Ganoderma Stem Rot Of Oil Palm: Epidemiology, Diversity And Pathogenicity

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GANODERMA STEM ROT OF OIL PALM: EPIDEMIOLOGY, DIVERSITY AND PATHOGENICITY

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FOR MUM AND DAD

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Abstract

Basal stem rot (BSR) of oil palm, caused by *Ganoderma boninense*, a white-rot basidiomycete is the main threat to oil palm cultivation in South East Asia. It can kill more than 80 percent of stands by the time they are halfway through their normal economic life. Attempts to control the disease have been hampered by the lack of understanding of the pathogen, how it spreads and infects and interacts with its host. Therefore this study was designed to better understand its epidemiology, diversity and pathogenicity. Reproducible root infection was obtained using small mycelium-infested rubber wood block inoculum and alternative infested wheat grain inoculum. This revealed that close contact between palm roots and the inoculum are important and that a smaller and alternative grain inoculum has potential for resistance screening. *G. boninense* was shown to remain viable for up to 4 years through the formation of melanised mycelium, which has implications for plantation management. Basidiospores clearly play a critical role in BSR infections. They were detected in abundance (ca. 3-5000/m^3) in aerial samples from plantations in North, South, East and West of peninsular Malaysia. *G. boninense* was revealed by RAMs, SSRs and somatic incompatibility to be highly genetically diverse within and between those plantations. High genetic diversity would arise from sexual production of basidiospores as this study confirms *G. boninense* to be heterothallic and tetrapolar with multiple alleles at both mating type loci. Basidiospores applied to cut frond (petiole) surfaces were taken up into xylem vessels where they germinated and must have formed invasive heterokaryons, as on occasion colonisation progressed beyond the anatomical barrier of vessel end walls. Efficacy of detection by *Ganoderma*-selective medium, DNA and RNA are compared and discussed. Scanning electron microscopy revealed that spores also can adhere, germinate and apparently penetrate oil palm root surfaces, revealing that superficial roots might provide another route of entry. Putative pathogenicity factors are represented by production of wide array of extracellular cell wall degrading enzymes (CWDE) including the three main groups of ligninases and

diverse polysaccharidases. All were produced during saprotrophic (on palm wood) and parasitic (root invasion) phases of the life cycle. Regulation *in vitro* was by nitrogen or carbon metabolite repression respectively and by aromatic or oligosaccharide inducers respectively. This study also showed production *in vitro* of peroxide and related reactive oxygen species-degrading enzymes, which could be linked both to lignin breakdown and protection from host defences. Preliminary evidence is presented for toxin production. The approaches and knowledge gained from the research described above culminated in the design of model systems, representing the life cycle of *G. boninense*, in order to facilitate a collaborative transcriptomic analysis. Long overdue, in depth understanding of *G. boninense* potentially will improve disease control through finding novel fungal targets and *via* effectoromics locating and assessing resistance genes in the oil palm.

CONTENTS

1 General Introduction

1.1 Oil palm

1.1.1 Taxonomy and botany

The oil palm, (*Elaeis guineensis Jaq*.) belongs to the family of Arecacea, tribe Cocoseae and subtribe Elaidinae. The genus name *Elaeis* is derived from the Greek word ‘elaion’, meaning oil and the species name guineensis was attributed to its origin in the Guinea Coast (Jacquemard, 1998). There are two species of *Elaeis*, the economically important *E. guineensis* (origin West and Central Africa) and *E. oleifera* (origin South America) (Hartley, 1988; Singh *et al.* 2013; Corley and Tinker, 2015).

The oil palm is a large, pinnate-leaved palm (35-60) having a solitary columnar stem with short internodes and can grow over 20 to 30m in height (Corley and Tinker, 2015). A projecting bole is developed at the base of the crown where fronds and inflorescences originate. Normally, it is unbranched but when the apical bud is damaged, branching may be induced (Nair, 2010). The stem is completely enclosed by frond (leaf) bases after several years. Leaf bases begin to fall away when the palm reaches about eleven years old (Turner and Gillbanks, 1974) after which the stem formation becomes apparent (Nair, 2010). The history of oil palm in Malaysia dated back in 1911 where the first palms of *E. guineensis* Deli origin were planted in Rantau Panjang, Kuala Selangor as an ornamental plant (Hartley, 1988). The first commercial planting of oil palm Deli dura in Malaysia started in 1917 at the Tennamaran Estate in Selangor, which gave the foundation for the current oil palm industry (Corley and Tinker, 2015).

1.1.2 Genetic background: Monogenic inheritance of the shell gene

Oil palm fruits consist of a hard kernel inside a shell (endocarp) that is surrounded by a fleshy mesocarp. The mesocarp contains about 49 percent palm oil and the kernel about 50 percent palm kernel oil (Nair, 2010). Crosses between the thick shell Dura (D) parent palm and the shell-less (usually female sterile) pisifera (P) parent, would generate 100% thin-shell tenera (T) palms (Fig. 1), thus showing the monogenic inheritance of the shell gene (Beirnaert and Vanderweyen, 1941). The T × T (F_1 × F_1) cross will give rise to segregating F_2 progenies in the classical Mendelian ratios of ¼ Dura, ¼ Pisifera and only ½ of the suitable Tenera (Nair, 2010). Currently D x P hybrids are the dominant commercial planting materials (Ngando-Ebongue *et al.*, 2012). In spite of a lot of efforts to widen the genetic base of breeding populations worldwide, particularly in Malaysia and Indonesia, the dura (Deli) is still considered the best in seed production (Nair, 2010; Ngando-Ebongue *et al*., 2012). Recently, clonal palm materials have gained a lot of interest from the industry as difficulties associated with its production are progressively being overcome. Tissue culturing is superior to conventional breeding by rapid multiplication of plantlets with uniformity having desired characteristics. However availability of commercial clonal palms still in limited quantities as compared to the total demand for oil palm seeds worldwide (Ngando-Ebongue *et al*., 2012).

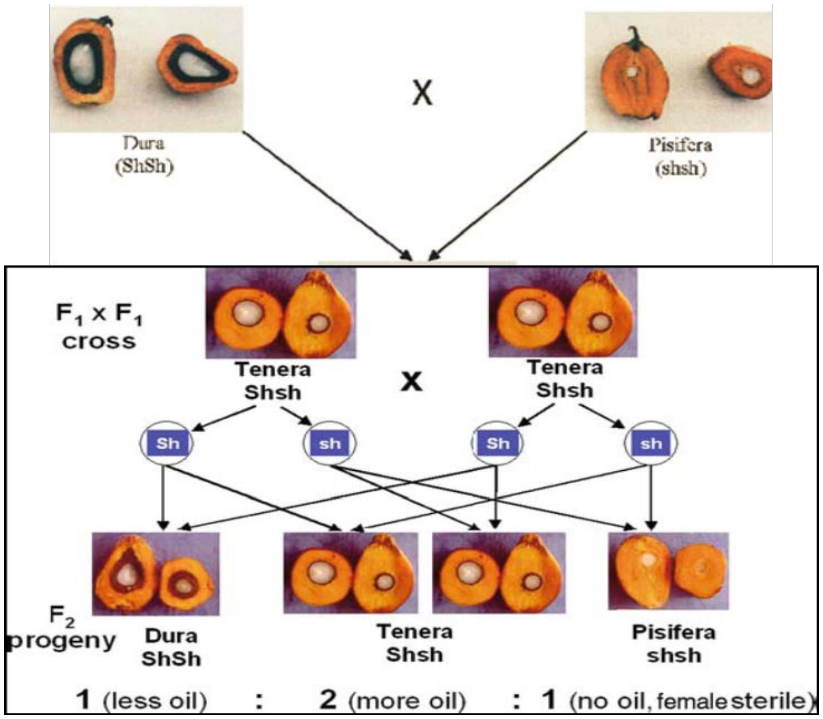

Figure 1. Monogenic inheritance of shell thickness: F2-segregation (Nair, 2010)

1.1 Oil palm industry and economic importance

Oil palm is one of the most important oil tree species in the world and a valuable economic crop in tropical regions due to the high yield of edible oil it produces. It constitutes 35% of total edible oil production worldwide and Malaysia and Indonesia are the two major producers in the world (Oil world, 2014). Two grades of oil can be produced from oil palm fruits: crude palm oil (CPO) comes from grinding fruit pulp to release standard quality oil (cooking oils, margarine and biscuits) and crude palm kernel oil (CPKO), which is obtained from grinding the seed to release high quality oil used in pharmaceuticals (Ngando-Ebongue *et al.,* 2012).

Palm oil is the largest internationally traded vegetable oil with its main markets in China, European Union, Pakistan, India, Japan and Bangladesh (Basiron, 2007). It is mainly used in food (80%) and the remaining 20% is used as oleochemicals (Basiron, 2007). Oil palm has been the major oil crop in Malaysia for the past 20 years and currently the oil palm industry is the important economy sector of Malaysia with export earnings of palm oils and oil palm products around £16 billion per annum (MPOB, 2012). The increasing petroleum prices have led to many countries considering biodiesel production from renewable resources which opened up a new application for palm oil (Basiron, 2007). Recently, Malaysian palm oil board (MPOB) has launched the Envo Diesel (5% palm oil and 95% diesel) produced from the MPOB integrated palm oil biodiesel plant in Malaysia (Johari *et al.,* 2015).

Oil palm has remained the world's leading oil crop since 2006 with a production of 37.1 million tons of palm oil and more than 4.3 million tons of palm kernel oil, as opposed to 35.3 million tonnes of soybean oil (Oil World, 2014). This is as a result of the increased in the oil palm cultivation areas. In Malaysia, oil palm planted areas grew from 54,000 hectares in 1960 to 4.05 million hectares in 2007 (Razali *et al*., 2012). Oil palm cultivation areas in Indonesia also has grown enormously from just under 2000 ha of oil palm production in the 1970s increasing to almost 14 million ha in 2005 (Nair, 2010). However pests and fungal diseases have remained a major threat to oil palm cultivation worldwide.

1.2 Fungal and oomycete diseases of oil palm

Fungal and oomycetes diseases of the oil palm can cause serious losses in production of the palm oil. It has been reported that the economic loss caused by these pathogens is up to 500 million USD a year (Ariffin, 2000; Ommelna *et al*., 2012). The most important diseases are basal stem rot (BSR) caused by *Ganoderma boninense*, vascular wilt caused by *Fusarium oxysporum f. sp. elaeidis* (Foe), sudden wilt (*Phytomonas staheli*) and bud rot (*Phyptophthora palmivora*) (Turner, 1981; Ariffin, 2000; Corley and Tinker, 2015). Devastation

has been caused by so called bud rot disease (more accurately considered as a spear rot) in Latin America. Torres *et al.* (2010) claimed the pathogen to be *Phytophthora palmivora* but many true fungi and bacteria have been linked with the syndrome and it may have many causes (Corley and Tinker, 2015). While, vascular wilt disease is the main challenge in west and central Africa oil palm cultivation, in South East Asia, Malaysia and Indonesia, the most serious and major threat to the oil palm industry is BSR disease. Some of these diseases can be very devastating and are directly responsible for hampering development of oil palm cultivation in countries where they occur.

1.3.1 Vascular wilt

Vascular wilt disease, also known as lemon frond, wilt disease and *Fusarium* wilt, is the most important disease of oil palm, affecting western and central Africa comprising the Ivory Coast, Ghana, Nigeria, Cameroon and Democratic Republic of Congo (Turner, 1981; Cooper, 2011; Corley and Tinker, 2015). The disease has also been reported in South America such as in Surinam, Brazil, Ecuador and Colombia (de Franqueville and Renard, 1990; Godswill *et al.,* 2012). The causal agent of vascular wilt is the soil-borne pathogenic fungus *Fusarium oxysporum* f. sp. *elaeidis*. The pathogen was isolated from palms suffering vascular wilt and disease symptoms were artificially reproduced on inoculated palm seedlings which confirmed its pathogenicity (Fraselle, 1951). The disease is thought to occur when healthy roots contact dead or infected roots or debris containing Foe chlamydospores, which can survive extreme environmental conditions but can germinate under favourable conditions, such as in response to root exudates (Schippers and Van Eck, 1981).

The pathogen normally invades intact roots, develops in xylem vessels and can become systemically distributed to all parts of the palm by conidia carried

in the transpiration stream (Mepsted *et al.,* 1995; Cooper, 2011; Corley and Tinker, 2015). Invasion of xylem causes water stress and hormonal imbalance with consequent severe yield loss and palm death (Cooper, 2011; Godswill *et al.*, 2015). Stunting and wilting of yellowed fronds are typical symptoms of the disease (Flood, 2006). Two forms of symptoms are usually observed on vascular wilted palms: the typical or acute symptoms through which the palm dies three to four months after their appearance, and the chronic symptoms which maintain the palm alive, weaken and render it almost unproductive (Godswill *et al.*, 2015). The internal symptoms include vascular browning which is diagnostic of *F. oxysporum* f.sp. *elaeidis* and host defence attempts to limit pathogen spread through the production of gums and tyloses (Godswill *et al.,* 2015).

This disease also can affect nursery palm and contaminate seed and pollen (Turner and Gillbanks, 2003). However the disease is very rare in the nursery, and is exceptional at the pre-nursery stage. Rapid destruction of oil palm cultivation caused by vascular wilt disease occurs in replanted areas (Corley and Tinker, 2015) and disease incidence might occur less than a year of planting in a previously infected area (Renard and de Franqueville, 1989).

Various cultural practices have been attempted to control the disease include removal of diseased palms; changing the cover crop; planting at distance from the old stumps. However it is not very effective thanks to the perennial habit of oil palm and persistence nature of Foe (Cooper, 2011). The only practical and sustainable approach is by breeding and selection for resistant cultivars as well as plant quarantine. Screening for resistance in nurseries was introduced to many plantations and research stations, and successful breeding programmes in West Africa notably in Ivory Coast, have resulted in more resistant oil palm material becoming available and large reductions in losses from this disease (Flood, 2006; Cooper, 2011). Introduction of resistant cultivars in Cameroon plantations successfully reduced losses in some areas from 20-30% to less than 3% (de Franqueville and Renard, 1990; Flood, 2006).

1.3.2 Bud or spear rot

Bud or spear rot (BR) disease is the most destructive disease of oil palm in South America comprising Ecuadorian Amazon, Brazil, southwest Colombia and Suriname. The disease also has been reported in India (Van Hall, 1922) and parts of Africa (Godswill *et al*., 2012). Since 1964, the disease has destroyed numerous plantations in Colombia. Recently, more than 30,000 ha plantation in south west Colombia and 10 000 ha in the Central Zone were lost (Benítez and García, 2015). BR in oil palms can manifest differently depending on the prevailing environmental conditions. In the Ecuadorian Amazon, Brazil, south west Colombia, and Suriname, BR is mostly lethal to oil palms, while in the drier eastern plains of Colombia, BR is often non-lethal, and a high rate of recovery is observed (Corley and Tinker, 2015).

In the detection of the causal agents, there has been an abundance of hypotheses, arguments, contradictions and conclusions for the responsibility to the organisms involved in this disease. Previously, *Thielaviopsis* spp., *Fusarium* spp., *Erwinia caratovora* and oomycetes from the Pythiales order were strongly associated with the disease (Nieto *et al*., 1996), but none were identified as the causative agent. Sarria *et al.* (2008) reported that *Phytophthora sp.* is the causal agent of this disease in Colombia and recently, Torres *et al.* (2010) reported to isolate the Oomycete, *Phytophthora palmivora* from palms suffering bud rot using baiting technique in Colombia and claimed successful in artificially reproduced bud rot on palm seedlings and confirmed its pathogenicity. However, re-isolation and detection of infected bud rot tissue in Ecuador failed to isolate any *Phytopthora spp* instead *Fusarium spp* and *Erwinia spp.* was isolated and pathogenicity test using *Fusarium spp.* reported to produce typical bud rot symptoms (Narvaez, 2012). Therefore, it may be possible that several pathogens are capable to cause bud rot disease of oil palm in Latin America.

Bud rot disease initial infection develops a lesion in unopened spear leaves and move through the central core of palm meristem, producing disease symptoms. The first symptom of bud rot is the discoloration and possibly the wilting of the spear leaf (youngest, unopened leaf). New youngest leaves may also appear discolored and wilted. The leaves will be a lighter green color than normal, or they will be chlorotic (yellow). Eventually, these leaves become desiccated, turn brown, and collapse. A close examination of the leaves, especially the spear leaf, often reveals blighted areas on the blade and leaf base often has distinct brown or necrotic (dead) areas. As the disease progresses, the spear leaf can be easily pulled from the bud, and the leaf base rots and has a foul odour. This part of the palm shows decay of soft tissues with different degrees of severity, ranging from relatively rapid recovery of the production of new spears and, in less serious cases with the possibility of recovery, to cases in which rot reaches the meristem and a ¨crater¨ of decomposed tissue is formed causing the death of the palm (Navia *et al.* 2014).

Screening for resistant varieties for disease control are difficult with confirmed pathogen, since no standard protocols exist for the infection and limited understanding on the spread of the disease could result in false identification of possible resistant varieties (Benítez and García, 2015). Nevertheless, there are several hybrid material *Elaeis oleifera x E. guineensis Jacq*. has been identified as a potential for disease resistance by expressing less severe symptoms and a slower progression of the disease in infected tissue (De Franqueville, 2003; Durand-Gaselin *et al.,* 2005; Navia *et al.,* 2014).

Additionally, these new variety hybrids (*E. oleifera* × *E. guineensis*) with similar maternal genitors and different paternal genitors (Coarí × La Mé and Coari × Pobé) have been released into high incidence (60%) of bud rot affected areas looking for survival (Navia *et al.,* 2014). OxG hybrids largely successful presumably due to the use of *E. oleifeira* as female parent crosses, however this assisted pollination is rather expensive and it may be impractical to be implemented at commercial scale (Chinchilla *et al.,* 2008).

Other limitation of commercial scale planting with these hybrids are that they present some agronomic disadvantages, such as vegetative disuniformity, excessive vegetative growth, very thick stems, long leaves (less plants can be planted per area unit), thick petioles (making harvesting more difficult) and less oil content. (Chinchilla *et al.,* 2008).

1.4 *Ganoderma* Stem Rot of Oil Palm

As far as the disease problem to oil palm in South East Asia is concerned, basal stem rot (BSR) and upper stem rot (USR) caused by *Ganoderma* spp. are the only diseases requiring an urgent solution in Malaysia. BSR has been known to attack oil palm since the early years when the crop was introduced into this country which manifest as a decay of the bole and is a major threat to oil palm cultivation. The occurrence of USR disease where decay occurs higher up the stem (above 1–1.5 m) has been considered as a relatively minor disease compared to BSR in oil palm plantation (Abdullah*,* 2000; Rakib *et al.,* 2014). Losses due to BSR and USR occur through the direct reduction in oil palm numbers in the stand and also through the reduction in the number and weight of fruit bunches from both diseased palms and those with sub-clinical infections (Turner, 1981; Ariffin *et al.,* 2000; Flood *et al.,* 2002; Pilotti*,* 2005; Rees *et al.,* 2009; Hushiarian *et al.,* 2013). This project serves to focus on *Ganoderma boninense* as the causal agent of oil palm stem rot diseases BSR and USR in Malaysia, Indonesia and Papua New Guinea.

1.4.1 History

Basal stem rot (BSR) caused by *Ganoderma boninense* was first described in 1915 in the Republic of Congo, West Africa as a disease of senescing palms (Wakefield, 1920). The disease was first reported in peninsular Malaysia in 1931 infecting oil palms of over 25 years (Thompson, 1931). As such BSR was considered not to be economically important. Originally Thompson (1931)

identified the causal agent of BSR as *Ganoderma lucidum*. Later, Ariffin *et al.* (2000) identified six *Ganoderma* species isolated from oil palm fields, *G. boninense*, *G. miniatocinctum*, *G. chalceum*, *G. tornatum*, *G. zonatum* and *G. xylonoides*. As with the genus *Ganoderma* in general, considerable uncertainty surrounding identity of the causal agent of BSR has led to many authors simply referring to the disease as '*Ganoderma* rot of palms' (Corley and Tinker, 2015). However, recent studies have indicated that *G. boninense* is the primary cause of the disease in South East Asia and other identified species are synonyms or due to secondary invasion of existing infections (Ariffin *et al*. 2000; Pilotti and Bridge, 2002; Pilotti*,* 2005; Chong *et al.,* 2011; Wong *et al.,* 2012).

BSR incidence has increased since its large scale introduction in the 1960s, leading to the obvious suggestion that monoculture of oil palm has had a role in rising disease incidence (Utomo and Niepold, 2000). Most severe losses from BSR occur in Indonesia and Malaysia, while less severe losses being recorded in the Democratic Republic of Congo, Ghana, Nigeria, Cameroon, Angola, Zimbabwe, Tanzania, Papua New Guinea and the Solomon Islands and are not economically important. The disease has now also been reported in Thailand where cultivation of the crop has occurred more recently with low disease incidence (0.1%) in 20-year old oil palms showing typical BSR symptoms (Likhitekaraj and Tummakate, 2000). Previously BSR disease was considered not to be economically important as it was attacked older palms (25 year old), due to replanting (Thompson, 1931; Ariffin *et al.,* 2000). However as palm became prominent as a plantation crop, BSR incidence increased and younger palms (10 – 15 years old) were infected (Ariffin *et al.,* 2000). Moreover, increased incidence in much younger palms has been reported in Malaysia, where Singh observed infected palms as young as 12-24 months after planting with increased rates in 4-5 year old palms in coastal alluvial soils (Singh, 1991). Furthermore, Lim *et al*. (1992) reported an average of 50% yield losses from 80% of 13 year old plantings in Malaysian coastal areas. A survey by Rao *et al.* (2003) further demonstrates the increasing problem of *Ganoderma* stem rot of oil palm. They reported typical disease levels of 30%

by the time palms were 13 years of age in inland and peat soils of Malaysia. Whereas in the coastal areas incidence of the disease has been known for many years, these inland areas have had typically low or negligible incidence of BSR or USR previously, and losses reaching levels comparable to those on the coast increases concern for sustainability.

The threat of *G. boninense* to the oil palm industry in this country warrants new and more aggressive approaches in finding solutions to the disease. The situation is made even more critical with the active replanting of second-generation oil palms that is currently being carried out in some plantations, including areas with a previous history of *G. boninense*.

1.4.2 Biology

Symptoms of *G. boninense* infection can vary depending on age of the palm and environmental conditions. Foliar symptoms usually only appear after more than 50% of the area of the basal region of the palm has been decayed. The symptoms first manifest as opened fronds (leaves) being smaller and paler than normal (Singh, 1991). Followed by multiple unopened fronds, termed ‘spear leaves’ and flattening of the crown. As infection advances the palm looks pale in appearance and affected leaves eventually die with leaves drooping and fracturing at the base (Fig. 2).

Figure 2. Infected and healthy mature oil palms. A. BSR infected tree with drooping fronds and one-sided chlorosis, typical of advanced infection B. Healthy canopy of mature uninfected oil palm. Fronds are uniformly green and arrayed to maximise light capture. Images taken from Bt. Lintang Estate (AAR, Pahang).

