the environment, with Turner (1981) for example listing 15 species of *Ganoderma* associated with BSR in oil palm (Idris, 2009). Ho and Nawawi (1985) concluded that all BSR associated *Ganoderma* is *Ganoderma boninense*.

Ganoderma spp. basidiospores differ with *G. boninense* and *G. zonatum* differentiated according to spore size (Pilotti e*t al*., 2004). Spores of both species are yellow to brown with the apical germ pore commonly visible (Fig. 4) (Pilotti e*t al*., 2004). *Ganoderma boninense*

spores are ellipsoid while *G. tornatum* spores are ovoid. Spores of *G. boninense* are slightly longer, approximately 10 µm, while *G. tornatum* spores are 7 µm (Pilotti e*t al*., 2004). Single basidiospores germinate to form monokaryotic mycelium; the fusion of two or more monokaryotic mycelia forms a dikaryon. *Ganoderma boninense* mating type has been proven to favour outcrossing via anastomosis of monokaryotic hyphae (Pilotti *et al*., 2003). Hyphae of dikaryon will form clamp connections that are easily identified using microscopy (Deacon, 2006a). Clamp connections are extensions of increased cytoplasmic volume from backward-folding of the lead hyphae and are a characteristic of hymenomycete heterozygosis (Fig. 5) (Deacon, 2006a).

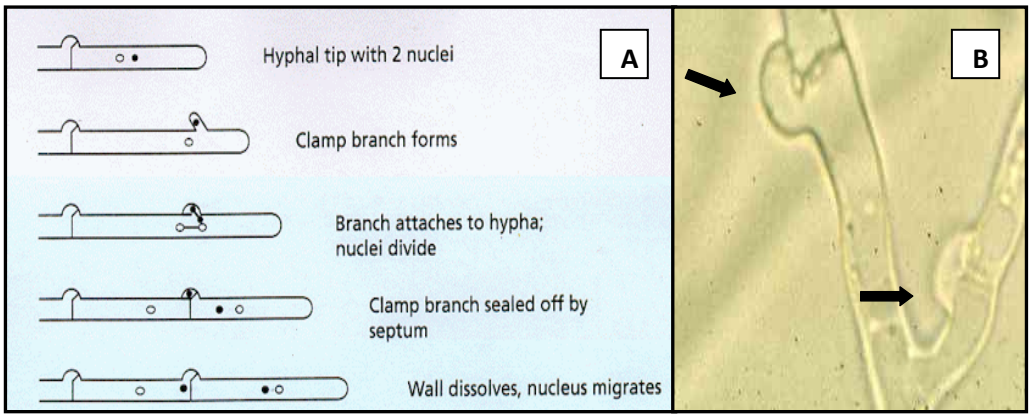


Figure 5: Clamp connections of Basidiomycota formed to maintain dikaryotic state. (A) Steps leading to the formation of clamp connections. (B) View of clamp connections (indicated by arrow) under light microscope (Deacon, 2006a).

1.2.2 Epidemiology of BSR disease

1.2.2.1 Plant hosts and life cycle

Ganoderma spp. are wood-rotting fungi that grow on perennial, palm and conifer hosts (Ariffin *et al*., 2000). *Ganoderma* spp. have been known to infect all palm species, including Salba palm, date palm and coconut (Latiffah *et al*., 2002; Elliott and Broschat, 2006; Karthikeyan *et al*., 2007). In the classification for Polypores, brown rotting fungi usually degrade the white (cellulose) tissue at a higher rate than brown (lignin) parts (Volk, 2000). However, *Ganoderma* spp. are white rot fungi that preferentially degrade lignin and cellulose (Volk, 2000; Elliott and Broschat, 2006; Paterson *et al*., 2009). Other than oil palm, *Ganoderma* has been reported as a pathogen in rubber, tea, betel nut palm, coconut, cocoa, stone fruits, grapevines and forest trees such as *Acacia* spp. (Idris, 2009). *Ganoderma* spp. produce a lignin degrading enzyme complex (LDE) that is efficient in the breakdown of both hard and soft wood (Ariffin *et al*., 2000). In a study carried out by the Malaysian Palm Oil Board (MPOB), 224 isolates of *Ganoderma* spp. from coconut, rubber, coffee, cocoa, *Oncosperma, Areca,* fruit trees, forest trees and tea plants were collected and tested for virulence on oil palm. Between 78 and 80% of isolates from coconut and other palms caused BSR in oil palm; and approximately 30% of isolates from other plants also caused BSR (Idris, 2009). Leguminous cover crops planted to protect soil respiration in oil palm estates were also tested for their resistance or susceptibility to *G. boninense*. Fifteen months post-inoculation, all species were infected, with *Mucuna brateata* having the lowest disease incidence (DI) of 11.7% (Idris, 2009). This work proves that *G. boninense* has a wide host range

and disease epidemics are caused by the increase in fungal inoculum in infected soil. Hyphae within infected root tissues proliferate to form a network of mycelium within plant vascular tissues (Idris, 2009). However, recent findings suggest that there is no early colonisation of vascular tissues but the presence of pseudo-sclerotia enveloping roots functions as a scaffold to protect *Ganoderma* spp. from dehydration and microbial attack (Rees *et al*., 2009). There have been observations of the infection strategy switching from biotrophy to necrotrophy (hemibiotrophy) as initial colonisation of host intracellular cytoplasm by large hyphae eventually leads to active enzymic cell wall degradation (Rees *et al*., 2009). The formation of melanised mycelium in thick pseudosclerotia is thought to be the third stage of infection where *Ganoderma* spp. protects itself from host defences (Rees *et al*., 2009). Substantial colonisation is observed with the presence of a sporophoreat the basal stem of the infected palm. In an environment that is conducive, such as in hot, humid and shaded areas of the estate, spores are steadily released throughout the day (see Section 1.2.2.3). Germination of a single spore will form monokaryon mycelium. Two or more vegetatively compatible hyphae must fuse (anastomosis) and create a dikaryon. It has been suggested that only dikaryotic mycelium can cause disease. From mating type relationships, it has been postulated that each breeding cycle for *G. boninense* is approximately 4-7 years with estimated disease incidence to death in mature palms taking place in 3-4 years (Pilotti *et al*.,

2003). BSR infection on wounded sites progresses faster than that in intact tissues (Flood *et al*., 2005; Idris, 2009).

1.2.2.2 Virulence factors

The natural mode of infection by *Ganoderma* spp. is through contact between infected and uninfected plant roots, through wounds or through contact of live tissues with colonised dead tissues (Idris, 2009). Following *Ganoderma* infection, affected roots become friable and powdery with the discoloration and breakdown of internal tissues such as the cortical tissue (brown) and stele (black) (Idris, 2009). In histopathological studies, *Ganoderma* was initially shown to invade the endodermis layer but, as the disease progresses, xylem, phloem, pith and parenchyma cells contain fungal hyphae (Idris, 2009). *Ganoderma* hyphae grow to extend across the diameter of the basal stem with cross sections of tissues revealing brown, yellow and black zones (Idris, 2009). Turner (1981) postulated that the black zones are 'reactive zones' for *Ganoderma* virulence factors while the yellow zones are a result of a plant defence mechanism of containment of the pathogen. The yellow zones also smell of fermenting (rotting) palm oil (Idris, 2009).

Ariffin *et al*. (1989) stated that the black zone or 'black lines' seen clearly with the naked eye in rotting tissues are thick-walled and swollen resting hyphae. A black line originated from a single mycelium, indicating its fungal nature and the location of black lines on one side of the black line indicated no competing fungi (Ariffin *et al*., 1989). These lines were

due to gum deposition following wounding or parasitic invasion (Ariffin e*t al*., 1989). Black lines have been reported in other wood-rotting fungi, such as *Armillaria* and *Stereum hirsutum,* but the term used to describe these lines was 'zone lines' and 'pseudosclerotia' (Lopez-Real e*t al*., 1975a). The stages involved in the formation of black lines are hyphal growth, hyphal swelling, hyphal aggregation and pigmentation (Lopez-Real e*t al*., 1975a). These deeply embedded 'resting structures' are a result of the initial aerobic environment for mycelium proliferation and, later, high carbon dioxide stress on hyphae (Lopez-Real e*t al*., 1975b). Black lines prevent recognition and attack by antagonistic fungi such as *Trichoderma* spp. and *Penicillium* spp., and ensure the long-term survival of hymenomycetes in wood debris or soils (Lopez-Real *et al*., 1975b). The presence of these 'pseudosclerotia' hyphae is an indication of the accumulation of inocula over a long period of time in oil palm plantations (Ariffin *et al*., 1989). In addition to the hard mycelial crust of the 'black lines' in infected palms, *G. boninense* also produces lignin degrading enzymes (LDEs) that may contribute to its pathogenic abilities as a white rot fungus (Paterson *et al*., 2007). LDEs play a role in the breakdown of lignin, lignocellulose, hemicellulose and pectin as observed in roots attached to mycelium removed one month post inoculation (Rees *et al*., 2009).

1.2.2.3 Mode of dispersal

There have been several hypotheses for the dispersal of *G. boninense* spores and mycelium. *Ganoderma boninense* does not produce chlamydospores that survive in soil, unlike other *Ganoderma* spp. such as *G. lucidum* and *G. weberianum* (Chang, 2003). It was proven that chlamydospore production from mycelium increased the survival of *Ganoderma* spp. from 12 weeks to 52 weeks in soil (Chang, 2003). However, the findings also revealed that infested woody debris will ensure long term survival of *Ganoderma* spp., even if they do not produce chlamydospores (Chang 2003). Most commonly, *G. boninense* infection is spread through the contact of infected wood debris with oil palm roots and between infected and uninfected roots (Pilotti *et al*., 2002; Rees *et al*., 2007; Idris, 2009). In a related study, naturally infected wood stumps, mounded with soil, placed in specific areas in an estate resulted in the infection of nearby oil palm seedlings (Flood *et al*., 2000). It was also found that un-mounded wood debris did not promote disease in 5 years and that the distance of 0.5 m between infected stump and seedling permitted disease spread and symptoms in 27 months as compared to 1.0 m, where it took 38 months for symptom development (Flood *et al*., 2005).

Evidence for secondary root-to-root infection has been largely controversial because, when tested for genetic lineages (Pilotti *et al*., 2002; Miller *et al*., 1999), *G. boninense* had distinct genetic diversity, indicating a role for basidiospores (Pilotti *et al.,* 2003). Somatic incompatibility tests revealed that multiple infections within the same

palm are common, thereby indicating air-borne inoculum (Pilotti *et al*., 2003). The presence of *G. boninense* on cut-frond bases of healthy palms adjacent to infected stumps was tested using a molecular detection system (Bridge *et al*., 2001). Although, the seedlings did not show any symptom of infection, *G. boninense* DNA was detected in the samples, indicating a potential route of infection via cut-frond bases. This also supported the hypothesis of spore-induced infection as cut-fronds are not in contact with soil (Bridge *et al*., 2001). Using spore traps, it has been estimated in one study that *G. boninense* produces approximately 10^6 spores per minute from a 100 mm x 50 mm basidiocarp (Sanderson, 2005). Accumulation of air-borne basidiospores within plantations in Sumatra in Indonesia showed continuous and high production over 24 h (range *c*. 2–11 000 spores m^{-3}) with maximum release during early evening (Rees *et al*., 2011) In another study, *Ganoderma* spp. basidiospores were reported as significant bio aerosols, as they were found on 95% of the days in a five months period (Craig and Levetin, 2000). *Ganoderma* spp. spores disperse through closely arranged pores, vertically downward into turbulent air (Deacon, 2006b). Infection by spores also depends on their dikaryotization via anastomosis because monokaryotic mycelia generated from *Ganoderma* spp. basidiospores were unable to cause BSR in ornamental palms (Lim and Fong, 2005).

1.2.3 Detection of *Ganoderma* spp.

1.2.3.1 Classical methods

BSR infection has a long latent phase as much of the *Ganoderma* mycelium can form hard-shelled pseudosclerotia to survive deep in the oil palm trunk and basal stem, making detection difficult (Ariffin *et al*., 1989). In field evaluations, BSR is detected on young and mature palms by the presence of *Ganoderma* spp. Basidiomata (sporophore) on the basal stem and roots of oil palm (Ariffin *et al*., 1991) (Fig. 6A, Chung, 2011). Several *Ganoderma* spp. have been associated with BSR in oil palm (Table 1) (Miller *et al*., 2000). Accompanying symptoms include the abscission of mature and green fronds and appearance of dry and brittle roots (Fig. 6B, Chung, 2011). Other significant 'early' symptoms include unopened spear fronds or new spear fronds that are smaller and yellow (Idris, 2009). Since there is no significant variation in climate all year round in Malaysia, symptoms can be present at any time in a calendar year (Singh, 1990). In many plantation practices, routine plant health surveys by managers and field officers are helpful for 'early' detection of BSR but, since the reports for BSR must be complemented by the presence of *Ganoderma* spp. sporophores, this is the stage of infection when a palm is near death (Ariffin *et al*., 2000). In young palms, symptoms such as yellowing and necrosis, develop on one side of the palm (Singh, 1990). Once detected, the palm trunk is marked by tape or paint to clearly differentiate such an infected palm in the plantation. This method of detection could lead to two outcomes; the continuous observation of the health of particular palms especially if the trees are bearing fruit, or in severely infected palms, the removal of the palm stand

using machinery (Chung, 2011). Although these are preventive methods, it has been established that sanitation based on early observation of *Ganoderma* is the most effective method to date to prevent the spread of BSR (Idris, 2009; Susanto, 2009; Chung, 2011). Other methods of detection include dissection of the fallen tree (Chung, 2011), confirmation of diseased tissue in *Ganoderma* Selective Medium (GSM) (Ariffin and Idris, 1992) and the use of isoenzymes and somatic incompatibility (Gottlieb *et al*., 1998; Miller *et al*., 1999; Latiffah and Ho, 2005) to indicate the relationship between isolates and molecular markers to accurately identify isolates from infected and non-host samples (Abdullah, 2001; Latiffah *et al*., 2002; Utomo *et al*., 2005; Karthikeyan *et al*., 2007).

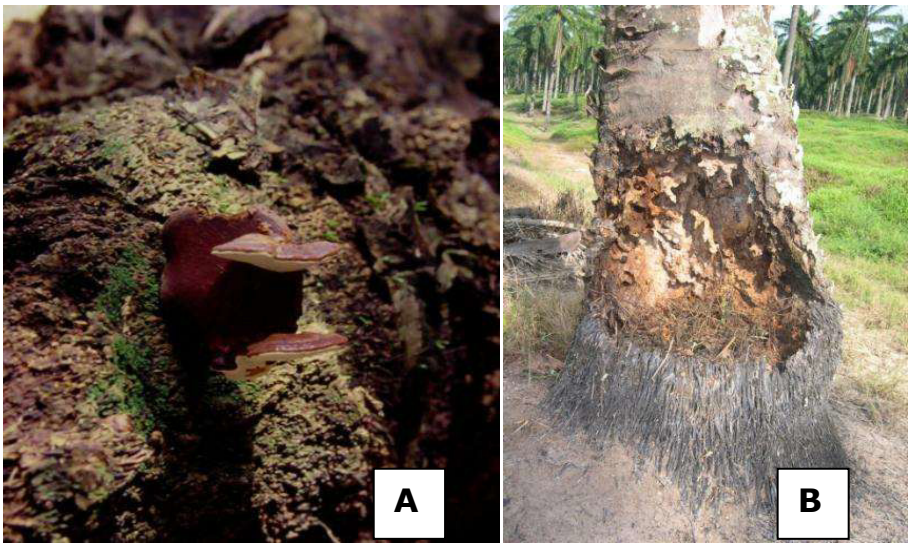


Figure 6: Advanced infection symptoms of BSR. A: *Ganoderma* spp. sporophoreon oil palm trunk. B: Dry rot and decay symptoms on basal stem and roots (Chung, 2011).

Table 1: *Ganoderma* spp. as pathogens for basal stem rot disease. Adapted from Miller *et al.* (2000).

Ganoderma species	Synonym	Occurrence
G. applanatum	*Fomes applanatus*	Angola
		Benin
		Indonesia
		Ivory Coast
		Malaysia
		Principé
		San Tomé
		Zaïre
G. boninense		Malaysia
G. chalceum		Malaysia
G. cochlear		Indonesia
G. colossum		Nigeria
G. fornicatum		Zaire
G. laccatum		Indonesia
G. lucidum	*Fomes lucidus*	Angola
		Ghana
		Indonesia
		Malaysia
		Principé
		San Tomé
		Tanzania
		Zaïre
		Zimbabwe
G. miniatocinctum		Malaysia
G. pediforme		Zaïre
G. pseudoferreum		Zaïre
		Malaysia
G. tornatum	*F. applanatus* var. *tornatum*	Cameroon
	G. applanatum var. *tornatum*	Malaysia
	G. australe	Zaire
G. tropicum		Indonesia
G. xylonoides		Zaire
G. zonatum	*G. tumidum*	Ghana
		Nigeria
		San Tomé
		Tanzania
		Zaïre
Ganoderma spp.		Colombia
		Malaysia
		Zaïre

1.2.3.2 Artificial infection

Methods to accurately identify *Ganoderma* spp. were being investigated in the late 1980s and early 1990s. However, at that time it was not possible to reproduce a disease cycle artificially. One reason for this was that the isolates identified varied according to palm type, age and field conditions. There was also confusion that several species of *Ganoderma* were pathogens of BSR, namely *G. boninense, G. miniatocinctum, G. tornatum* and *G. zonatum* (Idris, 2009). It was also particularly difficult to correctly identify *Ganoderma* spp. according to their aggressiveness as different estates presented different selective pressure for fungal evolution. Therefore, Sariah *et al*. (1994) proposed a standard artificial inoculation technique that can reproduce the infection of young oil palm with known *Ganoderma* spp. until symptoms, such as sporophore formation at the base of the stem, wilting of leaves and brittle roots, are visible. This method was a success and, to date, it is used as the 'standard' method to determine the virulence of field isolates of *Ganoderma* spp. The technique involves the sterilisation of rubber wood blocks (RWB), 5 cm in diameter and 15 cm in length, mixed with malt extract broth. Nutrient coated RWB are inoculated with *Ganoderma* spp. via 10 mm fungal plugs grown in PDA (Sariah *et al*., 1994). Variations of the RWB artificial infection methodology have since been published (Kartikeyan *et al*., 2007; Izzati *et al*., 2008; Rees *et al*., 2009). Perhaps the advantage of this method is the reproducibility of its results, but one disadvantage is that it takes 6 to 12 months to observe *Ganoderma* BSR symptoms such as wilting of leaves or the presence of basidiomata on the

stem of oil palms. Confirmation of Koch's postulates using these artificial inoculation techniques requires the re-isolation of *G. boninense* on *Ganoderma* Selective Medium (GSM) as recommended by the Malaysian Palm Oil Board (MPOB) (Ariffin and Idris, 1992).

1.2.3.3 Somatic incompatibility

Out-breeding potential and the production of clamp connections in hymenomycete fungi led to the understanding of genetically similar but vegetatively incompatible fungal isolates (Fig. 7). It was recommended that both vegetative incompatibility and DNA identification methods were used to confirm the identity of *G. boninense* (Abdullah, 2001). This approach became crucial when it was realised that *Ganoderma* does not propagate via clonal reproduction as *Ganoderma* populations from infected palms were distinct individuals ('genets', sensu Rayner) (Miller *et al*., 1999). These individuals are categorised in 'somatic incompatibility groups (SIGs)'. In one plot, out of 39 isolates, 34 were 'genets' or individuals (Miller *et al*., 1999). Sexual recombination has been identified as the leading cause for high out-breeding potential and basidiospores increase the chances of genetic recombination during mating (Miller *et al*., 1999). In another related study, 182 *Ganoderma* basidiomata isolated from infected oil palm trees and coconut stumps in Sime Darby Plantations across Peninsular Malaysia and Sabah , the same and adjacent trees provided samples for somatic incompatibility (Latiffah and Ho, 2005) and confirmed that all pairing combinations (except for self-pairing)

showed incompatible reactions, confirming that *Ganoderma* from the same palm or from the same estate were genetically individual and not clones (Latiffah *et al*., 2005). Somatic incompatibility by interfertility testing has been used to analyse mating type alleles to determine the spatial distribution of *G. boninense* mating alleles (Pilotti *et al*., 2003). It was found that basidiospores may play a role in random dispersal as local clustering of mating type was not observed (Pilotti *et al*., 2003). In comparison with other fungi, *Ganoderma* mating behaviour is closer to that of *Schizophyllum commune* which does not exhibit allelic repeats in close or distant sporophore. However the out-breeding of *Ganoderma* is different when compared with other wood rotting fungi, such as *Heterobasidion annosum* and *Armillaria mellea,* because the latter use natural vegetative, root-to-root contacts and thus have reduced genetic diversity (Pilotti *et al*., 2003). The role of lignin degrading enzymes (LDEs) in somatic incompatibility isolates was investigated (Goh *et al.*, 2014). Three complete somatically incompatible *G. boninense* isolates were tested for the ability to produce LDEs (Goh *et al*., 2014). Lack of antagonism in the three pairings could be due either to non-self recognition in compatible mating, or "switching off" of putative incompatibility genes in these isolates after fusion of their hyphae by unknown mechanisms (Pilotti *et al*., 2002). It was confirmed that *G. boninense* was highly heterozygous and the assumption of individualism among the isolates was confirmed (Goh *et al*., 2014).

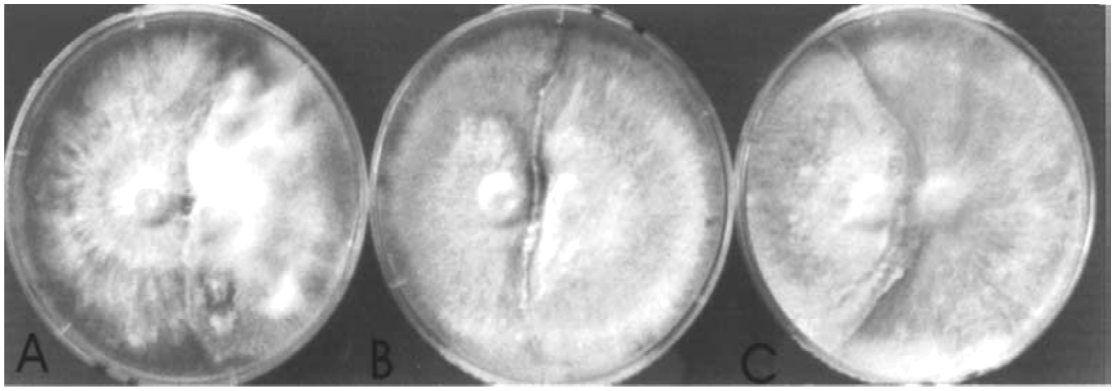


Figure 7: Somatic incompatibility between mycelium cultures of *G. boninense* during an inter fertility test. (A) Weak (B) Medium and (C) Strong (Pilotti *et al.,* 2002).

1.2.3.4 Isozyme detection of Ganoderma enzymes

Isozymes are multiple molecular forms of an enzyme that have the same enzyme activity (Miller *et al*., 2000). Fungal isozymes show polymorphism which can be the basis for taxonomic differences especially if the micromorphology structures remain the same or are plastic (Miller *et al*., 2000). Pectinase isozymes have been differentiated based on isoenzymes for fungi on palm hosts (Miller *et al*., 2000). For *Ganoderma lucidum*, production of endo-polygalacturanase and endo-pectin methyl trans-eliminase was reported; however, *G. boninense* pectinase isozyme profiles did not show significant differences between isolates tested (Miller *et al*., 2000). Several intra- and extracellular laccase isozymes have been used to differentiate *Ganoderma* spp.; however, the wide genetic heterozygosis found provided only a limited link with the host of origin (Miller *et al*., 2000). In another study, electrophoresis was used to categorise eight isozymes of 134 dikaryotic *Ganoderma* isolates (Gottlieb *et al*., 1998). In order to limit the dikaryotic mycelium to individual

groups, somatic incompatibility tests were performed, which limited the number of test isolates to 53 based on strong host evidence (Gottlieb *et al*., 1998). However, the outcome was inconclusive (Gottlieb *et al*., 1998). In a later study, *Ganoderma* species were successfully distinguished based on isozymes of glucose-6-phosphate dehydrogenase using cellulose acetate gel electrophoresis (CAGE), this method was suggested as an important diagnostic tool for *Ganoderma* species (Smith and Sivasithamparam, 2000).

1.2.3.5 Molecular detection of *Ganoderma*

The importance of accurate identification of *Ganoderma* BSR became a concern once the RWB artificial inoculation technique had been established. There were several driving factors for accurate identification of the causal agent (*Ganoderma* versus other fungal samples) from field isolates (Utomo and Niepold, 2000) to differentiate saprophytic and necrotrophy *Ganoderma* (Latiffah *et al*., 2002), to track changes in virulence between isolates in pre-recorded field samples (Bridge *et al.*, 2001) and to map the origins of isolates of *Ganoderma* based on ancestral DNA information (Utomo and Niepold, 2000; Zheng *et al.,* 2009).

In an experiment on field epidemiology of BSR, bait seedlings were grown near diseased oil palm stumps. Stumps were recognised as the major source of fungal inoculum (Flood *et al*., 2005). Around each stump, eight oil palm seedlings were planted and the experiment had eight replicates. After 28 months, 76% of seedlings showed disease symptoms

(Flood *et al*., 2000). In a related experiment, stump size was found to be positively related to disease occurrence with higher numbers of disease symptoms near to larger stumps (Flood *et al*., 2005). Also, sanitation practices via removal of 'infected' soil reduced disease incidence (Flood *et al*., 2000). However, in these experiments, detailed analyses to confirm BSR infection in palms without symptoms were required. Molecular fingerprinting using ITS3/GanET primers provided the test in a polymerase chain reaction (PCR) when pure mycelium of field *Ganoderma* isolates was used to extract total DNA (Fig. 8) (Miller *et al*., 1999; Flood *et al.,* 2005). A combination of restriction fragment length polymorphism with mitochondrial DNA produced a profile showing that identical isolates were present naturally on infected stumps and on bait seedlings (Flood *et al*., 2000). The heredity of mitochondrial DNA (mtDNA) provided a close genetic link between parent and progeny and although *Ganoderma* mtDNA is highly polymorphic, the similarities found in this experiment indicated close contact between isolates via root-to-root infection. The use of a third molecular tool in this study, the amplified fragment length polymorphism (AFLP), confirmed earlier results of the relationship between the natural resident *Ganoderma* on stumps and those found in bait seedlings (Flood *et al*., 2000). Use of several molecular approaches has not only redefined the methods used for *Ganoderma* detection from field isolates but provided a tool to link progenies of *Ganoderma* associated with BSR which otherwise would remain as a 'knowledge gap' due to lack of early disease symptoms on infected palms.

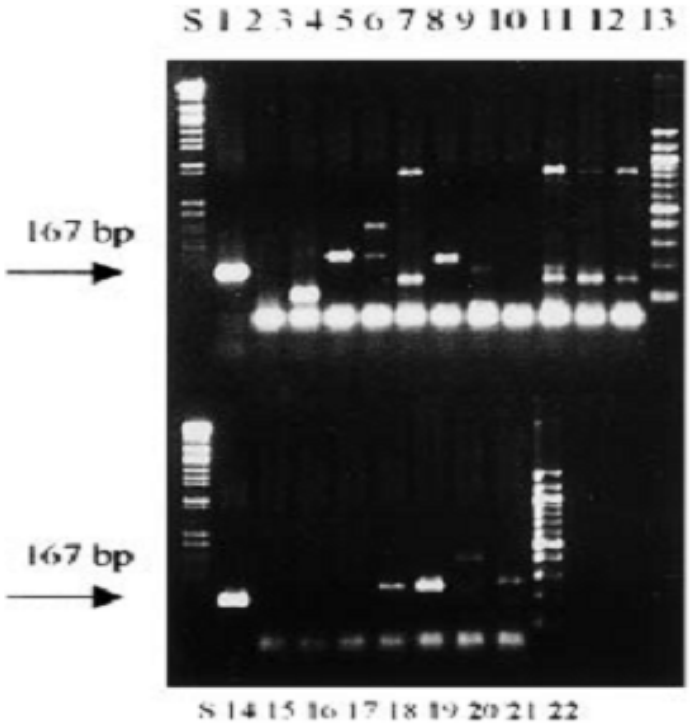


Figure 8: Specific primer detection of a single band, 167 bp from field isolates of *Ganoderma* spp. and other soil fungi using conventional PCR reactions (Utomo and Niepold, 2000).

1.2.3.6 Genomic and transcriptomic data on *Ganoderma*

The oil palm genome was fully sequenced and published in 2013 (Singh *et al*., 2013). The genome consist of 1.8 Gb of the *Elaies guineensis* (African oil palm), of which 1.535 Gb of assembled sequence and transcriptome data was derived from 30 sample tissues; at least 34,802 genes were predicted (Singh *et al*., 2013). The oil palm genome was compared to that of *Arabidopsis thaliana, Musa acuminata* (banana) and *Phoenix dactylifera*

(date palm) with the highest similarity with date palm (1698 genes) (Singh *et al*., 2013). The differences are greater when compared with *Zea mays* (corn) and *Oryza sativa* (rice) genomes (Fig. 9).

From understanding the oil palm genome, other researchers looked into the details of the transcriptome of oil palm during BSR. Oil palms that were artificially infected with *G. boninense* using the rubber wood technique were evaluated for transcriptome data for the up-regulation of genes linked to BSR (Tee *et al*., 2013). Out of 3,748 transcripts, 61 genes were significantly up-regulated or down-regulated during the 3 to 6 weeks post infection (Tee *et al*., 2013). Isoflavone reductase, an enzyme responsible for the production of flavonoid phytoalexins was profiled using qRT-PCR, and the expression data facilitated development of potential biomarkers involved in the transcriptome of BSR-infected oil palm roots (Tee *et al*., 2013).

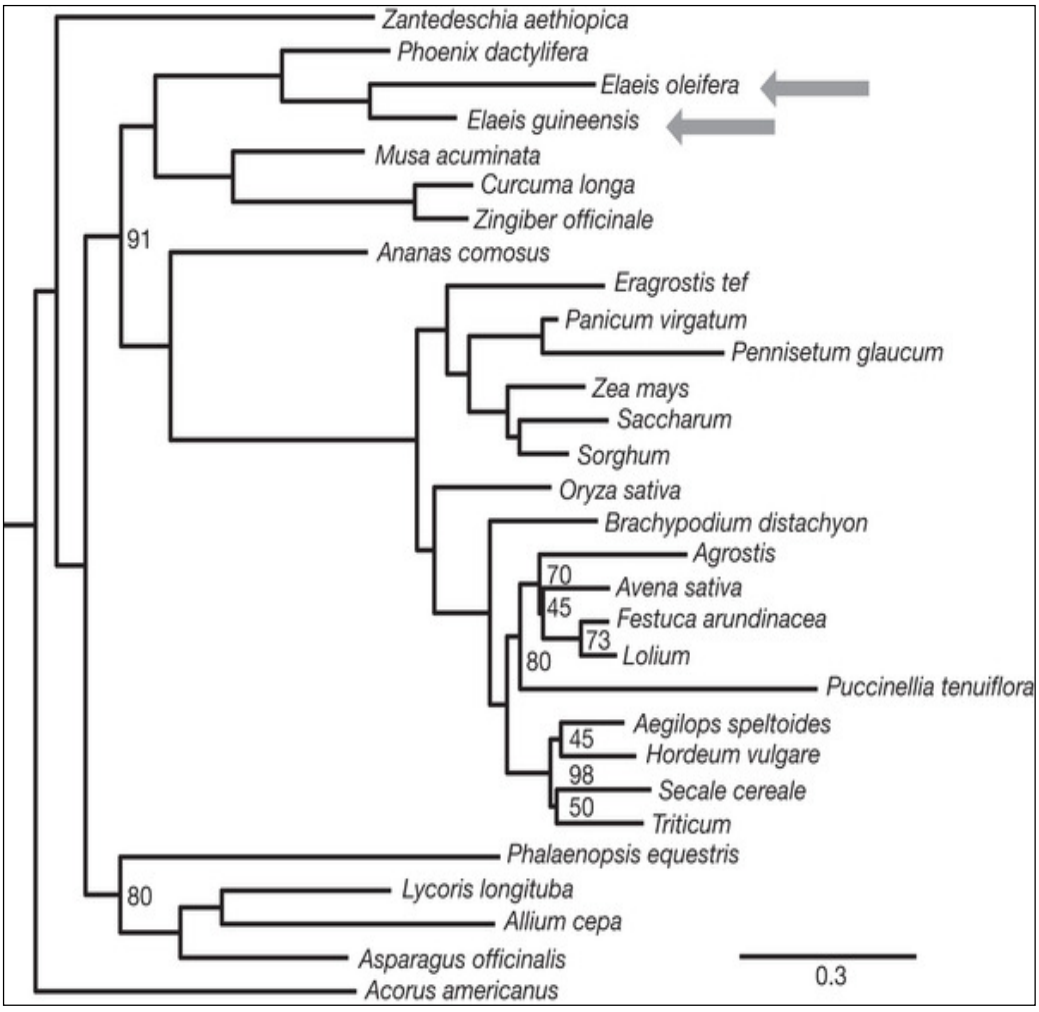


Figure 9: Evolutionary tree diagram for oil palm (Singh *et al*., 2013).