Infected young palms will normally die within 6-24 months after first appearance of symptoms, whereas mature palms can take 2-3 years or more to die. However, these symptoms are not diagnostic of BSR as similar symptoms can also be seen during water stress (Turner, 1981). In fact, palm may be heavily infected with 60% of the stem degraded with no foliar symptoms or basidiocarps. Therefore there is not always a correlation between foliar symptoms, formation of basidiocarps and degree of infection, making it difficult to diagnose early infections. In Malaysia it was reported that 13-22% of palms showing no foliar symptoms or yield loss in 22-year old palms were actually infected with BSR on inspection of bole transverse sections (Corley and Tinker, 2015). Hasan & Turner (1998) also noted the prevalence of sub-clinical infections during experiments using bait seedlings where comparisons were made between infection rates of seedlings planted adjacent

to BSR and apparently healthy trees. In one instance, 40% of seedlings planted adjacent to an apparently healthy mature palm (two palms distant from the nearest BSR infected neighbour) became infected within 2 years, with later manifestation of symptoms on the standing mature palm indicating that the palm may have had a sub-clinical infection (Ariffin *et al.*, 2000). Such infections are difficult to identify and may go unnoticed for long periods. The only truly diagnostic feature of the disease remains the presence of basidiocarps at the palm base or higher up the stem (1–1.5 m) for USR.

Basidiocarp appearance on the stem surface indicates extensive internal rotting; analysis of cross-sections through rotting areas shows soft discoloured areas of rot where extensive colonisation of the tissue and degradation of cell wall polymers has occurred. There is a distinct dark brown margin at the advancing edge of invasion and has been termed the 'reaction zone' (Turner, 1981). Pearce (1996) describes a reaction zone as 'a static barrier that serves to restrict or slow fungal ingress through a combination of factors including deposition of phytoalexin-like compounds such as phenolics and polyphenolics as well as microenvironmental factors'. The dark coloration of reaction zones may in part be due to fungal stress responses inducing formation of pigmented, thick cell walled hyphae. In saprophytic decay wood, margins of colonisation are often delimited by 'pseudo-sclerotial plates' and have been shown to be produced by other *Ganoderma* species including *G. australe*, responsible for white-rot of wood tissue in Chile (Agosin *et al*., 1990) and similar formations can be formed by *G. boninense* on oil palm blocks (Rees *et al*., 2009). Melanized pseudo-sclerotial plates also known as reaction zone (Ariifin *et al*., 2000) have been suggested to play a role in separating decay tissue, to secure the nutrient sources and provide protection for the fungus itself, against competitors in saprophytic wood degradation. Immediately in front of this reaction zone, a pale yellow colour stem tissue is observed which may indicate a reaction mechanisms of the host defence.

The decay patterns of BSR are disorganised with multiple patches of highly defined compartmentalized reaction zones (pseudo-sclerotia) in the infected stem. This indicates the possibility of multiple points of infection and high inter

and intra-specific competition in the basal lesion. In contrast, USR rot decay pattern are more organised demonstrating a successive wave of rot with ever-expanding layers, each delimited by the reaction zones from the point of infection (frond base) (Flood *et al.,* 2002). This extensive rotting of stem tissues restricts the uptake of water and nutrients to the fronds due to a disrupted vascular system, causing chlorosis and subsequently compromised structural integrity of the palm causing toppling or palm death.

1.4.3 Epidemiology

After decades of research, knowledge on the epidemiology of basal stem rot (BSR) remain incomplete. Mechanism of infection by *G. boninense* in BSR is still poorly understood, and identification of definite *G. boninense* infection biology urgently need to be established.

The *G. boninense* route of infection has remained a debatable issues among oil palm research community (Ariffin *et al.,* 2000; Pilotti*,* 2005; Rees *et al.,* 2012; Hushiarian *et al.,* 2013). *G. boninense* is a facultative parasite and is capable of living saprophytically on rotting stumps and roots, and become pathogenic when suitable substrate becomes available (Rees *et al.,* 2009). The primary route of infection of BSR disease has long been considered to be root contact with inoculum sources in the soil. Turner (1981) claimed *G. boninense* inoculum on stumps and trunk tissue left in the field as the source of root infection, particularly on land previously planted with oil palm and coconut where the inoculum has built up. BSR disease incidence increased when oil palm was under planted with coconut stumps left to rot (Ariffin *et al.,* 2000) and disease incidence was higher (70%) in areas where oil palm being planted very near to each other compared to moderate planting densities (Azahar *et al.,* 2011). In addition a field study by Idris *et al.* (2004) showed that all oil palm seedlings growing near *G. boninense* colonised stumps (0.3 m distance) in the field were severely infected within 18 months. By contrast oil palm seedlings remained healthy, when diseased stumps were removed by excavating to 1.5x1.5x1.5 m pit (Idris *et al.,* 2004). Furthermore, the physiology

growth of oil palm primary roots, can spread horizontally more than 6 m in the soil of oil palm plantings (Jourdan *et al.,* 1997), may well increase BSR disease spread by root contact between healthy and diseased palms (Miller *et al.,* 1995). Various infection trials using oil palm seedlings and large *Ganoderma*-colonized rubber-wood blocks have provided data supporting this view (Navaratnam and Chee, 1965; Lim *et al*., 1992; Sariah *et al*., 1994; Hasan and Turner, 1998; Lim and Fong, 2005; Breton *et al*., 2006). Additionally, Rees *et al*. (2007) showed that by attaching infested wood blocks to roots, much smaller inoculum can be used, allowing infection to occur through unwounded roots and progression and rate of invasion to be followed.

Disagreement against root infection as the primary means of BSR comes from genetic studies which show a high degree of genetic diversity of *G. boninense* in oil palm plantations (Miller *et al*., 1999; Pilotti *et al*., 2003; Pilotti*,* 2005; Rees *et al.,* 2012). Vegetative compatibility studies by Miller *et al.* (1995) and Ariffin *et al.* (1996) indicated that *G. boninense* isolates collected from the same infected palms were genetically different. This observation may suggests the role of basidiospores in disease dissemination in palm plantations. The occurrence of USR rot strongly indicated basidiospores infection as it happened 1.5 m away from the roots and infected palm will collapse at the point of infection (Miller *et al.,* 1995; Pilotti*,* 2005; Sanderson, 2005; Cooper *et al*., 2011; Rees *et al.,* 2012). There is no direct evidence thus far, that infection from basidiospore inoculum occurs in the field. Field observation by Ho and Nawawi (1986) found that although abundance of basidiospores were released from *G. boninense* basidiocarps, the majority of oil palms remain uninfected, suggesting basidiospores may require a specific condition to establish infection (Ho and Nawawi, 1986; Sanderson, 2005).

Oil palm fronds are cut regularly as a standard management practice for pruning and harvesting oil palm fruit bunch and provide a large wound surface (Cooper *et al*., 2011). Basidiospores may enter through the cut frond bases Pilotti and Bridge (2002), which later invade the stem trunk of oil palm. However, artificial inoculation of basidiospores on oil palm has so far failed to

produce BSR or USR infection (Turner, 1981; Hasan and Flood, 2003; Rees *et al.,* 2012). Clearly this aspect of BSR requires resolution and it will be considered in detail later (Chapter 3).

1.4.3.1 Disease Control

Effective control strategies for BSR of oil palm has so far proved difficult; the best means of combating infection remains the maintenance of sanitary conditions at replant through rigorous land preparation. To minimise losses from *Ganoderma* infections both long and short-term strategies for disease control are necessary.

1.4.3.2 Cultural practice

Cultural control practices such as trenching have been recommended since the early twentieth century. Trenching has been extensively used in the oil palm industry and is designed to separate the root system of infected palms from neighbouring healthy palms; this assumes spread of the pathogen occurs through the root system (Idris*,* 2009, Hushiarian *et al.,* 2013) The method has been largely unsuccessful in preventing proliferation of the disease perhaps because of insufficient depth of the trenches or failure to maintain trenches once in place. Furthermore, digging trenches not only severs roots of infected palms but also roots from healthy palms. Should spores play a role in direct infection of oil palms these cut roots would represent another wounded surface for basidiospore germination and development of disease. Also, dissemination of infection by basidiospores rather than by root contact would render the digging of trenches inappropriate.

Mounding of soil around infected palms has also been utilised as a method of extending the productive life of infected palms. This stimulates renewed root development from stem tissue above the infected region and provides added stability, which compensates for reduced stem structural integrity due to decay

by *G. boninense*. However, this treatment simply extends the life of the palm but does nothing to prevent the course of infection or transmission of the fungus (Ariffin *et al*., 2000).

1.4.3.3 Chemical control

Effective use of chemical control for treatment of BSR infected palms is problematic since both symptomatic and subclinical palms may harbour advanced infections (Sariah and Zakaria, 2000). Contamination of palm oil by fungicides and fumigants may also preclude widespread use within the industry. Fungicide resistance of the fungus has not been a concern and numerous fungicides have been shown to be inhibitory to *Ganoderma in vitro*: hexaconazole, cyproconazole and triadimenol gave ED50 (amount required to produce an effect in 50% of tests) values of 0.03, 0.043, and 0.06 ppm active ingredient respectively (Ariffin *et al.*, 2000).

Fungicide application such as soil drenching, trunk injection of systemic fungicides and pressurised trunk injection (Idris *et al.,* 2004) in the field has proven difficult to be delivered over the infected stem area as lesions are often very large. Additionally, lesions are predominantly at the base and high-pressure injection often results in passage of the chemical into the soil (Ariffin *et al*., 2000; Naher *et al.,* 2013; Hushiarian *et al.,* 2013).

Correct application technique and fungicides helps to reduce progress of BSR reported by Idris *et al.* (2004) using pressurised trunk injection with hexaconazole prolong the production life of the infected palm. However Tey and Ahdly (2007) reported this pressurised trunk injection was not effective to reduce progress of the disease based on the assessment and comparison of disease severity score between treated and untreated palm over one and half year.

Although chemical treatments are considered as the immediate short term control measures of BSR, implementation in the plantation has been limited because of inconclusive evidence for efficacy at reducing disease incidence or prolonging productive life of infected trees and the high cost of fungicides (Chung, 2011). For fungicides and fumigants to become a realistic method of disease control, more research is required to develop delivery systems for accurate application of the agents.

1.4.3.4 Biocontrol

Various studies have been done to study the possibility of biological control of BSR in oil palm. Potential antagonists to *G. boninense* such as *Trichoderma* spp., *Gliocladium viride*, *Pseudomonas* spp., *Bacillus* sp and *Penicillium* species have been isolated and applied (Sariah *et al*., 2005; Susanto *et al*., 2005). Whilst antagonistic activity towards *Ganoderma* is shown in culture, and some reduction of BSR has been obtained in nursery seedling palms, there are no reports of successful utilisation of biocontrol in the field.

1.4.3.5 Resistance or Tolerance

Progress on genetic resistance has been limited and research is complicated due to problems associated with the long breeding cycle of oil palm. Subsequent plantings of new crosses are often subjected to different land preparation and management practices in order to minimise disease risk. Also, crop history has an effect on disease incidence in the field, making it difficult to determine resistance from other factors. Nevertheless one long-term study involved field observations of material of known origin in North Sumatra, Indonesia. *Elaeis guineensis* material of deli origin from Malaysia and Indonesia was more susceptible than African material (Durand-Gasselin, 2005), indicating possible genetic resistance within populations. However, the authors note the above considerations and point out the necessity for further research and development of an effective screening process.

Development of a rapid screen for resistance has been hampered by the requirement for a very large inoculum and several months for symptoms to manifest (Navaratnam and Chee, 1965). To establish a successful screening programme, a smaller inoculum source and more rapid infection is necessary before investigation of genetic resistance can be comprehensively investigated. One approach for the development of a rapid screening method has involved inoculation of oil palm at the germinated seed stage, which allows observation of symptoms after 3 months (Breton *et al*., 2006) and reports encouraging results in terms of differential susceptibility between crosses. Availability of the oil palm (*Elaeis guinensis*) genome sequence (Singh *et al.,* 2013) should benefit research on genetic resistance of palm against BSR.

1.5 *Ganoderma* spp. and related basidiomycete species of tree pathogens

In Asia, species of *Ganoderma* are the most widely reported basidiomycetes fungal pathogens associated with important root rot diseases of various tropical crops such as coconut, tea, acacia etc. Basidiomycete fungi are also responsible for economically important root rot diseases of conifer trees and perennial crops in temperate regions in the northern hemisphere for example *Heterobasidion annosum* (Woodward *et al*., 1998) and *Armillaria* spp. (Onsando, 1997).

1.5.1 *Ganoderma* in coconut

Basal stem rot of coconut, also known as Thanjavar wilt, *Ganoderma* wilt, and Anabe Roga disease, is a major problem in India and losses can reach as high as 31% (Kandan *et al.,* 2010). Several *Ganoderma spp.* namely *G. lucidum, G. boninense, G. applanatum* have been implicated as the causal agent of the disease and has also been reported as a parasite of palms in the Punjab and Sindh regions of Pakistan (Nasir, 2005; Kandan *et al.,* 2010). A reddish brown viscous fluid oozes from the stem of infected palms, termed as bleeding. Drooping of leaves, reduction in leaf production, reduction in leaf size and tapering are characteristic symptoms of the disease (Bhaskaren, 2000). Pathogenicity of isolated *Ganoderma* isolates was established by experimental infection of palms resulting in symptoms of disease. *G. applanatum* has also been isolated from infected palms but was unable to cause infection experimentally. *G. boninense* was reported to be responsible for the disease in Sri Lanka (Peries, 1974), but this was based on morphological characters that have been shown to phenotypically polymorphic. Therefore, taxonomic study on these isolates is required using molecular and cultural characteristics with interfertility studies for confirmation

of species. Coconut in Malaysia and Indonesia are not considered susceptible to infection by *Ganoderma* spp. and is considered to exist in symptomless coconut palms, possibly as an endophyte. However, where poisoned or felled and replanted with oil palm *G. boninense* will colonise and sporulate from remaining logs and stumps, thus becoming a dangerous reservoir for *Ganoderma* isolates infectious to oil palm. Many surveys indicated that the disease incidence of BSR is highest (40%-50%) in oil palm areas previously planted with coconut, especially where the coconut stumps had been retained in the ground (Turner, 1981; Abdullah, 2000; Rees *et al.,* 2009; Hushiarian *et al.,* 2013). Furthermore, *G. boninense* infection in this area may become apparent on palm as early as 4–5 years old (Ariffin *et al*., 2000).

1.5.2 *Ganoderma* in rubber

Ganoderma pseudoferrum (synonym *G. philippi*) causes red root disease of rubber and contributes to widespread root decay of rubber. The distribution of the disease is worldwide and is especially serious in China and reported as the second most significant root disease in Malaysia and Indonesia (Ogbebor *et al*., 2010). Transmission of the fungus is believed to occur through root contact with inoculum in wood debris, although a number of insect species, in particular the tipulid flies, *Limonia nervosa* and *Limonia Umbrata*, are regarded as potential vectors for the pathogen (Ariffin *et al.*, 2000).

1.5.3 *Ganoderma* in Acacia

Acacia magnium is indigenous to the far islands of Indonesia, the western province of Papua New Guinea and northeast Queensland, Australia. It grows rapidly and is cultivated in plantations throughout South East Asia for wood production. Up to 40% tree mortality has been recorded in surveys of root-rot disease in Malaysian *A. mangium* plantations (Lee, 2000; Glen *et al.,* 2009). In second rotation commercial plantations in Indonesia, root-rot incidence has

been recorded between 3 and 28% in *A. mangium* plantations aged from 3- to 5-years old (Glen *et al.,* 2009).

Two distinct root diseases are problematic to *Acacia* production: brown-root disease caused by *Phellinus noxius* and red-root disease thought to be caused by a *Ganoderma* spp. Several *Ganoderma* species have been considered responsible for the red-root disease such as *Ganoderma philippii* (Bres. & Henn.) Bres., *Ganoderma mastoporum* (Lév.) Pat., *Ganoderma aff. steyaertanum* B.J.Smith & Sivasith, based on morphological identification of basidiocarps as well as molecular detection of the isolates from butt and root diseased tissues (*Glen et al.,* 2009). Recent phylogenetic analyses by Coetzee *et al.* (2011) showed that all *Ganoderma* isolates collected from symptomatic roots belong to the same *Ganoderma* sp identified as *G. philippii* and claimed as the major causal pathogen of *Ganoderma* root rot of *A. magnium* in Indonesia.

Disease infected roots are covered by a wrinkled, reddish-brown mycelial mat rhizomorph that becomes evident when the root is washed clean of soil, and white mycelium can be seen on the underside of the bark having a very characteristic odour (Mohamed *et al.,* 2006; Coetzee *et al.,* 2011). Typical symptoms of this disease include crown dieback and reduced growth of the tree and diseased trees tend to be clustered in a circular patches (Mohamed *et al.,* 2006) which may suggest vegetative spread of the pathogen in the soil

There are so far no reports of successful methods of controlling the disease and control is difficult as the pathogen survives on woody material in the soil (Glen *et al.,* 2009; Coetzee *et al.,* 2011). Resistant planting materials may be the best solution for the management of the disease.

1.5.4 *Heterobasidion* root and butt rot

This well-studied basidiomycete disease system has many clear analogies with BSR and could provide a useful model to help progress our understanding of *Ganoderma* spread and infection. The root and butt rot disease caused by the white rot basidiomycetes *Heterobasidion* spp. are the most economically damaging forest diseases in temperate forests in the northern hemisphere (Asiegbu *et al*., 2005). In Europe, the disease is responsible for the loss of 800 million euros annually, and it is also widespread in forests in the USA. *Heterobasidion* spp. have been reported in almost 150 species of trees, including some angiosperms (Hodges, 1969). Species of *Abies, Juniperus, Larix, Picea, Pinus, Pseudotsuga* and *Tsuga* are the major hosts of the disease. *Heterobasidion* spp. causes primarily a root rot and kills the trees within several months to years after infection. However, spruces, firs, larches, hemlocks and Douglas fir are primarily damaged by butt rot which may extend for several metres up the stem and it may kill young trees (Hodges, 1969). The pathogen was long considered to represent a single species. However, genetic studies revealed host specialized inter-sterile groups (IGs) within *H. annosum*. Norway spruce, Scots pine and Douglas fir serve as a host for three different *Heterobasidion* spp: *H. annosum*, *H. paviporum* and *H. abietinum*. (Niemelä and Korhonen, 1998).

The pathogenicity of the *Heterobasidion* butt and root rot is very well understood. The disease primary infection is mediated by basidiospores that infect fresh stump surfaces or wounds on the roots or stem (Rishbeth, 1950; Redfern and Stenlid, 1998). Rishbeth (1950) demonstrated conclusively that freshly cut surfaces could be infected by airborne basidiospores. The basidiospores will adhere, germinate and form special structures for penetration of stump when there are suitable conditions. Successful penetration will result in mycelial colonization and infection progressing from stump to the roots at about 1m per year (Hodges, 1969). Moreover, infected stumps act as an infection centre where the fungus spreads via root contacts or grafts from infected to healthy trees. The vegetative spread of the fungus via the root systems within forest stands could produce >100-year-old

expanding disease centres with one individual genet (clone) capable of occupying areas about 50 m in diameter (Stenlid, 1985; Lygis *et al.,* 2004; Garbelotto and Gonthier*,* 2013). Moreover *Heterobasidion* spp. produces a vast array of cell wall degrading enzymes for infection and colonization.

Trees that have root infections of *Heterobasidion* spp. will often show no above ground symptoms, but below ground the roots will have lesions. In some species, these lesions will form in the sapwood, phloem, and the cambium, girdling the tree and killing it (Asiegbu *et al.*, 2005a; Garbelotto and Gonthier, 2013). White mycelium may be found growing underneath infected bark, and white pockets containing black specks may be present in the cambium. In an advanced stage these white pockets will cause the wood to become soft and stringy in texture. Dying trees will have browning foliage, stunted needles or tufted needles, chlorosis, and reddish to purplish bark (Garbelotto and Gonthier, 2013). Wind thrown trees are also characteristic of infection by *H. annosum*. The presence of basidiocarps of *H. annosum* around the base of the stump or butt of the tree are also a characteristic of the disease. Basidiocarps can appear on an infected stump after 1.5 to 3 years, and can form just a slight layer of tubes over the surface of a log or stump, or can grow up to 30 cm in diameter (Asiegbu *et al*. 2005a; Garbelotto *et al*., 2013).

Recent molecular and genomic approaches have provided in-depth understanding on the disease development and infection by *Heterobasidion* spp. the genome was reported by Dalman *et al.* (2013). Transcriptome studies such as EST and RNA seq have started to provide an overall view of events and pathogenicity genes that occur during *Heterobasidion* growth and invasion (Asiegbu *et al*. 2005b). The *Heterobasidion* spp. disease epidemiology, distribution, genetics and pathogenicity research have been well documented and could be used as a potential model for BSR disease of oil palm, as will be discussed later.

1.5.4 *Armillaria* root rot

Armillaria root rot disease also known as Mushroom Root Rot, Shoestring Root Rot, Honey Mushroom Rot is found throughout temperate and tropical regions of the world. Hosts include hundreds of species of trees, shrubs, vines, and forbs such as *Eucalyptus*, *Pinus*, *Acacia* and *Cupressus* that are utilized in plantations (Wargo and Shaw 1985; Hood *et al*., 1991; Sinclair and Lyon, 2005). *Armillaria* spp. have been regarded as primary pathogens, which live as parasites on living host tissue or as saprophytes on dead woody material for decades (Wargo and Shaw, 1985; Baumgartner *et al.*, 2011). From this food source, the fungi spread to living hosts. Spread occurs when rhizomorphs, growing through the soil, contact uninfected roots or when uninfected roots contact infected ones.

Rhizomorphs can grow for distances of up to 10 feet (3 m) through the upper soil layers, and they then penetrate roots by a combination of mechanical pressure and enzyme action. The rhizomorphs growth and ability to penetrate roots depend upon the specific fungus, the type and amount of the food source, the soil environment, and the host species (Solla *et al.,* 2002; Prospero *et al.,* 2004; Prospero *et al.,* 2006; Baumgartner *et al.,* 2011; Hood *et al.,* 2015). When uninfected roots contact infected ones, the fungal mycelium invades uninfected roots without forming rhizomorphs. Such spread is common in dense stands where root contact is frequent. Vigorously growing trees often confine the fungus to localized lesions and limit spread up the roots by secreting resin and rapidly forming callus tissues. But when infected trees are in a weakened condition, *Armillaria* spreads rapidly through the roots (Rosso and Hansen, 1998; Morrison *et al*. 2001; Hood *et al*., 2015). Common symptoms of *Armillaria* infection include branch die back, wilting and yellowing (chlorosis) of tree tops, crown thinning, resin exudation, as well as the occurrence of white mycelial fans rhizomorph under the bark of infected trees (Morrison and Mallett*,* 1996; Prospero *et al.,* 2006; Baumgartner *et al.,* 2011).

Armillaria root rot can be easily recognized by clusters of short-lived, yellowish to honey brown basidiocarps at the base of infected trees. These structures are usually formed when there are cool, moist conditions during autumn and winter, or occasionally in late spring.. The epidemiological importance of resulting basidiospores is thought to be low, relative to colonization by a resident mycelium. In orchards, vineyards and timber plantations, a disease centre is typically inhabited by one to a few diploid individuals (i.e. resident mycelia) that originate from the previous forest stand (Rizzo *et al.*, 1998; Baumgartner and Rizzo, 2002; Prospero *et al.*, 2008; Labbe *et al.,* 2015; Xing *et al.,* 2015). Furthermore, inoculation attempts with basidiospores in the field often fail (e.g. Rishbeth, 1970) and the haploid mycelia from germinated basidiospores are rarely detected in nature (e.g. Peabody *et al.*, 2000). By contrast, other studies suggest that basidiospores are important. First, the genetic diversity of *Armillaria* founder populations is probably a result of sexual reproduction (Prospero *et al.*, 2008; Baumgartner *et al.,* 2010). Second, in some temperate hardwood and conifer forests, a disease centre among naturally established host plants is inhabited by multiple, intermingled, diploid individuals of *Armillaria* (Ullrich & Anderson, 1978; Rishbeth, 1988; Legrand *et al.*, 1996). Finally, in conifer plantations of New Zealand, basidiospores of *A. novae-zelandiae* have been shown to colonize freshly cut wood of *Pinus radiata* (Hood *et al.*, 2008).