In another related study, 11 putative defence related genes from oil palm that were infected with *G. boninense* were analysed at 3 to 12 weeks post infection (Tan *et al*., 2013). Using qRT-PCR, two candidate genes, early methionine-labeled polypeptide (EgEMLP1) and metallothionein-like proteins (EgMT), were significantly different in palms that were *G. boninense* treated, untreated and treated in the presence of a biocontrol agent (Tan *et al*., 2013). Further BSR-linked research was recently published on the use of simple sequence repeat markers to detect tolerance and susceptibility of oil palm progenies to BSR (Hama-Ali

et al., 2015). In this work, 58 SSR markers were tested with three progenies of oil palm for a molecular map for the association to BSR (Hama-Ali *et al.,* 2015). Only two alleles were linked to BSR and one progeny, KA4G1 remained tolerant to BSR (Hama-Ali *et al.,* 2015).

Some of the advantages of genome-wide data were proven when non-BSR research, such as the somaclonal variations leading to mantled fruits in oil palm were explained (Ong-Abdullah *et al*., 2015). This phenomenon has puzzled estate owners and the research fraternity for decades. The phenomenon is of an oil palm raised from clonal material with uniform genetics but when grown in soil, some plants produced mantled fruits with low oil yield. It was long suspected as an epigenetic outcome and the discovery of *Karma* genes at the MANTLED loci where hypomethylation was linked to mantled fruits proved the hypothesis (Ong-Abdullah *et al*., 2015).

1.2.4 Control of BSR

Based on MPOB reports, as BSR has spread at an alarming rate in inland and coastal estates, plantation officers have designed counter measures to try to control and eradicate the disease (Idris, 2009). A typical approach includes mounding of soil to cover *Ganoderma* sporophore at the stem base marking of BSR trees to prevent unnecessary contact and early prevention by removal of diseased stumps

and surrounding soil (Fig 10A-D) (Idris, 2009; Chung, 2011). Soil mounding does not inhibit the progress of BSR although it was found to prolong the lifespan of oil palms at fruit bearing age (Chung, 2011). Tree surgery to remove part of the basal stem was only useful on young palms as older palms did not recover and they toppled faster (Fig 10B) (Chung, 2011). Digging trenches (100 cm deep x 50 cm wide) around BSR infected palms has successfully prevented the spread of BSR to neighbouring palms (Fig 10C) (Chung, 2011). Prior to the 1990s, burning was used to incinerate diseased tissues in plantations. Government regulations have prevented burning so there has been an increase in *Ganoderma* in roots and soil (Chung, 2011). In order to remove diseased trees, a 2 m x 2 m x 1 m hole is dug around the palm to loosen the soil before toppling the palm (Chung, 2011). It was reported that BSR incidence is 93.3% in estates that retained diseased stumps compared to 6.7% in estates that had diseased material removed (Chung, 2011). To date, the best control measure is sanitation practices that remove the disease and prevent its spread. Other approaches for BSR control include the use of biological control agents such as *Trichoderma* spp. (Susanto, 2005; Susanto *et al.,* 2009) and application of hexaconazole, a chemical fungicide, via trunk injection (Idris, 2009). While all the methods increase the life span of the oil palm, none are curative to control BSR.

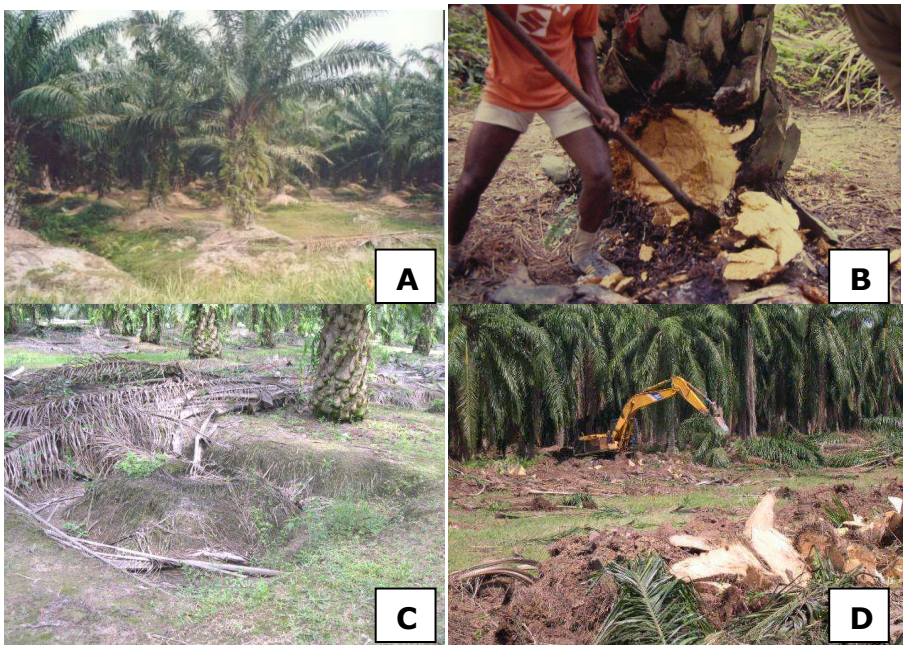


Figure 10: Control strategies for BSR in oil palm estates. A: Mounding, B: tree surgery using a chisel, C: trenching and D: clean-clearing using an excavator (Chung, 2011).

1.3 Artificial infection assay techniques

Underpinning humanpathology research are the principles of Koch's Postulates that state that a hypothesis of infection and disease by an organism has to be proven by successful artificial inoculation, infection of a pure culture of the pathogen on a host organism and the successful of re-isolation of the same pure culture organism in the diseased organism (Agrios, 2005). The symptoms of disease should represent natural infection symptoms and the same pathogen should be re-isolated from artificially infected host tissues. The principle safeguards the process of investigation and proves that a pathogen causes the hypothetical symptoms and disease on plants. At present, the rubber wood block (RWB) inoculation method, recommended by MPOB for BSR takes 6 to 12

months for the appearance of symptoms (Sariah *et al*., 1994; Ganeson, 2015) (Fig. 11). Therefore repeat experiments are time consuming. In a related RWB paper, Izzati *et al*. (2008) described the colonization of *G. boninense* on RWB takes 8-10 weeks. The blocks are then co-cultivated with 3 month old oil palm seedlings in a pot filled with soil (Fig. 11) and only after 20 weeks, a high disease rate of 70% was achieved (Izzati *et al.,* 2008). Though this method clearly differentiates pathogenic and non-pathogenic *G. boninense* isolates, the duration is a hindrance for effective BSR management. In other epidemiological models for disease, rapid inoculation-infection assays have proved useful for the analysis of early disease symptom development (Baumgartner *et al*., 2010).

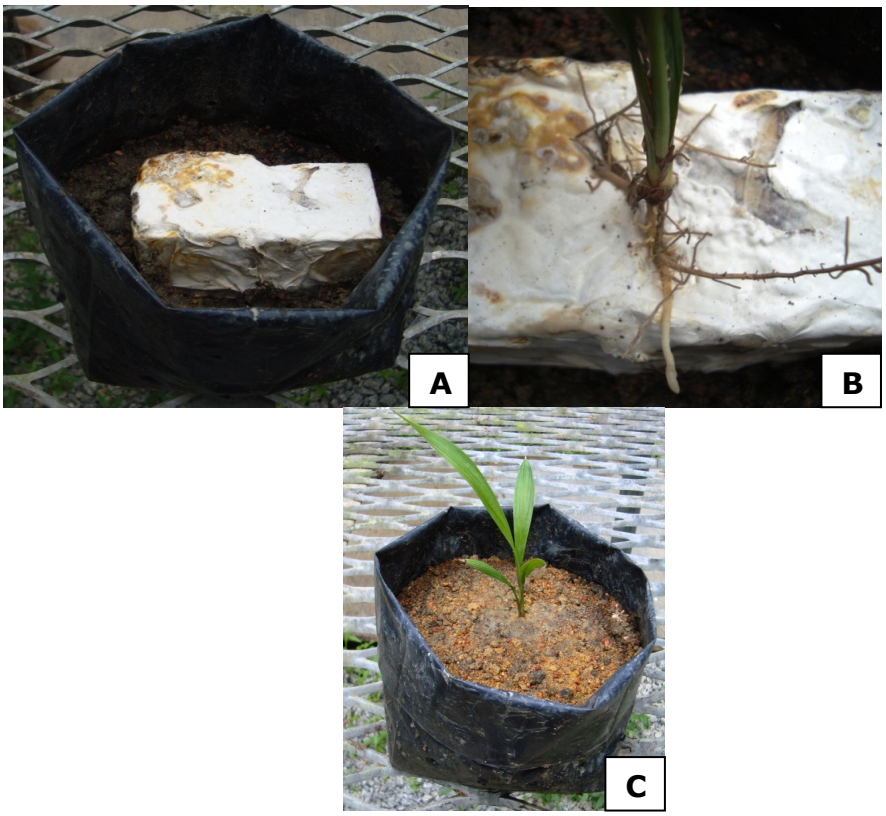


Figure 11: Rubber wood block inoculation technique on oil palm seedlings with Ganoderma isolates. RWB measuring 6 x 6 x 12 cm was washed and autoclaved before inoculation (1 cm) with *Ganoderma* spp. inoculum in

MEA medium in polypropylene bags (A) RWB inoculums were transferred into polypropylene bags containing unsterilized soil (B) Roots of oil palm seedlings were transplanted directly in contact with blocks to avoid root contact with external sources (C) Inoculated oil palm seedling maintained under shade house condition (Ganeson, 2015).

1.3.1 *In planta* infection strategy

An artificial inoculation-infection of a pure fungal culture will provide evidence of its virulence on a host plant, often reproducing the symptoms first observed in a naturally infected host. However, if the host is a perennial plant, the symptoms could take longer than non-perennial species to establish, hence increasing the testing period for the suspected fungal pathogen (Baumgartner *et al.,* 2010). In a natural infection environment, the collection of samples to study the effect of entry, colonisation and infection by a fungal pathogen will be confounded with environmental factors such as climate, rainfall and the presence of biotic stress agents such as insects (Nielson, 1976). Although disease epidemiology studies should reveal the nature of what caused the pathogen to spread in the first place, an artificial infection strategy provides an even, uniform and reproducible platform for a closer look into the events leading to the disease development (Nielson, 1976). Artificial infection assays performed *in planta* have been useful in fungal-plant interaction studies on oil palm (see Section 1.2.3.2), spruce (Deflorio *et al*., 2011) and beech (Olbrich *et al*., 2010). While in oil palm, the reproducibility of the virulence trait by *Ganoderma* was a concern (Section

1.2.3.2), in spruce and beech, the closeness of the infection to its natural form for the induction of defence responses was a priority (Olbrich *et al*., 2010; Deflorio *et al*., 2011). *Heterobasidion annosum*, a fungal pathogen that causes root and butt rots in gymnosperms, was artificially inoculated on Sitka spruce for the induction of defence responses such as phenolics, chitinases and lignin. Early disease resistance was detected in bark and sapwood samples before and three day post inoculation. The results enhanced the potential use of artificial inoculation-infection assays in pathology studies (Deflorio *et al*., 2011).

Assays have been developed for shade house grown plants to represent the natural plant growth environment as well as mimic temperature and humidity of field situations. These methods facilitated a virulence study for small grain and erect panicle (SGP) in rice, a symptom of nematode infestation (Liu *et al*., 2008). Nematodes were confirmed to cause SGP and although the environmental factors were difficult to simulate, inoculation at a site between leaf sheath and culm of rice reduced effects of environmental factors, hence promoting further experiments based on artificial inoculation and infection (Liu *et al*., 2008). Detached leaves, stems, roots and fruits have also been subjected to pathogen infection. This is an extension to artificial infection of whole plants in a shade house or in their natural environment. Besides providing the necessary tissues for fungal invasion, detached explants reduce temporal and spatial aspects related to whole plant infection, such as plant growth facilities which could be impractical for forest species

(Denman *et al*., 2005). In a study to determine infectivity of *Phytophthora ramorum*, the causal agent of Sudden Oak Death (SOD) disease in broad-leaf tree species in the US and UK, detached leaf assays or 'leaf dip assays' were used successfully to differentiate aggressive and non-aggressive isolates based on disease incidence, disease severity and infection potential (Denman *et al*., 2005). These results supported an earlier study that looked at the potential of using artificial versus natural infection in screening for *Phytophthora* spp. (Hansen *et al*., 2005). Also, the 'leaf dip' assay was incorporated into 'pest risk assessment' for 'quarantine-listed' *Phytophthora ramorum* (Denman *et al*., 2005).

1.3.2 *In vitro* explant assay

Using axenic explants to develop inoculation-infection assays has been useful for rice (Koga *et al.,* 2004), grape rootstocks (Baumgartner *et al*., 2010), rye grasses (Scheffer and Tudzynski, 2006), banana (Jie *et al*., 2009), soybean (Bressano *et al*., 2010) and pine (Kabir *et al*., 2013). *Armillaria mellea* infection on grape rootstocks was initially studied in greenhouses but the method used was slow and unreliable (Baumgartner *et al*., 2010). Although, *Armillaria* spreads root-to-root via vegetative mycelium, similar to that of *Ganoderma* spp., it has a lower percentage of genetic polymorphism indicating minimal involvement of genetically dissimilar spores. Therefore uniform mycelium as produced via culture methods were introduced to axenic grape rootstocks in tissue culture (Fig. 12) (Baumgartner *et al*., 2010). This study also used molecular methods

to quantify the load of fungal DNA on infected samples, collected at designated time intervals. The method was both novel and time sensitive for routine testing of grape rootstocks for resistance to *Armillaria* spp. (Baumgartner *et al*., 2010).

In the case of *Pinus radiata- Dothistroma* needle blight (DNB), an urgent reliable artificial infection study was needed to determine susceptibility to the pathogen (Kabir *et al.,* 2013). The available controlled virulence assay was reported to produce infection rates as low as 10% (Kabir *et al.,* 2013). Therefore, the microclimatic conditions for disease development were adjusted to achieve 80% infection rates (Kabir *et al.,* 2013). Plants were kept in a modified (39.5 cm×69 cm×45 cm) humid chamber with a home-made water-fogger and an airspace of 30 cm x 20 cm on the top, to minimise fungal growth (Kabir *et al.,* 2013). Spray inoculation was most reliable to achieve 28.5 ± 14.1% infection rates during the 14 day observations (Kabir *et al.,* 2013).

In a more advanced study, where a combination of axenic and greenhouse plants was used, the impact of the natural endophytic fungal population was assessed to reduce disease severity in banana plantlets (Jie *et al*., 2009). Tissue culture plantlets of banana were artificially inoculated with pure cultures of natural occurring endophytes found in a banana plantation (Jie *et al*., 2009). Subsequently, plantlets were transferred to a greenhouse where they were inoculated with *Fusarium oxysporum* f. sp. *cubense* race 4 causal agent for *Fusarium* wilt in banana

(Jie *et al*., 2009). Introducing endophytes reduced disease incidence by 67% in *Fusarium*-infected banana plantlets. Since commercial production of banana relies on tissue cultured plantlets, the introduction of endophytes could be done quickly and with ease to prevent the devastating *Fusarium* wilt disease (Jie *et al*., 2009). Sowik *et al*. (2001) studied the functionality of an *in vitro* infection assay for *Verticillium dahliae* infection on strawberry. The objective was to elucidate the mode of action of *V. dahliae* during virulence, the hypothesis being that there was toxin production and blockage of the plant's vascular system. The outcome proved that there were no toxins produced during the interaction and that it was easy to test homogenates of fungus for virulence. They also mentioned that an *in vitro* method was beneficial for the selection of strawberry clones, especially somaclonal variants and hybrids that were resistant to the fungus. This study indicated the importance of a simple, quick and reliable artificial inoculation-infection assay for the study and control of plant pathogens.

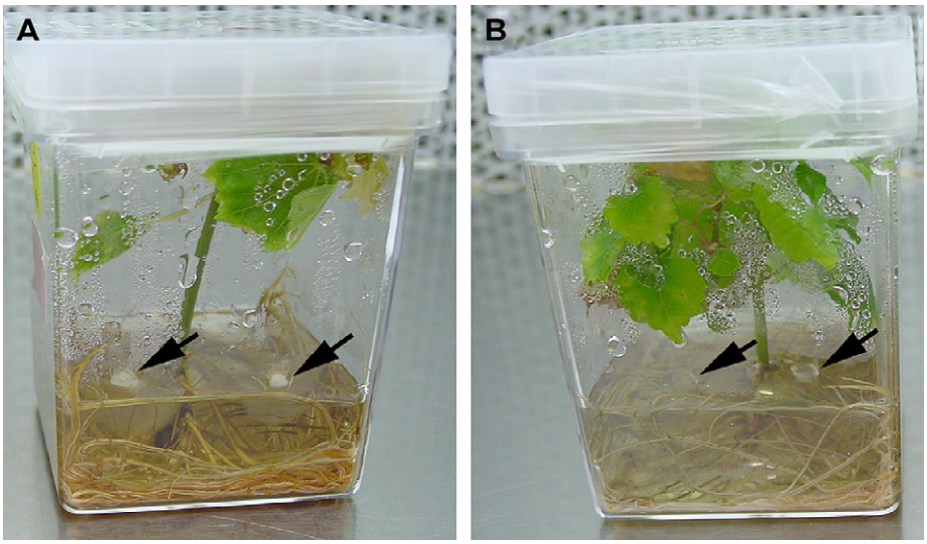


Figure 12: Axenic infection assay for grape rootstocks infection by *Armillaria* spp. Arrows point to fungal inocula (A) and agar inocula without fungus (B) (Baumgartner *et al*., 2010).

1.4 Oil palm lignin and its role in plant-pathogen interactions

The genera of white rot fungi (WRF) comprise several hymenomycete species, of which *Ganoderma* spp. are significant members (Adaskaveg *et al*., 1990). WRF decay lignin and polysaccharides in wood, deriving energy from exposed cellulose reduced to glucose (Adaskaveg *et al*., 1990; Paterson *et al*., 2008). Although, *Ganoderma* spp. have been recorded as white rot fungi, the presumed correlation with BSR has been made only recently in 2007 (Paterson *et al.,* 2008).

1.4.1 Lignin degradation by white rot fungi (WRF)

White rot fungi (WRF) such as *Heterobasidion* spp., *Armillaria* spp. and *Ganoderma* spp., have been classified according to their host and habitat (Dai *et al*., 2007). Of 102 species of pathogenic wood decaying fungi collected in China, 88 species were white rot fungi while 14 were brown rot fungi (Dai *et al*., 2007). The results of this 12-year study revealed that 86 of the 102 wood decaying species were polypores in the Basidiomycota phylum. *Ganoderma* spp. were found to be pathogenic on 20 angiosperm host trees, while *Fomitopsis pinicola* was found in various gymnosperms and angiosperms (Dai *et al*., 2007). *Phanerochaete chrysosporium* Burds is a WRF directly cause the mineralisation of lignin

under laboratory conditions, while in the 1980s 'ligninases' representing lignin-degarding enzymes were characterised (Hatakka, 1994). Since then ligninases such as laccase and manganese peroxidase became important for industrial biodegradation of lignin such as bio pulping and pulp bleaching (Hatakka, 1994). Common extracellular lignin-degrading enzymes include laccases (Lac: EC 1.10.3.2), manganese peroxidases (MnP: EC 1.11.1.7) and lignin peroxidases (LiP: EC 1.11.1.7) (Hatakka, 1994). Among the species of WRF that readily produce Lac and MnP but not Lip are *Coriolopsis polyzona* (Pers.) Ryv, *Stereum hirsutum* (Willd. ex Fr.) Fr., *Ganoderma valesiacum* (Boud.) Pat. and *Ganoderma australe* (Fr.) Pat. (Hatakka, 1994). It was known that no fungi, WRF included, could use lignin as their sole carbon source of energy but the depolymerisation of lignin provides cellulose and hemicellulose nutrient carbon sources (Cullen and Kersten, 2004). In contrast brown rot fungi efficiently mineralize crystalline cellulose while slow degradation of lignin takes place (Cullen and Kersten, 2004).

1.4.2 Potential role of *Ganoderma* spp. lignin degrading enzymes (LDEs) in BSR

Oil palm trunk fibre contains 17.1% lignin (dry weight) and high content of starch that impacts degradation by fungi (Paterson *et al*., 2008). Although a direct link between *Ganoderma* spp. LDEs and BSR has only been suggested in review papers (Paterson *et al*., 2007; 2008; 2009), the potential degradation and delignification of wood by *Ganoderma* spp. has been extensively reported (Adaskaveg *et al.,* 1990; Varela *et al*., 2000; Maeda *et al*., 2001; Haddadin *et al.,* 2002; de Souza Silva *et al*., 2004). In a study to screen for ligninases, all *Ganoderma* spp. decolorized Remazol Brilliant Blue R (RBBR) dye (de Souza Silva *et al*., 2004). The greatest reduction of RBBR colour was recorded in *Ganoderma* strains testing positive for Lac than MnP (seven times more Lac than MnP) and 23 times more Lac than LiP (de Souza Silva *et al*., 2004). This result was in contrast to strains that had the lowest RBBR colour reduction, representing three times more LiP compared to Lac and almost 18 times more LiP as compared to MnP, further implicating laccases in RBBR decolourisation (de Souza Silva *et al*., 2004). Laccase has been identified as the main enzyme in the breakdown of synthetic dyes and is a preferred enzyme in the textile industry as it does not require peroxide for substrate oxidation and has broad substrate specificity with certain modifications (Murugesan *et al*., 2007).

1.5 Aims and objectives of the work

The main aim of this research was to analyse the plant-pathogen interaction between young oil palm and *Ganoderma* spp. using a novel

artificial inoculation-infection assay. A preliminary study on the disease incidence and severity (DI/DS) trends in four estates in Peninsular Malaysia provided crucial preliminary evidence of the rates of infection in the selected fields. Evaluation of DI/DS also facilitated the collection of *Ganoderma boninense* isolate GBLS that was used in this study. Although studies elsewhere have mentioned the virulence, disease and control of BSR, a closed system artificial infection and interaction of pathogen and oil palm has not been reported. Also, the presence of LDEs and their role(s) in the development of BSR has not been reported in any original research, though it was suggested in a review (Paterson *et al*., 2009).

Therefore, the novel aspects of this work are

1. The development of an axenic *in planta* artificial infection assay.
2. The use of LDE-encoding genes of *Ganoderma* spp. to detect the rates of BSR infection and to understand the role of laccase and manages peroxidase during infection.

It was hypothesized that an innovative assay platform as compared to conventional virulence testing for BSR would reduce the time taken for screening oil palm clones bred through a tissue culture process for the susceptibility to the pathogen. To determine the efficiency of the infection assay technique, quantity of fungal DNA on infected and uninfected samples was analysed using qualitative and quantitative PCR (qPCR) using interspace transcribed regions, ITS 1 and ITS 4 primers that

produce a 167 bp fragment (Utomo and Niepold, 2000; Karthikeyan *et al*., 2007). The study also set objectives to sequence a laccase gene fragment in virulent *G. boninense* and to determine its expression during the oil palm-*Ganoderma* interaction *in vitro*. Laccase is a lignin degrading enzyme produced by common white rot fungi such as *Ganoderma* spp. Laccase encoding genes could be developed as biomarkers/diagnostic tools to analyse gene expression in *Ganoderma* spp. infecting young oil palm. The efficiency and sensitivity of laccase biomarkers was compared with existing diagnostic markers such as non-coding ITS fragments of *Ganoderma* spp. in qualitative and quantitative PCR analyses. The overall link between individual experiments is presented in a chart form (Fig. 13).

Therefore, the specific objectives of the study are:

1. To survey, isolate and identify *Ganoderma* spp. associated with basal stem rot disease of oil palm in Malaysia.
2. To develop a time-sensitive and efficient *in planta* infection assay for *Ganoderma* spp. in young oil palm *in vitro*.
3. To design a biomarker to determine the virulence of *Ganoderma* spp. based on a laccase gene fragment from virulent *G. boninense.*
4. To determine the role of laccase during the oil-palm-*Ganoderma* interaction.

This study is organised into four experimental chapters that is collection, isolation and identification of *G. boninense* GBLS (Chapter 3), an artificial infection assay for GBLS-oil palm interactions (Chapter 4), isolation of a gene encoding laccase (Chapter 5) and the the role of lignin degrading

enzymes, laccase and MnP during the virulence of GBLS on oil palm (Chapter 6).

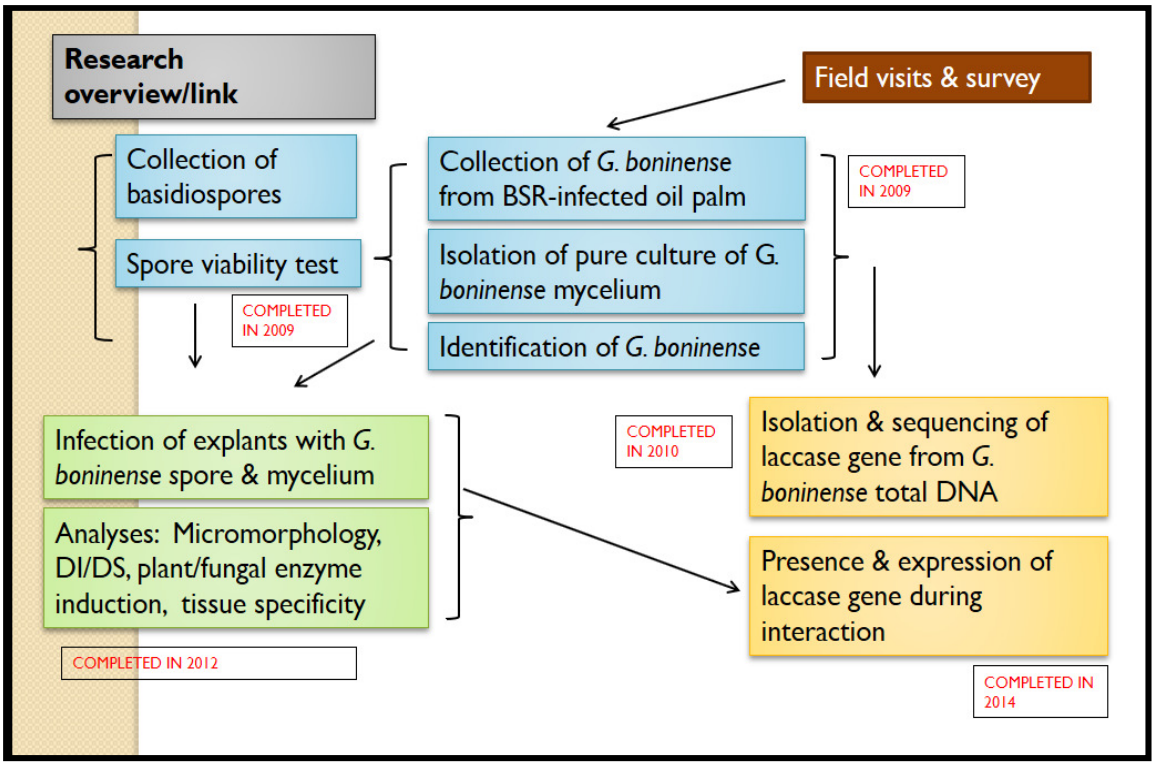


Figure 13: Overview of research experiments of this work.

2. CHAPTER 2: GENERAL METHODS

2.1 Plant and fungal source

2.1.1 Oil palm

Oil palm (clone AA68) was obtained *ex vitro* from Applied Agriecological Research Sdn Bhd, Sg. Buloh, Selangor, Malaysia (AARSB). Axenic rooted ramets were collected from AARSB in standard tissue culture tubes.

2.1.2 *Ganoderma boninense*

Ganoderma spp. sporophores were collected from Balau Estate, Selangor; Lian Seng Estate, Tangkak, Johor and Melaka Tong Bee Estate, Melaka in 2007. Each sporophore was dusted off to remove estate debris and insects and packed individually into a sealable plastic bag. Bags were stored in a cooler box during transport. In the laboratory, the sporophores were stored at room temperature, in the dark for up to one month.

2.2 Culture medium

All culture media were sterilized by autoclaving (Tomy ES-315, USA) at conditions of 121°C for 15 min unless stated otherwise. Agar medium at temperatures between 45°C and 60°C, were poured into 9 mm Petri dishes in a Class II Biosafety Cabinet (ESCO, Singapore), allowed to cool to room temperature and sealed using Parafilm strips.

Solidified agar media were used immediately or stored in a chilled cabinet at 10°C for a maximum of two weeks.

Potato dextrose agar (PDA) medium was prepared by adding 39 g of PDA (Merck, Germany) powder into one litre of purified water. For liquid medium, 24 g of potato dextrose broth powder (Oxoid, UK) was weighed and dissolved in one litre of purified water. Sterile medium was used immediately for experiments.

Czapek Dox is a synthetic fungal medium designed to provide minimal nutrient for fungal growth. One litre medium was prepared by adding 49 g of CDA (Merck, Germany) powder into one litre of purified water.

Ganoderma Selective Medium was formulated for selective growth of *Ganoderma* spp. (Ariffin and Idris, 1992). The medium contained parts A and B (Table 2). Constituents of Part A were stirred on a hot plate stirrer at 70°C until dissolved and the mixture was autoclaved. Individual components of Part B were mixed at room temperature and not sterilized. Part B components were added into Part A to complete GSM agar medium.

Table 2: Composition of *Ganoderma* selective medium (GSM) (Ariffin and Idris, 1992).

Part A	**Amount per litre**
Bacto peptone	5.0 g
Agar	20.0 g
$MgSO_4.7H_2O$	0.25 g
K_2HPO_4	0.50 g
Distilled water, pH 5.5	

	900 ml
Part B	**Amount per litre**
Streptomycin sulphate	300 mg
Chloram phenicol	100 mg
PCNB, pure	285 mg
Ridomil (25% wettable powder)	130 mg
Benlate T20	150 mg
Ethanol, 95 % v/v	20 ml
Lactic acid, 50 % v/v	2 ml
Tannic acid	1.25 g
Distilled water, pH 5.5	80 ml

One litre of malt extract broth medium was prepared by adding 48 g of malt extract (Merck, Germany) into one litre of purified water.

Water agar is a minimal nutrient medium that is useful in determining fungal spore germination and proliferation of primary hyphae. Water agar medium was prepared by adding 15 g of Microbiological Agar (Merck, Germany) into one litre of purified water. Sterile medium was poured into 9 mm Petri dishes which were stored until use.