The whole genome of *Armillaria mellea* has been sequenced recently (Collins *et al.,* 2013). The genome comprises 58.35 MB, contains 14473 gene models of average length 1575 bp (4.72 introns/gene) using next generation sequencing. Proteomic analysis of this genome sequence revealed a rich reservoir of carbohydrate degrading enzymes, laccases, and lignin peroxidase in the *A. mellea* proteome (Collins *et al.,* 2013).

1.6 *Ganoderma boninense* pathogenicity

1.6.1 Fungal infection strategy and pathogenicity

There is a vast body of information on mechanisms of pathogenicity of plant pathogenic fungi (and oomycetes) (Deising *et al.,* 1991; Salanoubat *et al.,* 2002; Toth *et al.,* 2006; Kamper *et al.,* 2006; Spanu *et al.,* 2010; Baxter *et al.,* 2010; Duplessis *et al.,* 2011; O'Connell *et al.,* 2012; Olson *et al.,* 2012; Collins *et al.,* 2013). Much of this comes from model systems, or those which show a relatively tractable interaction with their hosts. Clearly *Ganoderma* and oil palm represent the opposite extreme case, with a weak pathogen requiring an enormous inoculum to infect, a very large and slow growing host and a disease that takes months to develop. Nevertheless *Ganoderma*-oil palm BSR research has lagged way behind and needs to take on board the approaches and information gained from the rapidly advancing field of plant-pathogen interactions. One such advance has been the discovery in virtually all microbial pathogens of effector proteins combatting stages of host innate immunity (Birch *et al.,* 2006; Truman *et al.,* 2006; Cunha *et al.,* 2007; De Wit *et al.,* 2009; Block & Alfreno., 2011; Pais *et al.,* 2013).

Phytopathogenic fungi have developed different modes of interactions and lifestyles with their hosts for nutrient absorption. Some phytopathogenic fungi live in nutrient provided from living host cells, often by producing special nutrient-absorbing structures called haustoria that tap into host's tissues (Voegele *et al.,* 2001; Horbach *et al.,* 2011). Other phytopathogenic fungi synthesize and secrete low molecular weight toxic metabolites, kill host cells and decompose the organic compound for nutrient. These contrasting modes of nutrition are referred to as biotrophy and necrotrophy (Horbach *et al.,* 2011; Koeck *et al.,* 2011; Gan *et al.*, 2013). However, some phytopathogenic fungi sequentially exhibit both of these lifestyles and nutritional strategies. These other types, known as hemibiotrophs, initially grow biotrophically and this stage is followed by a transition to necrotrophic lifestyle.

Biotrophic phytopathogens such as the mildews, rusts and smuts live in close contact with their hosts and differentiate special infection structures for penetration and colonization of living plant cells (Voegele *et al.*, 2001; Horbach *et al.,* 2011). Appressorium formation often initiates infection by penetrating plant epidermis or cuticle by means of force (Takahashi *et al.,* 1985), lytic enzymes (Pryce-Jones *et al.,* 1999) or vesicles (Swann and Mims, 1991) without causing degradation and death of the host cells. The rust fungus *Puccinia triticina* forms a primary and appresorial germ tube and an appresorium 24h after inoculation (Zhang *et al.,* 2003), which penetrates into epidermal cell to form haustorium for penetration of host cell wall (Bolton *et al.,* 2008). Although the host cell wall is breached, haustoria are not truly intracellular as they remain separated from host cytoplasm by the extrahaustorial membrane, a derivative of the host plasma membrane that tightly envelops the haustorium (Panstruga and Schulze-Lefert, 2003; Szabo and Bushnell, 2001)., The haustorium functions for nutrient acquisition from host cell to the fungal thallus. Haustoria are involved in long term suppression of host defences by effector protein secretion and the redirection of host metabolism (Voegele and Mendgen, 2003). More recently this structure has emerged as a sites of translocation and secretion into host cells of a class of pathogen virulence proteins known as effectors during infection (Whisson *et al.,* 2007; Rafiqi *et al.,* 2010; Petre and Kamoun, 2014). These effectors are secreted proteins of the pathogen that can manipulate plant processes to promote host infection and colonization (Petre and Kamoun, 2014).

In contrast, infection strategy of necrotrophic pathogens is less complex than that of biotrophic fungi. For example, *Alternaria, Botrytis, Cercospora, Fusarium, Helminthosporium, Sclerotinia, Armillaria, Heterobasidion* species penetrate and quickly damage or kill plant cells, subsequently decompose the plant tissue and absorb the nutrients. Disabling the host cell is mediated by different strategies such as secretion of low molecular weight or peptide toxins, endo-pectinases or by eliciting cell death in the host by secretion of reactive oxygen species (ROS) (Friesen *et al.,* 2008; Daub and Chung, 2009; Tudzynski and Kokkelink, 2009). For example, in addition to toxins, reactive

oxygen species play an important role in the grey mould fungus *Botrytis cinerea*. Cytological studies suggest that the fungus actively contributes to the elevated levels of ROS detected at infection sites (van Kan, 2006). In addition, many necrotrophic fungi secrete an array of cell wall degrading enzymes to cause significant tissue damage. The cell wall degradation presumably serves two purposes, i.e. support of penetration and provision of carbohydrates. The genomes of necrotrophs *Fusarium graminearum* and *M. oryzae* harbour 103 and 138 genes encoding hydrolytic enzymes respectively (Kamper *et al.,* 2006). In comparison, the genome of biotrophic corn smut fungus *Ustilago maydis* is poorly equipped with genes encoding only 33 such hydrolytic enzymes (Kamper *et al.,* 2006). Cell wall degrading enzymes and fragments resulting from cell wall degradation may elicit defence responses and the fact that such genes are maintained or even duplicated strongly argues that cell wall degrading enzymes are indispensable for full virulence of plant pathogenic fungi (Walton, 1994).

Hemibiotrophic phytopathogenic fungi are a destructive group of filamentous plant pathogens, which have a devastating effect and global distribution on a wide range of plants important to natural ecosystems and agriculture (Jupe *et al.,* 2013). For example, *Phytophthora infestans (*the causal agent of late blight in potato and tomato crops), *Colletotrichum truncatum* (causal agent of lentil anthracnose), *C. higginsianum* (causal agent of crucifer anthracnose) and *Magnaporthe oryzae* (causal agent of rice blast), which exploits a stealth bi-phasic infection strategy to colonize host plants (Bhadauria *et al.,* 2011). These pathogens have a lifestyle that features a biotrophic phase, followed by a switch to necrotrophy (hemibiotrophy) which is critical in symptom and disease development (Koeck *et al.,* 2011; Horbach *et al.,* 2011; Jupe *et al.,* 2013).

These pathogens penetrate the host cell wall, colonizing the intercellular space using feeding structures such as haustoria to absorb nutrients, suppress host defenses without disrupting the plasma membrane (biotrophic). Then in a later stage they switch to overpowering the host by utilizing a variety of secreted pathogenicity and virulence factors throughout infection and to

proliferate (necrotrophic) (Koeck *et al.,* 2011; Bhadauria *et al.,* 2011; Horbach *et al.,* 2011; Jupe *et al.,* 2013).

Advances in molecular 'omics' study has provided valuable insight into the molecular mechanisms of biotrophy to necrotrophy transition during pathogenesis in the host plant. Genome and transcriptome analyses have identified numerous effector candidate genes during pathogenesis in the hemibiotrophic fungi e.g. *Colletotrichum* spp. (O'Connell *et al.*, 2012; Gan *et al.*, 2013). Comparative analyses of the effector candidate genes suggested that each *Colletotrichum* species has a large set of unique effector genes that likely have crucial roles in the manipulation of each host plant's condition (Irieda *et al.,* 2014). Several studies have pointed out hemibiotrophic pathogens secrete effectors to avert the HR cell death or host defences and alter cellular metabolism to suit the needs of the invading pathogen during the biotrophic phase. For instance, the anthracnose pathogen *Colletotrichum spp*. secreted consecutive wave of effectors before, during and after penetration from the appressorial penetration pore that indicate distinct suites of effectors are deployed at each infection stage (Kleeman *et al.,* 2012).

The potato late blight pathogen *P. infestans* effector Avr3a has been shown to inhibit cell death triggered by the INF1 elicitor, a cell death inducing protein from *P. infestans* (Lee and Rose, 2010). Similarly, the soybean pathogen *Phytophthora sojae* effector Avr1b can suppress PCD triggered by the mammalian BAX protein, which is a positive regulator of apoptosis (Dou *et al.,* 2008). These results imply that these secreted effectors play an important role in suppressing PCD or host defenses and camouflage during biotrophic interactions (De Jonge *et al.,* 2011; Vleeshouwers and Oliver, 2014).

Transition from biotroph to necrotroph in hemibiotrophic fungi has been considered to be highly regulated by a transcriptional programming of effectors which act antagonistically between the two phases (Lee and Rose, 2010). Recent transciptome analyses of *P.infestans* have found the SNE1 effector specifically expressed during biotrophic growth of *P. infestans* suppressed the

action of necrosis-inducing effectors PiNPP 1.1 and PsojNIP (Nep1-like proteins) and sequentially these necrosis effectors are secreted during necrotrophic growth (Lee and Rose, 2010). Similarly transcriptome analyses of *Colletotrichum* spp by Kleeman *et al.* (2012) found expression of many effector genes is plant-induced and distinct sets of effectors are deployed in successive waves by particular fungal cell-types supporting pathogenic transitions (Kleeman *et al.,* 2012). The first wave of ChEC genes is induced in unpenetrated appressoria *in planta*, exemplified by *ChEC7* and*ChEC9*.

Similarly, *ChEC3*, *ChEC3a*, *ChEC4*, *ChEC6* and *ChEC36* are also induced in unpenetrated appressoria *in planta*, but their expression continues into the early biotrophic phase (wave two). Of these, ChEC6 had the highest relative expression level of all ChECs tested, suggesting an important role in early pathogenesis. In contrast, *ChEC13*, *ChEC34*, *ChEc51, ChEc56, ChEC88 and ChEC89* are specifically induced during penetration and establishment of biotrophic hyphae (wave three). The last wave of effector genes, exemplified by *ChNLP1* and *ChToxB*, is induced only during the switch to necrotrophy, suggesting that their toxic products contribute to terminating the biotrophic phase for subsequent necrotrophic growth (Kleeman *et al*., 2012).

In depth understanding of *Ganoderma* lifestyle and pathogenicity may provide hints for more effective control. For example some effector proteins from fungi and oomycetes are being used to find and characterize resistance genes, so called effectoromics (Jonge *et al.,* 2011; Rafiqi *et al.,* 2012; Bozkurt *et al.,* 2012; Du *et al.,* 2014).

1.6.2 *Ganoderma boninense* infection of oil palm roots

The only critical information concerning *Ganoderma* pathogenicity comes from a study on invasion of oil palm roots and bole by Rees (2006) and Rees *et al.* (2007; 2009). *Ganoderma boninense* may behave as a hemibiotroph in newly colonized root and bole tissues according to ultrastructural analysis (Rees *et al*., 2009). Invasion of living root cortex and stem with intracellular invasive wide hyphae occurred in cells still with fully intact cell walls, plasma membrane and intact cytoplasm. The infection may thus be comparable to the lifestyle of hemibiotrophic fungi such as *Colletotrichum* spp. and *Magnaporthe oryzae*. Comparably, the invasion then switched into an aggressive necrotrophic stage associated with extensive host cell wall degradation. Cell wall-degradingenzymes are thus far the only putative pathogenicity factors recognized in *G. boninense*.

G. boninense is a white rot fungi capable of degrading lignin polymers (Paterson, 2007). Rees (2006) detected *G. boninense* enzymes production that correspond to lignin and other major structural cell wall polymers of oil palm *in vitro* and *in vivo*. Penetration of root cortex and stem parenchyma cells resulted in extensive degradation of all cell wall layers, indicative of simultaneous cell wall attack. This contrasts with selective lignin degradation, where lignin in the secondary wall and middle lamella is almost entirely removed, whereas less degraded polysaccharide components of the S1 and S2 cell wall layers are left intact (Agosin *et al.,* 1990). This aspect of *G. boninense* pathogenicity will be covered in more detail later.

Starch represents another major polysaccharide in oil palm tissue. *G. boninense* appeared to utilise starch as a source of carbon during pathogenesis. Transmission electron microscopy confirmed substantial depletion of starch in host bole cells in plants invaded by *G. boninense* during early infection (Rees *et al*., 2009).

1.6.3 Genome and transcriptome of *G. boninense*

With even previously intractable pathogenic fungi such as obligately biotrophic rusts and mildews, genomics and transcriptomics has facilitated considerable progress (Mendgen and Hahn, 2002; O'Connell *et al.,* 2012; Hacquard *et al.,* 2012) However until now there is no published *G. boninense* genome sequence and there is also a lack of critical research on the differential gene expression of *G. boninense*. Although there are efforts by the Malaysian Palm Oil Board (MPOB), ACGT Sdn. Bhd., Malaysian Genomics Resource Centre (MGRC) and Felda Agricultural Services Sdn. Bhd to sequence the genome of *G. boninense* in recent years, the sequence has not been made available in the public domain.

Nevertheless the published genome of white rot basidiomycetes *G. lucidum* (Liu *et al*., 2012), *Phanerochaete chryosporium* (Martinez *et al*., 2004) and pathogenic basidiomycete *Heterobasidion irregulare* (Olson *et al.,* 2012) are providing insights into those genes encoding wood degradation, toxins, effectors, and other pathogenicity genes during host colonisation. Genomes of pathogenic basidiomycete rusts and smuts are also now available. Based on background genome information, transcriptomes of some of these fungi are revealing much about the sequence of gene expression during infection stages (Duplessis *et al.,* 2011; O'Connell *et al.,* 2012; Wollenberg and Schirawski, 2014; Petre and Kamoun, 2014; Hubbard *et al.,* 2015; Taniguti *et al.,* 2015). Preliminary stages of this approach will be one component of the following thesis with the ultimate aim of enabling us to develop more effective strategies to control BSR and improve screening for resistance varieties.

1.7 Research Objectives

In view of the many unanswered questions underlying infection and epidemiology of *G. boninense*, this research has several aims with the ultimate goal of improved control of BSR.

Overall, the main objectives in this research are:

1. To study the mode of infection and survival by *G. boninense* mycelium
2. To determine the infectivity and survival characteristics of *G. boninense* basidiospores for disease establishment of BSR and USR.
3. To determine the extent of genetic variability of *G. boninense* in Malaysia and by implication, the possible role of aerially transmitted basidiospores in its spread.
4. To study potential pathogenicity factors of *G. boninense.*
5. To establish *Agrobacterium-Ganoderma* GFP gene tagging transformation method for future use in visualizing hyphae during infection and in the study of fungal pathogenicity genes at the molecular level, either for creating targeted or random gene disruptions.
6. To develop a model system of pathogen-host interaction for the study of the transcriptome of *G. boninense*.

2 MATERIALS AND METHODS

2.1 Plant materials and growth

Oil palm seedlings were supplied by the Malaysian Palm Oil Board (MPOB), Malaysia. Germinated seeds were transplanted to seed trays (300 x 220 x 50 mm) filled with compost (Levingtons F2 + sand, Levingtons M2, Perlite 1:1:1) and maintained in a controlled environment cabinet [28ºC, 80% RH and 12 hour photoperiod, with lighting (240 µ mol m^{-2} sec^{-1} photo flux density) PFD]. Propagator lids were placed over the trays to maintain 100% RH. The RH was reduced after 2 months to ca. 80% when the seedlings (two leaf stage) were transplanted into black polyethylene bags (LBS Horticulture) (8.9 cm x 17.8 cm x 17.8 cm) containing 1.2 L of compost and transferred to the glasshouse. Conditions in the glasshouse were maintained by careful use of shading and artificial lights (Camplex 500 W metal halide). Light levels was maintained between 800 and 500 µ mol m^{-2} sec^{-1} with a day length of 14-17 hours; RH and temperature ranged from 60 to 90% and 20ºC to 35ºC respectively.

After 3 months, palms were transferred into larger black polyethylene bags (15.2 cm x 125.4 cm x 25.4 cm). The number of plants per trough was reduced from 10 to 6 to prevent overcrowding. The palms within each treatment were randomized between the troughs. Once a month, palms were watered with liquid fertilizer (BHGS; 1 in 45 dilution, containing N, P, K. in the ratio of 8:3:3 and trace elements). Compost pH ranged from 5.0 at the beginning of the experiment to 6.4 after 6 months. Plants were watered from below on alternate days.

2.2 *Ganoderma* and other fungal isolates

Five *G. boninense* isolates PER71, FM, UB, SH1 and SH2 and two *Trichoderma* strains were obtained from Malaysian Palm Oil Board (MPOB) and FELDA, Malaysia (Table 1). The *G. boninense* isolates were collected from the ground tissue of basidiocarps on infected oil palm trunks using *Ganoderma* selective medium (GSM) (Ariffin and Idris, 1991). Importation of all strains was covered under a DEFRA Licence PHL 188A/6287. All isolates were grown on malt extract agar slants in universal bottles covered with a layer of sterile distilled water or 10% glycerol (v/v) and stored at 4°C as the original stock cultures.

Many other isolates were obtained from basidiocarps for molecular analysis of genetic diversity as described in section 4.2.1 Spores were collected from basidiocarps in the field for basic germination studies and for plant inoculations, and this procedure is given in section 3.2.8.

Table 1. *Ganoderma boninense* strains and *Trichoderma* used in this study

Genus	Species	Code	Origin
Ganoderma	*boninense*	PER71	Malaysian Palm Oil Board (MPOB)
		FM	FELDA Mempaga, Malaysia
		UB	FELDA Ulu Belitung, Malaysia
		SH 1	FELDA SertingHilir 1, Malaysia
		SH 2	FELDA SertingHilir 2, Malaysia
Trichoderma	*spp.*	TPP4	CABI UK
		T1-203	CABI UK

2.3 *Ganoderma* standard growth media

2.3.1 Solid plates

Nine mm plugs excised from the growing edge of *G. boninense* mycelia using a sterile cork borer were placed onto malt extract agar (MEA) (SIGMA) (Appendix 1) on a 90 mm pre-sterilised petri dish and incubated inverted at 25°C for 7 days. All culture dishes were sealed with parafilm to avoid contamination.

2.3.2 Liquid cultures

Malt extract broth (MEB) (Appendix 2) culture medium 100 mL was inoculated with two plugs (9 mm) of *G. boninense* in sterilized 250 mL conical flasks and incubated at 25°C at 200rpm or static for 14 days. All conical flasks were closed with a cotton stopper for aeration. Mycelium was used for DNA extraction (see section 3.2.15) or after homogenization for inoculating liquid cultures used in biochemical studies (see section 5.2). *Ganoderma* was also grown static in shallow liquid culture in Nunclon^{TM} cell culture flask for production of cell wall degrading enzymes and other putative pathogenicity factors. Details are given later in section 5.2

2.3.3 Other fungal isolates

Other fungal isolates used were grown on potato dextrose agar (PDA) in petri dishes (90 mm diameter) at 27^{o}C unless otherwise stated.

2.4 *G. boninense* inoculum preparation

2.4.1 Woodblock materials

All rubber wood blocks (RWB) and oil palm wood blocks (PWB) were obtained from Institute Tropical of Agriculture, Universiti Putra Malaysia (UPM), Malaysia. Woodblocks were cut to varying sizes, heat-treated at 70°C for 48 hours to kill most vegetatively growing organism and transported in a secured box to the University of Bath. Ganoderma-infested wood blocks were used for inoculation of palms and also to provide external mycelium for mRNA preparation for transcriptomic analysis.

2.4.2 Preparation of woodblock inoculum

RWB and PWB were soaked in distilled water overnight at room temperature in a 36 x 26 x 9cm tray. Wood blocks in varying sizes were autoclaved twice for 30 min at 121°C. One block each was put in a heat-resistant polypropylene bag (15 cm x 33 cm x 0.05 cm thick materials) and 100 ml of molten MEA was added as supplementary nutrient for *G. boninense*. Since inoculation of the blocks with *G. boninense* plugs can introduce contamination, the polypropylene bags were closed by drawing its open end through 4 m diameter polyvinyl chloride (PVC) tubing 2 cm long and the remaining hole plugged with cotton. The bags each with wood block and molten MEA were autoclaved at 121°C, 15 psi for 30 min. After sterilization and cooling, the rubber wood blocks were rotated to ensure they were well covered with the agar before solidification. When the agar had solidified, five plugs (10 mm each) were taken from five day-old *G. boninense* culture and inoculated onto each of the blocks. Polypropylene bags were then incubated in a dark cabinet at 28 ± 2°C for four weeks until they were colonised by *G. boninense* mycelium .

2.4.3 Preparation of wheat grain inoculum

Untreated wheat grains were immersed overnight at room temperature in distilled water in a 500 ml conical flask, then drained and autoclaved at 121˚C for 45 min. Five mycelial plugs (1 cm^2) were placed randomly into the wheat grains. The wheat grains were incubated at 27˚C in the dark for 2-3 weeks. Sterile distilled water (SDW) was used to maintained sufficient moisture for growth of *Ganoderma*. Wheat grain inoculum was used to provide alternative methods of infecting palms (section 3.3.2) but also to facilitate early colonization of palm roots *in vitro* for trasnscriptomic analysis (section 5.3)

2.4.4 Inoculation of oil palm roots

Oil palm roots were carefully uprooted and colonised wood blocks (4 weeks old) were placed in close contact with roots, which were then covered with soil in polybags. Uninoculated plants served as controls. The inoculated and uninoculated plants were watered twice daily using tap water. Alternative methods of inoculation using colonized wheat grain and dowels are described in section 3.2.3 and 3.2.4.

2.5 RNA and DNA Extraction

Note that mRNA extraction for pathogen detection and for transcriptomic analysis are detailed in sections 3.2.13 respectively.

2.5.1 Fungal CTAB-based method

DNA of *G. boninense* was isolated using a modified CTAB method described by Manicom *et al*. (1987). Approximately 200 mg mycelium and 10-15 glass beads (Sigma, 1-2mm) were added to 1.5 ml tubes, and cells were disrupted by vortexing for 45 secs. Suspensions were combined with 500ml hot extraction buffer (1% Cetyltrimethylammonium bromide [CTAB], 50mM Tris-

HCl [pH 8.0], 0.7M NaCl, and 10mM EDTA [pH 8.0]) and vortexed again before incubation at 65ºC for 40 min. The suspension was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), vortexed, and centrifuged at 13000 g for 10 min. The upper aqueous phase was transferred to a clean tube, and mixed with an equal volume of cold isopropanol to precipitate nucleic acids at -20ºC overnight. The DNA was pelleted at 13000 g for 30 min, washed twice by centrifugation with 100 ml of cold 70% ethanol and taken up in 100 ml of sterile MilliQ water. DNA purity and concentration was determined using the ND-100 Nanodrop spectrophotometer (Thermoscientific) according to the manufacturer's protocol.

2.5.2 PCR for target sequence amplification

Reactions were prepared in sterile 0.2 ml thin-walled PCR tubes and consisted of approximately 100 ng DNA template, 2.55 µl 10X DNA polymerase buffer, 2.5 µl of 25 mM MgCl2, 1.0 µl of 20 mM dNTPs, 0.5 µl of 10 mM forward primer, 0.5 µl of 10 mM reverse primer, 0.1 µl of DNA polymerase (Promega) and SDW to 25 µl reaction mixtures. Reactions were cycled in a PT-200 Peltier Thermal Cycler (MJ Research) at 94ºC (5 min) followed by 35 cycles of 94ºC (1 min), 55ºC (1 min), 72ºC (1 min), and a final step of 72ºC for 10 min. The annealing temperature was adjusted to approximately 5ºC below the melting temperature (Tm) of the primers and within the range of 50-60ºC. Amplification products were visualised by agarose gel electrophoresis.