One litre of Luria Bertani medium was prepared by the addition of 10 g of tryptone powder, 5 g of yeast extract powder and 10 g sodium chloride into 800 ml of purified water. The mix was adjusted to pH 7.0 using 5 N NaOH and to a final volume of one litre with purified water. The medium was autoclaved (Tomy ES-315*,* USA*)* at 121°C for 15 min.

One litre of 2XYT medium was prepared by the addition of 16 g of tryptone powder, 5 g of yeast extract powder and 10 g of sodium chloride into 800 ml of purified water. The mix was adjusted to pH 7.0 using 5 N

NaOH and to a final volume of one litre with purified water. The medium was autoclaved (Tomy ES-315*,* USA*)* at conditions of 121°C for 15 min.

MS (Murashige and Skoog) medium were based on Murashige and Skoog (1962) and was prepared according to manufacturer's recommendation (Duchefa Biochemie, Product M0222, The Netherlands). MS powder, 4.4 g and 30 g/L of sucrose was dissolved in 800 ml of distilled water at room temperature with constant stirring. The pH of the MS medium was adjusted to 5.7 using a pH meter (Eutech Instrument, pH510) with dilute hydrochloric acid. Next, 3.2 g of Phytagel powder (Sigma, USA) was dissolved in the medium. MS medium was then autoclaved (Tomy ES-315*,* USA*)* at conditions of 121°C for 15 min.

2.3 Chemicals and buffers

2.3.1 CTAB buffer for plant and fungal DNA extractions.

CTAB buffer was used for the breakdown and removal of cell walls and cytoplasmic membranes of living organisms at the lysis step of DNA extraction. CTAB buffer is made up of 100 mM Tris-HCl pH 8, 1.4 M NaCl, 2 % v/v CTAB, 20 mM EDTA and 1 % v/v PVP. CTAB buffer was added to the fungal mycelium for DNA extraction.

2.3.2 TBE buffer for Electrophoresis.

TBE buffer was used to dissolve agarose powder and as electrophoresis buffer. A five times (5X) concentrated TBE buffer stock was prepared with 54 g of Tris base powder, 27.5 g of boric acid powder and 20 ml of 0.5 M EDTA (pH 8.0). All components were dissolved in one

litre of purified water and autoclaved (Tomy ES-315, USA) at 121°C for 15 min. A 1: 5 dilution was prepared for working stock of 1 X TBE.

2.3.3 TE buffer for storage of nucleic acids.

TE buffer was used as the final buffer to dissolve and store nucleic acids such as DNA and RNA at -20°C. TE buffer was prepared by mixing 10 ml 1 M Tris-Cl pH 7.5 and 2 ml 500 mM of EDTA, pH 8.0. The mix was adjusted to one litre. The buffer was autoclaved (Tomy ES-315, USA) at 121°C for 15 min.

2.3.4 Agarose gel for electrophoresis.

Agarose gels were used to visualise DNA and RNA products under ultraviolet (UV) light conditions. A concentration of 1.0% w/v agarose gel was prepared by the dissolution of 1 g agarose powder into 100 ml autoclaved 1 X TBE buffer in a microwave oven. Liquid agarose was added with 1 X Sybr Safe solution (Invitrogen, Inc) and poured into a gel casting tray fitted with suitable comb. Once solidified, the gel was used immediately in electrophoresis.

2.3.5 SOC buffer for cloning and transformation.

SOC buffer is used to increase the transformation efficiency of bacterial infection such as *E. coli* transformations. The buffer contained 2% w/v tryptophan, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 20 mM sucrose. All ingredients except sucrose were mixed,

dissolved and autoclaved. Sucrose was filter-sterlized through 0.25 µM membrane filters (Sartorius, Germany).

2.3.6 IPTG and X-Gal for cloning and transformation in *E.coli*.

Pre-made IPTG and X-Gal solutions were purchased from supplier (Fermentas) and used according to the manufacturer's recommendations.

3. Chapter 3: Collection, isolation and identification of *Ganoderma boninense* from infected oil palm estates.

3.1 Introduction

Ganoderma boninense is a Basidiomycota fungi found in all soil-types in oil palm plantations. A preliminary survey was conducted by Microgreen Bio-Industrial Sdn Bhd in collaboration with oil palm estates. Estates visited during the survey were in Johor, Sg Gerchang Estate, Achi Jaya Plantations at Chaah (GPS Coordinates : 2.261063, 103.066263) and Ladang Boustead Eldred at Sg Bekoh (GPS Coordinates : 2.291840, 103.134584), and in Melaka, Melaka Tong Bee Estate at Air Kuning (GPS Coordinates : 2.497943, 102.495218). Collection and identification of *G. boninense* from oil palm estates was conducted according to Sariah *et al*. (1994). The collection of sporophore provided pure and reproducible mycelium cultures that were used for the study. Identification of the fungus *G. boninense* was crucial to limit the pathogen pool to known isolates of the fungus.

3.2 Materials and Methods

3.2.1 Collection of *Ganoderma* spp. basidiocarps

BSR infected oil palm trees at selected estates had multiple *Ganoderma* spp. basidiocarps situated between 0 and 5 m above ground. Infections were confirmed by visual presence of the basidiomata, which also indicated aggressive colonisation by the fungi (Ho and Nawawi, 1986a). In a preliminary study by Microgreen Bio-Industrial Sdn Bhd, severity of disease in Sg. Gerchang Estate, Achi Jaya Plantation Sdn Bhd (ex-Socfin estates) was recorded using a 13 point field evaluation form

(Table 3) that is processed into four categories of disease severity (DS) index of 1 to 4, with 4 representing a healthy tree (Table 4). Sporophoreof at least 30 mm x 20 mm were carefully removed from the stem of oil palms and dusted off to remove oil palm debris and insects. Each sporophorewas carefully examined for *G. boninense* characteristics such as shiny surface, stipe and pileus and a cream, off-white underside with pores (Ho and Nawawi, 1985; Seo and Kirk, 2000).

Table 3: An evaluation sheet for *Ganoderma* spp. BSR disease incidence (DI) based on 13 visual symptoms for oil palm No. 1 to No. 10 at Achi Jaya Plantation, Sg, Gerchang Estate, Johor. Source: Microgreen Bio-Industrial Sdn Bhd (with permission).

Evaluation Of The Efficacy of MG Plus™ (Variants A, B, C, D and E) in Oil Palm Estate of Achi Jaya Plantation Sdn. Bhd.										
Name :										
Date :										
Symptom	Palm Number									
	1	2	3	4	5	6	7	8	9	10
Ganoderma spp. fruiting body on trunk or base										
Ganoderma spp. fruiting body on soil around tree										
Drooping fronds (more than 50% of fronds)										
1-5 Drooping fronds										
3-5 unopened fronds (spear)										
1-2 unopened fronds (spear)										
Mature fronds –brown										
Mature fronds –green										
Spear fronds –brown										
Spear fronds –green										
Root – brittle										
Root – strong										
Healthy Oil Palm										
Comments										

Table 4: Disease severity (DS) assessment based on numerical index for oil palm No. 1 to No. 10 at Achi Jaya Plantation, Sg, Gerchang Estate, Johor. The test period was October, 2004 to October, 2005. Source: Microgreen Bio-Industrial Sdn Bhd (with permission).

Sg.Gerchang: Categorization Based on Evaluation								Attachment 2	
	1:Severe	2:Bad	3:Moderate	4: Healthy					
Palm Label	Oct	Dec	Feb	Apr	Jun	Aug	Oct	Total	Comments
1	3	4	4						
2	3	4	4						
3	3	4	3						
4	3	4	4						
5	4	4	4						
6	4	4	4						
7	2	4	4						
8	3	4	4						
9	4	4	4						
10	2	4	4						

3.2.2 Isolation of *Ganoderma* mycelium from sporophore

Isolation of mycelium from *Ganoderma* sporophore was conducted according to Ho and Nawawi (1986 a,b) with modifications. Sporophores were washed gently under running tap water for one hour. A 500 ml solution of 0.5 % (v/v) sodium hypochlorite was prepared using commercial bleach. Sporophores were immersed into the solution for one min and washed with sterile, distilled water three times. Sterile scalpels and forceps were used to make longitude incisions on the sporophore, exposing the inner layers of tissue (Utomo *et al*., 2005). A section of 5 mm x 5 mm x 5 mm was cut from internal tissues (Fig. 14) and placed at the centre of PDA amended with 250 µg/ml of streptomycin sulphate. Inoculated Petri dishes were incubated at room temperarure for 5 -7 days in the dark, to facilitate the growth of vegetative mycelium. Mycelium was observed as white, cotton-like structures growing from the outer margin

of the sporophore (Fig. 15). When white mycelium extended out from a cultured sporophore, a 5 mm cork borer was used to bore mycelium at the advanced growing region of PDA medium. This plug was transferred to fresh PDA and GSM. Isolation procedures for mycelium cultures were conducted in triplicate.

3.2.3 Identification of *Ganoderma* spp.

Mycelium growth was observed as concentric circles on PDA, until complete colonisation of PDA in 7-10 days. Pure cultures were maintained by transferring agar plugs onto fresh PDA medium every two weeks. Uniform mycelium culture was transferred onto GSM for the confirmation of *Ganoderma* spp.

Figure 14: Sporophore pieces (0.5 cm x 0.5 cm) cut out from a mature sporophore of *Ganoderma boninense* GBLS.

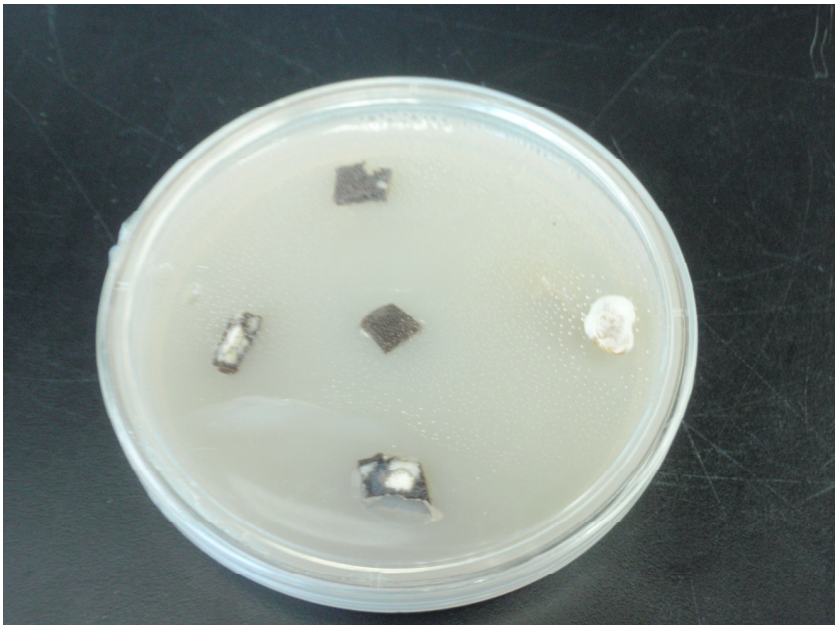


Figure 15: Sporophore pieces (0.5 cm x 0.5 cm) cultured on Ganoderma Selective Medium (GSM). White mycelium germinates from 7 day cultures of sporophores.

3.2.3.1 Microscopic analyses of mycelium

A sterile plastic loop was used to pick up mycelium tissue from advanced growing regions of the pure mycelium cultures and placed onto a glass slide. Heat fixed mycelium samples were stained with lacto phenol blue (Priyadarsini *et al*., 2011). Stained samples were examined under light microscopy at 40 x and 100 x magnifications (Nikon) (Fig. 18). Photographs of images were captured using a Nikon camera.

3.2.4 DNA extraction from pure mycelium cultures

DNA extraction was conducted according to Goes-Neto *et al.* (2005) with modifications. *Ganoderma boninense* pure mycelium, 7 to 10 days of age was scraped from Petri dishes into an autoclaved pestle and mortar. Liquid nitrogen was used to grind the mycelium into fine powder. One milligram of mycelium powder was transferred into a sterile 1.5 ml polypropylene tube. Pre-warmed 200 µl CTAB buffer was added and

mixed with the mycelium powder. The mixture was incubated at 60°C for 1 hour. The mixture was centrifuged at 10,000 g to separate the liquid and solid content. Supernatant was transferred into a fresh 1.5 ml tube. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the liquid and mixed gently. After separation by centrifugation at 10,000g for 2 min, DNA was precipitated using 2.5 volumes of cold isopropanol for 1 hour at -20°C. A DNA pellet was produced via centrifugation at 10,000g for 20 min at 4°C. The DNA pellet was washed by centrifuging DNA with 1 ml 70% v/v ethanol and the procedure repeated twice. The DNA pellet was dried and dissolved in 30 µl of nuclease free water with 100 µg/ml of RNAse A. DNA concentrations were estimated in triplicate using a Nanodrop DNA quantification system (Thermo Fisher). Presence of DNA was visualised in 1% w/v agarose gel with 1 X Sybr Safe dye (See section 2.3.10). Photographic data were collected using a Gel Documentation System (Biorad, USA).

3.2.5 PCR amplification of specific ITS region

Oligonucleotide primers were synthesized using internal transcribed spacer region, ITS 1 and 4 of ribosomal DNA for *Ganoderma* spp. according to Utomo and Niepold (2000) and Kartikeyan *et al*. (2006). The expected PCR fragment size was 167 base pairs. Oligonucleotide synthesis was carried out at First Base Laboratories Sdn Bhd, Selangor, Malaysia. Primers were designated as GbF for 5' forward primer and GbR for 5' reverse primer respectively.

Forward primer, GbF, 5'- TTG ACT GGG TTG TAG CTG – 3' (Tm 42.9 °C)

Reverse primer, GbR, 5'- GCG TTA CAT CGC AAT ACA – 3' (Tm 40.6 °C)

Concentration of primer stocks were 100 µM while working concentrations were 10 µM (1:10 dilution). Primers were used in a PCR reaction mix with (final concentration) 1 X Green reaction buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 0.2 mM dNTP (Promega), 0.8 µM of each forward and reverse primers, 60 ng of template DNA and 0.125 Units of *Taq* polymerase (Promega). All components for PCR were kept at -20°C and prepared fresh for each PCR. PCR was carried out in a final volume of 20 µl in 0.2 ml plastic PCR tubes. Conditions for PCR amplification were initial denaturing at 95°C for 5 min, followed by 35 cycles of 94°C for 40 seconds, 45°C for 40 seconds and 72°C for 45 seconds. Final extension step was performed at 72°C for 12 min. PCRs were conducted using a GStorm Thermal Cycler (GStorm). PCR products were separated in a 2.5% (w/v) agarose gel (7 x 10 cm) prepared with 1 x TBE buffer and 1X Sybr Safe DNA dye. Electrophoresis was run at 80V for 1 hour in a submerged horizontal electrophoresis system (Bio Rad). Agarose gel products were visualised and recorded using UV/Vis Gel Documentation System with CCD a camera (Bio-Rad).

3.3 Results

3.3.1 Collection of *Ganoderma* spp. basidiocarps.

A survey for *Ganoderma* BSR DI/DS in selected oil palm estates in Johor and Melaka was carried out between the years 2004 and 2006, while visits to Balau Estate in Semenyih took place in 2007, 2008 and 2009. Most estates reported a few cases of *Ganoderma* disease incidence (DI) of 3-5%, 2004 to 2006. The rare presence of *Ganoderma* made the collection of *Ganoderma* spp. sporophores from infected trees difficult. Other problems related to sample collection were short palms that were covered with wilted and desiccated fronds (Fig. 16). In Lian Seng Estate, Tangkak, Johor, a higher DI (more than 5%) was recorded in a field survey in 2007 but the 23 years old palms were rapidly showing symptoms of BSR and the potential spread between infected and non-infected trees. At Balau Estate, Semenyih, Selangor, there were no designated 'infected' plots in the years 2006-2009, but individual trees with sporophores were found scattered around the estate. According to estate management at Balau Estate, Semenyih, there were no new BSR infected palms but due to the heavy commercial development around the estates, the oil palms were showing signs of stress which could easily be confounded with BSR symptoms (Mohd Rafli, Manager, Balau Estate, pers comm). Microgreen Bio-Industrial Sdn Bhd observed that plots amended with suitable fertiliser combinations (treatments A5 to E20) had higher percentages of improved palm health as compared to unattended plots (Fig. 17). The severity of disease was recorded as percentages of changes as 240 individual trees were recorded for severity values every two months for 8 months (October 2004 to June 2005). MeanImp is an

abbreaviation given to palms that has improved severity scores, i.e. became healthier in the course of the observations. MeanWors indicates percentage of palms as a ratio of the total number of palms that deteriorated in the months of observation. Mean NC shows percentage of the number of palms that show no significant difference in plant severity scores at the test plot. Scoring was completed using evaluation forms described in Table 3 and 4. Visual confirmation of *Ganoderma* spp. sporophores was made using published sources of shape and colour of *G. boninense* (Fig. 14).

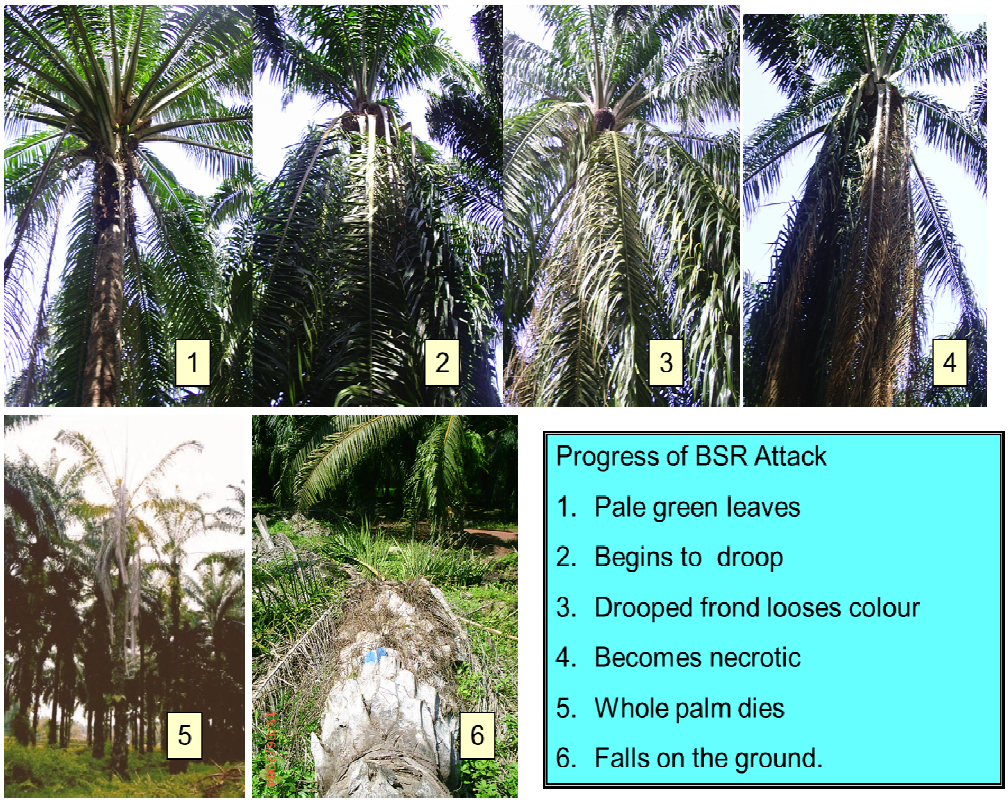


Figure 16: Symptoms related to BSR disease of oil palm. Microgreen Bio-Industrial Sdn Bhd (with permission).

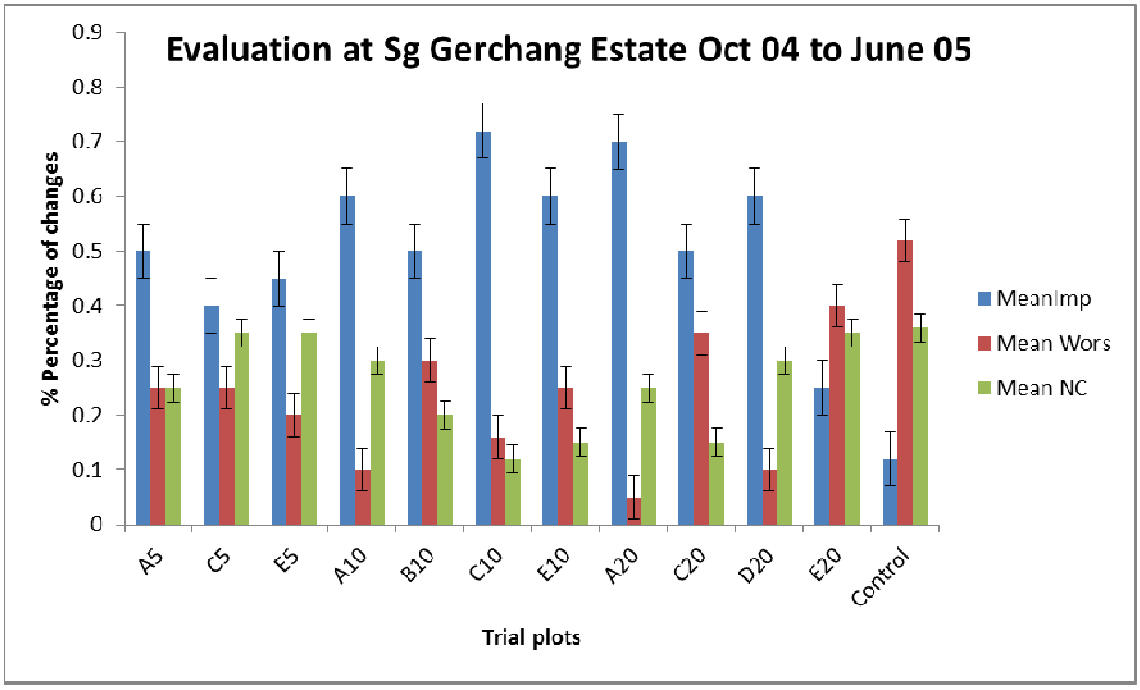


Figure 17: Mean percentage of changes from initial tree physiological observation survey using numerical Disease Severity Index (Index 1 represents poor health; Index 4 represents healthy plant) at Sg Gerchang Estate for 8 months. Trial plots represent designated plots for an ongoing ferilizer efficacy trial. Mean Imp: Index number had increased; Mean Wors: Index number had reduced; Mean NC: Index number had remain unchanged. Error bar indicates standard error. Microgreen Bio-Industrial Sdn Bhd (with permission).

3.3.2 Isolation and identification of *Ganoderma* mycelium from basidiocarps.

White, cotton-like mycelium extending from cut pieces of *Ganoderma* sporophores were observed on PDA medium in 4-7 days (Fig. 15). Petri dishes that were contaminated at this step commonly contained filamentous fungi such as *Trichoderma* spp. or *Aspergillus* spp. *Ganoderma* spp. isolates were confirmed as *G. boninense* using Ganoderma selective medium (GSM) (Fig. 15). Confirmation of Basidiomycota fungi was obtained from lacto phenol stained mycelium showing clamp connections (Fig. 18). DNA extraction produced an

average of 500 ng/µl of total DNA in each *Ganoderma* isolate (Table 5). However, the concentration of DNA was sufficient for PCR. PCR revealed the predicted single band of 167 bp ITS DNA fragment that identifies *Ganoderma* from infected oil palm and coconut species (Fig. 19). In this study, a fragment of 635 bp of ITS 1 and 4 fragments that identified GBLS, was deposited in GenBank with the accession number KF164430.1. Further sequencing results yielded a 635 bp ITS fragment of GBLS that is found to have 581 bp that are 100% similar to PER71, pathogenic *Ganoderma* spp identified by MPOB.

Table 5: DNA quantification result for *Ganoderma* spp. total DNA extraction using CTAB method according to Goes-Neto et al., 2005. Arrow: The concentration of DNA was between 1 and 1500 ng/µl. The average amount of DNA was lower than 500 ng/µl.

Test Type	Nucleic Acid	Date/time	10/25/2010 4:14 PM	Page #	1				
T	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
3:43 PM	0.66	0.017	0.030	0.58	8.10	50.00	230	0.002	-0.012
3:43 PM	-2.79	-0.056	-0.031	1.81	3.18	50.00	230	-0.018	-0.011
3:44 PM	712.69	14.254	7.007	2.03	1.71	50.00	230	8.345	1.507
3:45 PM	705.13	14.103	6.888	2.05	1.82	50.00	230	7.739	0.462
3:46 PM	142.77	2.855	1.498	1.91	1.09	50.00	230	2.619	0.072
3:47 PM	136.68	2.734	1.425	1.92	1.25	50.00	230	2.187	0.056
3:47 PM	851.05	17.021	9.078	1.87	1.11	50.00	230	15.365	6.814
3:48 PM	1137.34	22.747	13.440	1.69	0.94	50.00	230	24.276	18.172
3:49 PM	353.41	7.068	4.123	1.71	1.02	50.00	230	6.901	4.604
3:50 PM	314.76	6.295	3.560	1.77	1.04	50.00	230	6.048	3.118
3:51 PM	1574.40	31.488	15.470	2.04	2.10	50.00	230	14.963	0.287
3:52 PM	1588.74	31.775	15.651	2.03	2.07	50.00	230	15.357	0.964
3:52 PM	1120.67	22.413	11.135	2.01	1.86	50.00	230	12.022	1.992
3:53 PM	1065.84	21.317	10.518	2.03	1.93	50.00	230	11.043	0.516
3:54 PM	288.93	5.779	3.327	1.74	1.18	50.00	230	4.887	4.362
3:55 PM	326.37	6.527	3.805	1.72	1.10	50.00	230	5.947	5.363
3:56 PM	88.41	1.768	0.897	1.97	1.09	50.00	230	1.622	0.078
3:57 PM	109.03	2.181	1.204	1.81	0.95	50.00	230	2.291	1.023
3:57 PM	57.53	1.151	0.623	1.85	0.70	50.00	230	1.650	0.021
3:58 PM	51.93	1.039	0.572	1.82	0.69	50.00	230	1.502	0.046
3:59 PM	15.82	0.316	0.212	1.49	0.64	50.00	230	0.496	0.012
3:59 PM	16.16	0.323	0.201	1.61	0.55	50.00	230	0.588	0.020
4:00 PM	205.62	4.112	2.170	1.90	1.29	50.00	230	3.194	0.206
4:01 PM	211.95	4.239	2.264	1.87	1.16	50.00	230	3.668	0.149
4:02 PM	336.55	6.731	3.805	1.77	1.22	50.00	230	5.509	4.792
4:02 PM	323.79	6.476	3.599	1.80	1.19	50.00	230	5.435	2.188
4:03 PM	102.07	2.041	1.011	2.02	2.03	50.00	230	1.006	0.065
4:04 PM	23.50	0.470	0.248	1.90	1.48	50.00	230	0.318	0.014
4:04 PM	-3.00	-0.060	-0.013	4.78	1.71	50.00	230	-0.035	-0.015
4:05 PM	43.62	0.872	0.477	1.83	1.04	50.00	230	0.836	0.044

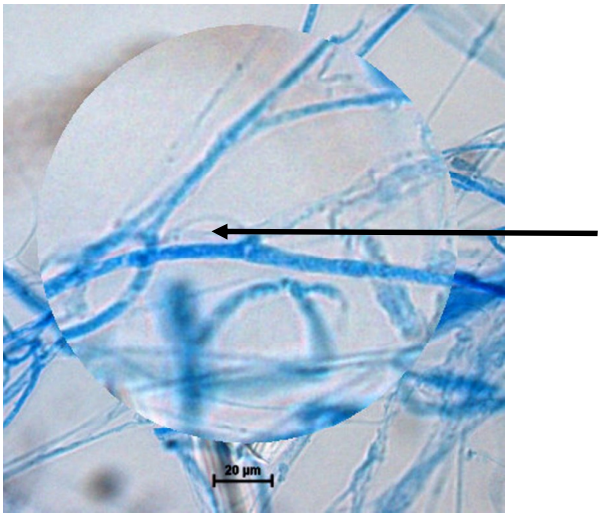


Figure 18: Microscopic observation of white mycelium of *Ganoderma boninense* GBLS. Fungal cell wall was stained with Lactophenol Blue dye.

Highlighted area represents a network of mycelium and arrow points to a clamp connection. Clamp connections are characteristics of dikaryotic mycelium of the Basidiomycota fungi, whereby each mycelium cell contains two sets of nuclei.

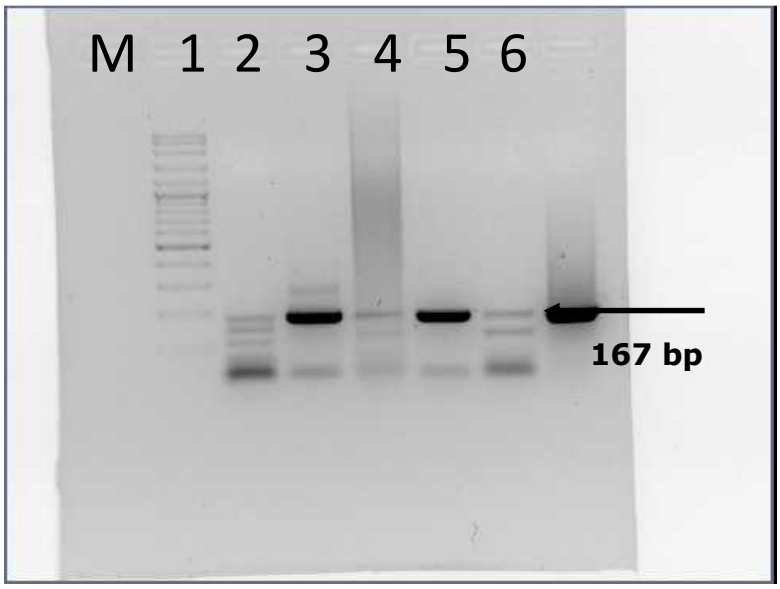


Figure 19: Confirmation of *Ganoderma* spp. using Gan1 and Gan 2 primers according to Utomo and Niepold, 2000. Lane M: 100 bp marker (Fermentas), Lanes 1, 3 and 5: negative control, Lanes 2, 4 and 6: GBLS genomic DNA.

3.4 Discussion

Preliminary surveys conducted by Microgreen Bio-Industrial Sdn Bhd indicated field BSR attacks on Malaysian oil palm. Although the field areas included in the survey were limited to inland areas of Peninsular Malaysia, the area had abundant Ganoderma spp. that revealed the ability of the BSR pathogen, *Ganoderma* spp. to spread from its original sightings at coastal areas and in ex-coconut plantations (Ariffin *et al*., 2000; Karthikeyan *et al.,* 2006; Idris, 2009). It was also reported that *Ganoderma* spp. originated from natural forest, mainly waterlogged areas and inoculum retained in the soil (Ariffin *et al*., 2000). The proliferation of *Ganoderma* spp. was largely attributed to its ability to produce millions of

spores each minute in a suitable estate environment (Sanderson, 2005; Rees *et al*., 2012). Although oil palm was suggested as preferred host plants for *Ganoderma* spp., monocropping and all year round climate patterns have been blamed for the onset and progression of BSR disease (Rees *et al*., 2007). The findings of the survey conducted between October 2004 and August 2005 at Sg Gerchang Estate correlated with other field observations (Singh, G., Retired Senior Research Advisor, United Plantation, Malaysia, pers. comm) that indicated an increase in disease severity in trial plots. Oil palms in trial plots were 17 years old and of the Dura x Pisifera (DxP) breed. Although there were no new 'sightings' of sporophores in the 230-palms trial plot that tested the efficacy of a fertiliser product, trial plots with standard fertiliser treatment (control) showed a more than 50% increase in DS (Fig. 17). The aggressiveness of the pathogen at the trial plot suggests that it was a suitable site for the collection of *Ganoderma* spp. sporophores for further research.