2.5.3 Agarose gel electrophoresis

PCR products of DNA fragments were analysed by agarose gel electrophoresis. Gel tray was assembled according to manufacturer's instructions (Biorad). 1% w/v agarose (Fisher Scientific) was weighed and added into 1x TBE buffer (20% v/v 5x TBE stock: 0.4M orthoboric acid, 0.3M Tris-base and 0.5M EDTA pH 8). The mixture was heated in a microwave oven until all the agarose dissolved to give a clear solution. It was left to cool to

<50^{o}C before ethidium bromide (EtBr) was added to a final concentration of 0.67 g/ml. The solution was swirled to ensure equal distribution of EtBr prior to pouring into the gel tray and a comb was inserted to mould the wells. Once the gel was set, the gel tray was transferred into a gel tank containing 1x TBE buffer. The comb was removed and DNA samples in a Blue/Orange 6x loading dye (Promega) were loaded alongside a 100bp DNA ladder (Promega). Electrophoresis was carried out at a constant voltage (typically 80V) until the DNA had migrated approximately three quarters through the gel. The gel was then visualised on a White/Ultraviolet transluminator (UVP) and the image was captured using Canon G10 and Remote Camera DC software.

2.6 Statistical analysis

All statistical analysis was carried out using PASW Statistics18 (software). Means were separated using contrast statements at significance level of $P < 0.05$. Comparison between means of treatment was made using T-test or Tukey HSD.

3 Infection and Epidemiology of *Ganoderma* Basal Stem Rot

3.1 Introduction

Ganoderma causes basal stem rot and upper stem rot infection of oil palm and the former is the most frequently found and devastating disease in oil palm cultivation in Indonesia and Malaysia. The primary route of infection through root contact has been generally regarded as the sole mode of infection by the pathogen. Other root-infecting basidiomycete diseases of tree and perennial crops are spread by vegetative growth. After basidiospores of *Heterobasidion* spp. colonise stump surfaces, the subsequent spread of the fungus from infected stumps to neighbouring trees occurs *via* root contacts. This vegetative infection of trees creates a clonal spread over a wide area (Stenlid*,* 1985, Garbelotto *et al.,* 2013). *Armillaria* species cause serious root diseases of a wide range of trees and other woody species. For example in Kenya and Indonesia they affect tea plantations (Lehmann-Dazinger, 2000). This fungus can spread by root contact and by cord-like rhizomorphs, which can directly penetrate healthy roots to cause disease (Baumgartner *et al.,* 2011). In contrast, various studies using vegetative compatibility and molecular analysis of *Ganoderma* isolates indicate high genetic variability within oil palm plantations and raise an argument on the issue of disease epidemiology.

Currently there are three opinions on the mode of infection: i) Healthy palm roots encounter a source of inoculum, such as infected roots from adjacent palms; this become infected and mycelial growth progresses along or through the root towards the bole leading to stem rot of the base of the palm. ii) Basidiospores are dispersed throughout the plantations onto wounded palm tissues and cause direct infection, for example via wounds created on

harvesting or pruning of palm petioles (fronds). iii) Basidiospores land on and colonize palm debris to create an inoculum to be contacted by roots. There is evidence to support the first two routes, which complicates disease control (Rees *et al.,* 2007; Cooper *et al.,* 2012; Rees *et al.,* 2012; Hushiarian *et al.,* 2013; Ho and Tan, 2015). Therefore understanding *G. boninense* infection and disease spread are critical to develop a successful disease management against BSR.

3.1.1 Root infection of oil palms

BSR of oil palm was claimed to occur as a result of contact between healthy roots and diseased tissues left buried in the soil (Turner, 1981). In addition it was also believed that wounded tissues or dead roots promote infection by *Ganoderma*. The fungus then grows along the infected root and reaches the bole of the palm causing the base of the stem to rot. The role of mycelial contact in disease establishment was primarily proved with several artificial inoculation methods with large inoculum which induced infection of oil palm seedling roots in a control environment; the fungus was then re-isolated from disease lesions. Navaratnam and Chee (1965) artificially inoculated 22 oil palm seedlings with very large 750 cm^3 blocks of naturally infected oil palm tissue and 15 of them were infected with BSR after 9 months. They claimed that a large inoculum source was essential for *G. boninense* infection (Navaratnam and Chee, 1965). Later Lim *et al.* (1992) used dikaryotic *G. boninense* colonised oat grain inoculated onto a single root and obtained 100% infection of palm roots after 9-10 weeks. He reported that the quality and purity of *G. boninense* inoculum determine the infection potential of the inoculum and not the inoculum size. However there are no further reports of success using this method. Root Inoculation Technique (RIT) was developed by Ariffin *et al.* (2000), which involved inoculating primary roots of oil palm seedlings in a polybag which were exposed and inserted into a test tube

containing *Ganoderma* inoculum grown in POPW medium (mixture of oil palm wood sawdust, paddy, ammonium sulphate, calcium sulphate, sucrose and peptone). They reported that *G. boninense* progression of root was < 2 cm/month and mean death of various progenies was 26 % after 12 months inoculation with the technique. Currently, inoculation method involve growing *G. boninense* on sterilized rubber wood blocks (RWB) (*Hevea brasiliensis*) which are then placed beneath seedlings growing in soil polybags and roots are wrapped over the RWB inoculum (Khairudin, 1990; Sariah, *et al*., 1994; Rees, *et al*., 2007; Mohd Aswad *et al.,* 2011; Kok *et al.,* 2013; Nur Sabrina *et al.,* 2012; Najihah *et al.,* 2015). It was first introduced by Khairuddin (1990) using a 750 cm^3 colonized RWB with dikaryotic *G. boninense* mycelium, which gave successful infection of oil palm in Malaysia (Khairudin, 1990). Similarly, Sariah *et al.* (1994) obtained 100% infection of oil palm seedlings with 5x5x15 cm RWB inoculum. In Indonesia Rees *et al.* (2007) showed that all oil palm seedlings inoculated with 6x6x6 cm RWB inoculum were severely infected 12 month after inoculation. However this artificial inoculation of seedlings requires a large RWB. This has been evident from many studies which have used sizes ranging from 6x6x6, 6x3x3, 5x5x15 and 6x6x12 cm^3 (Sariah *et al.,* 1994; Rees *et al.,* 2007; Kok *et al.,* 2012; Idris *et al*., 2006). Rees *et al.* (2007) reported that only 30% of seedlings were infected with a small 6x3x3 RWB inoculum after 15 months. However, by attaching *G. boninense* RWB inoculum with parafilm closely onto roots a smaller inoculum 3x3x3 cm^3 managed to induce infection (Rees *et al.,* 2009; Cooper *et al.,* 2011).

Currently RWB technique is used widely for infection by *G. boninense* (Sariah *et al.,* 2004; Rees *et al.,* 2007; Kok *et al.,* 2012; Nur Sabrina *et al.,* 2012; Tan *et al.,* 2013; Muniroh *et al.,* 2014) to study various control measures in the nursery (Alizadeh *et al.,* 2011; Nur Ain Izzati and Faridah*,* 2008; Najihah *et al.,* 2015). It is unknown whether these methods provide the optimum methods for the key behind finding disease resistance i.e. screening new crosses for BSR resistance. The RWB methods are all slow, inoculum preparation requires much time and space. Futhermore the enourmos size of RWB used might overcome any potential resistance of oil palm. A simple and more rapid

infection technique, with a small inoculum size would provide a better method for resistance screening of BSR disease to facilitate oil palm breeding.

BSR in the field has sometimes been linked with multiple, natural infections of different roots by *G. boninense* within a single palm (Ariffin *et al*., 2000; Flood *et al.,* 2005; Rees *et al*., 2007). The source of disease inoculum is important in root to root contact for development of BSR disease in the field (Hassan and Turner, 1998; Flood *et al*., 2005). Bait seedlings planted close to BSR infected palm stumps became infected within 6-28 months and eventually died. Stumps left in the ground were more infectious than felled trunks left on the surface and buried shredded palm tissue was more infectious than shredded palm tissue left on the surface (Hassan and Turner, 1998). Untreated stumps left in the ground resulted in 38% infection of seedlings adjacent to stumps from BSR palms compared with 6% infection in seedlings planted adjacent to healthy palm stumps. Higher infection rates of seedlings adjacent to diseased stumps and trunk tissue, suggests root contact as a likely route for infection. Also, it is probable that palm roots come into contact with buried colonised oil palm material more readily than with surface material. In addition, Flood *et al*. (2005) showed young palms would become infected quickly by BSR when exposed to large amount of infected debris close to their planting points in the field. Seedlings showed 60% infection after 52 months when planted 0.5 m from buried trunks, 25% infection at 1 m distance from buried trunks and 0% infection at 1.5 m distance. All seedlings planted adjacent to non-buried trunks remained uninfected within the observation period (Flood *et al.,* 2005).

It is important to note that the survival of *G. boninense* inoculum in soils needs to be addressed. How long inoculum can survive in the soil and in what form the pathogen survives have never been determined before. However, Rees *et al.* (2009) observed that *G. boninense* forms an extensive, tough, melanised mycelium or pseudo-sclerotium around colonised roots and suggested that this melanised structure could play an important role in survival in soils (Rees *et al.,* 2007; Cooper *et al.,* 2011). Therefore determination of the role of

melanised structures in the survival of *G. boninense* may provide insights on how *G. boninense* inoculum in the field (i.e. colonised debris or roots) from previous planting contributes to new infections.

Shading and consequent soil temperature in the plantation have a dramatic effect on BSR infection. Severe disease occurred after 8 months on inoculated seedlings under shade, but not on seedlings exposed to sun. Soil temperatures in sunlight frequently rose above 40°C and reached 45°C, whereas in shade they never exceeded 32°C (Rees *et al.,* 2007). *G. boninense* mycelia with a growth optimum of 25-28°C cannot tolerate high temperature for growth or survival and therefore fails to establish infection under exposed conditions. The need for intimate contact between *G. boninense* mycelium and oil palm roots probably relates to the pathogen's inability to grow from a source of inoculum through the soil; it appears to be a very weak competitor against soil microflora (Rees *et al.,* 2007).

Penetration and colonisation of oil palm roots by *G. boninense* is essential for the pathogen to establish infection and develop BSR disease. Artificial inoculation methods such as RWB showed *Ganoderma* mycelium could penetrate the roots of oil palm seedlings on contact, colonised and reaching the bole, eventually causing typical symptoms of BSR (Sariah *et al.,* 1994; Rees *et al.,* 2007; Kok *et al.,* 2012). Rees *et al.* (2009) showed that colonisation of *G. boninense* can occur through unwounded roots following intimate contact. Histopathological investigations of *Ganoderma*-infected roots showed tissues like xylem, phloem, pith and parenchymal cells were all infected at the advanced stage of the fungal pathogenesis (Ariffin *et al*., 2000). In contrast, Rees *et al*. (2009) showed colonization by *G. boninense* progresses mainly through inner, thin-walled cortex and no colonization was observed in the vascular tissue of the roots.

3.1.2 Direct Infection and dissemination by basidiospores

Currently, the role of *Ganoderma* basidiospores in disease initiation and spread of infection remains unclear (Ariffin *et al*., 2000; Sanderson *et al.*, 2000; Rees *et al.,* 2012). The role of basidiospores was first suggested by Thompson (1931) following BSR incidence in first generation oil palms on cleared virgin jungle areas. Later Sanderson *et al.* (2000) also reported occurrence of BSR incidence from two oil palm planting areas in Solomon Islands, which were a forest previously. A strong evidence of the involvement of basidiospores was reported by Sanderson *et al*. (2000) based on high incidence of BSR in first generation oil palms, on land previously cultivated with paddy for 10 years. It was a commercial paddy cultivation area where heavy machinery was used to prepare the land and regular application of chemicals from the air was applied over the area. In this instance it is difficult to implicate roots coming into contact with *G. boninense* inoculum buried in the soil.

In other important basidiomycete tree pathogens such as *Heterobasidion annosum*, basidiospores initiate primary infection of *H. annosum* on freshly exposed wood surfaces such as stump tops or stem and root wounds and once established the disease spreads through root to root contact. *H. annosum* basidiospores play an important role in disease initiation and spread of the butt rot disease in coniferous forests of Northern Hemisphere (Garbelotto *et al*., 2013). *Ganoderma boninense* basidiospores may play a significant role in spreading BSR in plantations (Pilotti *et al.,* 2003; Sanderson, 2005; Rees *et al.,* 2012). It was reported that basidiospores were involved in the epidemiology of BSR in the field, based on genetic analysis involving somatic compatibility testing and molecular markers (Ariffin *et al*., 1996; Miller *et al*., 1999; Zakaria *et al*., 2005; Rees *et al*., 2012). These studies showed high genetic diversity of *G. boninense* within and between population of BSR in Malaysia, Indonesia and Papua New Guinea. High genotypic variation may have occurred through sexual recombination following dispersal of

basidiospores, which strongly implies involvement of basidiospores in BSR establishment (Miller *et al*., 1999; Zakaria *et al*., 2005; Rees *et al*., 2012).

Abundant *G. boninense* basidiocarps form around the stem of BSR-infected palms (Idris *et al.,* 2006; Rees *et al.,* 2009; Cooper *et al.,* 2011; Hushiarian *et al.,* 2013), which subsequently will release myriad basidiospores to the atmosphere within affected plantations. Sanderson (2005) recorded a stream of basidiospores released from a basidiocarp using a video camera with a telephoto zoom in an oil palm plantation in PNG. He reported that approximately 2 million basidiospores were released from a 100x50 mm basidiocarp per minute throughout the day from 7 am to 4 pm. He suggested that the astronomical number of basidiospores available in the plantation environment must contribute to BSR disease. Similarly, vast numbers of *G. boninense* spores 2-11 000/m^3 were sampled day and night in Indonesian plantations, which could potentially establish infection to wounds and colonize palm debris in the plantation (Rees *et al.,* 2012). However Ho and Nawawi (1986) observed that although abundant basidiospores were produced in the field, the majority of oil palms remain healthy and they suggested that basidiospores may require very specific conditions to establish infection (Hushiarian *et al.,* 2013). However there is currently no literature on the properties of *G. boninense* basidiospores that would influence their spread and survival as a viable propagules to establish BSR or USR in the field.

Wound sites created by plantation management may provide an opportunity for infection by *G. boninense* basidiospores along with physical damage by invertebrate pests such as *Oryctes rhinoceros* and also by rats. In order to harvest oil palm fruit it is necessary to sever the peduncle (fruit stalk) and petiole (frond) to free the fruit bunch. Several harvests are made from one tree in a year, causing multiple large wound sites. A tough abscission layer forms at the base of a cut frond and likely poses a considerable challenge for infection progress. However, as the tree grows in height fruit becomes more difficult to harvest and the probability of wounding the trunk becomes greater. This point of entry by basidiospores was further supported following detection

of *G. boninense* DNA in 10-15% (200 total) of palm tissue beneath the cut frond bases of oil palm (Bridge *et al.,* 2000; Panchal and Bridge, 2005). This may facilitate infection by allowing direct penetration of the pathogen into the palm trunk (Rees *et al*., 2007).

However direct infection of basidiospores on or into oil palm has so far failed to initiate infection of BSR and USR disease. Inoculation of cut frond bases (Turner 1965 b) and oil palm seedlings with *G. boninense* spores failed to establish BSR disease (Ramasamy, 1972; Idris *et al.,* 2006). Similarly Hasan *et al.* (2005) reported that basidiospores inoculation onto different oil palm tissues of cut young male inflorescence stalks, cut ripe bunch stalks, freshly cut fronds and fronds damaged by wedges in the field failed to initiate USR infection based on a 3 years observation. They also reported that although fresh basidiospores were used and considerable care using basidiospores was taken, infection did not occur (Hasan *et al*., 2005). Turner (1981) suggested that the spores may provide inadequate inoculum to establish infection, therefore requiring a suitable substrate to build up potential inoculum, such as cut stumps of trees or palms left to rot in the field, which may become infection foci (Flood *et al.,* 2005). Rees *et al.* (2012) showed by cryo-SEM that *G. boninense* basidiospores germinated within the xylem vessels of freshly cut fronds. They also showed that spores could travel down the xylem vessels and stop at vessel end walls with a maximum of c. 10 cm (Rees *et al.,* 2012). Spores contaminating cut frond surfaces are drawn into xylem as a result of negative tension within functional vessels (Cooper, 1981). These vessels would provide protection to the basidiospores from dehydration, microbial competition and solar radiation to germinate and potentially establish BSR following petiole colonization (Rees *et al*., 2012).

3.1.3 Detection of *Ganoderma*

In order to evaluate critically infection and colonization by *G. boninense*, sensitive detection or identification methods are required. Several methods have been developed for detection and identification of *Ganoderma* infection, for example *Ganoderma* selective medium (GSM), immunoassay, and DNA based techniques (Rees *et al*., 2012; Cooper *et al*., 2011; Hushiarian *et al.,* 2013). *Ganoderma* selective medium (GSM) was developed to isolate and confirm *Ganoderma* (Ariffin *et al.*, 2000). It is a useful technique to isolate the fungus from environmental samples of healthy and diseased tissue and provide a useful identification of *Ganoderma* without significant contamination. The mixtures of antibiotics, fungicides, tannic acids and nutrients in the medium provides the selective growth for *Ganoderma* and kills or inhibits the growth of bacteria and fungi associated with oil palm (Ariffin *et al.*, 2000). *Ganoderma* oxidises the tannic acid to produce brown pigmentation.

Currently most studies on infection, pathogenicity and collection of isolates from the field have relied on GSM for confirmation and pure culture isolation. Idris (2009) reported that with GSM it is possible to identify *G. boninense* in 5 to 16% of oil palms with no apparent BSR symptoms in the field. Rees *et al.* (2007) used GSM to re-isolate and track *G. boninense* infection progress along the inoculated roots of oil palm seedlings. Pilotti (2005) reported air-borne spore trapping by GSM and showed that basidiospores were able to grow on the medium. However this detection method is incapable of differentiating between pathogenic and saprotrophic *Ganoderma* species in the field.

Currently a more rapid and reliable identification method for confirming and detecting *Ganoderma* isolates is required. Immunological methods have been developed such as ELISA, using polyclonal antibodies (Utomo and Niepold, 2000) and monoclonal antibodies (Shamala *et al.,* 2006), Molecular methods involve PCR-based detection (Utomo and Niepold, 2000, Bridge *et al.,* 2000, Panchal and Bridge*,* 2005, Hushiarian *et al.,* 2013). The use of PCR detection for *Ganoderma* in oil palm have been frequently reported (Idris *et al.,* 2003; Bridge *et al.,* 2000; Utomo and Niepold, 2000; Rees *et al.*, 2012).

Ganoderma specific primer combinations for PCR include: PER44-123 and Lr1 primer which amplify a product at 580 bp (Idris *et al.*, 2003), GAN1 and GAN2 which amplify a product at 167 bp (Utomo and Niepold, 2000). Bridge *et al*. (2000) developed specific PCR detection with GanET primer and ITS universal fungi primer, which was able to detect presence of *G. boninense* in diseased standing palms. The specific PCR primer GanET was constructed from ITS region and used with ITS3 primer to produce approximately 361 bp product band, specific for detecting *G. boninense* isolates. The specificity of the primer combination was assessed against purified DNA from a collection of palm-associated fungi such as species of *Verticillium, Ascochyta, Phoma, Fusarium, Rhizoctonia, Psilocybe, Thielaviopsis and Phytophthora* and no cross-reactivity was detected. They also showed the diagnostic capabilities of the primer combinations to amplify the specific product from healthy and infected mature palm stem samples that contain saprotrophic microbes and invertebrates. Furthermore, PCR detection has been shown to be sensitive enough detection of *G. boninense* basidiospores. Idris *et al*. (2003) reported the sensitivity of Per44-123 and LR1 primer combination against a dilution series of basidiospores of *G. boninense*. PCR products were visible when 1000 or more basidiospores were supplied (Idris *et al.,* 2003).

3.1.4 *Trichoderma* as biological control for *G. boninense*

Infection of cut petioles by *G. boninense,* to be described later, suggested the potential to use a biological control agent (BCA) at the time of creating harvesting or pruning wounds in order to protect that large and vulnerable palm surface. There follows a brief overview of *Trichoderma* as the genus chosen with greatest potential as a BCA.

Trichoderma is a conidia producing Deuteromycete with a wide range of uses in industry and agricultural biotechnology (Carreras-Villasenor *et al.,* 2012). *Trichoderma* species are abundant in many soil types from temperate to tropical regions. Many strains interact with plants by colonizing roots, influencing the expression of many plant genes (Harman *et al.,* 2012). *Trichoderma* spp. are often able to penetrate the epidermis and outer cortex of roots, living as opportunistic avirulent symbionts (Harman *et al.,* 2004; Mukherjee *et al.,* 2011). Several *Trichoderma* species have long been recognised as BCAs, including *T. harzianum*, *T. virens* and *T. viride* (Chet and Inbar, 1994; Howell, 2003; Benitez *et al.,* 2004; Harman *et al.,* 2012). *Trichoderma* acts as a BCA through a number of mechanisms including mycoparasitism, antibiosis, substrate competition and the production of cell-wall degrading enzymes (Shi *et al.,* 2012).

Mycoparasitism is the predominant method used by *Trichoderma* to control plant pathogens, including *Rhizoctonia solani, Botrytis cinerea, Sclerotium sclerotiorum, Pythium* spp*.* and *Fusarium* spp. (Omann *et al.,* 2012). *Trichoderma* detects the presence of other fungi by producing low amounts of an extracellular exochitinase and then grows towards the host (Elad *et al*., 1983; Chet *et al*., 1998; Harman, 2006). Diffusion of exo-chitinases triggers the release of cell-wall oligomers from the pathogenic fungus which in turn induces expression of potentially fungitoxic endo-chitinases (Harman *et al.*, 2004). On contact *Trichoderma* may coil around the host's hyphae and forms appressoria before producing antifungal metabolites or enzymes (Chet *et al*., 1998; Harman, 2006).

Antibiosis was first discovered in *T. lignorum*, (now *T. virens*) by Weindling (1941). *Trichoderma virens* demonstrated toxicity towards *R. solani* and *Sclerotinia americana*. Peptaibols are antibiotic peptides, which are produced by a number of *Trichoderma* spp. They include trichokonic VI, which has recently demonstrated broad-spectrum antifungal activity and could be effective against a range of plant pathogens by inducing apoptotic programmed cell death (Shi *et al.* 2012). Also gliovirin is inhibitory to some pathogens (Strange, 2003).

Competition for nutrients and space is a major growth-limiting factor in natural environments and can be used by BCAs to control plant pathogens. *Trichoderma* is fast growing and can mobilise and take up soil nutrients increasing its competitiveness against many other soil microorganisms, including pathogens (Benitez *et al.,* 2004). *Trichoderma harzianum* uses competition for nutrients as a major mechanism to control *Fusarium oxysporum* (Vinale *et al.,* 2008).

Trichoderma has been shown to induce systemic or localised resistance, which might partly explain their lack of pathogenicity towards plants (Harman *et al*., 2004). Induced resistance can occur in response to many factors including microbial attack, physical damage and various chemical inducers. Pathways that induce resistance in plants usually lead to the production of pathogenesis-related (PR) proteins (Harman *et al.,* 2004). Induced host responses can include the production of antimicrobial compounds and physical barriers such as tyloses (Sun *et al.,* 2008). Tyloses balloon out from adjacent parenchyma into the vascular tissue and cause a blockage which prevents and halts the spread of pathogens. Induction of host responses by *Trichoderma* or wounding, leading to the formation of tyloses, could reduce *Ganoderma* establishment or progression within vessels during initial colonization of petioles (Rees *et al*., 2012).