Sporophores were confirmed as *Ganoderma* spp. based on visual comparison of the shape and colour to published sources (Risda, 2008). The colour of the sporophores was brown to black with shiny or dry surfaces and off-white to cream undersides (Fig. 6 and 14). The size of sporophorewas not uniform and was influenced by the condition of the host plant and the surrounding environment. Larger sporophores were observed on older and weaker palms and palm stumps as compared to multiple smaller button-like sporophores found on younger palms. The

number of sporophores on each palm varied and did not differ between young and mature palms. This observation was also reported in other field surveys that studied the differences between the species of *Ganoderma* found on oil palm (Idris *et al*., 1996). One study by MPOB concluded that there were four different species that could be clearly distinguished by morphology (Idris *et al*., 2000). However, in studies related to the *Ganoderma lucidum* complex, appearances were said to be poor measures of speciation (Monclavo, 2000). Microscopic observations showing clamp connections (Fig. 18) were useful as these are produced primarily by hymenomycete fungi after the fusion of mycelium to form dikaryotic colonies that will result in sporophores (Deacon, 2006b). Virulence experiments were necessary to differentiate pathogenic and non-pathogenic *Ganoderma* spp. (Sariah *et al*., 1994).

Ganoderma boninense isolate GBLS was successfully characterised. However, the virulence was only observed on its host oil palm. To determine the virulence of GBLS, the rubber wood block assay was performed as reported in other related studies (Goh *et al*., 2014; Ganeson, 2015). Physical and morphological characteristics were insufficient to determine *G. boninense* GBLS among other species isolated from infected oil palm (Monclavo, 2000). Low yields of DNA during extractions from sporophore were attributed to tough mycelium cell walls that required homogenisation for cell lysis (Goes-Neto *et al*., 2005). The use of DNA fingerprinting techniques such as RAPDs was able to differentiate *Ganoderma* spp. from oil palm and other host plants such as

rubber and wood trees (Idris *et al*., 1996). However, fingerprinting was not producing accurate detection of the disease agent, as compared to single band detection. Molecular confirmation was made when a single band of 167 bp generated from specific primers GbF/GbR amplified a fragment of the ITS 1 and 4 primers and this fragment was discovered unique to pathogenic *Ganoderma* spp. infecting oil palm (Utomo and Niepold, 2000; Utomo *et al.,* 2005). The reproducibility of the 167 bp fragment was proven when *Ganoderma* spp. causing basal stem rot in coconut were indentified using the same set of primers (Karthikeyan *et al.,* 2006; Kandan *et al*., 2009). The Malaysian Palm Oil Board (MPOB) has recommended the use of *G. boninense* PER71 as a standard strain and the ITS sequences were deposited in GenBank. However, since the virulence of GBLS was confirmed using the same standard method as PER71, it was deemed pathogenic (Ganeson, 2015). However, there is a need for biomarkers that clearly differentiate pathogenic and non-pathogenic *Ganoderma* spp. A potential biomarker for *Ganoderma* spp. could be specific primers that amplify the production and expression of LDEs in relation to the severity of infection. This is suggested as the mode of action for white rot fungi against oil palm, causing decay and damage to woody tissues (Paterson *et al*., 2009).

Therefore, in this chapter, *G. boninense* GBLS was successfully identified from sporophores present on infected oil palms. Each sporophoreprovided several isolates and sub-isolates of *G. boninense* mycelium in Ganoderma Selective Medium (GSM). Re-isolation in PDA

was necessary for the routine maintenance of the fungi. *Ganoderma boninense* spores and mycelium were distinguished based on descriptions in published reports. Clamp connections that were also characteristics of hymenomycete and *Ganoderma* spp. were clearly identified in lacto phenol-stained vegetative mycelium samples using light microscopy. PCR provided the molecular confirmation of *G. boninense* as the expected 167 bp band was detected in all pure cultures of mycelium. For the purpose of this study, *G. boninense* Pat will be referred to as *G. boninense* strain GBLS.

4. CHAPTER 4: NEW ARTIFICIAL INFECTION ASSAY FOR *GANODERMA* SPP. INFECTION OF YOUNG OIL PALM *IN VITRO*.

4.1 Introduction

Ganoderma boninense infects both young and old oil palms and since the factors affecting its virulence are temporal and spatial, recognition of disease and the understanding of aggressive behaviour of

some of the isolates over others have been difficult to study. To date, a rubber wood block (RWB)–mediated artificial inoculation assay has been used to screen potential tolerance and susceptibility in clones of oil palm as well as to differentiate pathogenic and non-pathogenic *Ganoderma* spp. (Sariah *et al*., 1994; Durand-Gasselin *et al*., 2005; Ganeson, 2015). The success of this artificial inoculation process on nursery seedlings has depended on factors such as inoculum size and potential, incubation and colonisation period of pre-inoculated RWB, the soil-RWB ratio and shade effect at nurseries (Breton *et al*., 2006). The process of infection is observed on oil palm seedlings as wilting symptoms of the foliar tissue, followed by drying and desiccation of leaves. Symptoms have been observed as early as 3.5 month post inoculation (Izzati *et al*., 2008). Although the method is reliable, the time to symptom development is lengthy for routine screening of aggressive *G. boninense* isolates. The method also confounds outcomes as soil-based assays face challenges in consistency, reproducibility and environmental uniformity for testing. In a recent review of the transcriptomics of oil palm-*Ganoderma* infection, the authors lament the inconsistencies around the artificially infected plants (Ho and Tan, 2015). The authors suggested that the use of *tenera* seed of oil palm with variable susceptibility to BSR and the destructive sampling according to time instead of degree of disease has created erroneous assumptions about the disease (Ho and Tan, 2015). Also, the paper suggested using clonal oil palm for consistent gene pool results and a better artificial inoculation method that has either consistent temporal

expression of genes or a method independent of temporal factors (Ho and Tan, 2015). In the wake of new technologies such as tissue culture and the 'omics' platforms, fast and accurate assays will become a requirement when testing virulence hypotheses. This fact has been made evident by recent work on grape rootstocks that were artificially inoculated with the root-rot pathogen *Armillaria mellea* in a controlled environment (Baumgartner *et al.,* 2010). Therefore, the aim of this chapter was to design and develop an efficient *in vitro* artificial inoculation system to induce BSR disease symptoms in one month old axenic oil palm ramets.

4.2 Materials and Methods

4.2.1 Plant and fungal material

Oil palm (clone AA68) from AARSB was collected as one month post-rooting axenic explants from Tissue Culture Laboratory, AARSB, Selangor, Malaysia. Plant materials were collected between June and December 2012. *Ganoderma boninense* strain GBLS was maintained as reported in Section 3.5.

4.2.2 Axenic *in planta* infection assay.

This technique was designed to enable the novel use of the axenic infection assay in the elucidation of virulence events during *Ganoderma*-oil palm interactions. Plastic tissue culture containers with the dimensions 72 x 72 x 100 mm from Incu Tissues, SPL Lifesciences, Korea were purchased and assembled as two containers connected with a plastic sleeve to achieve a height of 200 mm (Fig. 20). The containers were autoclaved separately and assembled in a Biohazard Class II cabinet

(ESCO, Singapore) under sterile conditions. A volume of 40 ml of MS medium (Section 2.2.8) was dispensed into each of the containers and sealed using Parafilm (Sigma, USA). The containers with axenic MS medium were stored for up to one week before use.

Each axenic oil palm clone was aseptically transferred to a culture jar from its original test tubes. All experiments were conducted in a Biohazard Class II cabinet (ESCO, Singapore) under sterile conditions. Three treatments were used:

T1: Non-treated experimental control,

T2: Infection control with artificial wounds,

T3: Artificially wounded and inoculated with GBLS.

Wounds on axenic plants were created using an aseptic syringe needle to puncture the basal stem region of the oil palm ramets (Goh *et al*., 2014). Basal stem was measured at 0.5 cm above the roots. In T3, aseptic syringe needles (TERUMO, 18G x 1.5”) were used to pick 7-14 day old GBLS mycelium grown on PDA which was applied onto the wounds created on the oil palm. Each treatment had 5 replicates and 3 plant units per replicate. Jars were arranged randomly in a CMP 6010 Conviron growth chamber (Conviron, USA) with the incubation specifications of 28°C and 50% relative humidity. The seven time points in this study were 0, 7, 14, 21, 28, 35 and 42 days post infection. Five plants per treatment were analysed at each time point. The entire experiment was repeated

four times. All results were analysed by Two-way ANOVA using GraphPad Prism programme version 5.02.

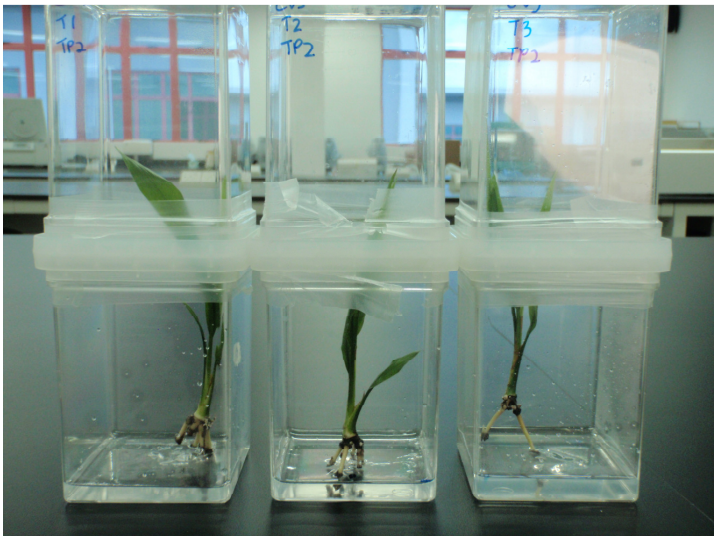


Figure 20: Tissue culture vessels were used for the growth and infection studies of *Ganoderma boninense* on oil palm ramets. From left to right: T1: non-treated control; T2: wounded control; T3: wounded and infected oil palms.

4.2.3 Analysis of disease severity in oil palms.

Oil palm ramets subjected to treatments of T1, T2, T3 were randomly arranged in the Conviron growth chamber to eliminate differences in exposure to light, temperature and humidity conditions in the chamber. At each time point, sample plants for each treatment were removed from the chamber and analysed for disease severity and physical changes. Disease incidence (DI) was not measured in this experiment because artificial inoculation and infection of GBLS had achieved 100% infection on T3 samples and no infection was found in T1 and T2. Disease severity in plants were measured using a modified severity index as

reported in Izzati *et al*. (2008) and Goh *et al*. (2014). The disease severity on oil palm seedlings after 42 days of incubation was assessed by calculating DSI values:

$$\text{Disease severity index (DSI)} = \frac{\sum (A \times B) \times 100}{\sum B \times 4}$$

Where, A is the disease class ranging from 0 to 4 according to the symptoms of infection (Table 6) and B is the number of plants showing that disease class per treatment. Photographic evidence of disease symptoms was compiled to provide visual comparison of the whole plant and cut sections of the internal tissue in relation to the infection (Fig. 21).

Table 6: Disease Severity Index: Scores of the symptoms of oil palm seedlings on a disease scale of 0-4 (Izzati *et al.* (2008) and Goh *et al*. (2014)).

Disease Class	**Symptom of infection**
0	Healthy plants with green leaves without appearance of fungal mycelium on any part of plants.
1	Browning of basal stem with or without chlorotic leaves.
2	Appearance of white fungal mass with chlorotic leaves (1-2 leaves) and browning of basal stem.
3	Appearance of white fungal mass with chlorotic leaves (> 2 leaves) and browning of basal stem.
4	Complete browning of basal stem and death of plants.

Physical measurements included height, weight (Sartorius, CP124S), number of leaves, width of the largest leaf measured using Vernier callipers (Mitutoyo America, 530-312) and leaf chlorophyll content

estimated using a SPAD meter (Konica Minolta, SPAD-502plus). Longitudinal sections of plants were cut and the appearance of basal stems and roots were photographed (Olympus Stylus TG-850 Tough, Olympus, Japan). After the physical measurements were conducted, the plants were quickly frozen in liquid nitrogen and stored at -80°C until further use. All the results obtained were analysed by two-way ANOVA using GraphPad Prism programme version 5.02.

4.2.4 Extraction of total DNA from GBLS and oil palm ramets.

Total DNA extraction from GBLS was described in Section 3.2.4. The DNA of GBLS was extracted from whole plant and not any specific tissues (Fig. 21). Total genomic DNA extraction from oil palm ramets was performed according to Moller *et al*. (1992) and Stewart and Via (1993) with modifications. Oil palm ramets were flash-frozen in liquid nitrogen before storage at -80°C. Prior to DNA extraction, plants were ground to fine powder in liquid nitrogen. Approximately, 1 g of plant powder was added to 1 ml methanol containing 0.1% v/v mercaptoethanol and phenolics were extracted three times by centrifugation at 10,000 g for 5 min each time (Eppendorf 5810R). The supernatant with plant phenolics was discarded and the remaining pellet suspended in 500 µl TES buffer and 2 µl of 0.1 mg/ml Proteinase K. The mixture was incubated at 60°C for one hour in a circulating water bath (Yih Der, BH320) for the digestion of proteins. The concentration of salt was adjusted to 1.4 M with the

addition of 140 µl of 5 M NaCl and 65 µl of 10% w/v CTAB buffer (Section 2.3.1). This lysis solution was incubated in a water bath at 65°C for 30 min with gentle mixing at every 10 min interval. To further remove polysaccharides and proteins, 700 µl of chloroform: isoamyl alcohol (24:1) (Biobasic Inc, USA) was added and the slurry mixed gently by inverting the tubes several times. Formation of DNA was clearly observed a colourless slurry above the layer of alcohol. After centrifugation at 10,000g at 10°C, DNA was collected into a new 1.5 ml tube and 225 µl 5 M sodium acetate and 510 µl ice-cold propanol added to precipitate the DNA. DNA pellets collected by centrifugation at 10,000g for 30 min were reconstituted with nuclease-free water and stored at -20°C. Sample GBLS and plant DNA was subjected to qualitative and quantitative analysis using Nanodrop 1000 (Thermo, USA).

4.2.5 Quantification using qPCR for GBLS ITS DNA in infected oil palms.

In Chapter 3, the ITS fragment of 167 bp was detected in pathogenic *G. boninense* GBLS strain (Section 3.2.5). In this chapter the 167 bp fragment was used to detect the quantity of GBLS DNA in infected and non-infected samples. The quantity determines the load of fungus post infection provides an indication of fungal activity within plant tissues. The primers for the amplification of the 167 bp fragment:

Forward Primer (**Gb F**): 5'-TTG ACT GGG TTG TAG CTG-3'
Reverse Primer (**Gb R**): 5'-GCG TTA CAT CGC AAT ACA-3'

Absolute quantification is a technique that quantifies the presence of a DNA fragment in ng/µl, calculated based on a standard curve (Zachar *et al.*, 1993). The quantity of DNA was measured by the extrapolation of the standard curve (Zachar *et al.*, 1993). In this study, absolute quantification was conducted via quantitative PCR according to manufacturer's recommendations (qPCR, Illumina), using GBLS DNA amplification of the 167 bp as standard curve. Concentration and quantity of GBLS DNA was measured using Nanodrop (Thermo, USA) and was diluted 10 fold to 10^{-6} dilutions. These dilutions were pipetted directly into a 48-well Ecoplate (Illumina, USA). Plant DNA from treatments T1, T2 and T3 at time intervals of 0, 7, 14, 21, 28, 35 and 42 dpi was extracted (Section 4.2.4) and quantified using Nanodrop. A concentration of <20 ng of each sample, with the ideal concentrations of 1-4 ng/µl, was pipetted directly into the remaining wells of the same Ecoplate. Control samples were GBLS standards created via serial dilutions (1×10^{1}, 1×10^{0}, 1×10^{-1}, 1×10^{-2}, and 1×10^{-3} ng/ul) and plate controls were non-template controls, where nuclease-free water was used to replace DNA. The qPCR reaction mix was prepared in a total volume of 10 µl containing 1 µl DNA, 0.2 µl each GbF and GbR primers (10 µM), 5 of KAPA SYBR FAST qPCR Mastermix (2X) Universal (KAPA Biosystems) and 3.6 µl nuclease-free water. Non-template controls (NTC) were prepared with 1 µl nuclease-free water replacing the DNA samples. A qPCR quantification was performed using an Eco Real-Time PCR System 110V (Illumina, USA). The qPCR protocol consisted of initial denaturation step at 95°C for 3 min,

followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. A melting curve profile was generated to determine primer-dimers at 95°C for 15 seconds, 55°C at 15 seconds and 95°C for 15 seconds. Three biological samples were run as one composite sample and each sample was run in triplicates for each run. All qPCR reactions were repeated three times.

Reaction C_q values were calculated using Eco Real-Time PCR v4.0 software (Illumina, USA) to show quantitative fluorescence signals rising above threshold levels during the early cycles of the exponential phase of PCR amplification. A standard curve was obtained by plotting the C_q value versus the logarithm of the concentration of each 10-fold dilution series of fungal genomic DNA. The relationship between C_q and DNA concentration was analysed by correlation analyses. Total amounts of GBLS DNA in infected oil palm seedlings were calculated by comparing the C_q values to the crossing point values of the linear regression line of the standard curve. The results were analysed by One-way ANOVA using GraphPad Prism programme version 5.02. Significant differences among treatments were tested by Boniferroni post test at ($P \leq 0.05$).

4.3 Results

4.3.1 Physical changes in oil palm ramets following an infection with *Ganoderma boninense* GBLS

Figure 21 shows the plants of T1, T2 and T3 treatments at day 7, 21, 35 and 42. Oil palm ramets at day 42 post inoculation with *G. boninense* were consistently observed to have suffered more damage to their overall health as compared to T1 and T2. It was also observed that in T2 plants, many adventitious roots were produced as compared to T1 plants. In this study, the oil palm maintained in MS medium for 6 weeks was able to survive with little damage to the overall structure and function of the oil palm ramets as observed in T1 plants.

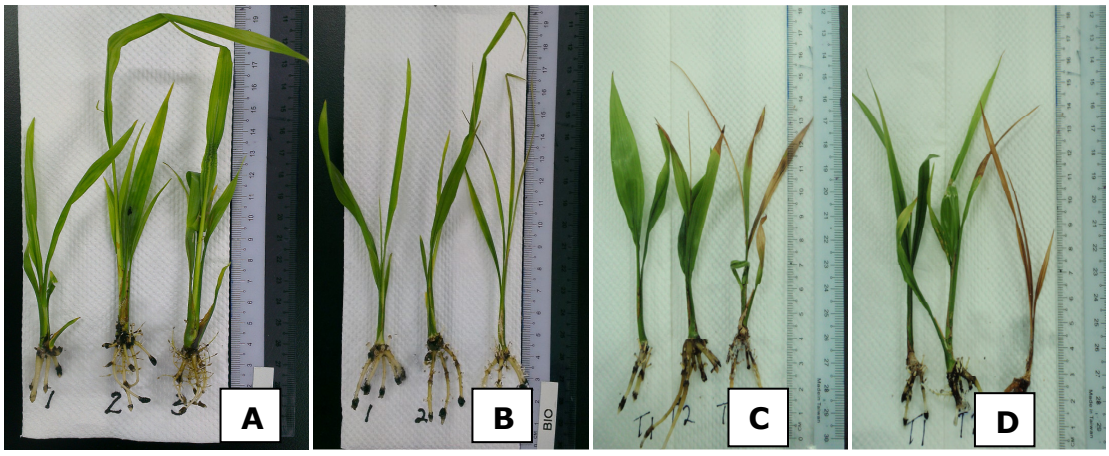


Figure 21: One month old, post-rooting oil palm ramets (Clone AA68, AARSB, Malaysia) from *in vitro* infection studies. T1: No treatment control; T2: wounded plants and T3: wounded and GBLS infected plants. A-D shows plant profiles taken at Day 7, 21, 35 and 42 post inoculations.

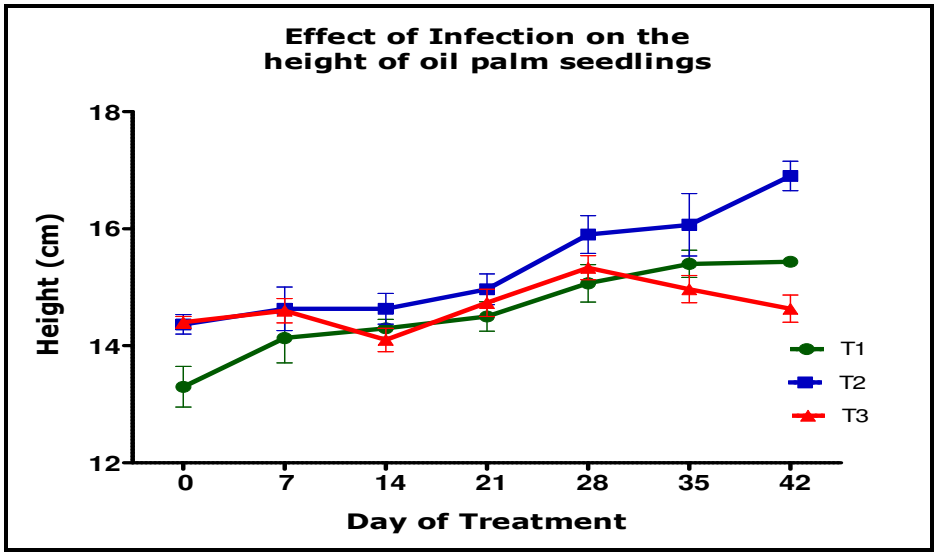
Heights of plants were not significantly different (P>0.05) for treatments at 35 days post inoculation (Fig. 22A). However, there were significant differences at day 42, where T2 plants were 1.5 cm taller than T1 (Tukey post-test; P< 0.001) and 2.3 cm taller than T3 plants (Tukey post-test; P< 0.001) (Fig. 22A). T3 plants experienced loss of vigour and were smaller at day 42 compared with T1 and T2 (Fig. 21). The heights recorded for control plants were between 12 cm and 14.5 cm, and not significantly different from T3 plants, with progressive increment during the experiment. T2 plants were significantly taller than T1 and T3 and increased the height from day 21 to day 42 post-treatment (Fig. 22A). Artificially inoculated palms struggled to survive in *in vitro* conditions when challenged with *G. boninense* (Fig. 21). These T3 plants showed minimum growth from day 28 to day 42. Significant reduction in growth, and stunting, were only observed in T3 plants. Most of the plants in this treatment turned brown and showed signs of rot by the end of the experiment.

The weight of oil palm ramets was not significantly different between treatments for the first 14 days post inoculation (Fig. 22B). From the start of week 3, T3 plants were of lower fresh weight than the other treatments. Plants in the other treatments showed similar trends to each other (Fig. 21). T1 plants were the same weight as T2 plants except at days 35 and 42 (Tukey post-test; P< 0.001) (Fig. 22B). T2 plants grew vigourously despite the injury at the wounded area. At day 28, T3 plants were of showed significantly lower weight than T1 and T2 (Tukey post-

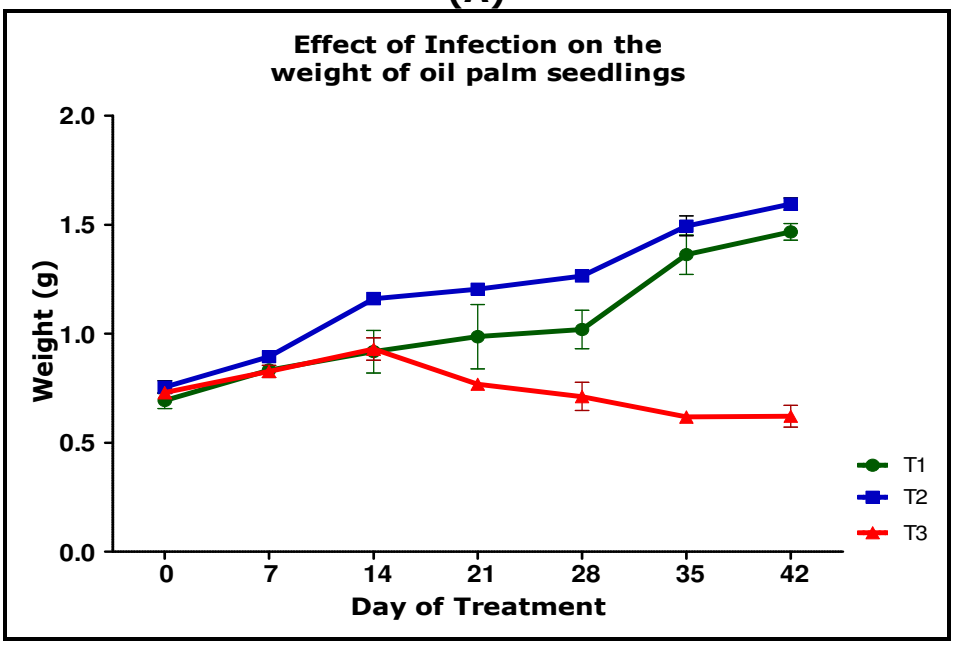
test; P< 0.05). However, T3 plants started to lose weight after day 28 and continued to do so until day 42 (Tukey post-test; P< 0.001) (Fig. 22B). Longtitudinal sections of T3 plants (Fig. 24) revealed hollow internal tissues and dead external tissues. Also, part of the loss was suspected due to low water content in tissues. The dead tissues observed in T3 were dry and lacked succulent interiors as compared to T1 and T2 plants. The wounded areas in T2 and T3 plants resulted in a browning of the outer stem (Fig. 24). The colour of the roots become more translucent with infection and in some samples the roots become stunted (Fig. 24).

The number of leaves for plants on each treatment was not significantly different throughout the experiment. However, the appearance of the leaves differed at day 28 post inoculation onwards to day 42 as T3 plants wilted and became dry (Fig. 23A). The colour and size of the leaves were significantly healthier in T1 and T2 plants. The width of the largest leaf was measured to reflect healthy growth in T1 plants and these results were compared with T2 and T3 plants (Fig. 23B). T2 plants had increased widths (1.60 g) at day 42 (Tukey post-test; P< 0.05) when compared to T3 plants (0.62 g). Differences in width were detected as early as day 28, but were significant at day 42 (Tukey post-test; P< 0.05) where T3 leaves were much narrower as compared to T1 and T2. Leaves on T3 plants had thinned and appeared dry compared to leaves on T1 and T2 plants. Control plant leves (T1) were not significantly wider than those on wounded plants (T2) but at day 42, T1 plants were significantly larger than T3 plants Tukey post-test; P< 0.05) (Fig. 23B). An overall rotting

and loss of vigour were observed for T3 plants. It was noted that T2 plants had longer, stronger and larger numbers of adventitious roots compared to T1 and T3 (Fig. 24). However, in T3 plants, roots lost colour and shape, remained stunted and turned white.



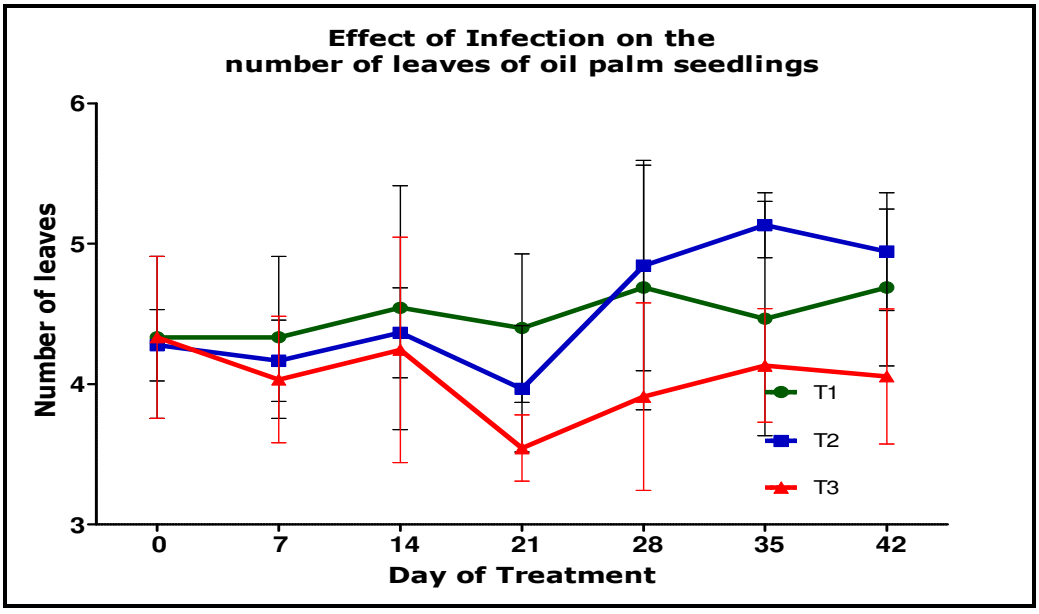
(A)



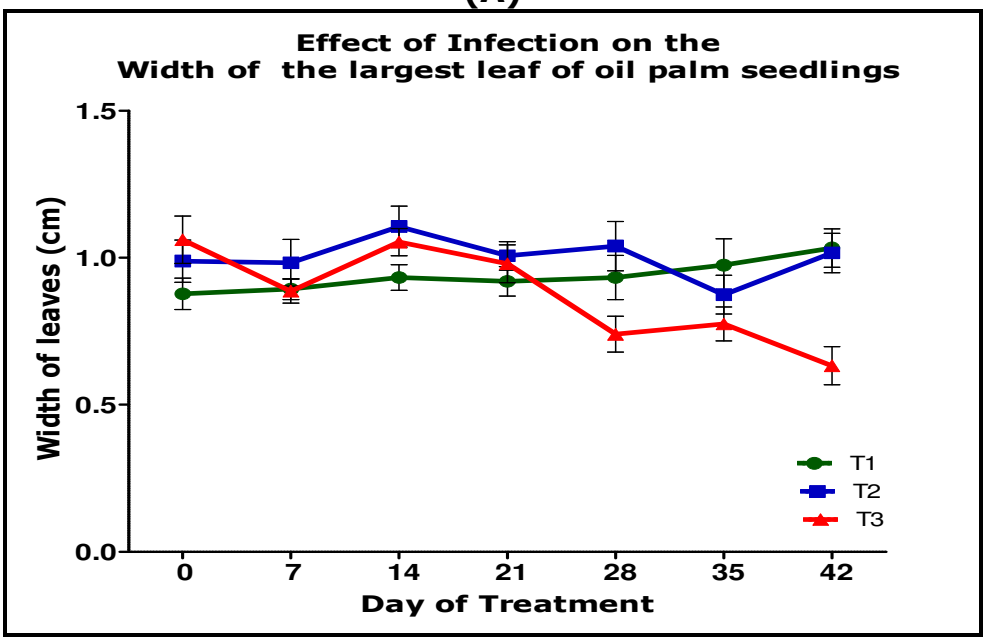
(B)

Figure 22: (A) Height, (B) weight of T1: non-treated control; T2: wounded but not infected and T3: wounded and GBLS-infected plants

within 42 days post infection. Vertical lines represent standard error of mean (SEM) (N=3; with 3 replicate experiments).



(A)



(B)

Figure 23: (A) Number of leaves, (B) average width of the largest leaf of T1: non-treated control; T2: wounded but not infected and T3: wounded and GBLS-infected plants within 42 days post infection. Vertical lines represent standard error of mean (SEM) (N=3; with 3 replicate experiments).

	Longitudinal Section View of stem bole and roots

	Treatment 1	Treatment 2	Treatment 3
Day 0			
Day 7			
Day 14			
Day 21			

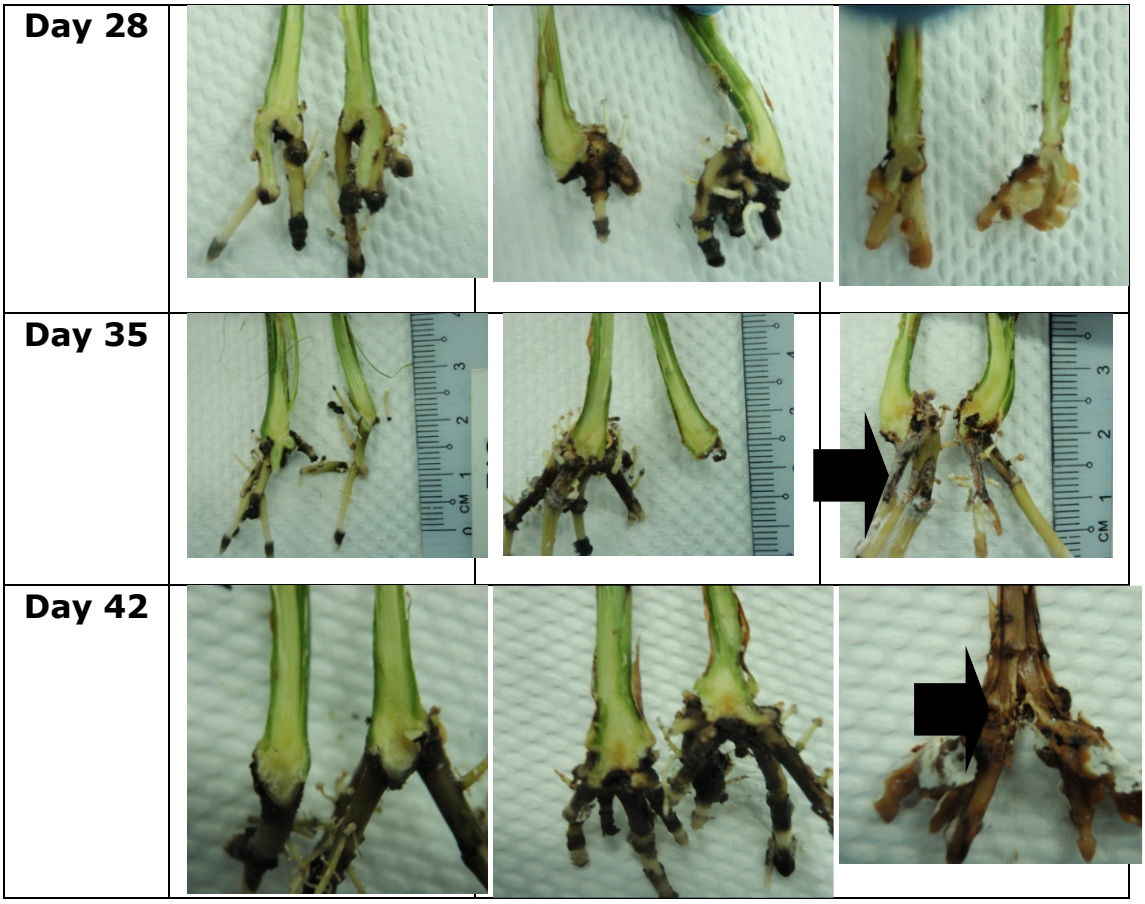


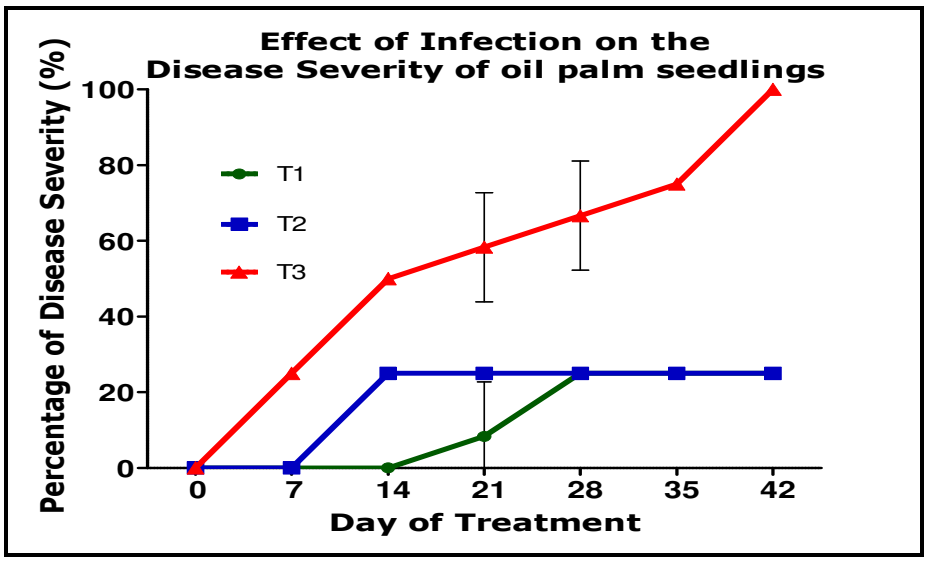
Figure 24: Images of longitudinal-section of stem base tissues for the observation of *Ganoderma* spp. infection in young oil palm. Sample plants from T1: non-treated control; T2: wounded but not infected and T3: wounded and GBLS-infected plants within 0-42 days post infection. Bold arrows indicate the browning and rotting of tissues.

4.3.2 Disease severity in *Ganoderma boninense* GBLS inoculated oil palm ramets.

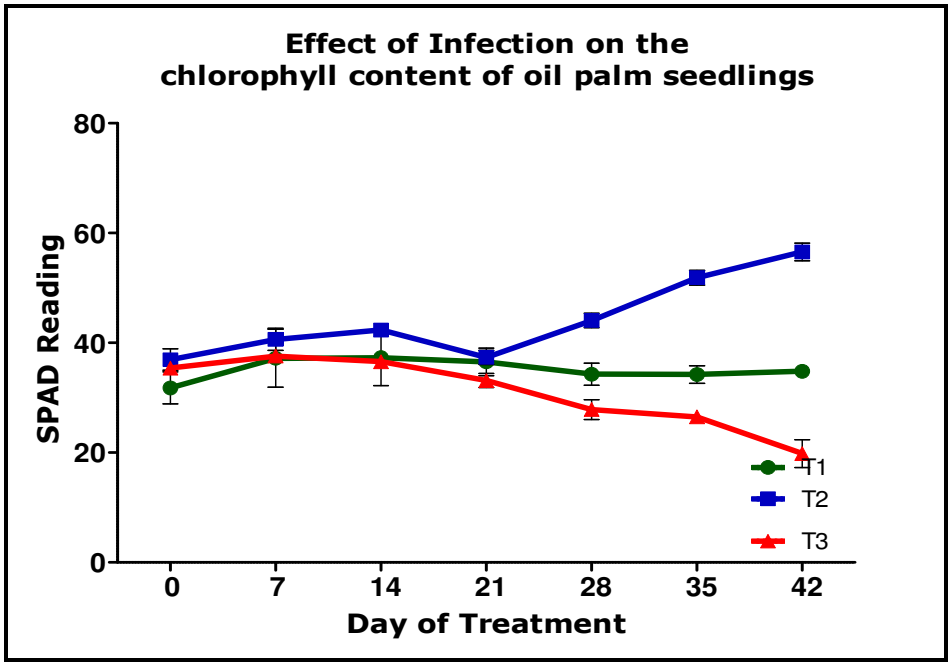
Disease severity increased consistently for T3 plants from Day 0 to Day 42. Disease severity increased significantly from day 14 in T3 as compared to T1 and T2 plants (Fig. 25A). Apparent DS for T1 and T2 levelled off at 20%, with no further browning of leaves after 2-3 weeks (Fig. 25A). DS increased most rapidly in the first 14 days after

inoculation, attaining 100% by day 42. By day 28, 70% of all T3 plants were showing signs of rotting and accumulation of fungal mass and by day 42 some plants were completely brown and dead (Fig. 24).

SPAD reading (SPAD 502 Plus Chlorophyll Meter, Spectrum Technologies, USA) is a measurement of green pigment within an index of -9.9 to 199.9 and to correlate the index of green pigment with the content of nitrogen in plants (Murdock *et al*., 2004; Lopez-Bellido *et al*., 2004; Fang Lin *et al*., 2010). The SPAD value at day 28 showed were significantly different between all treatments (Tukey post-test; P< 0.001), with readings of 34.30, 44.06 and 27.84 for T1, T2 and T3 plants respectively. The same trend was observed at days 35 and 42 (Tukey post-test; P< 0.001).



(A)



(B)

Figure 25: (A) Percentage of disease severity (%), (B) Chlorophyll content using SPAD meter for T1: non-treated control; T2: wounded but not infected and T3: wounded and GBLS-infected plants within 42 days post infection. Vertical lines represent standard error of mean (SEM) (N=3; with 3 replicate experiments).

4.3.3 Quantification of GBLS DNA in oil palm ramets.

Pathogen DNA was at its maximum at day 14 after inoculation (Fig. 26). The *Ganoderma* spp. 167 bp DNA fragment was successfully detected in each infected sample (T3). This fragment was not detected in T1 and T2 samples (Appendix 4). Fungal DNA was detected in plants on day 0, after mycelium was applied to the wound surfaces of T3 plants. However, in one week, the fungal DNA reached 250 ng/µl and peaked at more than 1000 ng/µl at day 14 (Fig. 26). The quantity of DNA dropped after day 14 to reduced growth and proliferation from day 14 to 35 (Fig. 26), increased at day 42.

The efficiency of the qPCR quantification for calculation of fungal DNA was at 94.2% and the R^2 was 0.986 for the standard curve (Fig. 27) (EcoStudy Software, Illumina, USA). The amplification was of a single band as observed by a single peak of the derivative melting curve (Illumina, USA) (Fig. 27).

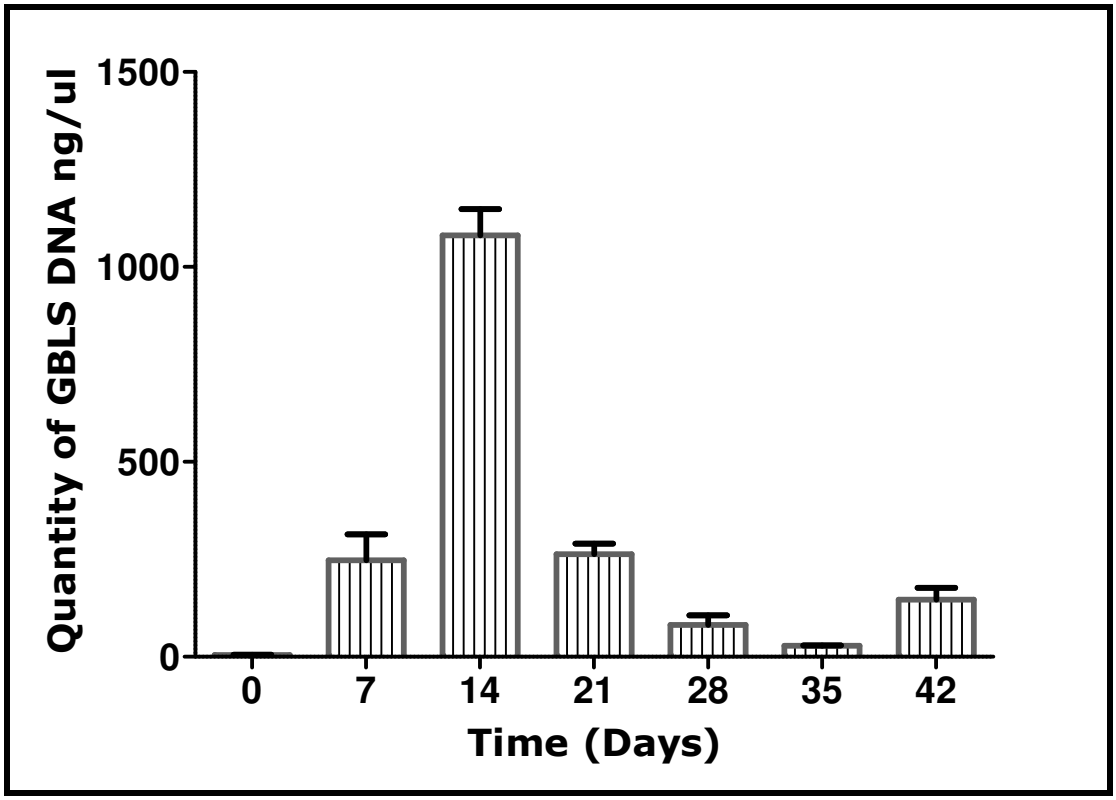
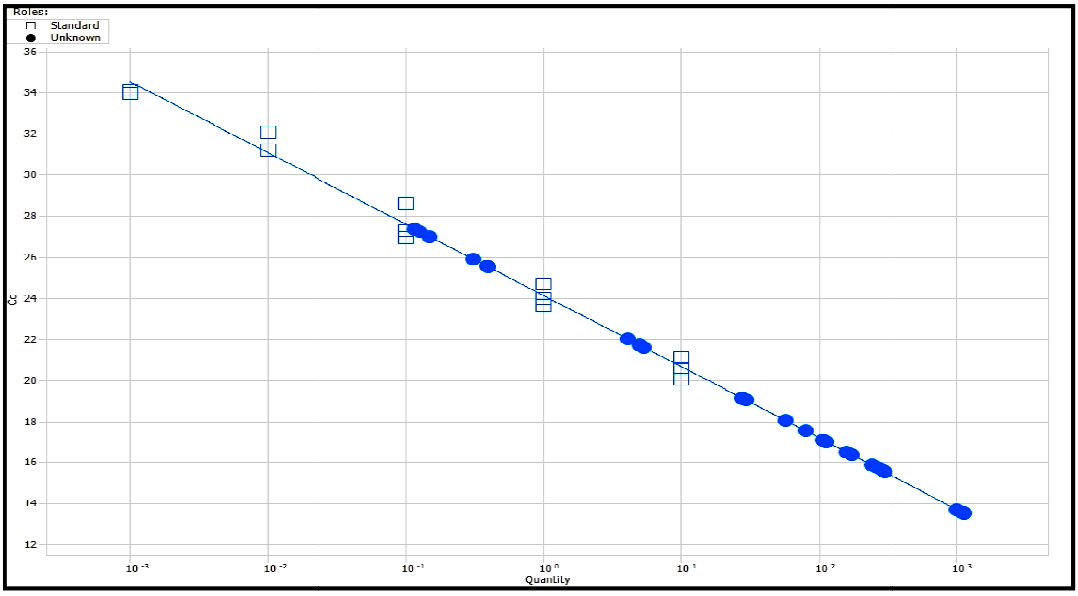
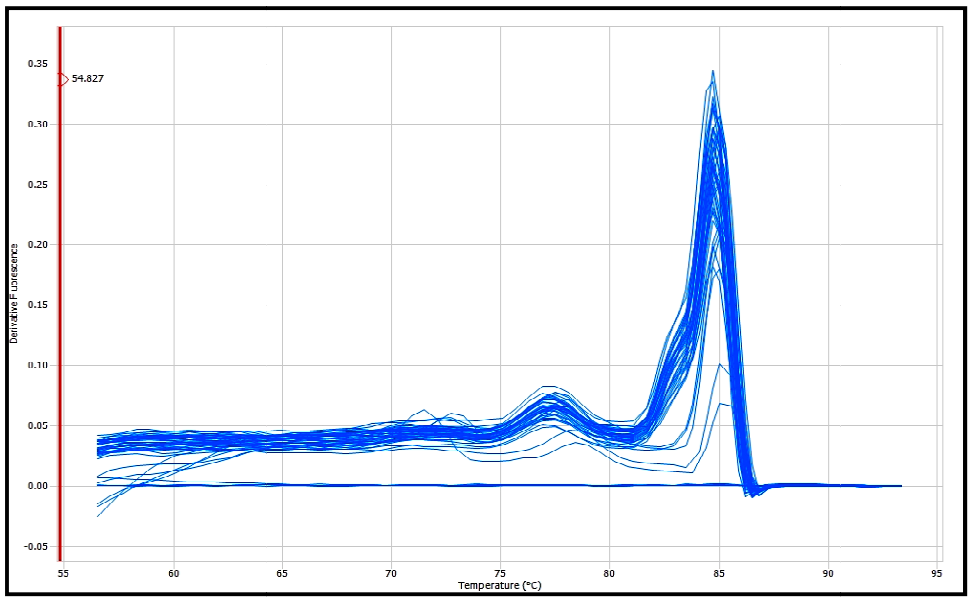


Figure 26: Changes in quantity of *Ganoderma boninense* DNA in young oil palm using GBLS DNA as standard quantity. Quantity of standard was measures using Nanodrop (Thermo, USA). T3: wounded and GBLS inoculated plants.



(A)



(B)

Figure 27: (A) Quantification of DNA using GBLS DNA as the standard. Quantity of standard was measured using Nanodrop (Thermo, USA). The efficiency of PCR was 94.2% with the R^2 of 0.986. (B) Melt curve analysis of DNA measured at 85°C for 5 seconds.

4.4 Discussion

The most widely used current method for the *Ganoderma*-oil palm artificial interaction is the rubber wood block (RWB) technique (Fig. 11). It

was first described by Sariah *et al*. (1994) and has not been modified since as it causes damage to the lower stem and bole and produces basidiomata, similar to that of natural *Ganoderma* spp. infection on mature oil palms (Izzati *et al*., 2008; Alizadeh *et al.,* 2011; Naher *et al.*, 2012a; Naher *et al.*, 2012b; Yeoh *et al*., 2012; Tan *et al*., 2013; Ganeson, 2015). However, the pertinent question is the use of rubber wood blocks and the rationale behind this practice. Perhaps, the understanding of *Ganoderma* spp. as white rot fungi leads to an assumption that requirement for *G. boninense* to be degrading wood before infecting oil palm, but why is this necessary when the oil palm used in the older studies were 3 months to 12 months old with lignocellulosic wood in their stems? Although the RWB method is highly reproducible and has been adopted as the recommended method by the *Ganoderma* Research Centre, GANODROP Unit at the Malaysian Palm Oil Board (MPOB), it has been used for 21 years, is outdated and should have been modified to fit the current understanding of the disease. There is an increase in disease incidence of *G. boninense* invasion in oil palm estates since 1990s (Naher *et al*., 2013). It was reported in a survery carried out every 4 years in Malaysia, that BSR disease incidence in estates doubled in 2002-2005 (Azahar *et al.,* 2011). Although many researchers subscribe to the RWB method, some are trying out other methods. Chong *et al.* (2012) reported that direct infection of *Ganoderma* spp. to oil palm tissue was possible. A suspension containing 20 ml potato dextrose broth, 50 µl of Tween 20 blended with total mycelium from one 9 cm Petri dish was prepared and

each oil palm root was sprayed with 17.5 ml of the suspension (Chong *et al*., 2012). Although the roots were planted in pots as with the RWB method, there were no wood blocks used and therefore it was one-to-one plant-pathogen interaction (Chong *et al*., 2012). Disease incidence was measured by the concentration of ergosterol and by 6 weeks, there was 3 times more ergosterol in inoculated plants compared to control plants (Chong *et al*., 2012). In the present study, the objective was to introduce a local virulent isolate, *G. boninense* GBLS into young oil palm using a new artificial infection assay. The virulence and symptoms in this one-to-one plant-pathogen interaction had to be similar to that of BSR infection in oil palm estates. However, the lack of strong woody tissues in young palms was possibly a disadvantage to the fungus because *G. boninense* is a member of efficient lignin degrading fungi (Paterson *et al.,* 2009). Also, for the fungus, the disadvantage is the use of axenic 14-day old cultures of GBLS as opposed to wild cultures, freshly isolated from an infected estate as it could affect its virulence over time. To overcome these disadvantages, the pathogen was cultured interchangeably in wood tissues, such as sterile rubber wood chips or saw dust and PDA. However, prior to infection, pure cultures were taken from PDA. Similar problems were encountered when using PDA for the growth and sporulation of fungi (Sharma and Pandey, 2010). While most pathogen mycelia grew well on PDA, sporulation was heaviest in ligno-cellulosic agar, a substrate representing plant cell structure and components (Sharma and Pandey, 2010).

In this work, the infection of GBLS on young oil palm was artificially introduced at 0.5 cm above the root zones so that GBLS will come into contact with plants before the medium. Although eventually the fungus will be exposed to the MS medium in culture vessels, it was important to note that the plants were colonised and decayed in this process, indicating fungal source of nutrient could be derived from the plants. This showed that *G. boninense* was an opportunistic pathogen of oil palm, whereby both plants and medium were selectively utilised as sources of energy. In the case of *Cryptococcus neoformans*, a human pathogen was able to exhibit parasitic behaviour in *Arabidopsis* and *Eucalyptus* grown *in vitro*. *Cryptococcus* was inoculated on the surface of the plants, away from MS and it was able to induce mating in the presence of plant myo-inositol and indole acetic acid (IAA) (Xu *et al.*, 2007). While usually this fungi undergoes mating process in the dark and mating was inhibited in the presence of light, when observed *in planta,* mating occurred in the light conditions, indicating virulent behaviour (Xu *et al.*, 2007). Another interesting observation was mating and disease on *Arabidopsis* and *Eucalyptus* when co-cultured with fungi on MS medium, where limited sporulation was observed when the pathogen was grown on MS medium alone, indicating *Cryptococcus* as a true pathogen that not only adapts to plant systems but alters its behaviour to cause disease in plants (Xu *et al.*, 2007).

In the present study, an *in vitro* dual inoculation assay is a controlled environment to create the one-to-one interaction between oil

palm and *Ganoderma* spp. This microclimate eliminates competition by soil microflora, fungistatic and selective nature of soil and/or the use of rubber wood blocks were removed. Although, one can argue that there is minimal similarity between this system and natural BSR infections of oil palm, many *in vitro* dual culture assays have been used to mimic plant-pathogen interactions at the cellular level (Denman *et al.,* 2005, Saxena *et al.,* 2007, Baumgartner *et al*., 2010, Kabir *et al.,* 2013). Also, it is a test for pathogen behaviour and infection potential on plants without the presence of soil microbes, as it was investigated in roses (Suo and Leong, 2002); banana plantlets (Jie *et al.,* 2009); soybean roots (Bressano *et al*., 2010); and grape rootstocks (Baumgartner *et al.,* 2010). The study of plant pathology has recent inoculation-infection assays conducted in controlled environments as well as the use of *in vitro* cultures as host plants.

The overall vigour of plants was reduced at 42 days post inoculation and this could be attributed to the maintenance of plants within the same medium condition for 6 weeks. It was learnt that oil palm can sustain growth in tissue culture conditions for as long as 12 weeks, before the medium had to be replenished (Ms Jenny Choo, Senior Officer, Tissue culture, AARSB Malaysia, pers comm). This could have been attributed to the growth adaptation in tissue culture conditions and could have been a critical time for plants to adapt and continue growth. The height and weight of the starting material were measured from one-month post-rooting oil palm ramets. The results observed were from

clonal oil palm with few genetic differences and all oil palm ramets were treated equally in the growth chamber to minimise other stresses such as light, humidity and heat.

In this work T3 plants were completely colonised by day 42 post-inoculation. However, disease severity was not the same throughout the infection period. The disease symptoms progressed steadily in T3 as compared to no-treatment control (T1) and wounded (T2) control plants during the 42 days of incubation and although the severity index also increased in T2 due to wounding created at day 0, the symptoms were different to those in T3. The T3 plants failed to increase in height (Fig. 22A) and remained stunted and turned brown with wilting. The longitudal sections clearly showed a hollow stem (Fig. 24) and the plant weighed a mere 0.5 g as compared to T1 (1.0 g) and T2 (1.2 g) (Fig. 22B). In other plant-pathogen interactions, loss of height and weight are often observed as critical parameters for disease. In barley infected with *Polymyxa graminis*, symptomatic plants grown in pots for 3 months were 30 cm shorter than asymptomatic plants (Thompson *et al*., 2011). The diseased plants had many tillers, had partial chlorosis and showed delayed physiological growth. Also the weights of symptomatic plants were 0.56 times that of asymptomatic plants. In peppers infected with Cucumber mosaic virus (CMV) and pepper mottle virus (PepMoV), plant heights were compared between day 1 and day 25 post-infection (Murphy and Bowen, 2006). There were significant reduction (highest 68.8%) in height of single and dual-infection, compared to control plants (Murphy and Bowen,

2006). The highest weight loss was 64.2% in dual-infection as compared to a zero infection control (Murphy and Bowen, 2006).

In this work, the average number of leaves at the beginning of the experiment was four per plant and in no-treatment control (T1) and wounded plants (T2), there was an increase in the formation of new leaves. Although, the changes were not significant (Tukey post-test, $P>0.05$), there was at least one new leaf in these treatments compared to *Ganoderma*-infected plants (T3) (Fig. 23A). In T3 plants, the number of leaves remained static and towards the end of the experiment, there was a loss in the number of leaves as dead leaves were not replaced by new ones (Fig. 23A). Just as in the estates, reduction in the number of leaves and the width of the largest leaf were observed in the dual culture system (Fig. 23B), indicating that this assay could represent some of the interactions of oil palm-*Ganoderma* spp. in the field. The importance of studying leaf conditions were emphasized by Filho *et al*. (1997), when determination of the width of the central leaf provided an accurate, reproducible and non-destructive technique to determine the overall health of diseased and control plants (Filho *et al*., 1997). Chlorophyll content could be used as one of the indicators of wilting and damage caused by BSR on soft tissues, affecting water and nutrient uptake processes. In the case of *Phoma* spp. infection on olive plants, the artificial infection assay observed that SPAD readings of infected plants were significantly ($P<0.001$) lower than that of control plants (Papachatzis and Vagelas, 2008). Chlorophyll content was also a

significant measurement for early detection of seedling leaf spots in cabbage where chlorosis and damage to the cabbage leaves were detected 8 hours earlier using chlorophyll fluorescence camera as compared to using a charge-coupled device (CCD) camera (Chiu *et al.,* 2015).

There are several tests developed to determine *Ganoderma* spp. in infected oil palm, such as isolation on GSM medium (Chapter 3), ITS-DNA-PCR (Utomo and Niepold, 2000, this study) polyclonal antibodies, measurement of volatiles and ergosterol quantity analysis (Tan *et al*., 2013). However, these methods are qualitative and while ergosterol and volatiles could be measured quantitatively, specific volatiles have to be indentified prior to using them to screen for pathogen or disease. The use of soil-based screening for a soil-borne pathogen should be specific for the pathogen, excluding the detection of other fungi. Unfortunately, ergosterol is produced by all other fungi and thus not specific to *Ganoderma* spp. (Tan *et al*., 2013). In this study, DNA detection was explored with quantitative PCR because the ITS fragment targeted is specific to *G. boninense*, so amplification of this fragment can be used as a measure of fungal colonization. A standard curve based on known quantity of fungal DNA was generated and amplified DNA fragments detected from infected plants. A similar strategy was used by Baumgartner *et al*. (2010) for *Armillaria* spp. infection in grape rootstocks and Debode *et al.* (2009) for *Colletotrichum acutatum* infection in strawberry leaves, when fungal load was quantified in infected plants.

In this work, the results from three biological replicates indicated that *G. boninense* GBLS was not detected at day 0 (day of inoculation) but reach an exponential stage at day 14. It was also important to note that while plants were showing severe symptoms, the quantity of GBLS reduced in the plant (Fig. 26). This decrease could be due plant barriers such as chemicals and oxidative responses that delay or reduce the growth of *G. boninense.* However, at day 42, there was an increase in measureable fungal DNA, indicating that either GBLS recovered from host defences or it had killed all the tissues and grew more extensively. Evidence for the oxidative burst of nitric oxide has been reported in *Ganoderma* spp.-infected palms. While insignificant amounts of nitric oxide associated 1 gene (*NOA1*) were detected in plants 28 days after inoculation, there was a 0.2 fold drop between 14 and 28 days and a 2.9 fold increase at day 96 post infection (Kwan *et al*., 2015). This finding clearly indicates a delayed oxidative burst in oil palm and thus could facilitate fungal growth. However, young palms in the present study could have similar responses but between day 28 and day 35 where GBLS growth was apparently low. In a related study, cDNAs for oil palm defence genes were down-regulated at 6 weeks post infection (Tan *et al*., 2013). This time was at the mid-point of a 12 weeks infection study and, in comparison to the present work, highest amounts of fungal DNA were detected at the mid-point of the study as well. This finding indicated that between inoculation and development of symptoms, there is a drop in plant defences, correlated with the increase in fungal mass. The pattern

of distribution for fungal growth was similar to other work such as *Colletothrichum acutatum* infection of strawberry leaves, where fungal growth shows a latent period until mid-point of the disease, and an increase in growth towards the death of plants (Debode *et al*., 2009). In this work, the effect of fungal load on the young oil palm was a contributing factor to the loss of overall vigour of the oil palm *in vitro*. Baumgartner *et al*. (2010) quantified fungal load by measuring the quantity of fungal DNA of *Armillaria* spp. in axenic infections of grape rootstocks. The fungal biomass was measured by the presence of nuclear elongation factor subunit 1-α (*EF1-α*) that was detected across many species of *Armillaria* spp. (Baumgartner *et al*., 2010). It was also reported that *Armillaria* spp. load of fungal biomass adversely affected the plants and that it was crucial to have measured the abundance of the fungal DNA (Baumgartner *et al*., 2010). The use of quantitative PCR was chosen over conventional PCR for the accurate quantification of the abundance of DNA, calculated using known concentration standards. The melt curve is important information because it confirms a single target for the primer (Fig. 27). Overall, the dual culture assay was a robust technique to show oil palm-*G. boninense* interactions over 6 weeks with a quantified pathogen load on infected plants. The assay could be further improved by replacing MS medium with sterile compost or compressed fibres to represent a "soil-like" substrate. However, the *in vitro* characteristics should be maintained for a direct, one-to-one interaction of the diease.

5. CHAPTER 5: ISOLATION OF A GENE FRAGMENT ENCODING LACCASE IN *GANODERMA BONINENSE GBLS*

5.1 Introduction

Ganoderma spp. are white rot fungi (WRF) that efficiently degrade polysaccharides and lignin (Adaskaveg *et al*., 1990). Factors that differentiate fungi within the WRF include substrate, as some WRF degrade all parts of wood, while others preferentially degrade lignin (Adaskaveg *et al*., 1990). The degree of decay also varies from 'pockets' of wood loss to complete breakdown of all woody material (Adaskaveg *et al*., 1990). The effect of white rot on host tissue is 'bleaching' (Worrall *et al*., 1997), while some fungi are able to change the structure and composition of wood (Blanchette *et al*., 1991). WRF utilize three LDEs; lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac). WRF are the only microorganisms known to selectively degrade all components of lignocelluloses with a combination of hydrolytic and oxidative enzymes (Elisashvili and Kachlishvili, 2009). WRF have been documented to completely breakdown lignin into carbon dioxide and water (Cullen and Kersten, 2004). One factor promoting the increase in lignin degrading capability of WRF is nitrogen starvation, presence of metals such as copper and the accumulation of ligninases (Paterson *et al.,* 2008). The oil palm trunk is 93% lignocelluloses and the main lignin monomer is syringaldehyde (Paterson *et al*., 2008). Compared to "hard"

wood that comprises mainly guaiacyl, 65% of syringaldehyde in oil palm wood makes it susceptible to WRF attacks (Paterson *et al*., 2008).

While hymenomycete fungi differ in the combination of LDEs used for lignin breakdown, *Ganoderma* spp. use all three enzymes, although limited published work cite the production of LiP in comparison to Lac and MnP (da Silva Coelho *et al*., 2010). The perception of *Ganoderma* spp. using all combinations of ligninolytic enzymes to colonize host plants could be extrapolated from its wide host range. It was reported that *Ganoderma* spp. in India were found on 144 host species, including four newly reported hosts and the report claimed that the list was not exhaustive (Sankaran *et al*., 2005) Although the pathogen was named *Ganoderma lucidum*, other studies verified similar shape, growth behaviour and molecular markers as *G. boninense* (Sankaran *et al*., 2005; Kandan *et al.,* 2009). The population of *Ganoderma* spp. in oil palm plantations (see section 1.2.2) contributes to the large pool of fungal biomass and a larger content of LDEs in the soil. Fungal laccases have been used as markers to understanding fungal soil ecology (Luis *et al*., 2004).