In summary, conjecture and uncertainty surrounding the mode of infection of oil palm by *G. boninense* is stifling attempts to develop adequate control strategies. In order to develop successful management practices, elucidating the route of infection is crucial. In this chapter basidiospore characteristics and infectivity, as well as other potential inoculum sources are investigated for their importance for spread, infection and eventual control of *G. boninense.*

3.2 Materials and methods

3.2.1 *Ganoderma boninense* isolate

Five *G. boninense* isolates PER71, FM, UB, SH1 and SH2 and two *Trichoderma* strains were obtained from Malaysian Palm Oil Board (MPOB) and FELDA, Malaysia (Refer Table 1, section 2.1). The *G. boninense* isolates were collected from the ground tissue of basidiocarps on infected oil palm trunks using *Ganoderma* selective medium (GSM) (Ariffin and Idris, 1991). Importation of all strains was covered under a DEFRA licence PHL 188A/6287. All isolates were grown on malt extract agar slant in a universal bottle covered with a layer of sterile distilled water or 10% glycerol (v/v) and stored at 4°C as the original stock cultures.

3.2.2 Preparation of wheat grains for oil palm inoculation

Wheat grains were soaked overnight in distilled water followed by autoclaving at 121°C for 40 minutes. The wheat grains were then transferred to 140 mm petri dishes (Thermo Scientific) and hydrated with 5 ml sterile distilled water. Each plate was then inoculated with 10 evenly spaced 6 mm diameter discs of *G. boninense.* The dishes were sealed and incubated at 25-27°C for 8 days with a further 5 ml sterile distilled water was added to each dish after 1, 2 and 4 days to maintain humid conditions. After this period the grains were colonized by an extensive mycelium.

3.2.3 Preparation of wheat grains on capillary mat for oil palm inoculation

Wheat grains were soaked overnight in distilled water followed by autoclaving at 121°C for 40 minutes. The wheat grains were then transferred on capillary mat (Lantor) in 140 mm petri dishes (Thermo Scientific) and hydrated with 5

ml sterile distilled water. Each plate was then inoculated with 10 evenly spaced 6 mm diameter discs of *G. boninense.* The dishes were sealed and incubated at 25-27°C for 8 days with a further 5 ml sterile distilled water was added to each dish after 1, 2 and 4 days to maintain humid conditions. After this period the grains were colonized by an extensive mycelium.

3.2.4 Preparation of dowels for oil palm inoculation

Commercial beechwood dowel and rubberwood dowel were soaked in a distilled water overnight at room temperature in a 36 x 26 x 9cm tray. Dowels were autoclaved for 30 mins at 121°C. 15-20 dowels were put in heat-resistant polypropylene bags (15 cm x 33 cm x 0.05 cm thick materials) and 10 ml of molten MEA was added as supplementary nutrient for *G. boninense*. Since inoculation of the dowels with *G. boninense* plugs can introduce contamination, the polypropylene bags were closed by drawing its open end through a 4 m diameter polyvinyl chloride (PVC) tubing 2 cm long and the remaining hole plugged with cotton. The bags with wood block and molten MEA were autoclaved at 121°C, 15 psi for 30 mins. After sterilization and cooling, the dowels in the polypropylene bag were rotated to ensure they were well covered with the agar before latter solidification. When the agar had solidified, five plugs (10 mm each) were taken from five day-old *G. boninense* culture was inoculated in each of the beechwood and rubberwood dowels bags. The inoculated dowels in their polypropylene bags were then incubated in a dark cabinet 28 ± 2°C for one weeks until they were colonised by *G. boninense* mycelium.

3.2.5 Collection of basidiospores and preparation of spore suspensions

G. boninense basidiospores were collected in the morning by placing petri dish with Whatman filter paper below the pores of an active basidiocarp and held in place by aluminium foil. The plate was left in place for 3-4 h until the paper was thickly coated in dark brown basidiospores. Spores were then taken to the laboratory and left to air dry for 10-15 min if the filter paper was slightly damp. The spore-coated filter paper was then cut into small pieces and added to SDW (pH 5.5) to make a suspension. Spore concentration was calculated with a haemocytometer and in all cases spores were used immediately after prepared or otherwise stated.

3.2.6 Biotester air sampling in Malaysia

A Biotest RCS centrifugal air sampler (Biotest UK) was used to sample the air concentration of spores within air in oil palm plantations. Water agar was loaded into the sampler and was run for 8 min. Agar blocks were then extracted and observed microscopically for trapped spores. Four samples were taken from each location to provide mean concentration of spores/m^3 using the formula: cfu/m^3 air = # colonies on agar strip x 1000/volume of air sampled (in an 8 min period 320 litres of air was sampled).

3.2.7 Inoculation of basidiospores and sampling from oil palm petioles

Following sterilisation of the oil palm epidermis and blades of the secateurs with 70% ethanol (v/v), the oil palm petioles were cut approximately 20cm from the base of the stem. A total of 21 petioles were inoculated with 500µL of a 2.9 × 10^7 spores/mL suspension, across 7 oil palms (3 petioles inoculated per

plant). This suspension was applied successively three times, to allow negative tension to draw the suspension into the xylem. Following full adsorption, aluminium foil was placed to cover the cut petiole (Fig. 3). Three petioles from a different plant were harvested at time zero, week 3, 6 and 9 of the inoculation period. This was achieved by cutting 10cm from the top of the cut petiole with ethanol-sterilised secateurs.

Figure 3. Petiole inoculation with *Ganoderma* boninense basidiospores. After the application of 500µL of a 2.9×10^7 spores/mL suspension, cut petioles were covered with aluminium foil.

3.2.8 Re-isolation of basidiospores from oil palm petioles

To identify if the basidiospores had infected the oil palm petioles and assess how far they had dispersed down the xylem vessel, cut sections of petioles were isolated onto GSM in sterile conditions. Firstly, each petiole was washed with 10% (v/v) sodium hypochlorite solution using cotton wool for 3 minutes,

followed by three rinses of the epidermis with sterile distilled water-soaked cotton wool. The petiole was then marked out in to centimetres before each centimetre section was cut off from the rest of the petiole with a flame-sterilised scalpel. This small section then had the entire epidermis removed using the scalpel, in order to avoid as much contamination as possible. The inner tissue of the petiole was presumed to have very low microbial content except for any *G. boninense* basidiospores present. This section without an epidermis was then cut in half, with half of the petiole being flash frozen in liquid nitrogen for use in RNA or DNA extractions, and the other half being cut in to 4 or 5 smaller sections. The small sections were then placed horizontally on to a sterile GSM plate, to allow the vessels to run parallel to the agar, touching a higher surface area.

The GSM plates were incubated for between 7-10 days at 28°C ± 2°C, to allow any *G. boninense* to grow and overcome any possible bacterial or fungal contamination. After this time, if *G. boninense* appeared to be present, it was re-isolated on to MEA to obtain pure isolates. These were confirmed as *G. boninense* by the identification of clamp connections at hyphal tips under a 200× magnification using an Olympus BH-2 microscope. This indicated growth by sexual reproduction.

3.2.9 DNA manipulations

3.2.9.1 Harvesting of *G. boninense* mycelia for DNA extraction

Ganoderma was grown on PDA for 1 wk. 1 cm^2 plugs were taken from the leading edge of the mycelium and placed in 60 ml of 3% malt extract (Oxoid) in 250 ml conical flasks. These were incubated at 28°C on a rotary incubator at 120 rpm, for 4-5 days. Mycelium was then extracted from the medium, filtered and washed in SDW to remove residue. Mycelium was placed directly into liquid nitrogen for immediate freezing and ground to a fine powder in a

mortar and pestle. 100 mg of the powder was then used for DNA extraction and the remainder was stored at -70oC for future extractions.

3.2.9.2 DNA extraction

DNA extraction was achieved using either the CTAB-based method described below or with the Qiagen DNeasy® plant DNA extraction kit as described in manufacturer's instructions.

3.2.9.3 CTAB-based method

100 mg of ground, frozen mycelium was transferred to a solvent-resistant centrifuge tube and 5 ml mercaptoethanol buffer was added. This was mixed by inverting and heated to 65ºC for 30 min in a water bath to lyse cell walls. Five millilitres of chloroform:isoamyl alcohol (24:1v/v) was added and mixed gently by rocking until homogeneous. The mixture was then centrifuged at 11000 g at room temp for 10 min and the upper aqueous layer was then collected with a wide bore pipette. DNA was precipitated by adding 0.54 (v/v) iso-propanol and collected by centrifugation at 11000 g for 5 min at room temperature. Isopropanol was carefully drained from the pellet and resuspended in 2 ml TE buffer. When dissolved, 20 units of ribonuclease A was added followed by incubation at 37°C for 30 min. A 1:1 concentration of chloroform:isoamyl alcohol was then added and mixed gently before centrifugation at 11000 x g at room temperature for 10 min. The upper aqueous layer was then collected using a wide bore pipette. Ammonium acetate was added at 1:5 (v/v) to give a final concentration of 1.5 M ammonium acetate. Absolute ethanol was added to 2:1 (v/v) to ppt DNA and this was collected by centrifugation at 11000 g for 5 min at room temperature. Ethanol was carefully discarded from the pellet and DNA re-dissolved in 500 µl of 200 mM ammonium acetate followed by a second precipitation with 2 x vol absolute ethanol. The pellet was then dried under vacuum using a SAVANT

svc200H speedvac and resuspended in 100-150 µl TE buffer.

3.2.9.4 DNA cleanup

The method used was adapted from Department of Agriculture: Subcommittee on Plant Health Diagnostics (2011) 200µL of chloroform:isoamyl alcohol (24:1 v/v) was added and the tube thoroughly vortexed, then centrifuged at 13,000 rpm for 15 minutes. The upper layer of the liquid was transferred to a new 1.5mL Eppendorf tube and an equal volume of cold isopropanol was added to this and immediately inverted. The tube was centrifuged at 13,000 rpm for 15 minutes and the supernatant discarded, prior to the addition of 100 µl of ice-cold 70% (v/v) ethanol. This was centrifuged again at 13,000 rpm for 1 minute and the ethanol was removed and the tube left to air-dry. To finalise, the pellet was resuspended in 50µL of TE buffer (or less dependent on the expected yield).

3.2.10.5 Polymerase Chain Reaction (PCR) of *G. boninense* specific primers GanET and ITS3

PCR amplification of samples was undertaken using the primer GanET [Bridge et l.,2000] together with the universal primer, ITS3. The reactions were undertaken in 50µl volumes in a reaction mixture consisting of 4 µl dNTPs (each at 5 mM), 2 µl (10 ng) DNA, 3 µl of 25 mM MgCl2, 5 µl buffer, 2.5 µl GanET (25 pmol), 2.5 µl ITS3 (25 pmol), 30.75 µl H2O and 0.25 µl Tth enzyme (0.5 U). PCR was carried out in a programmable thermocycler (MJ Research) with a programme comprising of 40 cycles of an initial denaturation at 95 °C for 1 min, followed by an annealing step at 50 °C for 1 min, and an extension step at 72 °C for 1 min. This was followed by 10 min at 72°C for the final extension. The PCR products were separated by electrophoresis in a 1.2% agarose gel in Tris–acetate–EDTA buffer (TAE), and stained with ethidium bromide.

3.2.10 RNA manipulation

3.2.10.1 RNA extraction.

RNA was extracted from 100mg of ground sample material. 2% β-mercaptoethanol was added fresh to the RNA extraction buffer [2% (w/w) CTAB, 2% (w/v) PVP-40, 100mM Tris-HCl pH8, 25mM EDTA, 2M NaCl] and was preheated to 65°C. 600µL of this solution was then added to each sample and incubated for 15 min at 65°C, vortexing every 5 min. The samples were then put on ice, 600µL of chloroform was added and each were vortexed and centrifuged for 15 min at 13,400rpm. The supernatant was transferred to a fresh tube and LiCl was added to a final concentration of 2M. The samples were then incubated at -20°C overnight. The next day, each sample was spun at full speed for 20 min, the supernatant was removed and 320µL of diethyl pyrocarbonate (DEPC) treated water and 20µL of β-mercaptoethanol were added to the pellet. Samples were vortexed and spun for 15 min at 13,400rpm. The supernatant was transferred to a fresh tube, an equal volume of phenol:chloroform was added to each and then they were vortexed and spun for 15 min at 13,400rpm. The supernatant was removed and transferred to a fresh tube and an equal volume of chloroform was added. Samples were then vortexed and spun for 15 min at 13,400rpm. The supernatant was transferred to a new tube and sodium acetate was added to a final concentration of 0.3M. 100% ethanol was added until the 1.5mL Eppendorf was full and then they were incubated at -80°C for 2h. The samples were then spun for 20 min at 13,400rpm, the supernatant was removed and the pellet then washed twice with 500µL of 75% ethanol, spinning for 8 min at 13,400rpm each time. The supernatant was removed and the pellet allowed to air-dry at room temperature for 5-10 min. Each pellet was then re-suspended in 40µL of DEPC treated water.

3.2.10.2 cDNA synthesis.

66ng of RNA was converted to cDNA using Moloney Murine Leukaemia Virus Reverse Transcriptase (Promega) according to manufacturer's instructions. A negative control was carried out for each sample to determine if there was DNA contamination.

3.2.11 Potential infection of basidiospores on detached live roots (mature and young) of oil palm seedlings

3.2.11.1 Basidiospore inoculation on cut roots

Basidiospore concentration for attachment studies was adjusted by dilution with sterile distilled water to give a final concentration of 10^6 spores ml except where otherwise stated. A total of five mature (brown) and young (white) roots of 18 month old oil palm seedlings were cut and washed three times with sterile distilled water to remove any soil debris before treated with sodium hypochloride for 5 mins. Sterile distilled water was used to remove any sodium hypochloride residue on the roots three times. The roots were inoculated with 200 µl of either spore suspension (10^6 ml^{-1}). After 24, 48, 72 and 96 h incubation in 27°C the roots were fixed and subjected for SEM observation as described below.

3.2.11.2 Preparation of samples for Scanning Electron Microscopy (SEM)

Preparation of samples was conducted in a fume hood. Samples were collected and fixed in 3.5% glutaraldehyde (agar scientific) in 0.05 M piperazine-N, N '-bis (2- ethanesulfonic acid) (PIPES) buffer at pH 8.0 to fix proteins. Tissue was then cut into 3x3x1 mm pieces and exposed to vacuum

for 16-20 h to extract trapped air. Samples were then viewed using 'low temperature scanning electron microscopy' on a JEOL SEM6310 model scanning electron microscope fitted with an Oxford Instruments cryotrans 1500 system attachment.

3.2.12 Assessing the control potential of Trichoderma in petiole vessels

3.2.12.1 Preparation of *Trichoderma* spore suspensions

Spores were collected from 7-day-old PDA plates of *Trichoderma*. Plates were flooded with 10 ml of sterile distilled water (SDW) and spores were scraped from the surface and dispersed with a sterile plastic spreader. 1 ml of this suspension was added to 9 ml of SDW in a centrifuge tube. Tubes were vortexed to ensure thorough mixing. A spore count was conducted with a haemocytometer then serial dilutions were carried out until a count of approximately $5x10^7$ spores/ml was achieved (exact counts are given with the relevant experiments).

3.2.12.2 Inoculation of cut oil palm petioles with *Trichoderma* spores and fungal re-isolation

0.5 ml of $5x10^7$spores/ml of spore suspension (<1 hour old) was applied directly onto freshly cut (sterile scalpel blade) surfaces of oil palm petioles using a pipette. The persistence of *Trichoderma* on the cut petioles and within the tissue was measured over an eight week period. Thee replicates were done each week for the two isolates used, TPP4 and T1-203. Two non-inoculated petioles were cut and left as controls. After inoculation, *Trichoderma* was re- isolated after 48 hours and then on a weekly basis. For re-isolation of *Trichoderma*, petioles were surface sterilised with 70% ethanol and transverse sections (TS) were cut with a sterile razor blade, every 5 mm down to 10 cm from the cut surface then placed onto *Trichoderma* Selective

Medium (TSM). Plates were incubated for 4-5 days at 25°C to observe characteristic growth.

3.2.13.3 Assessment of xylem vessel length

A suspension containing distilled water, eosin dye and fluorescent vinyl particles was prepared and applied to the cut surface of oil palm petioles. The suspension was added to the cut surface of five oil palm petioles at 15 min intervals maintaining an excess of suspension on the cut surface at least for 1 h. Thin sections were prepared every 1 cm using a razor blade and applied to slides for microscopy. Particles fluoresce red under UV and sections were examined on a Leica DMIRB microscope with fw4000 imaging software. Sections were viewed under an EVOS® fluorescence microscope using a 10X objective lens. The total number of vessels was counted under transmitted light. The number of vessels containing red vinyl particles was counted under red fluorescent light.

3.3 RESULTS

3.3.1 Root Infection and developing possible new inoculation techniques

3.3.1.1 Effect of inoculum size and root wounding on infection

Compared with the majority of fungal pathogens, *Ganoderma* infection requires a relatively enormous inoculum of colonised rubber wood blocks ranging from 6x6x12 cm to 3.5x3.5x3.5 cm for successful infection of oil palm seedling roots (Goh *et al.,* 2015). This procedure is laborious and involves a long period of incubation (4 weeks) to obtain fully colonized woodblocks. In addition, excessively high inoculum might overcome and therefore shield potentially useful resistance or tolerance. A smaller inoculum could clearly be advantageous for revealing useful resistance. Rees *et al*. (2009) reported successful infection using a smaller rubber wood inoculum (3x3x3 cm). The potential of reduced *Ganoderma* woodblock inoculum to induce infection on roots was investigated. Also it was necessary to confirm the pathogenicity of the isolate being used for this study and confirm that infection can occur via roots.

Rubber wood blocks (RWB) were cut with a table chainsaw to 2.5x2.5x2.5, soaked in a distilled water (overnight) and sterilised before inoculation with *G. boninense* mycelium in a polypropylene bag. Three 9 mm agar plugs were cut with a sterile cork borer from the advancing edge of 5 day old *G. boninense* cultures grown on malt extract agar (MEA) and plugs were placed over the RWB aseptically. Rubber wood was used as an inoculum source as these induce greater infection of seedlings than colonised oil palm wood (Rees *et al.,* 2007). Fully colonised RWB were attached individually by parafilm for direct contact with the roots of 15 month old seedlings. Uninoculated blocks were placed in contact with oil palm seedling roots by parafilm as positive control.

There were two treatments: 1) colonised rubber-wood blocks attached to wounded roots, 2) colonised rubber-wood blocks attached to unwounded roots. For each treatment five 15-month old palm seedlings were used and five spatially separated roots were inoculated with *Ganoderma* on each palm. Plants were then re-potted into (8 L) polybags and grown at mean 28°C in a glasshouse. Examination of inoculated roots and monitoring for symptoms of BSR was made monthly. Infection was clearly demonstrated from the small inoculum source in this study. First symptoms were observed after 4 months (Fig. 4) when wounded inoculated seedlings began displaying characteristic one–sided mottling of fronds. Levels of infection increased over the subsequent seven months until 100% of seedlings were affected and died. Based on disease incidence (DI), infection rapidly progressed in wounded roots with 60% infection over 6 months compared to 40% infection in unwounded roots. *G. boninense* PER71 progressed rapidly through the wounded roots at an average of 3.9 cm/month (Fig. 5). Thus, a small inoculum source could successfully induce infection of oil palm root and use of colonised blocks against wounded roots was shown to have greatest efficacy for infection.

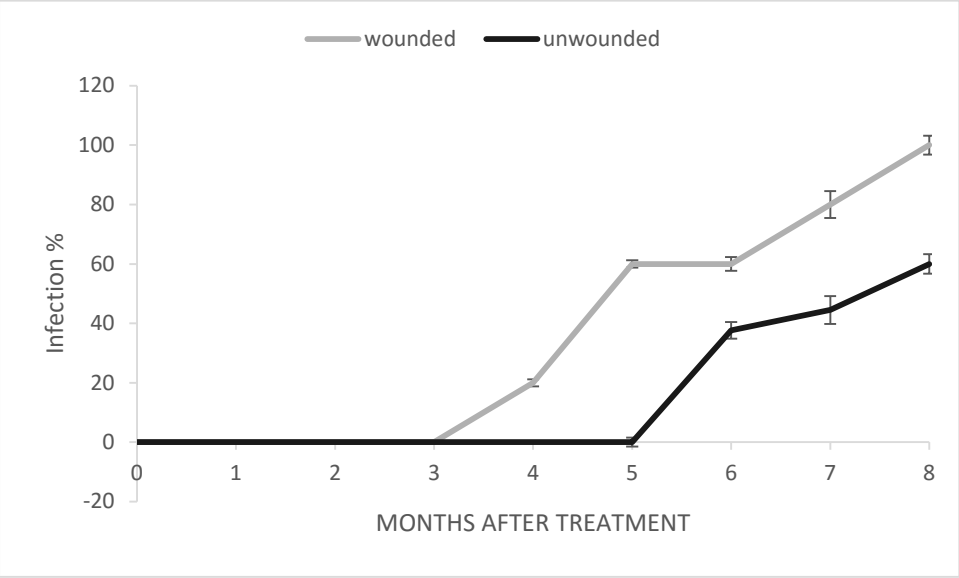

Figure 4. Basal stem rot disease development of oil palm seedlings using small rubber-wood block inoculum in the presence and absence of root wounding. Error bars represent standard deviation of means disease infection of five inoculated seedlings.

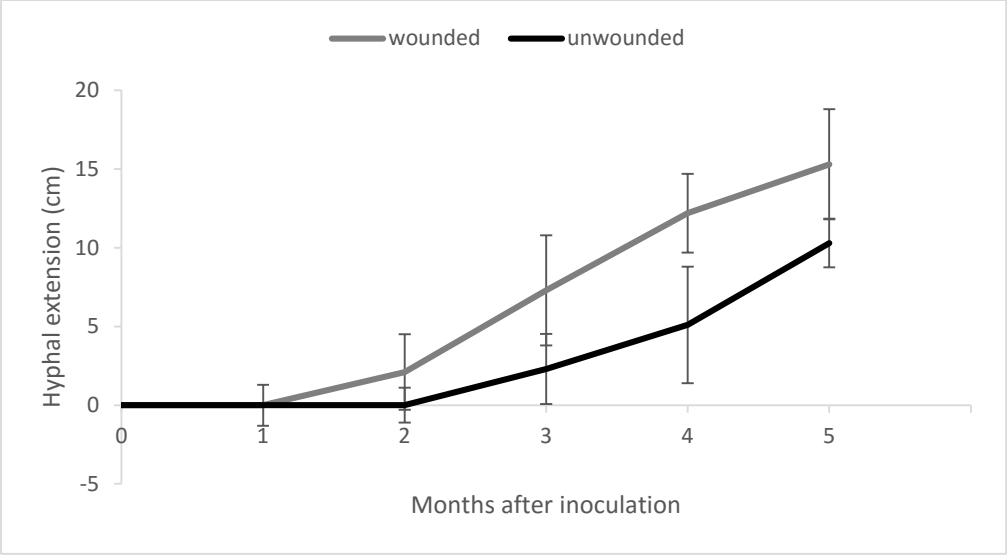

Figure 5. Advance of infection within infected roots of oil palm seedlings in the presence and absence of root wounding. *G. boninense* Per71 progressed along the root and after 5 months infection have progressed to the bole. *G. boninense* disease progression was followed by reisolation of infected root with *Ganoderma* selective medium (GSM).