Laccase (benzenediol: oxygen oxireductase EC 1.10.3.2) is a lignin degrading enzyme (LDE) that oxidises aromatic compounds with low ionization potential, and is most common in WRF and similar versions of LDEs have been found in brown rot fungi (D'Souza *et al.,* 1996). Almost all WRF produce several versions of the laccase enzyme (Baldrian, 2005). Laccase is also commonly known as blue copper oxidase that reduces

four-electron oxygen molecules to water via a one-electron oxidation of organic material (D'Souza *et al.,* 1996). Blue copper laccases are common fungal laccases but others such as 'yellow' and 'white' laccases lack a Cu atom, although it is suggested that these are not 'true' laccases (Baldrian, 2005). Laccases have been known to alter a wide range of phenolic substances such as polymeric lignin and humic materials (Baldrian, 2005). In fungi, laccases play a role in morphogenesis, pigmentation, host-pathogen interactions and lignin degradation (Baldrian, 2005). Most laccases are secreted as extracellular enzymes and are stable in the outer environment (Baldrian, 2005).

The aim of this experiment was to isolate a gene encoding laccase in *G. boninense* GBLS. The gene fragment would be cloned using standard cloning vectors and transformed in *E.coli*. The fragment would be sequenced to confirm the presence of laccase.

5.2 Materials and Methods

5.2.1 Fungal cultures, growth conditions and DNA extraction

Mycelium cultures of the GBLS isolate were prepared according to Section 3.2.2 and DNA extraction performed according to Section 3.2.4. Quantity and purity of DNA samples were observed in 1% w/v agarose gel in 1 X TBE buffer. Images of agarose gels were captured using BioRad Gel Documentation System (BioRad, USA). Concentration of DNA was checked using Nanodrop Quantification (Thermo, USA).

5.2.2 PCR amplification of laccase gene

5.2.2.1 Primer design

Primers were designed for laccase, manganese peroxidase and lignin peroxidase enzymes of hymenomycete fungi (Table 7). Degenerate primers for the amplification of the laccase gene were designed based on sequences of *Ganoderma* spp. reported by D'Souza *et al*. (1996). Alignments of GenBank sequences were performed using Clustal W analytical software (Clustal W, EMBI). A total of 22 laccase enzymes of copper oxidase-related protein sequences from *Ganoderma* spp. were aligned to determine the conserved regions for primer design (NCBI, USA). Nucleotide sequences of 20 *Ganoderma* spp. were also compared (NCBI, USA). Primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/).

D96Lac2F: Forward primer, 5'-**CAY TGG CAY GGN TTY TTY**-3'

D96Lac2R: Reverse primer, 5'-**RTC RCT RTG RTA CCA RAA NGT**-3'

Table 7: Primer sequences designed for the PCR amplification of lignin degrading enzymes in this study. *Lac* and *Cu* primers were designed for laccase enzyme, *lip* for lignin peroxidase and *mnp* for manganese peroxidase.

Primer name	**Primer sequences, 5'-3'**	**References**
D96Lac2F	CAY TGG CAY GGN TTY TTY CA	D' Souza *et al*., 1996
D96Lac2R	RTC RCT RTG RTA CCA RAA NGT	D' Souza *et al*., 1996
D96Lac1F	CAY TGG CAY GGN TTY TTY CAR	D' Souza *et al*., 1996
D96Lac1R	ACN TTY TGG TAY CAY AGY CAY	D' Souza *et al*., 1996
lacF	CAT TGG CAT GGC TTC TTT CA	AF297527.1, AF297526.1,

		AF297525.1;
lacR	GGC TGT GGT ACC AGA AGG TTC C	FJ858749,
CuF	CAC TGG CAT GGT TTC TTC CAA	AY839941.1, AY839940.1,
CuR	CTT TCT GGT ATC ACT CTC AC	AY839941.1, AY839940.1
lipF	CCC GAA CAT CGG TCT CGA CGA	AF140062
lipR	GGG ATC ACA TCC GAF CAG TCG	X54257.1
mnpF	GAC GGC TCC ATG CTC CTG TTC CC	AY442338.1, AY442337.1
mnpR	TGC CAG AAG CAC GCC GTG CG	AY442338.1, AY442337.1

5.2.2.2 PCR reaction for D96Lac2F and D96Lac2R primers

The PCR mix for D96Lac2F/2R consisted of 1 X green reaction buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 0.8mM dNTP mix (Promega), 0.8 pmol of each primer, 40 ng total genomic DNA of *Ganoderma* spp. and 0.125 units of *Taq* polymerase (Promega). PCR was performed in a thermal cycler (G Storm, USA) at conditions of 95°C for 5 min, followed by 35 cycles of 94°C for 40 seconds, 49.5°C for 40 seconds and 72°C for 45 seconds. A final extension step was performed at 72°C for 12 min. Gel electrophoresis was conducted according to Section 3.2.5. A single band of ~200 bp was observed in gel electrophoresis.

5.2.3 Cloning of laccase gene fragment into the pGEM-T-*E. coli* system.

Initially, each reaction tube from the PCR experiment was quantified to estimate the quantity of DNA. The average concentration per tube was

15 ng/µl. This amount was low and insufficient for cloning. In order to obtain a higher quantity of PCR product of the ~200 bp sequence, multiple samples were amplified via PCR and the products pooled into a single tube. A volume of 100 µl of product was separated in 1% w/v agarose gels. Purification of PCR product was performed using a Gel Extraction Kit according to manufacturer's recommendation (Fermentas). Purified ~200 bp DNA was quantified using the Nanodrop Quantification (Thermo, USA). The concentration of gel-purified DNA was 50 ng/µl. The cloning vector, pGEM-T Easy was digested with *Eco*R1 restriction enzyme to allow the ligation of the ~200 bp fragment. Ligation into the pGEM-T Easy cloning vector was performed in 1:1 ratio with 1 µl of DNA (50 ng) added to 1 µl of vector (50 ng), 5 µl of ligation buffer and 1 µl of T4 DNA ligase (Promega, USA). Total reaction volume was 10 µl. Ligation was performed at room temperature for one hour and 4°C overnight and the recombinant plasmid designated pGEMTGbLacL25. For cloning purposes, 5 µl of the reaction mix was added to 200 µl of competent JM109 *E. coli* (Promega) on ice for 30 min. The bacteria with ligated plasmid, pGEMTGbLacL25, were heat shocked for 45 seconds at 42°C and immediately placed in ice for 2 min. Then, 945 µl of SOC buffer was added to the bacteria mix and the tube gently inverted. The tube was incubated at 30°C for 3 hours with constant agitation at 200 rpm. The mix tube was then centrifuged for 15 min at 2500 rpm. The top layer of 700 µl was discarded and the remaining 300 µl of bacteria transformed with plasmid DNA was mixed well and spread onto duplicate LB agar Petri

dishes containing 50 µg/ml ampicillin and blue-white screening reagents, 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) and 3.2 µg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The lawn of bacteria was incubated at 37°C for 18 hours. Results were observed as blue-white bacteria colonies where 'blue' colonies represent the bacteria without the desired DNA insert, while 'white' colonies indicated the positive transformants that carried the targeted ~200 bp DNA fragment.

5.2.4 Restriction endonuclease and PCR confirmation of 208 bp fragment in *E. coli* clones

Selected 'white' colonies (20 colonies) of bacteria from blue-white screening were propagated in 10 ml LB broth supplemented with 50 µg/ml ampicillin for 18 hours at 37°C. The bacterial culture, approximately 5 ml, was centrifuged at 12,000 rpm for 2 min and placed in ice. Plasmid DNA, pGEMTGbLacL25, was isolated using Plasmid Extraction Kit (Fermentas, USA) according to the manufacturer's recommendations. Extracted and purified plasmid DNA was cleaved using *Eco*R1 restriction endonuclease to detect the presence of inserted ~200 bp within the plasmid DNA. A reaction buffer containing 1 µg plasmid DNA, 1 X reaction buffer (Promega, USA), 0.1 mg/ml BSA and 1 unit *Eco*R1 was prepared and incubated at 37°C for 2 hours. DNA samples for cleaved and intact bands were visualised on 1% w/v agarose gel in 1 X TBE buffer.

5.2.5 Glycerol stocks of bacterial clones

All 20 'white' colonies were confirmed to contain ~200 bp *G. boninense* in the recombinant plasmid, pGbLacL25 using the restriction enzyme assay. Selected clones were subcultured to LB agar supplemented with 50 µg/ml of ampicillin for future plasmid DNA extractions. Glycerol stocks of the positively transformed bacterial cultures were made by adding 12.5% v/v of glycerol (autoclaved, molecular biology grade) to 200 µl of bacterial culture in a 1.5 ml tube. The glycerol stock was gently inverted several times to facilitate the mixing of glycerol and bacterial culture. Stocks were labelled and stored at -80°C.

5.2.6 Sequencing of 208 bp fragment of *Ganoderma boninense* laccase

For sequencing purposes, 50 ng of pGEMTGbLacL25 was quantified using Nanodrop. Sequencing was done using ABI Prism Sequencer with a Big Dye Termination Kit at First Base Laboratories, Seri Kembangan, Malaysia.

5.3 Results

5.3.1 PCR amplification of laccase gene

Primer combinations were tested for the isolate of laccase enzyme from *G. boninense* total DNA. The number of bands and their relative size are listed in Table 8. Single band amplification through the combination of D96Lac2F/ D96Lac2R produced a ~200 bp size fragment. Although larger

size fragments were obtained in D96Lac1F/lacR combinations, these were not chosen for analysis by sequencing because there was more than one band in each sample (Sample L31, L38 and L40). These sets of fragments were amplified in temperature gradients and 'touchdown' PCR to obtain fewer and more significant bands (results not shown). Reproducibility of the ~200 bp from D96Lac2F/ D96Lac2R at 45°C was an important criterion for selection and purification for cloning purposes.

Table 8: Amplification of putative laccase fragments using specifically designed primer combinations.

Sample No.	Primer combination	Tm (°C)	Number of bands	Size of bands (bp)
L30	D96Lac1F/ D96Lac1R	45.5	0	No bands
L31	D96Lac1F/ D96Lac2R	45.5	4	1500, 550, 400, 200
L32	D96Lac2F/ D96Lac1R	45.5	2	600,300
L33	D96Lac2F/ D96Lac2R	45.5	1	~200
L38	lacF/lacR	45.5	3	1200,450,350
L39	lacF/ D96Lac1R	45.5	0	No bands
L40	D96Lac1F/lacR	45.5	3	1500,1200,200

5.3.2 Cloning and sequencing of *Ganoderma boninense* laccase gene fragment

The complete sequence obtained from cloning and sequencing experiments was 1181 bp (Fig. 28). Although the sequence is representing the pGEM-T vector section of pGEMTGbLacL25, the primer regions and restriction enzyme sequences present on the cloning vector sequence were clearly marked (Fig. 28). The result of sequencing with reverse primer, D96Lac2R, on pGEMTGbLacL25 produced a 208 fragment (Fig. 28). Although, the presence of a laccase gene fragment was detectable (Fig. 28), it was unconventional to sequence reverse-cloned fragments. Since the aim of this experiment was to clone and sequence a fragment of laccase, the orientation of the sequence in the cloning vector was not significant.

BLAST analyses of the 208 bp sequence revealed 89% similarity (or 2e-33 value) to uncultured hymenomycete laccase isolated from forest soil (AJ420341.1) and 86% and 84% similar to *G. lucidum* and *Ganoderma* spp. BAFC2488 respectively (Table 9).

Figure 28: Sequencing of 1181 bp of 5’-3’ pGEMTGbLacL25. Yellow highlighted section if the forward and reverse primers respectively, while green highlighted fragment refers to the reverse primer.

Table 9: BLAST search result of 208 bp of pGEMTGbLacL25. The fragment is conserved within the hymenomycete laccase gene family, with high homology to various hymenomycete fungi.

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
AJ420341.1	Uncultured Basidiomycete partial lac14 gene for laccase, exons 1-2	159	159	59%	5e-36	88%	
HM569745.1	*Ganoderma* sp. En3 laccase mRNA, complete cds	150	150	60%	2e-33	86%	
AY450406.1	*Flammulina velutipes* laccase mRNA, complete cds	150	150	60%	2e-33	86%	
AY485826.1	*Flammulina velutipes* laccase gene, complete cds	145	145	59%	1e-31	86%	
AJ420176.1	*Ganoderma lucidum* partial lac gene for laccase, exons 1-2	145	145	59%	1e-31	86%	
AY364842.1	*Ganoderma s*p. BAFC2488 laccase gene, partial cds	141	141	62%	1e-30	84%	
AY243866.1	Hypoxylon sp. HKUCC 3196 isolate Lcc14v2 laccase gene, partial cds	136	136	59%	5e-29	84%	
HE585214.1	*Ganoderma weberianum* partial lac2 gene for laccase, isolate B-18, exons 1-2	134	134	66%	2e-28	82%	
AY875864.1	*Coriolopsis gallica* laccase (lacg201) gene, partial cds	120	120	60%	4e-24	80%	
AY204503.1	*Trametes versicolor* putative laccase gene, partial cds	118	118	60%	1e-23	80%	
EU684156.1	*Pycnoporus cinnabarinus* strain BRFM 44 laccase gene, partial cds	114	114	59%	2e-22	80%	

5.4 Discussion

To the knowledge of the author, this is the first identified laccase gene fragment from *G. boninense*, pathogen of oil palm. The fragment will be used as a biomarker to identify the presence of laccase in cDNA samples from the oil palm-*Ganoderma* interaction. The interaction sample

will be used to determine if laccase was transcribed early during the infection of oil palm by *Ganoderma* spp. or if it has a role in the process.

A plant-pathogen interaction includes a single or a combination of mechanisms to successfully cause disease (Dickinson, 2003). Although some fungal pathogens use toxins, e.g. *Cochliobolus* spp., cell wall degrading enzymes, e.g *Colletothricum* spp.; anthracnose disease and structural lesions, e.g. *Magnaporthe oryzae*; rice blast disease, fungi, Basidiomycota generally rely on white or brown rot mechanisms to cause disease. The highly evolved hymenomycete fungi co-exist naturally with plants and the environment as organic degraders of plant biomass. Therefore, the change of biotrophic fungi to a necrotrophic state is a developmental process influenced by the host plant resistance (biotic) and the environmental (biotic and abiotic) stresses. *Ganoderma* spp. were found only on old and decayed oil palm in the 1970s but have altered behaviour to attack one year-old palms in areas previously planted with oil palm. While the cumulative inoculum potential of *Ganoderma* spp. and the monocropping of oil palm have been blamed for the increased virulence of *Ganoderma* spp. as a pathogen, the real mechanism remains elusive. *Ganoderma* spp. produce laccases as one of their LDEs the role of this enzyme family in virulence has been suggested (Paterson *et al.,* 2009).

Laccase encoding genes are highly conserved among hymenomycete fungi (Baldrian, 2005). Other fungi such as yeast do not produce laccase although multicopper oxidase with similar sequence and structural

homology to laccase was found in *Saccharomyces cerevisiae* (Baldrian, 2005). Cloning and sequencing of several fungal laccases has been reported because laccase has industrial importance to bleach wood pulp and breakdown azo dyes (Mayer and Staples, 2002). In the case of white rot fungi, *Pleurotus eryngii*, two laccases were cloned and produced heterologously in *Aspergillus niger* (Rodriguez *et al*., 2008). Laccase signal peptide, *pel3* was contructed using an *A. niger*, glucoamylase preprosequence to target secretion of laccase (Rodriguez *et al*., 2008). This work shows that though laccases are exclusive to the host plant and fungi, they are inducible in other fungi by means of heterologous expression.

Thus, the isolation of a laccase encoding gene will facilitate further analyses on its expression during virulence. This chapter described the isolation of a 208 bp fragment of a laccase gene from total DNA of *G. boninense* associated with basal stem rot disease. This finding was similar to D'Souza *et al*. (1996) as the predicted fragment size of laccase amplified by the primer set was between 120 to 246 bp (D'Souza *et al*., 1996).

In other published work, the full length gene was reported to contain 4357 bp with 9 introns (Joo *et al*., 2008). The full length cDNA was 1560 bp and codes for 520 amino acids (Joo *et al*., 2008). A unique *Ganoderma* spp. En3 isolated from the forest of Tzu-chin Mountain in China produced predictable laccase but not manganese peroxidase and lignin peroxidase (Zhuo *et al*., 2011). Cloning and heterologous

expression of the laccase enzyme showed the predictable 520 amino acids and 1563 bp cDNA region. This fragment was successfully expressed in *Pichia pastoris*, further confirming the conserved regions of laccase in *Ganoderma* spp. and hymenomycetes (You *et al*., 2014). The objective of this chapter was achieved through obtaining a single 208 bp fragment of the gene encoding laccase enzyme in *G. boninense* GBLS.

CHAPTER 6: THE ROLE OF LACCASE DURING THE INFECTION OF OIL PALM BY *G. BONINENSE* GBLS.

6.1 Introduction

Ganoderma boninense is a fungal pathogen that is well known in oil palm BSR in Malaysia and Indonesia. However, the mode of infection has been largely speculative as the life cycle of the pathogen is long: the appearance of disease on oil palm tissues can take months or years and is relatively inconsistent between age of palm, location of estates and soil condition and is also affected by weather patterns (Breton *et al*., 2006; Hushairian *et al*., 2013). In addition, other *Ganoderma* spp., such as *G. zonatum* and *G. miniatocinctum* have also been associated with BSR. In Sarawak, *G. zonatum* accounts for more than 70% of BSR cases in Betong and Miri (Rakib *et al*., 2014). Since *G. boninense* has been detected via the use of specific primers since 2005 (Utomo *et al.*, 2005), many cases of BSR have been narrowed to *G. boninense* as the pathogen. In this work, GBLS, a strain of *G. boninense*, was isolated from sporophores of *Ganoderma* spp. found on BSR infected palms. Since GBLS

was detected using standard G. boninnese primers (Chapter 3) (Ganeson, 2015), it was chosen as a virulent genet of the pathogen.

Lignin is a polymer made up of monolignol unit derived from the phenylpropanoid pathway (Campbell and Sederoff, 1996). The monolignols are guaiacyl (G subunit), parahydroxyphenyl (H subunit) and syringyl alcohol (S subunit) (Campbell and Sederoff, 1996). Understanding *Ganoderma* biology has demonstrated that this fungus is a good organic recycler, particularly lignin-based material, as it produces significant amounts of the LDEs laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) (Sharma *et al*., 2013). In a related study, LDEs of *G. boninense* were quantified and consistently lignin peroxidase was absent in cell cultures (Ganeson, 2015). This was also reported by Namoolnoy *et al*. (2011) that white rot fungi (WRF) in oil palm produced measurable laccase, manganese peroxidases and/or lignin peroxidases. While the production of MnP and LiP was subjective across 54 WRFs tested in this work, laccase was produced by every single isolate (Namoolnoy *et al*., 2011). Laccase was detected in a medium containing ABTS (2,2-azinobis) 3-ethyl-benzthiazoline-6-sulphonic acid), a standard substrate for inducing laccase production (Namoolnoy *et al*., 2011).

In the present work, laccase has been chosen as a potential candidate virulence factor for *G. boninense* and its role in BSR virulence was investigated. The laccase gene fragment cloned and sequenced in Chapter 5 was used as a biomarker which led to the design of new primers to

detect the presence of laccase in infected and control plants. Also, the DNA quantities of laccase enzymes present in diseased plants throughout the 42 days post infection period are detailed in this chapter. While the work described in this study was targeted at fungal laccases, specifically *Ganoderma* spp. laccase, it does not discount the fact that plants too produce laccases. Plant laccases play a major role in the lignification process, along with other oxidative enzymes such as catecholase, tyrosinase and peroxidase (Harvey, 1997). In fact, the earliest laccase recorded, by Yoshida in 1883, was when the lacquer-forming sap was found on Japanese lacquer tree, *Toxicodendron vernicifluum* (formerly *Rhus verniciflua*) (Harvey, 1997). Laccase was identified as oxidative coupling of cathecol, which led to the production of a black lacquer used as a wood finish (Harvey, 1997). Despite the importance of plant laccases, the majority of research was focused on fungal laccases and only in the 1960s was the importance of higher plant laccases identified (Harvey, 1997). However, there are many differences between plant and fungal laccases such as their optimum pH and the breakdown of specific substrates. It was reported in the 1970s that syringaldehyde is not detected by plant laccases as opposed to fungal laccases but in the 1990s sycamore and tobacco laccases were shown to utilize this substrate (Harvey, 1997). Therefore, besides the need to study the role of *Ganoderma* spp. laccases during the infection of oil palm, there is also a need for specific biomarkers that quantify the activity of fungal laccase in a mixed, plant-fungi total laccase sample. It was reported that the main

difference between plant and fungal laccases is the optimum pH required, whereby fungal laccases are optimum at pH 3.6-5.2, whilst the Chinese laquer tree, has a laccase with optimum pH range of 6.8 to 7.4 (Madhavi *et al*., 2009).

In this study, it is hypothesized that the induction of laccase in *G. boninense* will trigger the modification of oil palm total lignin content and that there will be a co-relationship between the activity of laccase and manganese peroxidase with the relative expression of laccase gene fragment in fungi. Enzyme activity detection using ABTS is highly specific for laccase, but it detects both plant and fungal laccases. Therefore, the difference between the total laccase activity in infected plant samples and control was measured. It is also hypothesized that there is a correlation with GBLS secretion of laccase and manganese peroxidase and the loss of lignin content measured using the LTGA assay. The LTGA assay was compared with other methods for total lignin content such the acetyl bromide and Klasson lignin methods (Moreira-Vilar *et al*., 2014). The acetyl bromide method was suggested to be faster and simpler than others and the thioglycolic acid (TGA) method was less efficient (Moreira-Vilar *et al*., 2014). However, in this work, the TGA method was used because neither the acetyl bromide nor the Klasson lignin methods were cost effective and reproducible results were obtained with the TGA method when tested on oil palm samples.

6.2 Materials and methods

6.2.1 Determination of total lignin content by LTGA assay

Total lignin content was quantified via the lingo-thioglycolic acid (LTGA) assay (Bruce and West, 1982; Mandal, 2010) with modifications. The LTGA assay converts lignin into base-soluble and acid-insoluble lignin thioglycolate through acid-catalyzed derivatization of lignin with thioglycolic acid (Kirk and Obst, 1988). Three oil palm seedlings (clone AA68, AARSB, Malaysia) infected with GBLS (Chapter 4), for each replicate were weighed and homogenized using a pestle and mortar in 80% v/v methanol (1 g plant whole plant: 10 ml of methanol). The mixture was filtered through filter paper (Whatman No. 4, USA) and filtrate discarded. Insoluble tissues were rinsed with 2 ml of 80% v/v methanol and the process repeated four times. Methanol-extracted tissues were air-dried overnight in a convection oven at 60°C. Approximately 50 mg of the plant residue was weighed, mixed with thioglycolic acid solution (0.5 ml of thioglycolic acid (Sigma Aldrich, USA) and 5 ml of 2N HCl) and boiled in a water bath (Yih Der, BH-320, Taiwan) for 4 hours. The mixture was allowed to cool to room temperature and centrifuged at 10,000g for 30 min. The pellet was rinsed in purified water twice, with centrifugation for 5 min each at 10,000g. The residue was dissolved in 5 ml 0.5 N NaOH overnight at 25°C, with agitation at 150 rpm (Yih Der, TS-520, Taiwan). Dissolved thioglycolic acid-lignin slurry was centrifuged at 10,000g for 30 min to separate the insoluble material. The supernatant was recovered in a fresh tube and

stored at -20°C until further use. The insoluble pellet was washed in 2 ml purified water, via centrifuged at 10,000g for 5 min. The supernatant was collected and 1 ml of 6M HCl added to precipitate LTGA at 4°C for 4 hours. The acidified solution was centrifuged at 10,000g for 10 min to precipitate the orange-brown LTGA pellet. The pellet was washed in 2 ml of 0.1N HCl, with centrifugation at 10,000g for 5 min, before neutrilising step with the addition of 2.5 ml 0.5N NaOH. The mixture was centrifuged for 3 min to remove insoluble matter. LTGA content was measured using a spectrophotometer at OD 280 nm and expressed as increase in TGA derivatives g^{-1} fresh weight of plant tissues.

6.2.2 Determination of laccase and manganese peroxidase (MnP) enzyme

GBLS had to be grown on malt extract broth (MEB) with rubber wood chips to induce enzyme production. Five mycelium plugs (5 mm^2) from 14 day old GBLS were subcultured into a 250 ml Erlenmeyer flask containing 5 g of rubber wood chips (Rubber Research Institute of Malaysia, Sg. Buloh, RRIM) and 30 ml of MEB. The culture was incubated for 7 days at room temperature in a rotary shaker at 120 rpm. After this period, 0.1 M of sodium acetate was added to the liquid medium at a ratio of 1:1. This mixture was shaken for 4-5 hours at 120 rpm and stored overnight at 4°C. In order to assay for laccase and MnP, solid and liquid portions of the sample were vacuum filtered through Whatman 4 filter paper and the filtrate centrifuged at 4000g for 30 min, twice. The supernatants were stored at -20°C until further use.

Laccase (p-diphenol: oxygen oxidoreductase; EC 1.10.3.2; p-diphenol oxidase; p-DPO; p-diphenolase) activity was determined by the oxidation rate of a 0.03% (w/v) ABTS (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid) solution as the substrate at 30°C (Murugesan *et al*., 2007). The 100 µl assay mixture contained 10 µl 100 mM sodium acetate buffer (pH 5.0), 80 µl 0.03% (w/v) ABTS and 10 of crude enzyme from the GBLS culture. Absorbance values for the detection of laccase were 420 nm, read at intervals of 1 min for 5 min. Laccase activity was determined by ABTS oxidation (molar extinction coefficient = 36,000M^{-1} cm^{-1}) as described previously by Murugesan *et al.* (2007).

Manganese peroxidase (MnP) activity was determined by the oxidation of guaiacol as substrate (Patrick *et al*., 2011). The 180 µl assay mixture contained 20 µl 0.5 M sodium succinate buffer (pH 4.5), 20 µl 4 mM of guaiacol, 20 µl 1 mM manganese (II) sulphate, 80 µl distilled water and 20 µl crude enzymes from GBLS culture with rubber wood chips. The mixture was incubated at 30°C in a water bath for 2 min before adding 20 µl of 1 mM hydrogen peroxide. Absorbance values of the reaction mixture were measured at 465 nm at 1 min intervals for 5 min. Control reactions were set up using purified water. Absorbance was measured using a kinetics enabled microplate reader (Varioskan Flash, Thermo Scientific). The absorbance value from three independent quantification assays was used to calculate the rate of colour change of substrate. One unit of enzyme activity was defined as the amount of enzyme required to oxidize

1 µmol of substrate per min. Each value presented was the mean of three replicates. The formula of conversion of absorbance value to µl unit:

Unit/L =

$$\mu l = \frac{(Ab_{Sample}) - (Ab_{blank}) \text{ x } TV}{\varepsilon \text{ coefficient value x } SV} \text{ x } 1000$$

where,

Ab_{sample} = Absorbance value of sample

Ab_{blank} = Absorbance value of blank

TV = Total volume of reaction mixture (unit = µl)

SV = Sample (enzyme extract) volume in reaction mixture (unit = µl)

ε coefficient value = Coefficient value of enzyme substrate (unit = mM cm^{-1})

To determine the relative quantity of fungal laccase produced by GBLS due to infection, the formula below was used:

GBLS Laccase= Laccase activity U/ml of T3 plants - Laccase activity U/ml of T1 plants

Laccase activity U/ml of T1 plants

To determine the relative quantity of plant laccase produced by oil palm due to wounding, the formula below was used:

Oil palm Laccase= Laccase activity U/ml of T2 plants-Laccase activity U/ml of T1 plants

Laccase activity U/ml of T1 plants

6.2.3 RNA extraction and cDNA synthesis

Preparations for RNAse-free material included the treatment of plasticware with 1 mM EDTA and 0.1 M NaOH and final rinsing of all

material with 0.1 % w/v DEPC-treated water. Non-disposable labware such as glass and metal were soaked in 0.1% w/v DEPC-treated water overnight at 37°C before sterilisation by autoclaving at 121°C for 15 min to remove residual RNAse.

Total RNA from GBLS was isolated using a Qiagen RNAeasy Plant Mini kit (Catalog no. 74903, 74904), according to manufacturer's recommendations (Qiagen, Germany). Pure cultures from 10-14 day old GBLS, grown on PDA were collected using a microbiological loop and extracted in liquid nitrogen. Powdered mycelium of approximately 0.1 g was placed into a 1.5 ml tube. The protocol for purification of total RNA from plant cells and tissues and filamentous fungi was used (RNAeasy® Mini Handbook, 2012, Qiagen, Germany).

Total RNA from oil palm seedlings was extracted using Trizol-based method as described by MacRae (1994), with modifications. Plants from the T1, T2 and T3 treatments (Chapter 4) were weighed to 0.1 g before grinding in liquid nitrogen. One ml of Trizol reagent (Invitrogen, USA) was to the homogenate after which it was vortexed and centrifuged at 10,000g for 5 min at 4°C. The supernatant was collected in a fresh tube and 200 µl chloroform added (Bio Basic Inc, USA). The mixure was shaken vigorously for 15 min in a Thermomixer (Eppendorf, Germany), before centrifuging at 10,000g for 15 min at 4°C. The upper aqueous phase was carefully pipetted into a fresh 1.5 ml tube. Next, 500 µl of ice-cold isopropanol was added to precipitate the RNA. The mixture was

handled gently by placing it in an ice bath for 10 min. Following centrifuging at 10,000g for 10 min at 4°C, the supernatant was discarded and 1 ml 75% v/v ethanol added. The mixture was centrifuged at 10,000g for 5 min at 4°C, and the same procedure repeated for a second time. Finally, the pellet was dissolved in 20 µl nuclease-free water (Promega, USA). The concentration and quality of RNA was determined using a Nanodrop (Thermo, USA). The quantity of RNA extracted was measured as f ng/µl and the quality (purity) determined by using the ratio of absorbance values at 260/230 nm Nanodrop (Thermo, USA).

Total RNA was used immediately after quality checks to minimise degradation. A cDNA synthesis step was set up using a Tetro cDNA synthesis Kit (Bioline, UK) according to manufacturer's recommendations. Specific components of the kit (5X RT Buffer, 10 mM dNTP mix and Oligo (dT) 18 primer mix) were thawed and briefly centrifuged. After this step, all reactions were conducted in an ice bath. The cDNA synthesis reaction consisted of 4 µl 5X RT Buffer, 1 µl 10 mM dNTP mix, 1 of Oligo (dT) 18 primer and 1 µl RiboSafe RNAse inhibitor. Then 8 µl extracted total RNA and 1 µl Tetro Reverse Transcriptase (200 ng/µl) were added. Finally, 4 µl DEPC-treated nuclease-free water was added. The mix was incubated at 45°C for 30 min. The enzyme reaction was terminated by incubation at 85°C for 5 min. Samples were allowed to cool and used immediately for qPCR quantification, or stored in a -20°C freezer.

6.2.4 A qPCR quantification for the abundance of transcripts encoding laccase in infected samples.

A total of 22 laccases (copper oxidase-related protein sequences) of *Ganoderma* spp. were aligned using Clustal W analytical software (Clustal W, EMBI) to determine conserved regions for primer design (NCBI, USA). Although the primer set used was successful in identifying a laccase DNA fragment in *G. boninense*, it did not amplify the fungal laccase in infected and control plant sampless. This was because the primer was designed to amplify laccase regions in DNA as opposed to RNA samples. However, the findings in Chapter 5 were important because it was the first such report of identification of a laccase DNA fragment in *G. boninense*. Therefore, in this chapter, full length cDNA fragments encoding *G. boninense* laccase were aligned (Clustal W, EMBI) and new primers designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/). Of the 10 primer pairings suggested, two primers Lac2Fa/Lac2Ra and Lac2Fc/Lac2Rc were able to amplify laccase fragments from GBLS pure cultures and GBLS-infected plant samples.

Sequence (5'->3')

Forward primer, Lac2Fa: **CTTACCCCGTCACCGTCAAT**

Reverse primer, Lac2Ra: **GTCACAGGTTCGACTGCGTT**

Forward primer, Lac2Fc: **AACTCTGCAATCCTGCGCT**

Reverse primer, Lac2Rc: **CGCTTCCGGACGGTAGTAAG**

A qPCR quantification was prepared using Eco 48-well plates (Illumina, USA). A 10 µl reaction containing 1 µl approximately 20 ng/µl of cDNA template, 0.2 µl each forward and reverse primers, 5 µl KAPA SYBR FAST qPCR Kit Master Mix (2X) Universal (Kapa Biosystems) and 3.6 µl nuclease free water was prepared. Negative controls were prepared with similar mix except the cDNA template was replaced with nuclease-free water. The nuclease-free water, primers and mastermix were pipetted first into Eco 48-well plates followed by the cDNA template. The KAPA mastermix contained SYBR Green detection dye and was light-sensitive; therefore reactions were not exposed to direct light. Eco 48-well plates were sealed using Eco adhesives (Illumina, USA) and the plates briefly centrifuged (Eppendorf, 5810R) at 1800 rpm before placing into the Eco Real-Time PCR System (Illumina Inc, USA). The thermal cycling conditions for cDNA template amplification were initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 5 seconds and 60°C for 30 seconds. A melting curve temperature profile was obtained by programming the Eco Real-Time PCR system for one cycle at 95°C for 15 seconds, 55°C for 15 seconds and 95°C for 15 seconds. Three individual reactions (technical replicates) were run for each biological sample of treated seedlings and the assays repeated three times for each targeted and reference gene. C_q values were calculated using the Eco Real-Time PCR v4.0 software (Illumina).

The cDNA of GBLS and GBLS-infected oil palm seedlings were quantified (Nanodrop, Thermo, USA) and diluted in nuclease free water to

ensure a final concentration of 20 ng. For the generation of standard curves, cDNA templates of GBLS cDNA in a 10-fold dilution series (1 x 10^{1}, 1 x 10^{0}, 1 x 10^{-1}, 1 x 10^{-2}, and 1 x 10^{-3} ng/ µl) were prepared in 100 µl volumes. Samples of oil palm cDNA from the T3 treatment (GBLS-infected) were prepared from four biological replication infection assays. Each set of cDNAs was run in triplicate (technical replicates). The C_q values were tabulated by Eco Real-Time PCR v4.0 software (Illumina Inc, USA) to detect significant fluorescence signals above set threshold levels during the early exponential phase of PCR amplification. A standard curve was obtained by plotting the C_q value versus the logarithm of the concentration of each 10-fold dilution series of GBLS cDNA. Interactions between C_q value and cDNA concentrations were analysed by correlation analysis. Total abundance of GBLS cDNA transcripts during the interaction with oil palm seedlings were calculated by comparing the C_q values to the crossing point values of the linear regression line of the standard curve.

6.2.5 Statistical analysis

Data were subjected to One-way ANOVA and tested for significant differences among treatments by Tukey Multiple Comparison post tests at $P \leq 0.05$ using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, (www.graphpad.com).

6.3 Results

6.3.1 Determination of total lignin content in infected oil palms.

The total lignin contents between T1, T2 and T3 were not significantly different at days 0, 7, 14 and 21 post-inoculation (Fig. 29). On day 0, wounding had induced higher lignin content in T2 plants but there was no immediate change in GBLS-infected T3 plants. On day 21, lignn content was similar in all treatments and T2 with an average of 93.34 TGA g^{-1}. Lignin content was significantly lower on day 35 (48.18 TGA g^{-1}) and day 42 (42.28 TGA g^{-1}) in T3 plants compared to T1 and T2 samples (Tukey post-test $P<0.001$). However, on the same day, there were no significant differences between T1 and T2 samples. Total lignin content in T3 plants increased sharply at day 21, but reduced significantly by day 42. Lignin content in T1 and T2 plants consistently increased in the first three weeks of the experiment and stabilised until day 42. No significant differences between T1 and T2 plants were recorded throughout the experiment (Tukey test $P>0.05$).

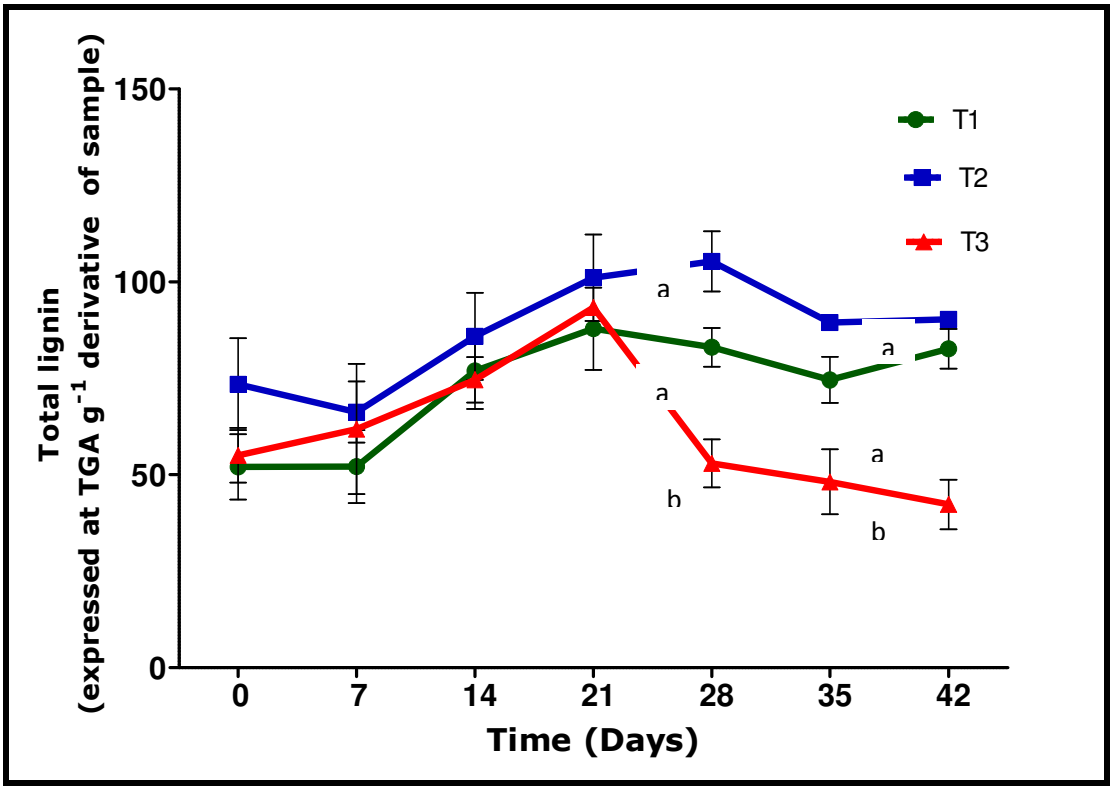


Figure 29: Total lignin content measured using LTGA assay for GBLS infection of young oil palm. Quantity of lignin was expressed as quantity of thioglycolic acid derivative per gram of fresh plant weight. Sample plants T1: Control; T2: wounded only; T3: wounded and GBLS-inoculated plants.

6.3.2 Laccase and manganese peroxidase activities in inoculated oil palm.

Laccase and MnP activity of three biological replicates in the T1, T2 and T3 plants showed no significant differences in laccase and MnP activity. The overall trends of laccase and MnP activities increased with time in all treatments (Fig. 30). To compensate for the quantity of laccase and MnP activity that could have been of oil palm rather than fungal origin, and to obtain relative laccase and MnP activities in the T2 and T3 samples, day 0 T1 (D0T1) samples were used as reference (Section

6.2.2). The values of all samples were re-calculated to deduct for mean of laccase and MnP activity of D0T1 samples. Following this deduction highest laccase activity, 12.93 fold higher than D0T1, was recorded at day 28 in GBLS-inoculated T3 plants (Fig. 30). Laccase activity was lower in the first 14 days for all three treatments, but increased consistently towards the end of the experimental period, specifically after day 28 (Fig. 30). Relative laccase activities in T3 plants were 3.86 times higher than the D0T1 sample for GBLS-infected plants on day 0 and 2.54 times on day 7 but peaked at 9.00 times higher on day 14 (Fig. 31). Activity reduced to 3.70 times at 21 days after treatment, before increasing again to days 35 and 42 (Fig. 31). When T3 samples were analysed separatedly, there were two peak inductions of laccase (Fig. 31) at day 14 and day 28 of the cycle. The range of MnP activity found was 0-5 U/ml. MnP activities were distributed unevenly throughout the time points with the highest reading recorded on day 21 to 35 in T1 plants (Fig. 32). When only T3 plants were analysed for MnP, there was an increased production of MnP on day 28 until 42, but not at other time points (Fig. 33). However, MnP activity was not significantly different at any time points. MnP production shows biphasic pattern with low enzyme quanitites in days 0 to 21 for all treatments and an increased enzyme production days 21 to 35 and a second reduction in activity at day 42.

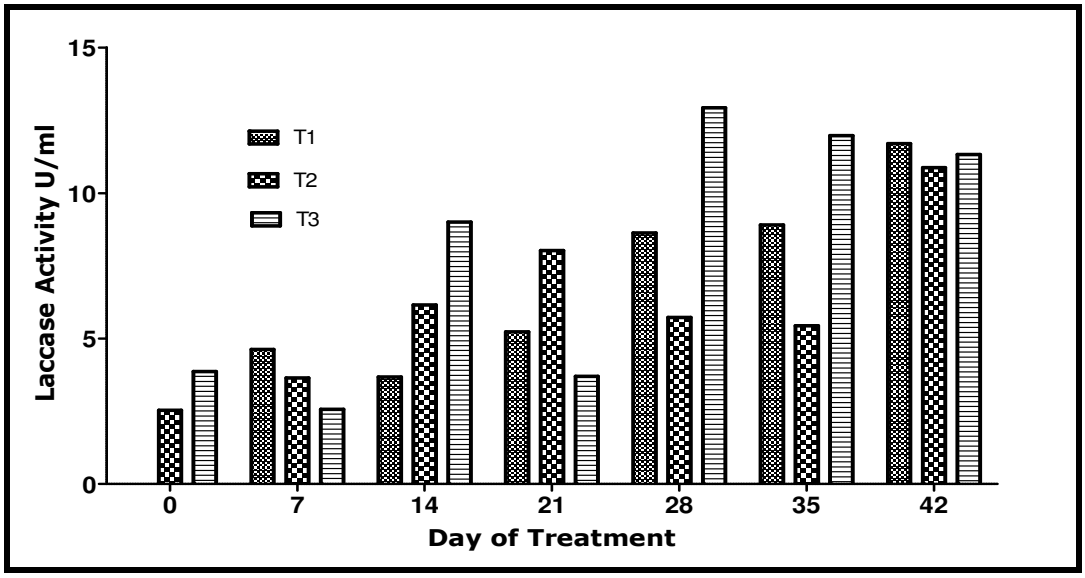


Figure 30: Relative laccase activity measured using control plants of day 0 as a reference sample. Laccase activity was expressed as the rate of oxidation of ABTS measured at 420 nM (^{TM}420 = 36,000 m^{-1} cm^{-1}). Sample plants T1: Control; T2: wounded only; T3: wounded and GBLS-inoculated plants.

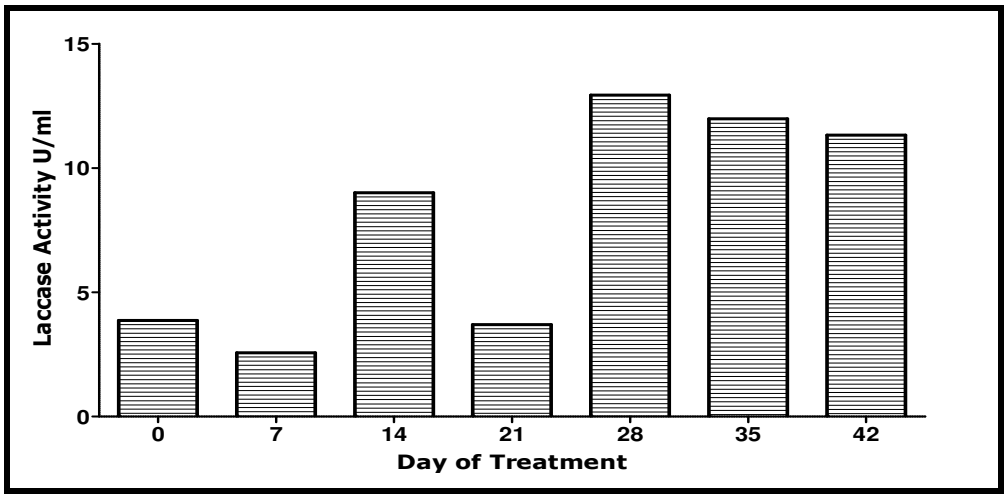


Figure 31: Relative laccase activity measured using control plants of day 0 as a reference sample. Laccase activity was expressed as the rate of oxidation of ABTS measured at 420 nM (^{TM}420 = 36,000 m^{-1} cm^{-1}). Sample plants T3: wounded and GBLS-inoculated plants.

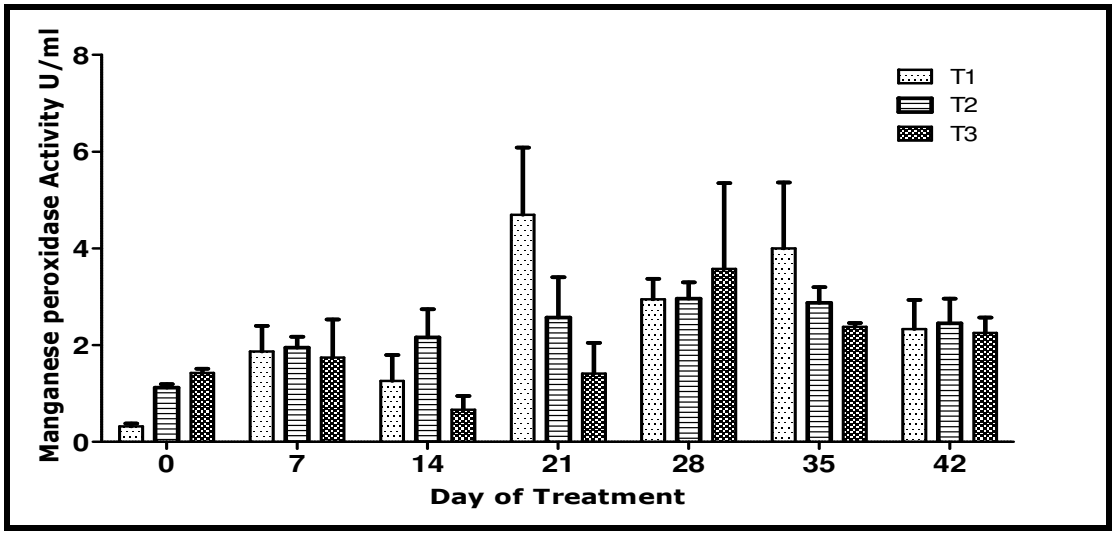


Figure 32: Relative MnP activity measured using control plants of day 0 as a reference sample. MnP activity was expressed as the rate of oxidation of guiacol measured at 465 nM (^{TM}420 = 12,100 m^{-1} cm^{-1}). Sample plants T1: Control; T2: wounded only; T3: wounded and GBLS-inoculated plants.

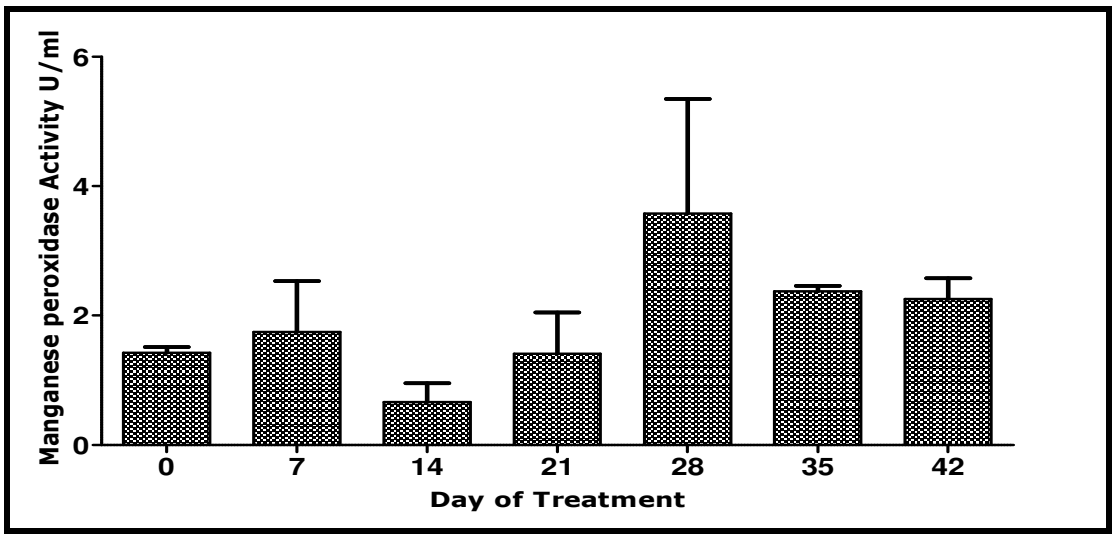


Figure 33: Relative MnP activity measured using control plants of day 0 as a reference sample. MnP activity was expressed as the rate of oxidation of guaicol measured at 465 nM (^{TM}420 = 12,100 m^{-1} cm^{-1}). Sample plants T3: wounded and GBLS-inoculated plants.

6.3.3 Laccase gene trancripts in inoculated oil palm at specific time points.

Primers Lac2Fa/Lac2Ra and Lac2Fc/Lac2Rc amplified a single band (Fig. 34) of approximately 200 bp. Analyses using qPCR for quantification of laccase gene transcripts showed Lac2Fa/Lac2Ra to produce a specific laccase fragment and therefore this set of primers was selected to determine the abundance of gene transcripts in the plant. The expression profile of the laccase gene unique to GBLS in T3 plants was determined using qPCR.

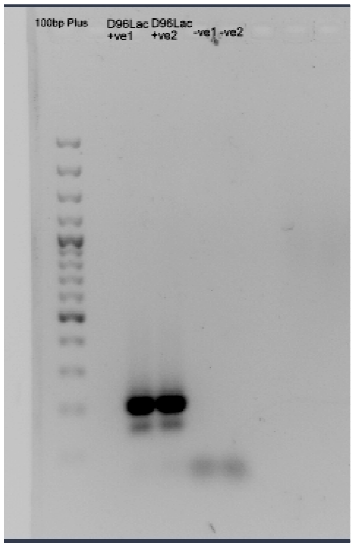


Figure 34: Primer Lac2Fa/Lac2Ra and Lac2Fc/Lac2Rc amplified a single band of 200 bp. The sequence of the band was identical to *Ganoderma* sp. En3 laccase mRNA, accession HM569745.1 and *Ganoderma lucidum* strain CGMCC5.0026 laccase (*lcc5*) gene, accession KC507939.1 in GenBank.

The qPCR was performed using Absolute Quantification Protocol (Illumina, USA) function to determine the quantity of laccase encoding transcripts in each sample. At each sampling time after treatment, abundance of laccase transcripts were analysed by comparison to a

standard curve generated using GBLS total cDNA. The overall R^2 obtained was 0.988 (Fig. 36). Laccase activity did not differ significantly across time points, but was produced at a higher quantity at days 35 and 42 (Fig. 35). Accumulation of laccase transcript was higher at day 35 and highest on day 42 with a quantity 0.74 ng per µl of T3 plant cDNA. Laccase transcripts were detected at very low quantity on day 21 (0.001 ng/µl). The results also indicate a bi-phasic production of laccase transcripts as shown in laccase enzyme activity (Fig. 30-31) (Section 6.3.2). A melting point analysis was performed to ensure that single amplification was detected in the qPCR quantification step using primers Lac2Fa/Lac2Ra for laccase (Fig. 37). There was no amplification in non-template control (NTC) samples (Fig. 37). All results were from experiments using three biological replicates.

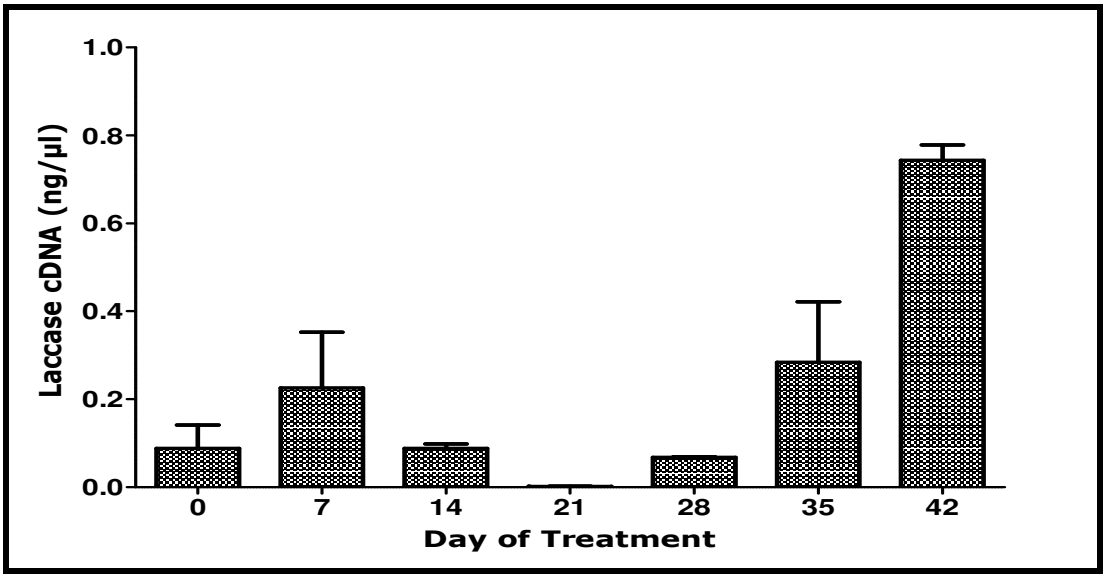


Figure 35: A qPCR quantification of laccase gene transcripts using laccase specific primers Lac2Fa/Lac2Ra on GBLS-inoculated T3 oil palms using qPCR (Illumina, USA). The unit (ng/µl) was calculated by using a standard

curve for GBLS cDNA amplification of the laccase encoding gene with known cDNA concentrations (Nanodrop, Thermo, USA).

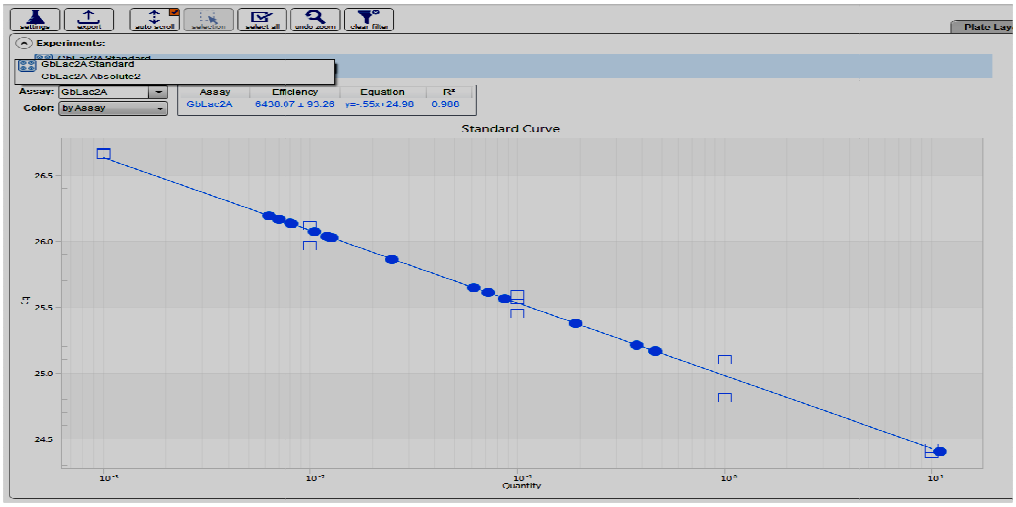


Figure 36: Standard curve for laccase generated with serial dilution of GBLS cDNA and amplification with laccase specific primers Lac2Fa/Lac2Ra. The graph was generated by Ecostudy software (Illumina, USA).

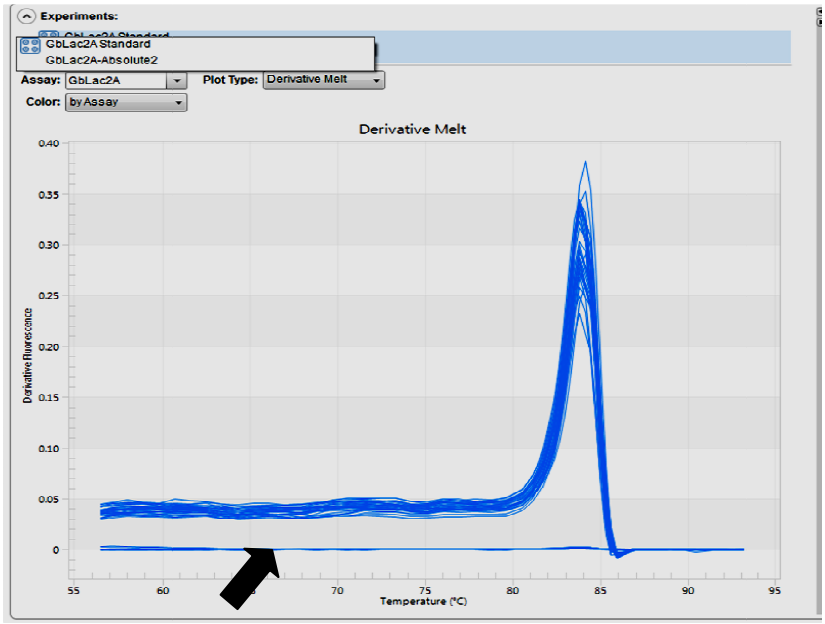


Figure 37: Melting point analysis of GLBS and T3 plants amplified with Lac2Fa/Lac2Ra primers. A single band at 200 bp for the detection of laccase was confirmed with a single peak on meltcurve. The template control was not detected during amplification (bold arrow).

6.4 Discussion

This is the first report on the role of *G. boninense* GBLS laccase on GBLS-infected oil palm. In this chapter, the role of laccase in the virulence of *G. boninense* on young oil palm was investigated. Laccases and manganese peroxidases are enzymes secreted extracellularly and therefore, were detected in liquid cultures of GBLS. In order to secrete these enzymes, the fungus was induced using lignin-containing material and in this study, rubber wood chips were used to induce laccase and MnP by *G. boninense in vitro*. Other inducers include cellobiose and peptone as medium and ABTS as inducer (Hou *et al.,* 2004) and copper and ammonium tartrate also increased laccase production (Cavallazzi *et al*., 2005). However, when the enzyme activities were measured in the *Ganoderma*- oil palm interaction, no induction was apparent. This experiment was to mimic the infection process in field, where lignin content of oil palm is sufficient for *G. boninense* to cause lignin degradation and BSR disease.

To determine if there was a loss of lignin during this interaction, total lignin content was measured using the LTGA method. Total lignin content in plants is subject to the types of lignin monomers present in the plant tissue. Hence, not all total lignin assays are efficient to exhibit the loss of lignin (Moreira-Vilar *et al*., 2014). In this study, the LTGA-based assay (Mandal *et al*., 2010) was used to determine thioglycolic acid residue per g of plant sample. Although the recovery of lignin was lower than that of

the Klasson lignin and acetyl bromide methods, it was highly reproducible (Moreira-Vilar *et al*., 2014). It was important to correlate the activities of lignin degrading enzymes (LDEs) to the loss of total lignin in young oil palms. Similar criteria were used to measure laccase and manganese peroxidase as well as the molecular expression of laccase, which were all perfomed on whole young oil palm. In this work, total lignin content between no-treatment control T1, wounded plants T2 and GBLS-infected T3 plants were not significantly different in the first 21 days after treatment. However, the situation changed from day 28 where lower lignin contents as compared to T1 and T2 were consistently recorded in T3 plants. Between T1 and T2 plants, there were also no major differences in lignin content. It is most likely GBLS reduced both lignin biosynthesis and accumulation in inoculated oil palm. Induction of lignin synthesis during virulence in T3 plants from day 7 until day 21 was part of the plant defence mechanism. Similar observations were made in cotton plants infected with *Verticillium dahliae* where total lignin content, measured using the Klasson lignin method, was higher in resistant cotton (*Gossypium barbadense*) as compared to sensitive cotton (*Gossypium hirsutum*) (Xu *et al*., 2011). The cross-sections analysis of both inoculated cotton stems indicated delayed development of xylem and decreased lignified xylem bundles, vessels, and interfascicular fibres in susceptible cotton compared to resistant cotton. Therefore, the lignin content and monomeric composition of the two different cotton lines were investigated. Lignin contents were estimated in cell wall residues (CWRs)

and at 14 days post inoculation (dpi), both cotton varieties had increased lignin content with the resistant variety reaching 105% and susceptible variety achieving only 103% in total lignin content (Xu *et al*., 2011). The subsequent measurement at 21 dpi showed that resistant cotton had a constant 105% increase, but all of the susceptible plants had died (Xu *et al*., 2011).

Lignin degrading fungi are divided into three groups, brown-rot, white-rot and soft-rot fungi (Chen *et al*., 1982). *Ganoderma boninense* is a white-rot fungus that may have evolved to producing specific LDEs to breakdown oil palm lignin. Lignin is broken down by cleaving C_1-C_α, β-aryl, C_α-C_β, and aromatic C-C bonds (Giardina *et al*., 2010). Due to the complexity of plant internal structures such as lignin, fungal lignolytic enzymes with broad substrate specificity such as laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP) were proposed to be expressed as a first line of attack on lignin (Janusz *et al*., 2013). Laccase and manganese peroxidase are common LDEs; however, their potential role in fungal virulence is poorly understood. It was reported that these enzymes have many functions within the host fungi and all fungi produce at least one isozyme of laccase (Baldrian, 2005). Fungal laccases are glycoprotein in nature and were also suspected to have a role in fungal morphogenesis, plant-pathogen interactions, stress defence and delignification of plants (Baldrian, 2005). Laccases are secreted as active enzymes and are stable extracellularly (Baldrian, 2005). Manganese peroxidase (MnP) is another strong, lignin-degrading enzyme that

produces effective oxidant, Mn^{3+} to breakdown aromatic components in plants via a redox mediator (Hofrichter *et al.*, 1999).

While laccases were implicated with white rot fungi and they have been proven to degrade aromatic bonds in plant lignin, their role as a virulence factor is poorly studied. This potential function is not to be confused with the large number of journal papers describing the isolation of laccase from plant pathogenic fungi for other purposes such as biotechnological applications, and not as a virulence factor with a role in disease (Rigling and Alfen, 1991; Binz and Canevascini, 1996; Wahleithner *et al*., 1996; Eggert *et al*., 1998; Litvintseva and Henson, 2002; Ryan *et al*., 2003; Iyer and Chattoo, 2003; Asiegbu *et al*., 2004; Kittl *et al*., 2012). Only the human pathogen *Cryptococcus neoformans* and plant pathogen *Botrytis cinerea* have been suggested as using laccases for pathogenesis (Manteau *et al.*, 2003; Zhu and Williamson, 2004), with some mentions of lignin degradation in wheat by *Fusarium proliferatum* (Anderson *et al*., 2005). *Cryptococcus neoformans* utilizes laccase as a virulence factor to convert mammalian substrates into reactive compounds that protect the fungus from oxidative stresses. Laccase derived reactive compounds damaged dopamine concentration by laccase oxidation of dopamine in the brain of mice, leading to death (Zhu and Williamson, 2004). In the case of *B. cinerea*, fungal laccases were expressed 50 fold higher during interaction with fruits and leaves of grape plants, to detoxify plant phenolics when there was a shift from pH 2.8 to pH 3.5 (Manteau *et al*., 2003). Therefore, senescense, like ripening,

induced the production of fungal laccase and in the case of *G. boninense*, may induce the breakdown of oil palm lignin.