Examination at root level revealed that hyphae grew around the surface of roots (Fig. 6) and mycelial growth resulted in the wood blocks becoming firmly attached to roots. From the point of inoculation, *G. boninense* grew steadily throughout roots associated with tissue degradation. Severely infected tissue was evident by brown discoloration of the cortex. The advancing edge of infections was determined by use of GSM to re-isolate *G. boninense*. Sometimes infection could be seen as *G. boninense* emerged from the infected tissue by breaking through the epidermis (Fig. 6B).

Infected bole tissue appeared necrotic and the perimeter of this region was often dark brown and has been termed the ‘reaction zone’ (Rees *et al*., 2009). Immediately in front of the infected area is a small area of yellow tissue, termed the ‘yellow zone’. Heavy colonisation of the basal tissue interrupts the vascular system, presumably leading to water stress and foliar symptoms.

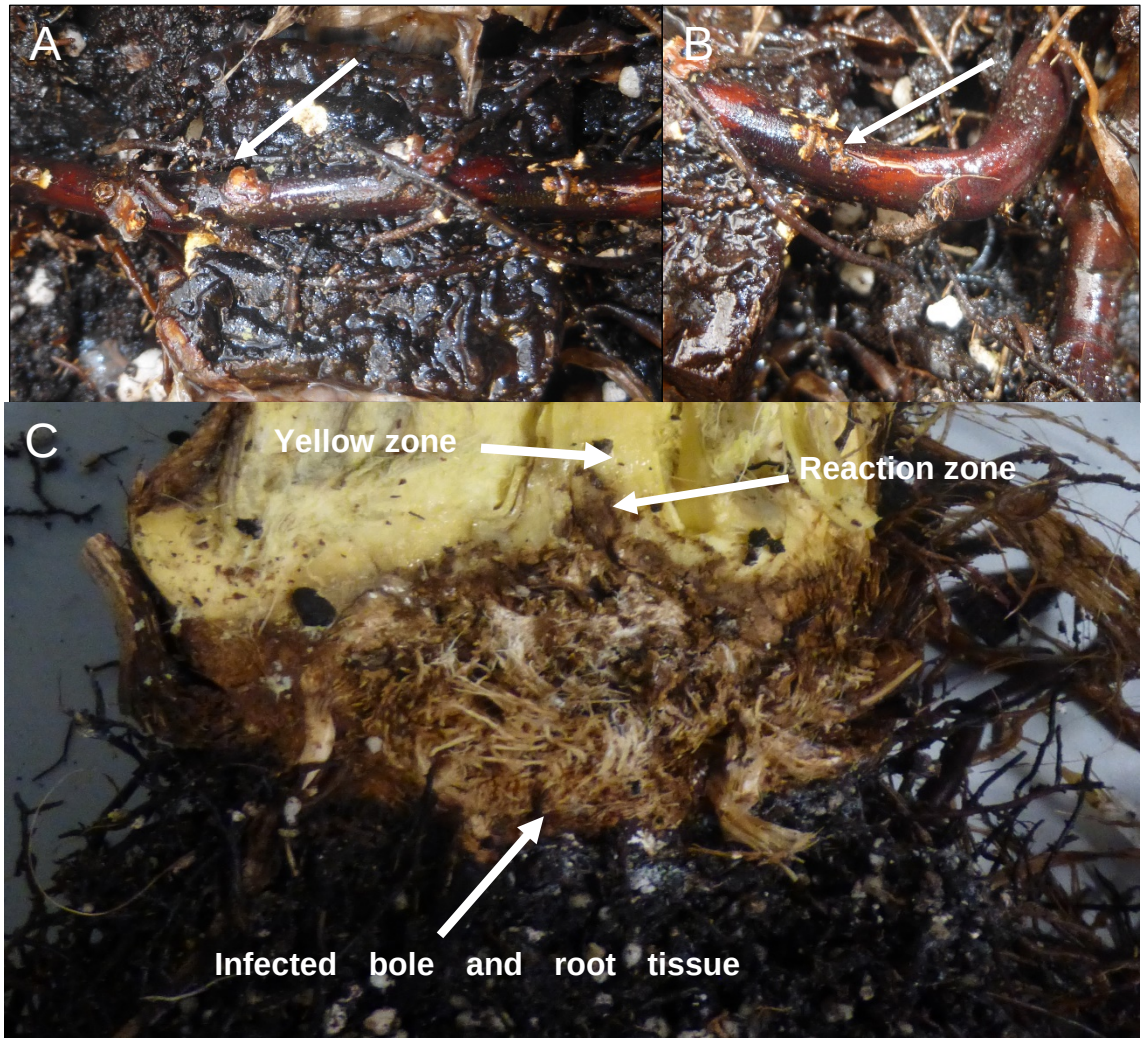

Figure 6. Infection of oil palm seedling roots and manifestation of disease in the bulb by *G. boninense*. A. Rubberwood block inoculum firmly attached to an oil palm root with mycelial growth around the root (arrow). B. *G. boninense* mycelium protruding through the epidermis from infected roots. C. Advance of the fungus into the bulb tissue. Tissue appears rotting (brown) and the perimeter of this region is often a dark brown termed the 'reaction zone'. Immediately in front of the infected area is a small area of yellow tissue termed 'yellow zone.

3.3.2 Effect of alternative inoculum source on root infection

Current root infection technique with rubber-wood block is labour intensive and time and space consuming. Also the availability of rubber wood is not always guaranteed. Hence, to overcome this situation, wheat grain was selected as an alternative substrate for inoculum. Wheat grain has been used widely as the inoculum substrate for pathogenicity testing of root rot fungal pathogens such as *Rosselinia necatrix, Armillaria spp., Fusarium spp., Rhizoctonia solani* (Mansila *et al.,* 2002). Furthermore, wheat grain can be prepared rapidly, it is easy to handle and is always commercially available.

In this study, the potential of wheat grain as an inoculum to induce infection was tested. Two different techniques were developed to test the effectiveness of wheat grain inoculum: 1) wheat grain (loose) and 2) wheat grain on a capillary mat used in horticulture (water holding capacity mat, Lantor). 1 cm agar plugs were cut from the advancing edge of 5 day old *G. boninense* Per71 grown on MEA and a single plug was used to inoculate 30g of wheat grain in 200 ml flasks until fully colonised. Alternatively, a plug of growing *G. boninense* mycelia was used to colonise wheat grain over a capillary mat in a petri dish (150 mm). The capillary mat formed a support base where the colonised wheat grain closely attached by the intermingling of hyphae with the mat fibre structures. Manipulation of the inoculum size on the mat could be easily cut and attach to palm roots using parafilm. Three millilitres of sterile distilled water was added weekly to maintain moisture content of the wheat grains and high RH. In addition, *G. boninense* rubber wood block inoculum was prepared as described previously and used as a control. These were then used to inoculate healthy 15 month old oil palm by attachment or direct contact to individual roots.

For inoculation, colonised wheat grains on capillary mats were cut into squares (3x5cm) with approximately 15-25 adhering grains and were attached directly to individual roots with parafilm. Wheat grain growing in a flask was shaken beforehand and approximately 30 g were placed around the palm roots 10-15 mm below the bole. All roots were washed twice with distilled water to remove soil and debris prior to inoculation. For each technique there were six treatments: 1) colonised wheat grains attached to wounded roots, 2) colonised wheat grains attached to non-wounded roots, 3) colonised wheat grain on capillary mat attached to wounded roots, IV) colonised wheat grain on capillary mat attached to non-wounded roots, v) colonised rubber-wood blocks attached to wounded roots (control). The degree of infection of *G. boninense* and the development of the disease were assessed monthly for 8 months.

Inoculated wheat grains and infested wood blocks induced infection within 8 months post-inoculation with associated external and internal symptoms (Fig.7). The experiment highlighted the potential of wheat grains to cause infection when 30 g of colonized wheat grains was applied, with more than 60% disease severity externally and internally. Capillary mat with low amount wheat grains failed to induce infection. RWB showed highest disease severity (>80%) among the treatments. These results indicate the potential of wheat grain as an alternative inoculum to RWB for achieving BSR infection, such as required when screening for resistance or studying host-pathogen interactions.

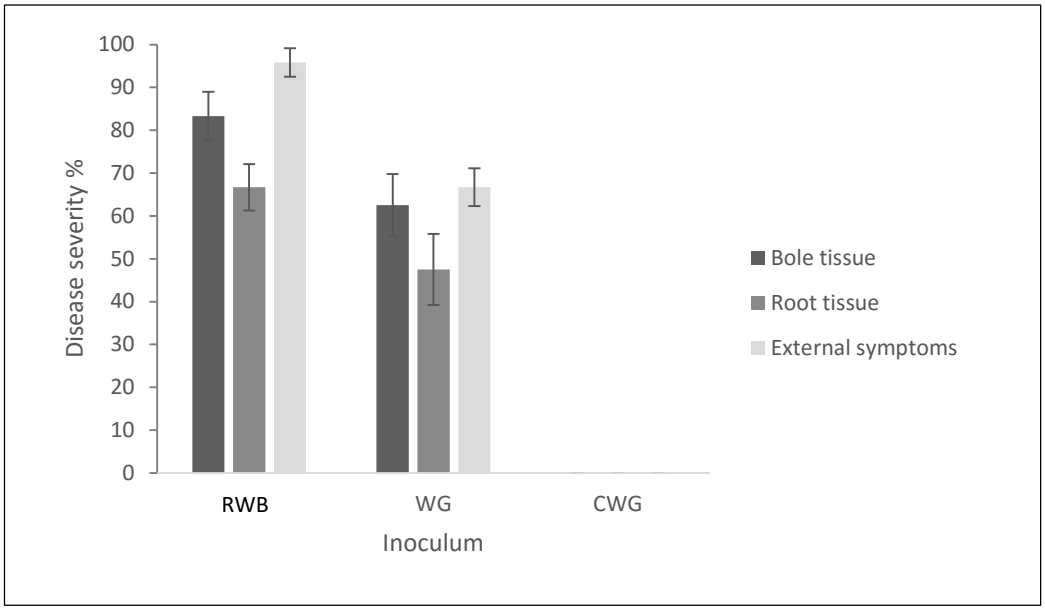

Figure 7. Basal stem rot disease development in oil palms inoculated with inoculum source after 8 months. Values are mean of 6 replicates and bars represent standard deviation. Control=positive control inoculation with RWB inoculum; WG= Inoculation with wheat grain inoculum CWG= Inoculation with capillary mat with wheat grain inoculum.

3.3.3 Direct inoculation of lower stem (bulb) to enhance infection rate

Ganoderma disease progression following root infection is slow, only reaching the bole of seedlings months after inoculation. Direct infection of palm bole tissue as an alternative inoculation technique was therefore assessed. There are precedents for by passing root infection and obtaining similar ranking of resistance or susceptibility, such as with *Verticillium* of cacao (Resende *et al.,* 1995).

Three inoculum sources: 1. Wheat grain, 2. Beech wood dowel (BWD), 3. Rubber wood dowel (RWD) were used in this study. All Inocula were soaked overnight in sterilised distilled water before autoclaving. For inoculation, sterilised beech wood dowels (BWD) and rubber wood dowels (RWD) (ca. 6 mm diam., 30 mm length) were immersed in malt extract broth for 2 h and then

placed on sterilised petri dishes. One cm agar plugs of PER71 grown on MEA for 7d were used to inoculate the dowels. Wheat grain inoculum preparation was as described above (section 3.2.2 and 3.2.3). Inocula were incubated at 28°C, 70% RH for 10-14 d for complete fungal colonisation. Oil palm boles were drilled below the meristem tissue (approximately 1 cm above ground level) and inocula were inserted carefully into pre-drilled holes. The drill bit was surface sterilised with 70% ethanol after each drilling. The hole was covered with parafilm to protect from airborne external inoculum sources.

There were four treatments: 1) BWD inoculated into bole 2) RWD inoculated into bole 3) Wheat grains inoculated into bole 4) Uninoculated bole (drilled bole covered with parafilm). All inoculated and uninoculated seedlings were watered twice daily. The oil palm plants were observed monthly for symptoms and the occurrence of root and bulb tissue rot were determined after 8 months. All inoculum sources failed to induce infection in bole tissue and all inoculated and uninoculated bole tissue remained healthy (Fig. 8).

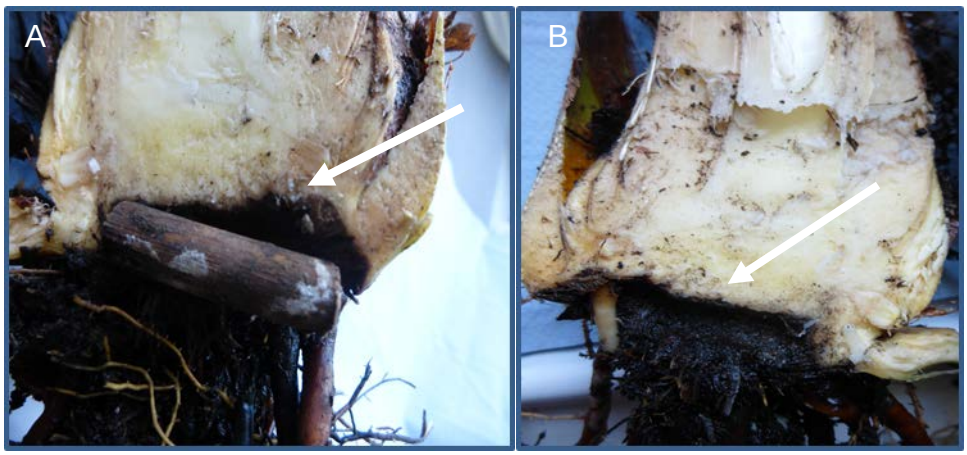

Figure 8. Alternative inoculation of oil palm bole showing no infection. (A) Inoculated bole tissue after 8 month dowel inoculation. Extracted piece of dowel in the bole showing presence of white mycelia of *G. boninense* (arrow). (B) Uninoculated bole tissue (control) remained healthy 8 month after inoculation. Longitudinal sections of drilled bole showing bole tissue remained healthy (arrow).

3.3.4 Mycelial growth of *G. boninense* in Malaysian soils and organic debris from plantation debris

3.3.4.1 Growth of *G. boninense* mycelia in soils

Ganoderma boninense has often been reported to be a soil-borne pathogenic fungus (e.g. Susanto *et al*., 2005) and the disease is established when mycelia come into contact with the roots of the oil palm. This dogma was investigated by studying *in vitro* growth of *Ganoderma* in soils collected from Malaysia.

Two type of soils (Munchong and Temang series) obtained from Malaysia Palm Oil Board (MPOB) and Felda Plantations were used. Half of each soil type was used directly and half was first sterilized (autoclaved 1 h at 121°C) before dispensing into 50 ml pre-sterilised tubes. Five Malaysian *G. boninense* isolates from BSR basidiocarps were obtained from MPOB and FELDA (Table 2.1, Chapter 2). *G. boninense* isolates grown on sterile wheat grains was used as inoculum to provide a sufficient nutrient base and approximately 2-4 g colonised wheat grain was placed at the bottom of each tube.

All non-sterilized soils were unable to support mycelial growth of *G. boninense*. In contrast, sterilized soils were colonized by all *G. boninense* strains, visible as characteristic mycelium emanating from the grain inoculum source at the base of tubes (Fig 9). Strains PER71 and FM were more vigorous in both soils with colonization ranging from 3.0 – 5.0 cm after 12 d incubation; UB strains showed lower colonization rate in both soils ranging from 2.5 – 3.0 cm (Fig. 10 and Fig. 11).

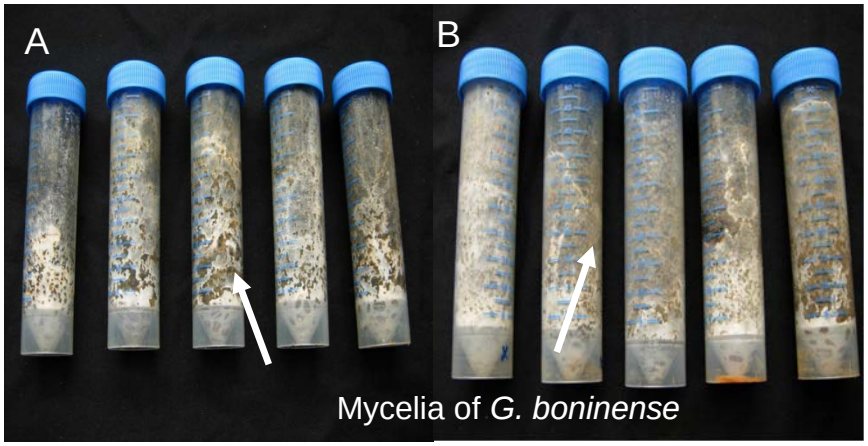

Figure 9. Colonization of *G. boninense* mycelia in sterilized MPOB (A) and FELDA (B) soils.

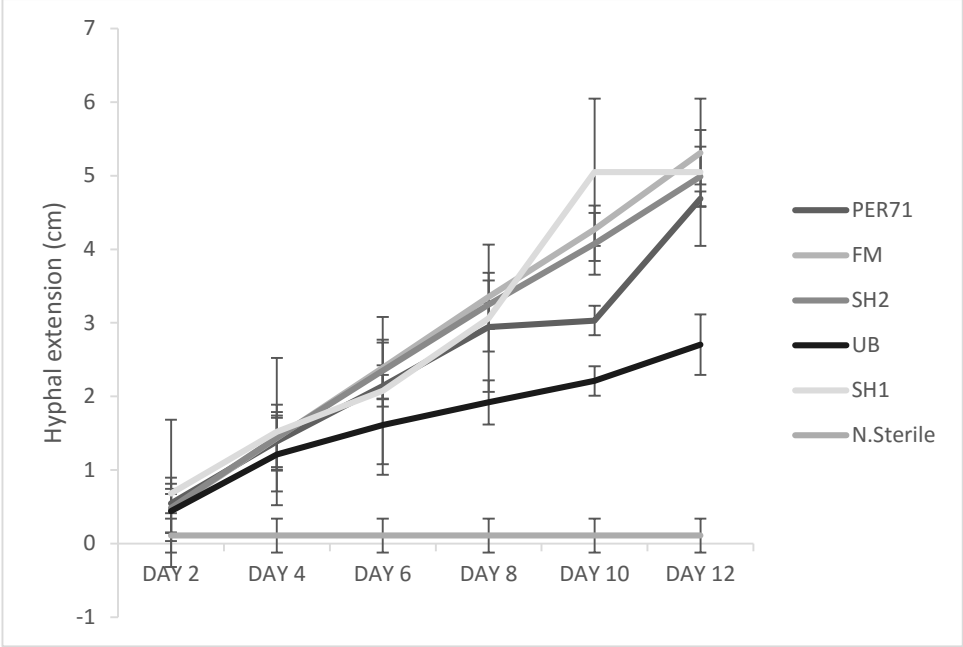

Figure10. Growth of five *G. boninense* strains in sterilized MPOB soil. Error bars represent standard deviation of mean from 5 replicates. Key represents *G. boninense* strains isolated from different location in Malaysia. Strain PER71= *G. boninense* stock culture from Malaysia Palm Oil Board, FM= Felda mempaga oil palm plantation, SH2= Serting hilir 2 plantation, UB= Ulu Belitung plantation, SH1= Serting hilir 2. N.sterile= not sterilize MPOB soil.

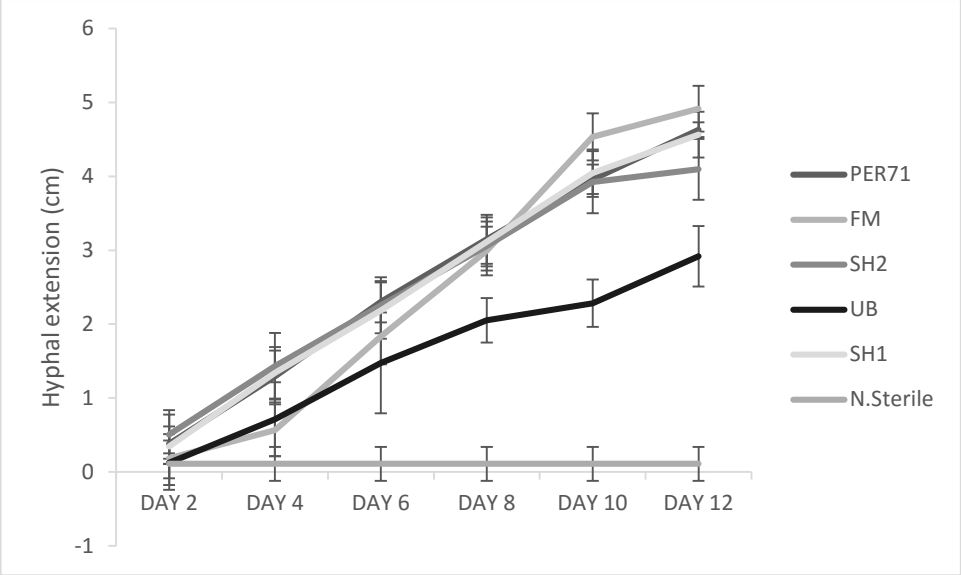

Figure 11. Growth of five *G. boninense* strains in sterilized FELDA soil and non-sterilized soil. Error bar represent standard deviation of mean from 5 replicates. Key represents *G. boninense* strains isolated from different location in Malaysia. Strain PER71= *G. boninense* stock culture from Malaysia Palm Oil Board, FM= Felda mempaga oil palm plantation, SH2= Serting hilir 2 plantation, UB= Ulu Belitung plantation, SH1= Serting hilir 2. N.sterile= non-sterilsed FELDA soil.

3.3.4.2. Growth of *G. boninense* mycelia in frond debris (FD)

There is an abundance of dead frond debris (FD) of oil palm on the soil surface in plantations. This FD could be a potential substrate for *Ganoderma* to establish an inoculum from mycelium or spores for infection. *In vitro* growth of *Ganoderma* was attempted in FD collected from three Felda plantations, Malaysia (locations SH, MAOKIL, UB) in Fig. 13 legend. Half of each FD was autoclaved (1h,121°C) before dispensing into 50 ml pre-sterilised tubes. *G. boninense* grown on sterile wheat grains was used as inoculum to provide a sufficient nutrient base. Non-sterilized FDs were unable to support mycelial growth of *G. boninense* Per71. In contrast, all sterilized FD were colonized rapidly, visible as characteristic mycelium emanating from the grain inoculum source at the base of tubes (Fig. 12), having progressed ca. 6-7 cm by 8d (Fig. 13).

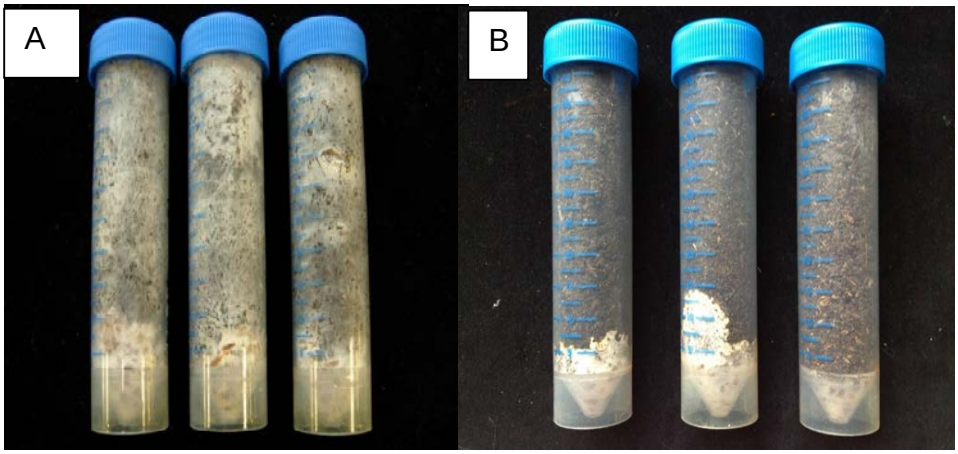

Figure 12. Colonization of *G. boninense* PER71 in frond debris (FD). A) *G. boninense* colonised sterilised FD and B) No growth in non-sterilized FD.

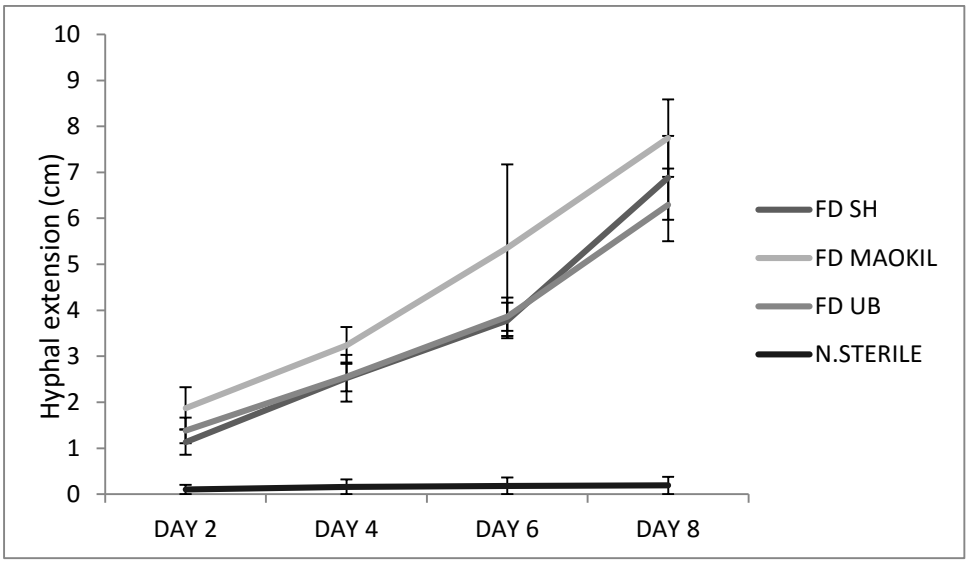

Figure 13. Growth of *G. boninense* PER71 in dead frond debris from three locations. FD SH= Frond debris Serting Hilir; FD Maokil=Frond debris Maokil; FD UB= Frond debris Ulu Belitung.N.Sterile indicates non-sterile FD, the others were sterilized. Error bar represent standard deviation of mean from 5 replicates.

3.3.5 Survival of *G. boninense* inoculum soil

Ganoderma boninense appears to be a poor competitor against other saprotrophic microflora as evident by its inability to spread from a nutrient base in plantation soil or in frond debris found on the soil surface of oil palm plantations. Growth only occurred on sterilized substrates. This raises a question as to how does *G. boninense* survive in the soil long term, if indeed it can do this. This ability is a potentially critical part of its epidemiology.

Non-autoclaved soil was placed directly into 50 mL pre-sterilised tube and inoculated with *Ganoderma*-colonized wheat grain, as described above, and incubated for more than 4 months. As in previous experiments, minimal or no growth occurred in the soils. After 4 months, melanized *G. boninense* mycelium was observed in the soil at the base of tubes corresponding to the original grain inoculum. The melanised mycelium were re-isolated onto GSM every 4 months and it showed continued viability only in wheat grain and frond debris up to 48 months (Table 2). However in soil melanised hyphae ceased viability after 12 months. This suggests the melanisation of mycelium as survival mechanism for *G. boninense*.

Table 2. Reisolation and detection of *G. boninense* from colonised wheat grain Melanin structure in different media over a period of 4 years. Reisolation of colonised wheat grains were done with *Ganoderma* selective medium (GSM) by isolating melanin like structure.

Media	Month											
	4	8	12	16	20	24	28	32	36	40	44	48
Melanized mycelium of *G. boninense* colonised in Wheat grain	+	+	+	+	+	+	+	+	+	+	+	+
Melanized mycelium of *G. boninense* colonised in frond debris (FD)	+	+	+	+	+	+	+	+	+	+	+	+
Melanized mycelium of *G. boninense* in soil	+	+	+	-	-	-	-	-	-	-	-	-

Reisolation was done in five replicate

+ = DETECTED

-= NOT DETECTED

Sampling was done randomly over the colonised wheat grains every 4 month. Black melanised mycelium structures produce *G. boninense* mycelia when it remained viable.

3.3.6 Basidiospore viability, survival and infectivity

Basidiospores are speculated to provide potential inoculum to infect directly via wound colonisation or indirectly following colonization of palm debris in plantations. Also wind dispersal might facilitate long distance spread of the pathogen. The characteristics of spore survival and germination were therefore investigated. *G. boninense* basidiospores are only produced on infected oil palms grown in the tropics; it is not practicable to produce basidiospores in the UK. Therefore experiments were done either in UK using spores recently imported from Malaysia or using freshly collected spores in the Malaysian laboratory at UPM. For example in one instance after 45 days storage at 20°C or 2°C germination was reduced from 55% when first received

in UK to 2.9 and 2.4% respectively (data not shown). Therefore storage was not a viable option for experimental purposes.

3.3.6.1 Basidospore germination studies

Basidiospore samples were collected from multiple basidiocarps from different infected oil palms within Coalfield Estate, AAR Malaysia. Spores collected on petri dishes were air dried in a laminar hood for 2 h and then plates were sealed. All spore plates were transported in a triple secured box to University of Bath.

3.3.6.2 Effect of different media on basidiospore germination

Factors favourable to spore germination may not necessarily be favourable to mycelial growth. To ensure the repeatability and comparability of experimental results, it is essential to optimise conditions for germination. In this study, water agar (WA), malt extract agar (MEA) and *Ganoderma* selective medium (GSM) were tested for the best germination medium for basidiospores. Five day old collected spore samples from three basidiocarps of different trees were prepared in sterile distilled water to give $1x10^5$ spores/ml and plated on each medium then sealed and incubated at 27°C, in the dark. Observations were made after 24, 48 and 72 h.

Germination on the three media ranged from 2-55% (Table 3). High germination occurred on WA (50-55%) after 42 h, while only 3-5% germination was observed on GSM after 72 h. High contamination levels occurred on MEA. WA was used as the germination medium for all future experiments.

Table 3. Effect of different media on basidiospore germination

Media	Germination %								
	Basidiocarp 1			Basidiocarp 2			Basidiocarp 3		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Water agar	0	55	55	0	51	51	0	50	50
Malt extract agar	4	HC	HC	5	HC	HC	2	HC	HC
Ganoderma selective medium	0	0	4	0	0	3	0	0	5

Germinated spores from three different basidiocarps presented in mean of five replicate of plates. HC: High contamination; 0: No germination. Germination was based on mean of five replicate of plates

3.3.6.2 Effect of heat treatment on spore germination

The viability of some imported batches of basidiospores was very low 3-4% (data not shown) and it was considered that *G. boninense* might exhibit constitutive dormancy as a strategy for providing germination over a long period of time. Fungal spore dormancy has been described in several basidiomycetes such as *Coprinus radiates* (Mills and Eilers, 1973), *Agaricus bisporus* (Feofilova *et al.,* 2004). Germination of dormant spores can be induced by certain chemicals and heat-shock treatment (Feofilova *et al.,* 2003; Deacon, 2005a).

Spore prints from four basidiocarps from different palms were combined and $1x10^5$ per mL spore suspensions were prepared in 1.5 mL tubes and incubated in water baths at 35°C, 40°C, 45°, 50°C and 55°C. Basidiospores germination were considerably stimulated only in the case of 25 min and 60 min treatments at 45°C when germination increased from 4% to 13% and 3% to 12% respectively (Fig. 14). Increased heating time for 2 h at 45°C decreased the number of germinated spores up to four fold; spores failed to germinate after 4 h at 45°C. Increased temperature of 50°C and 55°C inhibit basidiospores germination completely.

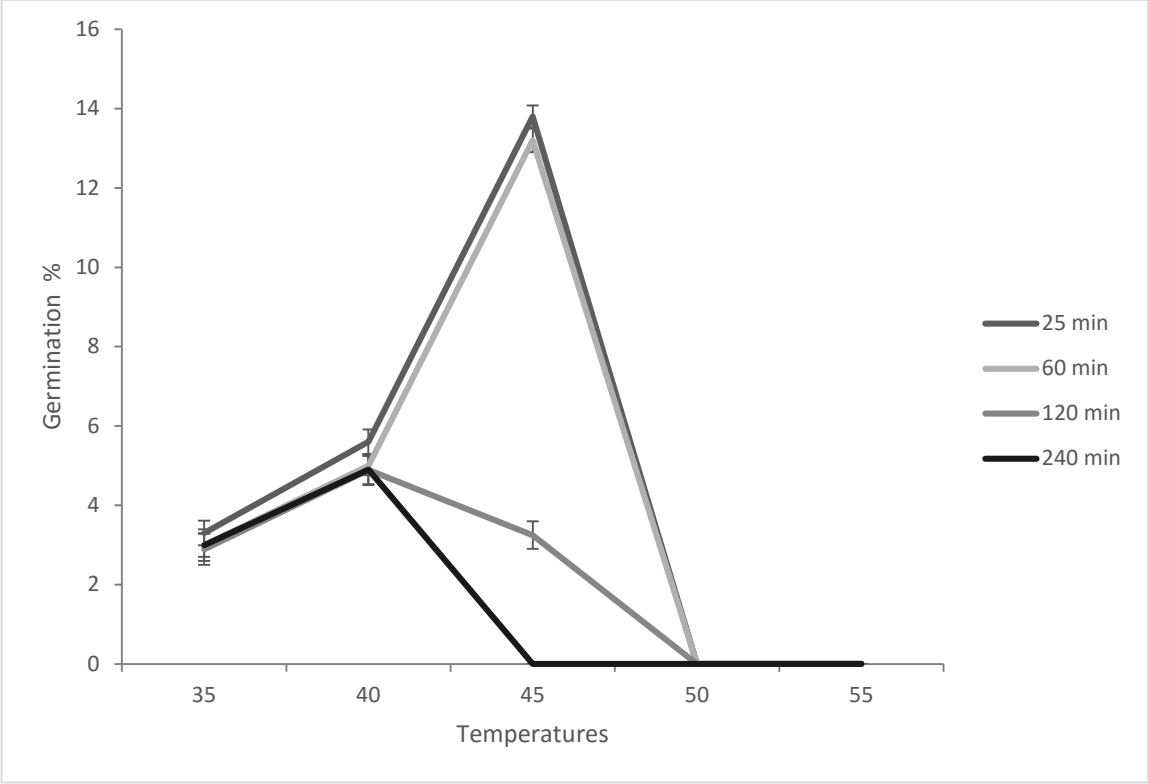

Figure 14. Effect of heat treatment and incubation time on germination of *G. boninense* basidiospores. Germination percentages based on three replicate plates.

3.3.6.3 Effect of UV on spore viability

Airborne basidiospores are produced abundantly in plantations (Rees *et al*. 2012). Oil palm cultivation receives typically 5-7 h per day of tropical sun. UV irradiation from sunlight could damage the basidiospore viability as they are dispersed by air currents and wind in the plantation from basidiocarps. In order to mimic possible conditions in the field, three basidiospore samples from spatially separated, infected trees were mixed on pre-sterilized petri dishes. The basidiospores were exposed directly to daylight, while unexposed spores were covered with aluminium foil in a glasshouse in Malaysia during April with average temperature ranges 28°C to 30°C and tropical weather conditions.

Basidiospores germinated after exposure to UV light (as natural daylight) (Fig. 15). However, viability rapidly declined, but was still more than 20% by 6 hours. The control (covered basidiospores) retained high germination throughout the trial period of 24 hours.

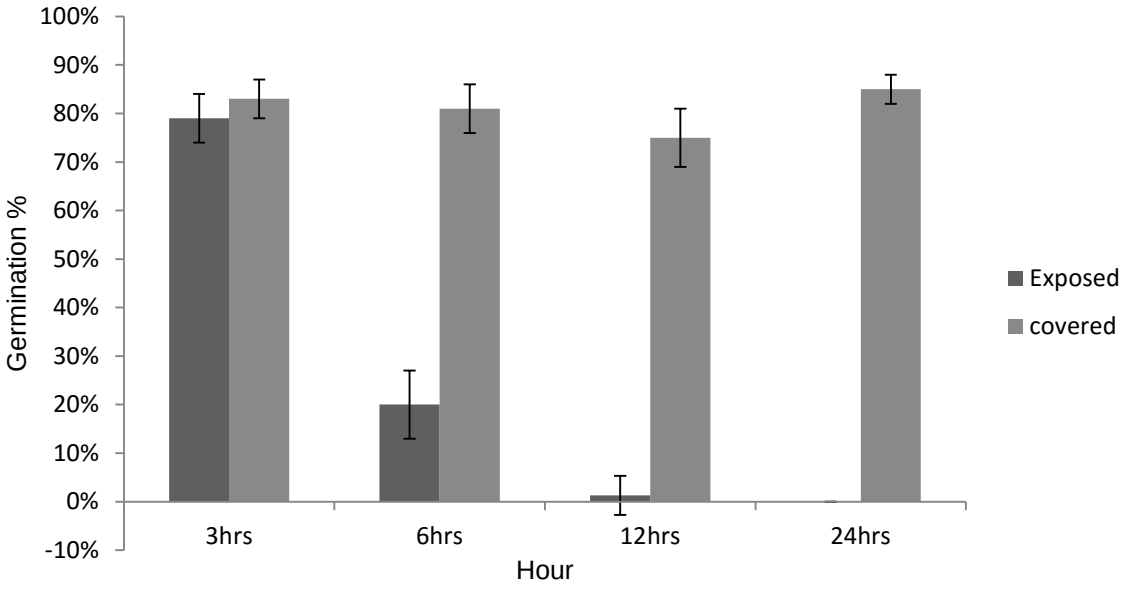

Figure 15.Influence of UV light (in Malaysia) on basidiospore germination. Error bars represent standard deviation of means of 5 replicate plates per treatment.

3.3.6.4 Effect of relative humidity (RH) on basidiospore germination

Hydration is an essential first step in spore germination. RH in Malaysian oil palm plantations is typically ~85-90% (Wahid *et al.* 2005). Therefore this study was conducted in Malaysia to examine the effect of RH on *Ganoderma* basidiospores. Basidiospores from different basidiocarps were collected at an AAR plantation (Coalfield Estate, Selangor, Malaysia) as described in section 3.2.5. Freshly harvested spore samples from three basidiocarps on different oil palms were combined and $1x10^5$ spores/ml suspensions were prepared in SDW, pH5.5. Approximately 1 mL spore suspension was pipetted and spread evenly onto water agar (WA) plates. Plates were then placed in desiccators at 27°C suspended over salt solutions to control RH at 33%, 53%, 75% and 97% in a moist chamber as described by Mandhavi (2009). Germination was observed after 30h, 48h and 72h.

Germination of *Ganoderma* spores was inhibited at lowest RH at 48 h and 72 h incubation and germinated less than 10% at 30 h incubation (Fig. 16). RH of 53% allowed spore germination (5%) only at 30 h, while basidiospores germinated more than 80% at the highest RH 95% and RH 75% after 48 and 72 h.

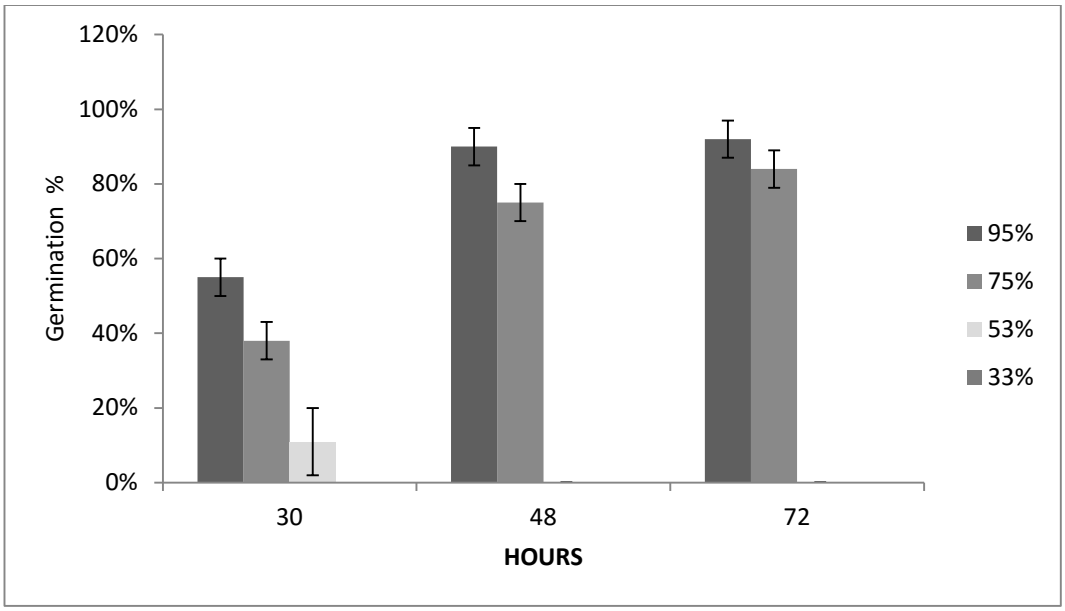

Figure 16. *Ganoderma* spore germination in different relative humidities (in Malaysia). Error bars represent standard deviation of means of 5 replicate plates.

3.3.7 Quantification of aerial basidiospores in Malaysian plantations

If basidiospores are important in disease aetiology, the quantity of and variation in basidiospores production within plantations requires monitoring. Rees *et al*. (2012) showed high numbers of aerial spores in BSR-affected plantations in Indonesia. However, quantification of basidiospores within plantations in Malaysia with different levels of BSR has never been determined.

In this study, air samples were taken from five oil palm plantations in four states of Malaysia; 1.Bt. Lintang estate (Kedah), 2. Sg. Jerneh estate (Pahang), 3. Coalfield estate (Selangor), 4. Sime Darby Banting plantation (Selangor) and 5. (Felda Kluang Plantation (Johor). These plantations were heavily affected by BSR with disease incidences ranges from 10% to 30 % (pers. comms. from estate managers). A total of five replicates of air samples were taken randomly around midday between oil palm planting rows within each plantation and

mean spore concentration/m^3 determined as described above using a Biotester.

High atmospheric basidiospore levels reflected *Ganoderma* basidiocarp frequency (Fig. 17). Basidiospore production of 3-5000 m^3 was recorded in all high incidence BSR plantations age 15 to 20 years old. Production of aerial spores in a Sime Darby plantation was low ca. 3 spores/m^3 despite high (16 to 20%, pers. comm.) BSR incidence within the plantation. Coincidentally, there was lack of basidiocarps observed within this plantation, which might be because of the soil mounding practise around infected trees' basal stems. These data indicate that there is an abundance of spores produced in oil palm plantations and they represent a potential inoculum source of BSR and USR

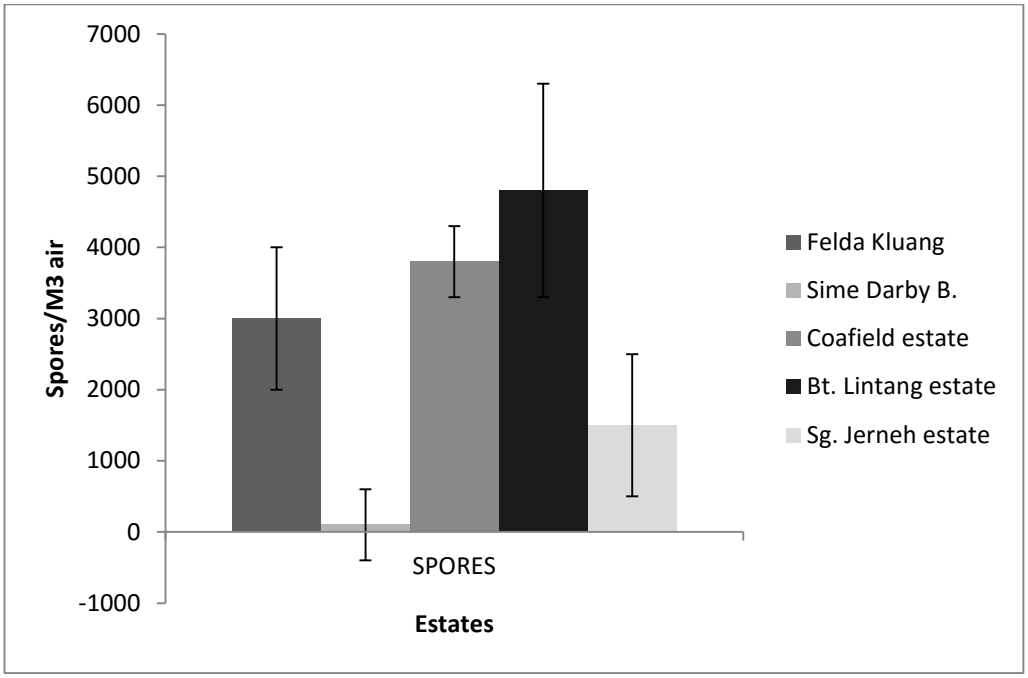

Figure 17. Aerial spore quantification of *Ganoderma* in oil palm plantations. Error bars represent standard deviation of mean of four samples at each plantation.

3.3.8 Colonisation of oil palm petioles by *G. boninense* basidiospore

All attempts to infect mature palms and seedlings with basidiospores have so far failed (Hasan *et al*., 2005; Sariah *et al*., 1994). Extensive wound sites are created by routine harvesting (severing fruit bunch peduncle) and pruning (general pruning of basal fronds plus of frond to free the fruit bunch), so this creates many potential extensive sites for spore infection. In this study oil palm seedling petioles were used to mimic wound sites in plantations.

Five day old basidiospores from five different basidiocarps on spatially separated, infected trees within a mature oil palm block were mixed, to encourage subsequent anastomosis and formation of potentially infective dikaryons, and applied concurrently to the cut surfaces of petioles. This study was conducted in the glasshouse in University of Bath, UK.

Petioles were cut by razor blade to leave a smooth, horizontal surface onto which 500µl of a suspension of $1x10^6$ spores/mL was immediately applied. Basidiospores were added immediately to cut petiole surfaces after wounding (three replicates per palm). Cut petioles were then covered with aluminium foil to protect the spores from UV and dehydration. Five oil palm seedlings aged one year old (commercial DxP) were used and three petioles from each seedling were assessed every 3 weeks by cutting 3 cm from the petiole surface and plating onto GSM.

Particles, including basidiospores applied to cut surfaces of petioles, get pulled in to xylem vessels as a result of negative tension in vessels (Rees *et al.,* 2012). A study of xylem functionality and of vessel length assessment (refer section 3.2.13) showed that vessels remain functional for at least 24h and that xylem vessel length of seedlings, as used in the research is between 3.5 and 5 cm. A study by Rees *et al.* (2012) using field palms showed some vessels extended to 10 cm. These properties will dictate 1) how long spores can be taken up after impacting on wounded petiole surface 2) what distance spores can travel within xylem by passive uptake within the first few hours.

Colonization beyond this length implies formation of an invasive (presumably dikaryotic) mycelium. Mean germination of basidiospores used in this study was 30% based on spores combined from 5 basidiocarps. The range of germination from those basidiocarps was 25-35% (data not shown).