Laccase has been identified in some hymenomycetes and some deuteromycete and ascomycete fungi (Piscitelli *et al*., 2011). Although the first laccase that was identified was a plant enzyme, the extensive reports only started in 1970s. There is a significant difference in the purpose of plant versus fungal laccases. The former are produced as plant defence system by the oxidation of unwanted lignin; the latter is an efficient lignin degrader (Harvey, 1997). Laccases may be secreted extracellularly or retained intracellularly in the fungi. The role of laccase has been postulated to aid melanin formation to protect fungi from UV radiation, detoxify phenolic plant defence mechanisms, breakdown lignin and in some cases aid in the conversion of Mn^{2+} to Mn^{3+} (Piscitelli *et al*., 2011). Plant laccases have been postulated to convert endogenous fungal phenolics into toxic quinones and accelerate the plant defence system (Harvey, 1997).

The level of laccase produced during the interaction between *Ganoderma* spp. GBLS and young oil palm was not significantly different across the 6 weeks of study although the highest production was recorded on day 28 when laccase activity was 12.93 times more than the activity recorded in T1 on day 0. All results were compensated for the T1 day 0 values to remove basal activity values of oil palm laccase. Plant peroxidases are widely distributed compared to laccases and were initially

thought to be the main plant enzyme family involved in inducing polymerisation of monolignols (Sterjiades *et al.,* 1992). However, it was later proven that oxygen added to spruce cambium extracts stimulated the production of the lignin-like molecule, DHP and that both laccases and peroxidases are important for the synthesis of lignin in plants (Sterjiades *et al.,* 1992). Wounded plants produce peroxidases which are invoved in the production of lignin, but fungi can breakdown peroxidases using superoxide dismutase and catalase to form CO_2 and H_2O, leaving the net amount of peroxidases lower than in wounded plants with no fungi (Zhao *et al*., 2013). It was reported that laccase activity in an induced environment peaks when the reducing sugars are minimal in medium (Spina *et al*., 2015). This explains the relatively high reading at the end of the experiment (day 28 to day 42) (Fig. 21) because the sugar content in MS media would have depleted as plants used this sugar source to grow *in vitro*. Although, the laccase production was not significantly different between each time point, the peaks indicated a bi-phasic production of laccase *in planta*.

Also, in the case of *Fusarium solani*, a wood- rotting ascomycete isolated from mangrove in Hong Kong, laccase was induced in high carbon and/or low nitrogen and/or the presence of inducers such as lignin where the results would be higher amounts of laccase (Wu *et al*., 2015). This could explain the higher activities of laccase recorded at day 35 and day 42 as compared to other days in the present work. Expression of laccases in plants and fungi has promotional functions towards the growth and

sustainability of the host producer. In *Arabidopsis thaliana*, several cell-specific functions such as *LAC4* in the formation of interfascicular fibres and seed coat columella, *LAC8* in the pollen grains and phloem and *LAC15* for formation of cell wall for seed coats, while *LAC7* was detected in hydathodes and root hairs (Turlapati *et al*., 2011). It was also suggested that the expression of these genes is tightly regulated at the transcriptional and post-transcriptional level of gene expression (Turlapati *et al*., 2011). Plant laccases are inducible and have undetermined roles beyond the boundaries of lignified tissues and many have cell/tissue type specific expression patterns (Tulapatti *et al*., 2011).

Lignin itself is a polymer made in Arabidopsis plants by the dehydrogenative polymerization of monolignols by peroxidases, laccases and polyphenol oxidases (Zhao *et al*., 2013). It was initially thought that peroxidases and not laccases catalyze efficient plant lignin polymerisation in Arabidopsis, and over-expression of peroxidases can result in ectopic lignin formation (Zhao *et al*., 2013). However, when a triple mutant for laccase was generated in *Arabidopsis thalina* to determine the evolution of lignin biosynthesis, the plants showed stunting indicating that plant laccases and peroxidases play a major role in lignification and do not utilise the same substrates (Zhao *et al*., 2013). In contrast, fungal peroxidases such as manganese peroxidase (MnP) and lignin peroxidase (LiP) work together with laccase in the biodegradation of lignin by destabilising lignin bonds from the inside of the lignin structure (Hofrichter *et al*., 1999). While all of fungi produce some form of

peroxidase, including laccase, some species of fungi produced MnP at a higher quantity as compared to laccase (Hofrichter *et al*., 1999). MnP and LiP were thought to oxidize phenolic compounds, generating phenoxy radicals while non-phenolic compounds are oxidized by cation radicals (D'Souza *et al*., 1999). The difference between laccase and MnP/LiP is that MnP oxidizes aromatic nonphenolics at lower oxidation-reduction potential as compared to LiP (D'Souza *et al*., 1999). However, in the presence of mediators, laccase too can oxidize aromatic nonphenolics at high oxidation-reduction potential (D'Souza *et al*., 1999). Therefore, the activities of MnP and laccase complement each other in this process. It was expected that MnP would be produced in a lower range as compared to laccase because MnP is regulated by the presence of manganese ion and may not be readily secreted in standard laboratory conditions (Morgenstern *et al*., 2010). As is the case with *Pycnoporus cinnabarinus*, besides laccase, there were no peroxidises detected when grown in lignolytic environment and *Phanerochaete sordida* where lignin peroxidase (LiP) was not detected (Morgenstern *et al*., 2010) In the present study, the release of MnP was not significantly different across the time points or between T1, T2 and T3 samples. In *Rigidoporus lignosus*, MnP and laccase worked synergistically to breakdown lignin; the *in vitro* de-polymerization of ^{14}C-labelled lignin was enhanced when both enzymes were present (Galliano *et al*., 1991). Leonowicz *et al*. (2001) claimed that fungal hyphae secrete laccase and MnP where the enzymes have synergistic activities particularly in the presence of mediators. Subsequently, the

mediator-generated radicals and chelators moved to the wood tissue, operating as enzyme "messengers" in lignin degradation (Leonowicz *et al*., 2001).

Fungal laccases could promote vegetative growth and fructification of fungus (Pezella *et al*., 2013). Specific expression of GBLS laccase was detected in all T3 plants but at low activities throughout the study. The highest fungal laccase expression detected in qPCR was at day 42 although laccase enzyme assays detected using ABTS solutions suggest that the highest accumulation was in day 28. On day 28, therefore, high laccase activities included more plant laccases than fungal laccases (plant laccase > fungal laccase) and the laccase activities detected on day 42 could have been due to more fungal laccases than plant laccases (plant laccase < fungal laccase). The induction of laccase gene transcription was bi-phasic, similar to the finding with secreted enzymes, but the time points of induction were shifted. The bi-phasic production of laccases could be related to lignin degradation, virulence and detoxification of the fungi (Baldrian, 2005). In this enzyme assay, laccase was induced at day 14 and day 28, while abundant laccase transcripts were detected on days 7 and 42. While the abundant transcription induction at day 7 could have contributed to the laccase activites on day 14, the induction on day 28 was a mix of plant and fungal laccases, although the net effect was a reduction of lignin content in oil palm. The expression of laccase to breakdown lignin has been reported in *Lentinula edodes*, *Agaricus bisporus* and *Phlebia radiata* (Mäkelä *et al*., 2006). Although laccase was

able to catalyse *in vitro* delignification of lignin, the resultant low molecular weight components were re-polymerized (Leonowicz *et al*., 1985). Therefore, the major function of laccase appeared to be polymerizing the oxidized products, but not in the oxidation of lignin itself. Laccases are resistant to toxic tannins and phytoalexins released from plants (Giardina *et al*., 2010).

Expression of fungal laccase genes was regulated in culture conditions and different isoforms might play different roles in the development of the fungus and attack on plant tissues (Pezella *et al*., 2013). In *Pleurotus ostreatus*, there was a strong up-regulation of laccase during the formation of sporophores (Pezella *et al*., 2013). However, due to overall low expression of laccase, there was a suggestion of redundancy of laccase transcripts and the *in vivo* functions remain a mystery (Pezella *et al*., 2013). The findings of Ander and Eriksson (1976) clearly supported a role for laccase in lignin degradation whereby the inability of *Sporotrichlm pulverulentum* laccase deletion mutants to degrade lignin was recovered in revertant strains. According to Thurston (1994), understanding the physiological role of laccase and the mechanisms of laccase activities during lignin degradation and its other physiological roles are not completely clear.

In this Chapter, the molecular expression of laccase transcripts seemed to indicate an expression that was induced early but reduced throughout the study until it was present in high quantity at the end of

the experiment. This finding indicates a possible role in virulence on oil palm, which is complete by day 42. Although, experiments on the specific expression of laccase in tissue specific samples as well as differentiation of plant and fungal laccases would be helpful, the preliminary role of laccase in the virulence of oil palm was presented here.

Chapter 7: General discussions and concluding remarks

Oil palm is the highest yielding oil crop and is an important source of edible oil to combat hunger and malnutrition. The majority of Asia, Africa and South America uses palm oil for cooking, making cost effective soap and candles and conversion to biofuel. In certain parts of Africa, palm oil is directly crushed and consumed as a source of Vitamin A due to the presence of tocotrienol derivatives (Poku, 2002). In Malaysia, oil palm has been grown commercially for over 100 years and been the top agriculture-based contributer to the national economy since the decline in rubber plantations. Malaysia also has the most advanced oil palm research centres in the world and has been actively supporting research on this crop for various uses of its products. However, for the last 50 years, basal stem rot (BSR) disease has been affecting yields and income from oil palm cultivaltion. The disease starts with the root contact between oil palm and *Ganoderma boninense*, reaches the soft tissues of the palm frond, causing wilt (Hushairian *et al*., 2013). The hymenomycete fungus is resistant to environmental conditions and the monocropping of oil palm has provided a good growth environment for *G. boninense*. The work described in this thesis started in the field of mature 17 year old oil palm in Johor, Sg Gerchang Estate, Achi Jaya Plantations at Chaah and Ladang Boustead Eldred at Sg Bekoh, and in Melaka, Melaka Tong Bee Estate at Air Kuning. Field surveys were carried out at bi-monthly intervals for 12 months to determine the progression of the disease. A 13 point criteria evaluation survey was generated and overall plant health

scored between 1 and 4, where 4 is a healthy plant. Although the survey was conducted as part of a fertiliser trial by a private company, estates with aggressive *G. boninense* were made available. For the isolation of *G. boninense*, an aggressive isolate from a field of infected oil palm was chosen. This was to ensure that virulence was sufficient to generate BSR symptoms during the artificial infection experiments. Sporophores that were collected from Lian Seng Estate at Tangkak Johor gave a pure culture of *G. boninense* that was named *Ganoderma boninense* Lian Seng (GBLS). The technique used for isolation and identification was a standard method recommended by researchers who studied BSR infections (Ariffin *et al*., 1992; Sariah *et al*., 1994; Karthikeyan *et al*., 2006; 2007; Kandan *et al*., 2009). Pure mycelium for GBLS was used to extract total DNA for the PCR amplification of the ITS fragment. Direct isolation of GBLS DNA from culture was less efficient that growing GBLS in liquid culture of malt extract broth for 4 days with agitation. The 167 bp ITS fragment generated using ITS 1 and 4 primers enabled the identification of *G. boninense* in the other studies and were used to identify the pathogen in this work (Karthikeyan *et al*., 2006; 2007; Kandan *et al*., 2009). Sequencing of a larger 635 bps fragments containing the same target showed 581 bps or 91.4% similarity to PER71, a strain of *G. boninense* recommended by the Malaysian Palm Oil Board (MPOB). The 635 bp fragment was deposited in GenBank under the accession number KF164430.1.

In this study, a new artificial infection technique was developed to reduce the test time from 6 months post inoculation to symptom development (Sariah *et al*., 2004; Izzati *et al*., 2008) to 6 weeks. However, the bigger impact of this artificial system was the omission of rubber wood blocks (RWBs) as inoculum source. As suggested by Chong (2010), the use of RWBs introduces contamination, as wood harbours microorganisms and may not be completely sterilized by autoclaving methods. Also, if a 6 cm x 6 cm RWB is used, it takes at least one month to colonise before it can be used as inoculum for BSR infection (Sariah *et al*., 1994; Ganeson, 2015). The actual infection method includes a young oil palm grown in soil and placed on an infected RWB, which is inaccurate because there is no enumeration of the true *G. boninense* inoculum load and the presumption that the RWB were fully colonised with living and virulent *G. boninense*. Often, competing microorganisms in the RWBs and soil slow down the true ability of *G. boninense* to cause symptoms on oil palm and cause BSR (Ganeson, 2015).

Due to many variables that could affect the outcome of the *Ganoderma*-oil palm interaction, an *in vitro* infection assay was designed and executed. The isolate was identified as *G. boninense* by microscopy, growth on Ganoderma Selective Medium (GSM) and sequencing of the ITS DNA fragment. The inoculation was conducted on one-month old, rooted ramets of an oil palm clone. For an *in vitro* experiment with controlled parameters, it was ideal to use clonal plants with uniform physical characteristics, as this would eliminate errors for the measurement of

physical and chemical responses to disease. The height, weight, number of leaves, width of the largest leaf and the SPAD readings indicated that the pathogen caused deterioration of plant health in 6 weeks. Specifically, one month post inoculation, the impact of the pathogen was observed on the plant, but only at day 42 were there significant differences between treatments for height and weight, the number of leaves and the width of leaves. Although, no extensive network of mycelium were observed during this work, the rotting and complete loss of live tissues were only observed in GBLS-inoculated T3 samples, and not in T1 or T2 samples. In other RWB studies, the plants had live tissues even though *Ganoderma* spp. sporophores were visible on them (Izzati *et al.,* 2008) but in the *in vitro* system, the plant had mostly degraded, implying the importance of the age of palm during *Ganoderma* spp. infections. Therefore, the *in vitro* system not only provides a one-to-one interaction opportunity for plant and fungus, but it is able to show infection until the oil palm dies, eventually. When the chorophyll content and the disease severity were measured, T3 plants were suffering from GBLS infection gradually from day 0 to day 42 of the disease. In this work, the timepoint to notice any symptoms was expected to be shorter than 14 weeks, and T1, T2 and T3 plants had similar severity scale until 2 weeks post treatment but severity increased exponentially in T3 until 6 weeks post infection. The disease severity scale did not change in T1 and T2 after 2 weeks post treatment. The overall observation on *in vitro* plants as stress in all treatments due to tissue culture conditions, narrowing of leaves in T3 due to wilting and

browning and decay of total plant mass in T3 due to *G. boninense* GBLS infection. Similar symptoms were observed in *in vitro* grape rootstocks infected with *Armillaria mellea* (Baumgartner *et al*., 2010). Perhaps in order to improve this system, the MS medium as nutrient source for oil palm could be substituted with compost or fibres infused with commercial fertiliser, as this would represent the field situation better. However, nutrient sources must be membrane-sterilized to remove any potential microbial contamination that could complicate a one-to-one plant-fungus interaction.

Another unique feature of the *in vitro* inoculation assay is the inclusion of a fungal load test. As recommended by Baumgartner *et al.* (2010), DNA quantification will indicate the proliferation of fungal mass in the infected samples. In the present work, *G. boninense* ITS sequences were used in quantitative PCR to measure the amount of fungus in GBLS-infected T3 plants. The quantity of DNA gave an indication of the proliferation rate of GBLS *in planta*. The highest quanitity of DNA of >1000 ng/ µl was detected on day 14 post inoculation and in days 7 and 21, significantly lower amounts of fungal DNA were detected and persisted until day 42 when the quanitity increased to 200ng/µl. This result suggests that while the largest amount of GBLS was found on day 14, it did not cause significant damage to the oil palms until two weeks later at day 28. The role of fungal LDEs such as laccase and MnP could not be excluded because the problem with BSR was probably white rot; the ability of the *G. boninense* to breakdown oil palm lignin was investigated

in Chapters 5 and 6. Lignin is a complex phenylpropanoid polymer derived from three common monomers or monolignols, synapyl (S), coniferyl (G) and ρ-coumaryl alcohol (H) subunits (Paterson *et al*., 2007). Oil palm trunk fibre consists of 41.2% cellulose, 34.4% hemicellulose, 17.1% lignin, 3.4% ash, 2.3% ethanol soluble and 0.5% of methanol extractives (Paterson *et al*., 2009). Therefore, oil palm is categorised as lignocellulose that comprises of a mixture of hemicellulose and lignin, with at least 12 different types of linkages including aryl ether and C-C bonds, making it one of the toughest woods, with limited biodegradability (Paterson *et al*., 2007).

However, white rot fungi, such as *G. boninense* efficiently mineralise lignin by the digestion of monomeric ring structures (Paterson *et al*., 2007). One of the most efficient LDE families is the laccases, and in this study it was hypothesized that GBLS was using laccase to access nutrients from oil palm by the removal of lignin to allow other fungal enzymes such as pectinase and amylase to access the sugar derivatives. In order to identify a laccase gene fragment from *G. boninense*, full length laccase DNA fragments were aligned using Clustal W programme (EMBI, UK). Although several laccase encoding genes were available in GenBank, they did not amplify a useful band in *G. boninense* GBLS. Therefore, the degenerate primer pair D96Lac2F/D96Lac2R was designed based on data from D'Souza *et al*. (1996) and a single band of 208 bp was obtained in 2009 (Chapter 5). The sequence showed 89% homology to fungal laccase and to the knowledge of the author it was the first

known highest similarity of *G. boninense* laccase described. The other study of potential *G. boninense* laccase described a 1617 bp fragment with 76% homology to *Trametes* spp. laccase, by Utomo in 2003 (GenBank). In order to investigate the role of laccase during virulence on oil palm, in this study, the quantity of laccase activities and cDNA transcripts were measured and described in Chapter 6.

Both plants and fungi produce laccases and since the efficiency of laccase was monitored over a time period *in planta*, the activity of plant laccases was subtracted from inoculated plant data to limit the reading to fungal laccases. Although in the three treatments tested, only T3 plants were exposed to *G. boninense* GBLS, the non-treatment control T1, and wounded plants T2, were also monitored for laccase activity. Over the period of 42 days, there were no significant differences in the laccase activities in all plants examined; the same results were also obtained in the biological replicates. Initially, expression of laccase during the infection of oil palm was to be measured but the reported house-keeping genes (HKGs) for *G. boninense* (Lim *et al*., 2014) were not accurate when tested and the design of new HKG for GBLS would have too long and increased the scope of this work. Therefore, the experiment was modified to measure the abundance of laccase cDNA transcripts present in inoculated plants to determine if indeed *G. boninense* laccase affected the outcome of the disease.

Quantitative PCR was used to determine the amount of specific GBLS laccase transcripts over 42 days post inoculation in T3 samples. The overall abundance of transcripts was less than 0.8 ng/µl on a standard curve generated using GBLS laccase cDNA. The highest laccase activity was recorded at day 42. Coincidently, at this timepoint, the total and complete degeneration of lignin was observed in oil palm (Table 7). The total lignin content also revealed the highest loss of lignin content was in T3 plants as compared to T1 and T2 plants and was also recorded at day 42 indicating the ability of GBLS to breakdown the total lignin content in T3 plants . Therefore, it can be concluded that oil palm ramets in this experiment were completely degraded by day 42. However, the role of laccases in these levels of damage was not clear. Although it cannot be ruled out that laccases were produced as a side effect of the growth and proliferation of *G. boninense in planta*, there is insufficient evidence to conclude that laccases were involved as virulence factors, for example in overcoming oil palm defence mechanisms. For this purpose, a further study to determine the expression of laccase *in planta* in oil palm with different inducing factors such as plant phenolics and or different quantities of lignified materials would give a clearer picture. At the moment, laccase has been implicated in the infection of wheat by *Fusarium proliferatum* (Anderson *et al*., 2005), *Botrytis cinerea* in grapevines and tomato (Manteau *et al*., 2003) and *Cryptococcus neoformans* in humans (Zhu and Williamson, 2004), and there is a growing body of evidence to implicate laccase as a virulence factor as

white rot fungi target younger oil palm plants and is no longer restricted to old plants with matured lignin contents.

This work gave an insight into the importance of using a reliable infection assay when *G. boninense* and oil palm interact and to determine if *G. boninense* GBLS was a pathogen. When interacted one-to-one, GBLS was able to induce oxidative damage leading to cell death. The experiment clearly showed the ability of GBLS to proliferate in oil palm and induce physical and chemical damage that led to plant death. This system is, therefore, reliable potentially more than the RWB method where sporophores were produced as proof of Koch's postulates but there was no explanation of the necrotrophic behaviour of *G. boninense* as it took more than 9-12 months for the plants to die and the quanitity of the pathogen was not measured (Izzati *et al.*, 2008). The present research was also a crucial step forward in understanding the presence and activities of LDE such as laccase and manganese peroxidases during the pathogenic interaction of *G. boninense* with young oil palm.

To further this work, improvements could be made to differentiate the roles of plant and fungal laccases, the ability of fungal laccase to accelerate lignin breakdown has been widely reported in other studies and the role in infection has also been suggested. Also, the inoculation-infection assat could be improved by using fibres instead of MS agar medium as a support for young oil palm. These fibres could be infused with commercial fertiliser commonly used for oil palm seedling to

generate a actual nursery experience for the oil palm. A digestibility assay for the enumeration of the breakdown of starch, pectin, hemicellulose, cellulose and lignocellulose could provide answers as a step by step breakdown on oil palm lignin material by *G. boninense*.

The results of this work on developing a better understanding of the epidemiology of the *Ganoderma*-oil palm interaction prior to the development of BSR symptoms and the role of LDEs as potential virulence factors, could contribute positively to the Malaysian and Indonesian governmental efforts to control the virulence of *Ganoderma* spp. disease on oil palm.

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APPENDIX 1

Preparation of chemicals and buffers

1. Tris-HCl, 1 M

Tris-HCl buffer was prepared with 121 g of Tris base powder dissolved in 800 ml of purified water. A pH value of 8.0 was adjusted by the addition of approximately 42 ml of concentrated hydrochloric acid (HCl). The buffer was autoclaved (Tomy ES-315*,* USA*)* at conditions of 121°C for 15 minutes.

2. EDTA, 0.5 M

EDTA is a powerful chelating agent that binds effectively to ion residues for the removal of contaminants during the lysis of cell for DNA extraction. A concentration of 0.5 M EDTA was prepared by the addition of 186.1 g of $Na_2EDTA.2H_2O$ into 700 ml of purified water. A pH value of 8.0 was adjusted using 10 mM of NaOH. The final volume was adjusted to one litre. The buffer was autoclaved (Tomy ES-315*,* USA*)* at conditions of 121°C for 15 minutes.

3. NaOH, 10 M

Sodium hydroxide of 10 M concentration was prepared by the addition of 400 g of NaOH powder into 450 ml of purified water. Once the powder has dissolved, the final volume was adjusted to one litre. The buffer was autoclaved (Tomy ES-315*,* USA*)* at conditions of 121°C for 15 minutes.

4. Sodium dodecyl sulphate SDS, 10 % w/v

SDS buffer was used in the lysis step of DNA extraction of E.coli during cloning experiments. A 10 % w/v of SDS was prepared by the addition of 100 g of SDS powder into 900 ml of purified water. The mix was heated up to 68 °C to aid the dissolution of powder before the adjustment to a final volume of one litre. The buffer was autoclaved (Tomy ES-315*,* USA*)* at conditions of 121°C for 15 minutes.

5. Calcium chloride, $CaCl_2$, 1 M

Calcium chloride of 1 molar concentration was used in the preparation of competent *E. coli* cells for cloning and transformation experiments. The buffer was prepared by the addition of 54 g of $CaCl_2.6H_2O$ into 200 ml of sterile, purified water. The mix was sterilized by syringe filter fitted with 0.22 µm disc filter (Sartorius). Sterile buffer was separated into aliquots of 1 ml and stored at -20°C prior to use.

6. Sodium chloride, NaCl, 5 M

Sodium chloride solution of 5 M was prepared by the addition of 292 g of NaCl powder into 800 ml of purified water. The final volume was adjusted to one litre. The buffer was autoclaved (Tomy ES-315*,* USA*)* at conditions of 121°C for 15 minutes.

7. Sodium acetate buffer (pH 5.0), 100 mM

Sodium acetate buffer was prepared by weighing 0.83 g sodium acetate powder into 100 ml purified water. The pH of the solution was adjusted to pH 5.0 with 1 M sodium hydroxide.

8. ABTS Solution and chemicals for laccase and MnP assays

ABTS solution contains 0.03 % 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), ~98% (ABTS) Buffer for laccase

ABTS powder (Sigma-Aldrich, USA), 3 mg, was dissolved in 10 ml of purified water. The solution was prepared freshly prior to use by filter sterilizing into a 15 ml falcon tube without autoclaving the solution.

125 mM sodium tartarate buffer (pH 3.0): 1.88 g tartaric acid powder (QRëC, New Zealand) was dissolved in 100 ml purified water. The pH of the solution was adjusted to pH 3.0 with diluted sodium hydroxide.

10 mM 3,4-Dimethoxybenzyl alcohol, ~96% (veratryl alcohol): 14.54 µl veratryl alcohol (Aldrich, USA) were dissolved in 10 ml purified water. The solution was prepared freshly prior to use by filter sterilizing into a 15 ml falcon tube without autoclaving the solution.

0.5 M sodium succinate buffer (pH 4.5): 5.91 g succinic acid powder (SYSTERM®, Malaysia) was dissolved in 100 ml purified water. The pH of the solution was adjusted to pH 4.5 with 1 M sodium hydroxide.

1 mM manganese (II) sulfate ($MnSO_4.H_2O$): 165 mg manganese (II) sulfate powder (SYSTERM®, Malaysia) was dissolved in 100 ml purified water.

10 mM hydrogen peroxide (H_2O_2): 11.30 µl H_2O_2 (Ajax Finechem, Australia) was dissolved in 10 ml of purified water. The solution was prepared freshly prior to use by filter sterilizing into a 15 ml falcon tube without autoclaving the solution.

4 mM guaiacol (2-methoxyphenol): 4.43 µl guaiacol solution (Acros Organics, USA) was dissolved in 10 ml of purified water. The solution was prepared freshly prior to use by filter sterilizing into a 15 ml falcon tube without autoclaving the solution.

9. Fungal Mycelium and Infected Oil Palm Seedlings DNA Extraction Buffers (Referred to Stewart and Via, 1993; Moller *et al.*, 1992)

1 M Tris hydrochloric acid (HCl) (pH 8.0): 12.14 g Tris base powder (Promega, USA) was dissolved in 100 ml purified water. The pH of the solution was adjusted to pH 8.0 with 6 M concentrated hydrochloric acid.

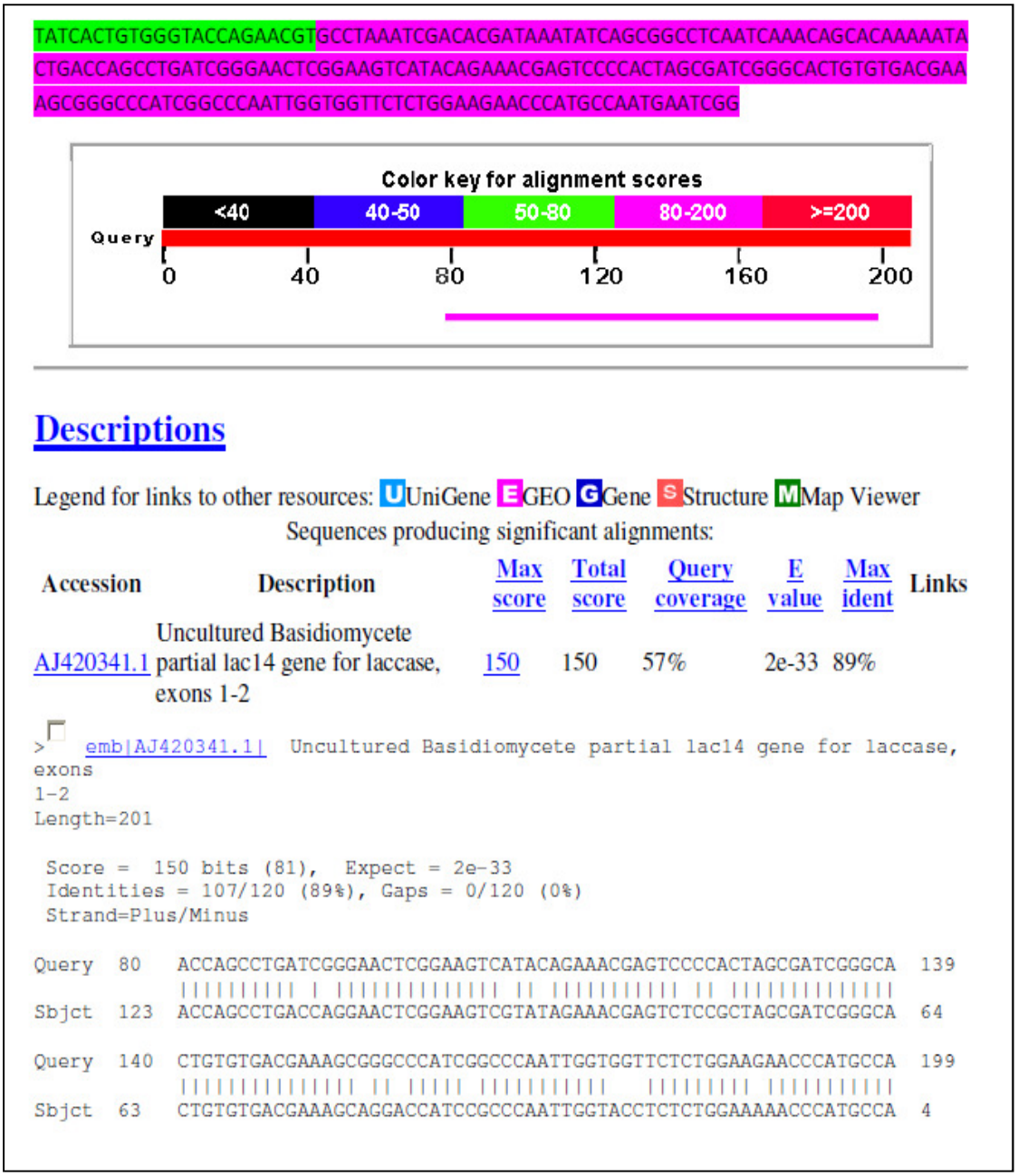
0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0): 18.16 g EDTA powder (Promega, USA) was dissolved in 100 ml purified water. The pH of the solution was adjusted to pH 8.0 with 10 M concentrated sodium hydroxide.

5 M sodium chloride (NaCl): 29.22 g NaCl powder (R&M Chemical, USA) was dissolved in 100 ml purified water.

10 % dodecyltrimethyl-ammonium bromide, ~99% (CTAB): 30.83 g CTAB powder (Acros Organics, USA) was dissolved in 100 ml purified water.

5 % polyphenolpyrollidine (PVP): 5.80 g PVP powder (Acros Organics, USA) was dissolved in 100 ml purified water.

Appendix 2: BLAST result for 208 bp laccase gene



Appendix 3: Disease severity Score

Disease Severity Score (0-4)									
Sample	T1			T2			T3		
R2D0	0	0	0	0	0	0	0	0	0
R2D7	0	0	0	0	0	0	25	25	25
R2D14	0	0	0	25	25	25	50	50	50
R2D21	25	0	0	25	25	25	50	75	50
R2D28	25	25	25	25	25	25	75	75	50
R2D35	25	25	25	25	25	25	75	75	75
R2D42	25	25	25	25	25	25	100	100	100

Appendix 4: Efficiency of qPCR quantification of GBLS ITS DNA.

Standard Curve Data			Eco-PCR Illumina Inc.			
Assay Name	Quantity Unit	Slope	Intercept	R2	Efficiency %	
GbFR		-3.46898	24.14825	0.986307	94.20908	

Appendix 5: Statistical table for Plant height, weight, number of leaves, width of leaf, SPAD reading data.

Documents are available as separate folder.

Appendix 6: Statistical table for laccase and MnP enzyme assay, LTGA assay and relative quantity of laccase.

Documents are available as separate folder.