3.3.9 Re-isolation of *G. boninense* from petioles

To identify how far into the xylem vessels that spores or subsequent mycelium had travelled, sections of petioles were re-isolated on to GSM (Fig. 18a-c), following each sampling. Petioles were sampled at weeks 3, 6 and 9 (and will continue to be sampled at later dates) by surface sterilizing with 90% ethanol before cutting 10 cm down the petiole from the initial inoculation site with a sterile blade. This was then divided into separate 1 cm sections that were analysed for the presence of *G. boninense.* Figure 19 describes an improved protocol for reducing bacterial contamination. Further re-isolation of putative *G. boninense* colonies on to MEA (Fig. 18d) was used to confirm that isolates were dikaryotic *G. boninense*, by the identification of clamp connections.

The presence of *G. boninense* in oil palm petioles is shown in Table 4. In the first sampling from week 3, the pathogen was identified in almost all of the first 6 cm of each petiole. However, the frequency of *G. boninense* presence decreased in both later samplings (weeks 6 and 9). Significantly, re-isolations from one of the week 6 petioles showed *G. boninense* present at 10cm distance along the petiole.

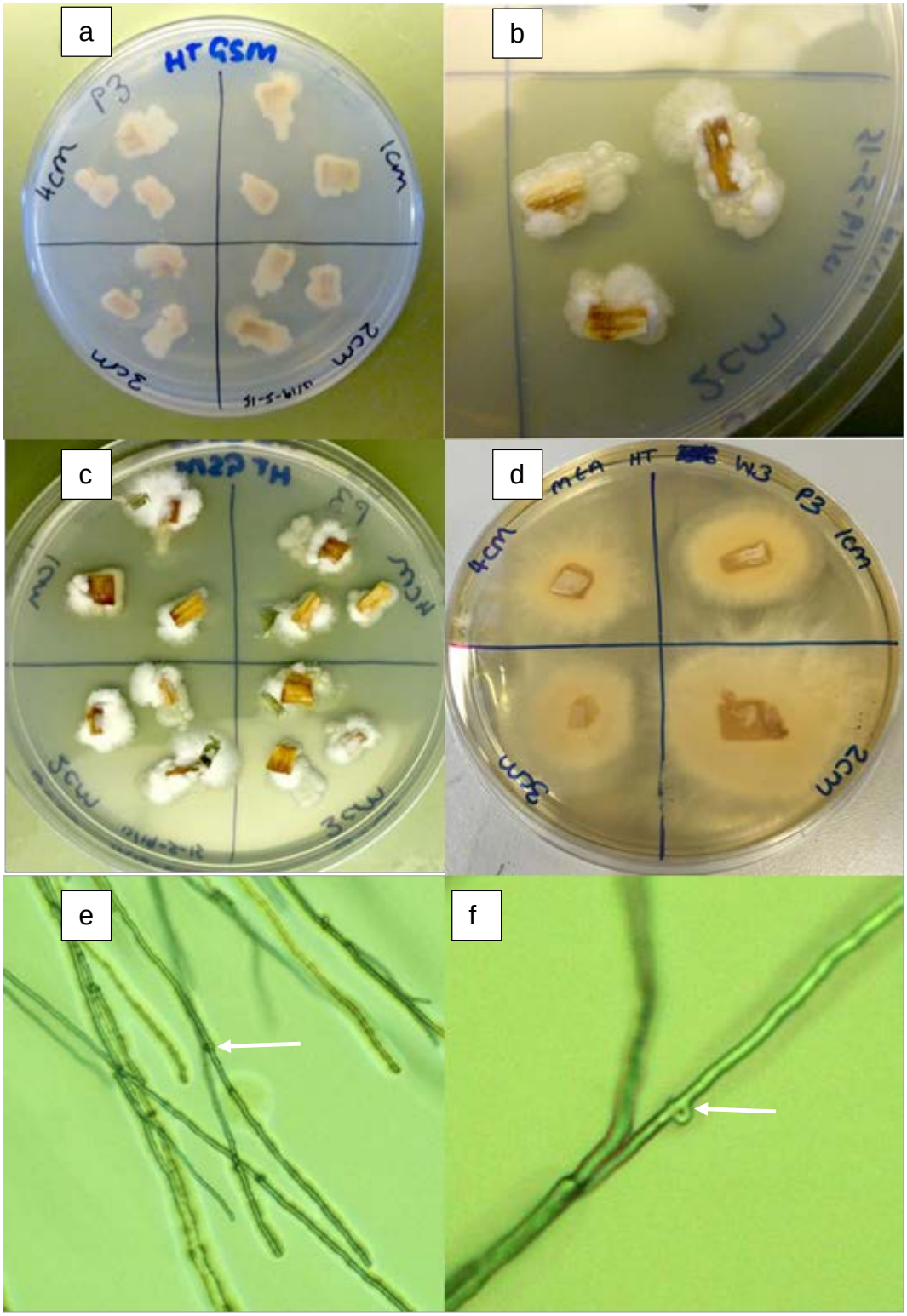

Figure 18. Representation of re-isolation of *Ganoderma boninense* from oil palm petioles. The figure shows a Week 3 re-isolation from 2 cm of the third petiole. a) Re-isolation of *G. boninense* on to GSM. b) Close up of *G. boninense* mycelial growth on GSM. c) Following re-isolation from GSM plate, showing *G. boninense* mycelia and cut away cubes used to re-isolate. d) Underside of re- isolation plate on MEA, in preparation for microscopic confirmation. e) Microscopic confirmation at 100X of clamp connections. f) Microscopic confirmation at 200X of clamp connections. Clamp connections indicated the presence of dikaryons and that anastomosis and nuclear exchange had occurred.

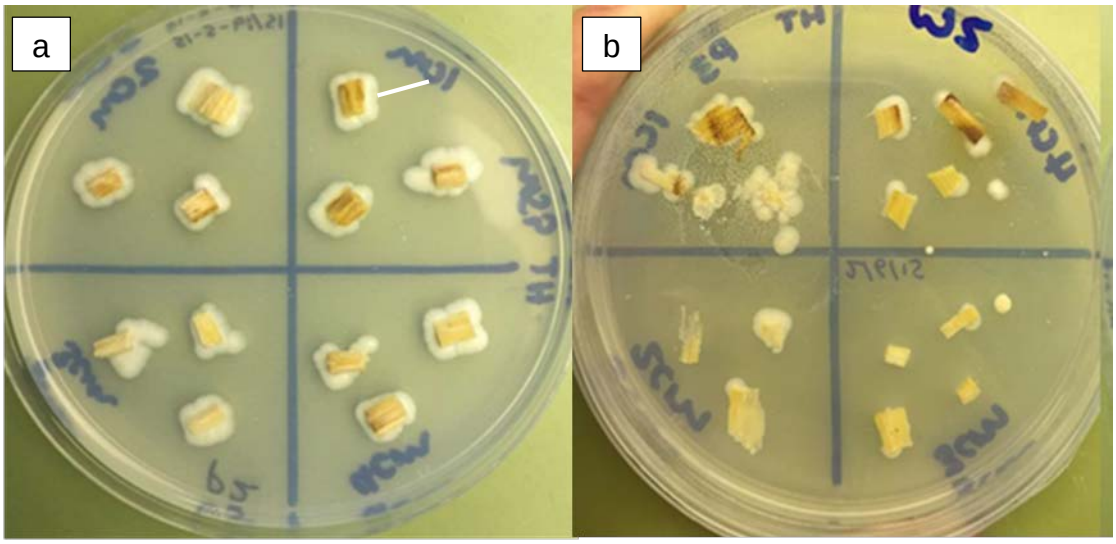

Figure 19. Optimisation of the oil palm petiole processing for re-isolation of *Ganoderma boninense***.** a) Bacterial contamination with the original method of leaving the epidermis of the petiole attached. b) Optimised method showing less bacterial contamination around pieces of petiole as the epidermis was first removed.

Table 4. Re-isolation of *G. boninense* from oil palm petioles. The presence of a tick in the cell indicates which week and petiole replicate that *G. boninense* presence was identified.

Week	Petiole Replicate	Presence of Ganoderma boninense in petiole (cm)									
		1	2	3	4	5	6	7	8	9	10
3	1	✔	✔	✔	✔		✔				
	2	✔	✔	✔	✔	✔	✔				
	3	✔	✔	✔	✔	✔	✔				
6	1	✔	✔	✔	✔						✔
	2	✔									
	3	✔	✔		✔	✔					
9	1	✔									
	2	✔									
	3	✔									

3.3.10 Development of a DNA detection system of *G. boninense* for petioles

Re-isolations of *Ganoderma* from spore-inoculated petioles onto GSM in this study, were laborious and problematic due to bacterial contamination from tissue samples. Moreover the germination of basidiospores on GSM is poor (see 3.3.8) and detection of *G. boninense* (presence or absence) on GSM was only possible after 14 days incubation.

Hence, it was considered that PCR-based detection might offer a more sensitive, reliable and fast detection procedure to determine presence of *G. boninense* in the inoculated petioles. PCR-based detection with the use of species-specific primers has been used widely for accurate identification and detection of pathogenic fungi in infected plants such as *Fusarium oxysporum*, *Heterobasidion annosum*, *Armillaria mellea* (Hantula and Vainio, 2003; Sicoli *et al.*, 2003). For PCR detection, Bridge *et al*. (2000) have developed a *G. boninense* specific primer, GanET for detection of *G. boninense* specific to oil palm. The combination of GanET and ITS3 (fungal universal primer) has been shown to be capable of detecting *G. boninense* DNA from oil palm tissue in the presence of other foreign DNA as well as oil palm DNA (Panchal and Bridge, 2005). Therefore, in this study PCR-based technique with *G. boninense* specific primers GanET and ITS3 (Bridge *et al.,* 2000) was developed for detection of *G. boninense* mycelium and basidiospores in inoculated petioles.

3.3.10.1 Optimisation of DNA extraction from *G. boninense* mycelia and basidiospore in the presence of oil palm tissue

PCR DNA detection of microbial pathogens is dependent upon extraction of products which are pure, intact and free of compounds that might inhibit amplification (Hushiarian *et al.,* 2013). However, DNA isolation of fungal pathogens from plants is usually compromised by excessive contamination by high amounts of polysaccharides, polyphenolic compounds and secondary metabolites that can interfere with DNA extraction and PCR amplification (Capoete *et al.,* 2012). These compounds often co-precipitate with fungal DNA thus reducing yield (Sarwat *et al.,* 2006).

In a preliminary study, PCR amplification failed to detect *G. boninense* mycelium in the presence of varying proportions of palm tissue (2, 5, 10, 90% w/w mycelium) based on the basic CTAB DNA extraction. Therefore, it was necessary to optimise the DNA extraction protocol for the detection of *G. boninense* in the presence of palm tissue. Optimization of DNA extraction was determined on two types of samples: 1) *G. boninense* mycelium with oil palm tissue, 2) *G. boninense* basidiospores with oil palm tissue. *G. boninense* was grown in a static liquid culture (MEB) for 5 days. Then mycelium was harvested by discarding all the liquid and washed three times with sterile distilled water before being weighed and cut to specific amount with sterile blade. Two year old healthy palm petiole was cut with a sterile blade and was used immediately or kept in a clean closed container at -20°C. The epidermis of the petiole was excised with a sterile blade to expose inner tissue prior to extraction.

Various proportions of *G. boninense* mycelium and oil palm tissue were weighed and combined (5, 10, 90% w/w mycelium) to reach a total of 100 mg and were subsequently ground into a powder with liquid nitrogen in a clean mortar and pestle. 100-mg of each sample were added to a series of 2-ml screw-cap plastic vials containing pre-heated (65°C) 500 µl modified CTAB buffer with β-mercaptoethanol and PVP-40 added. These components are effective for removal of plant phenolic compounds (PVP forms hydrogen bonds with the phenolic compounds) and is a strong reducing agent which can remove tannins and other polyphenols often present in the crude plant extract of DNA extraction (β-Mercaptoethanol). Suspensions were incubated at 65°C for 40 min in a water bath and subsequent extraction and DNA clean up procedure were done as described above. The purity and quantity of DNA was determined by nanodrop. PCR amplification of samples was undertaken using the primer GanET together with the universal primer, ITS3 (Panchal and Bridge, 2005). The reactions were undertaken as described in section 2.5.2.

Based on the above band detection, the modified CTAB-DNA extraction protocol was suitable to be used for the extraction of genomic DNA of *G. boninense* mycelium in the presence of palm petiole tissue. Bands were detected in all mycelial concentrations tested down to 5% (w/w) mycelium/petiole (Fig.20). The GanET/ITS3 primer pair amplified a 321 bp region of the ITS2 spacer from *G. boninense*.

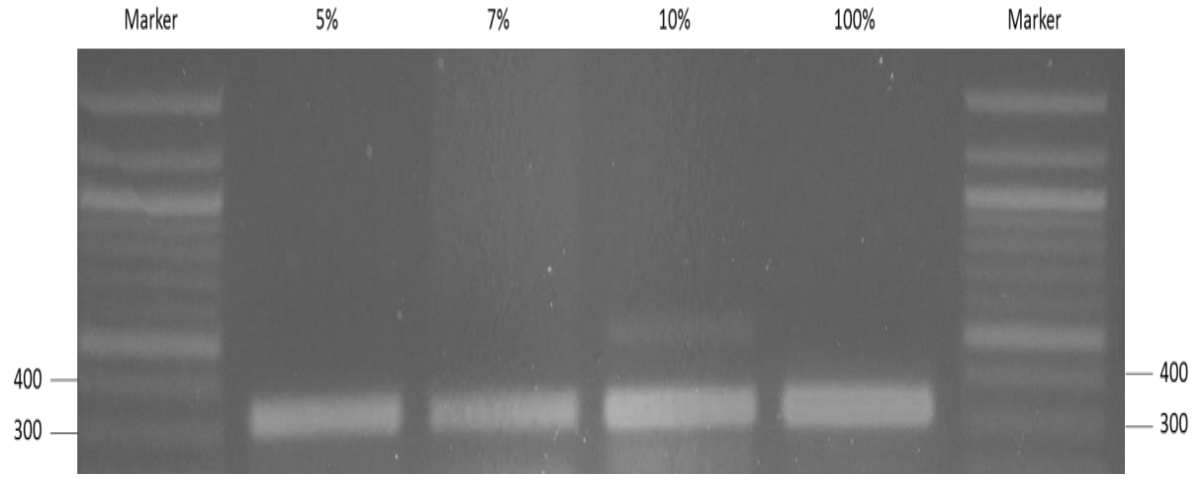

Figure 20. The modified DNA extraction protocol produces a *Ganoderma* specific primer product in the presence of high oil palm petiole concentrations. Percentages correspond to the total mycelial proportion in the final 100 mg used for subsequent DNA extraction steps. The remaining proportions were made up with oil palm petiole. 100% refers to a positive control with only mycelium present. A band was seen for all samples tested. No band was seen on negative control (palm petiole tissue only) (data not shown). A 100 bp DNA ladder (Promega) was used to determine the size of the band which ran between the 400 bp and 300 bp marker bands indicated on the figure.

The optimized CTAB-DNA extraction procedure was then used to optimize extraction of genomic DNA of *G. boninense* basidiospores, to detect basidiospores in the presence of oil palm petiole tissue. Spores from five different *G. boninense* basidiocarps were adjusted to give 100 µL of a $5x10^6$ spore suspension. The suspension was applied directly to a 0.5 cm sample of oil palm petiole (epidermis petiole excised with a sterile blade), then ground together with liquid nitrogen. 100 mg of resulting powder was then mixed with 500 µL of optimised CTAB buffer with a sterile ceramic beads (400-520 µm, Sigma) and vortexed for 60 sec.

In order to extract DNA exclusively from basidiospores, 100 µL of a $5x10^6$ spores/mL was pelleted by centrifugation (13,000 rpm, 5 min). Liquid nitrogen was then added directly to the pellet and once evaporated, the pellet was physically disrupted by a clean micro pestle (SIGMA) for 60 sec. Subsequently 500 µL of optimized CTAB buffer and sterile ceramic beads were added and vortexed for 60 sec. Both samples underwent DNA extraction and DNA clean-up followed by PCR amplifications as described above.

The optimized CTAB extraction protocol proved to be suitable for extraction of genomic DNA from basidiospores. A band at 321 bp was only visible, when basidiospores were present, with or without the presence of oil palm petiole tissues (Fig. 21). DNA homogenization with liquid nitrogen and subsequent beating with beads during extraction was shown to be sufficient for basidiospores extraction.

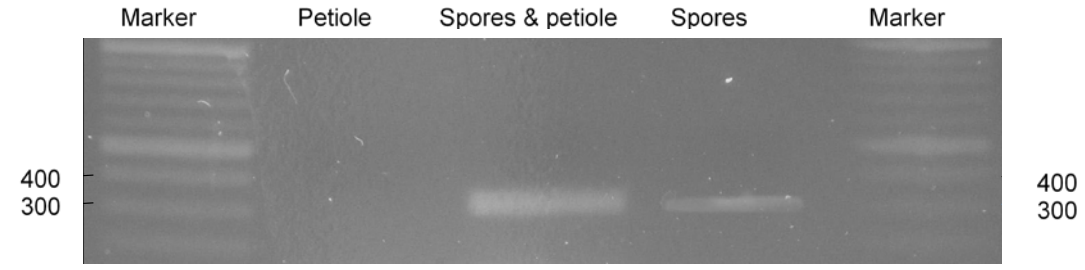

Figure 21. *Ganoderma*-specific primers can detect *G. boninense* spores. $5x10^6$ spores were added to a 0.5 cm sample of petiole tissues and ground in liquid nitrogen. The sample containing spores alone were powdered in liquid nitrogen bead-beaten in extraction buffer for 60 sec. A 321 bp band was only visible in the presence of basidiospores (with or without palm petiole). A 100 bp DNA ladder (Promega) was used to determine the size of the product indicated on the figure.

3.3.10.2 Determining the sensitivity of the detection system for *G. boninense*

Early colonization by *G. boninense* basidiospores will involve germination to form minute hyphae in the petiole xylem vessels. Sparse hyphae coupled with possible plant inhibitors of PCR may result in false negative detection; conversely, false positives can be caused by the presence of contaminating DNA or PCR products (amplicons) (Capote *et al*., 2012). Therefore, it was important to determine the sensitivity of detection to low levels of *G. boninense* in the presence of palm tissue for improved accuracy and reliability of detection.

3.3.10.2.1 Detection sensitivity of different concentration of *G. boninense* DNA

The minimum concentration of DNA that was detectable by the optimised PCR protocol was established using a serial dilution series of purified *G. boninense* DNA ranging in concentration between 1 ng/µL and 0.001 ng/µL. Five day old, *G. boninense* Per71 mycelium grown in MEB, was ground into a powder and 100 mg was re-suspended in 500 µL of previously optimised CTAB buffer. Subsequent DNA extraction and DNA clean-up was performed as above. Concentration of DNA was quantified by checking 1 µL DNA sample on a NANODROP. Sterilized milli-Q water was used to prepare the concentration series of DNA from a known amount of high quality *G. boninense* DNA. PCR amplification was performed using *G. boninense* specific primers GanET and ITS 3 as above. A specific *G. boninense* product 321 bp was detected in all the DNA concentrations tested from low to high (1ng/µL to 0.001ng/µL) (Fig.22).

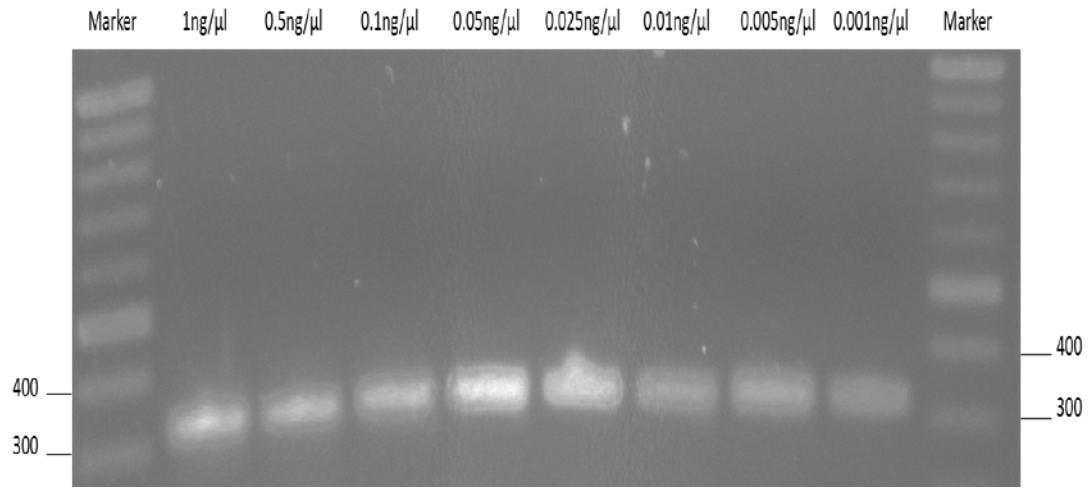

Figure 22. *Ganoderma*-specific primers are sensitive to all mycelial DNA concentrations tested. PCR was undertaken with decreasing concentrations of *G. boninense* mycelial DNA, which were detectable down to 0.001ng/µL. A 100bp ladder marker was used to determine the size of the bands which ran between the 400bp and 300bp marker bands as indicated on the figure. No band was detected in negative control (oil palm petiole tissue).

3.3.10.2.2 Detection sensitivity to low levels of *G. boninense* mycelium in the presence of palm tissue

Although PCR was capable of detecting low DNA concentrations of *G. boninense*, it was necessary to determine the sensitivity of the method at detecting *G. boninense* mycelium in the presence of oil palm petiole to establish its suitability for identifying early stages of infection of *G. boninense* in the petiole. To mimic trace amounts of hyphae within a palm petiole, *G. boninense* mycelium from a 7 d shaken culture (MEB) was homogenised to create a 0.1 g/mL suspension using a sterilized T8 ULTRA TURRAX® (IKA®) homogeniser. A concentration series of this homogenous mycelial suspension was added to 1 cm of petiole and then were ground into a powder. 500 µL of optimised CTAB buffer was added to 100 mg of each sample for subsequent DNA extraction and DNA clean-up as above. For samples of 5 mg and under, the reaction was carried out in triplicate to compensate for the irregularity of the homogenous mycelial suspension. A minimum of 5 mg (wet weight) of *G. boninense* mycelium was necessary to produce a band which ran between the 400 bp and 300 bp marker bands (Fig.23). For 5 mg of mycelium, a band was only visible for two of the three replicates.

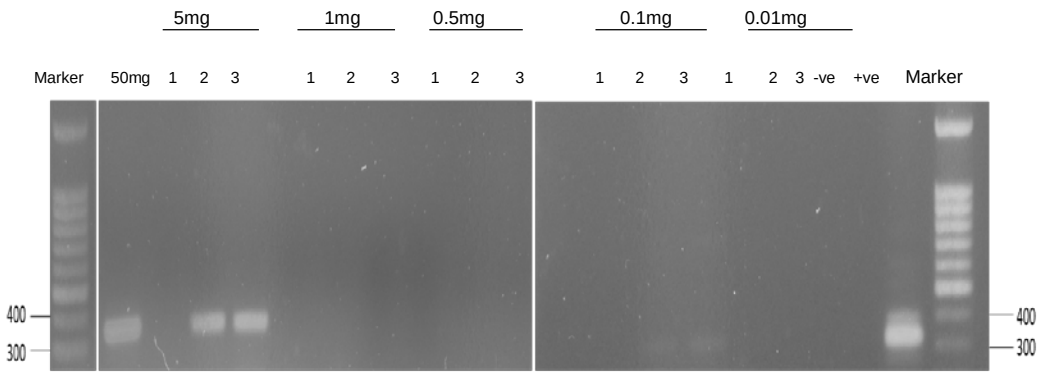

Figure 23. Detection system is relatively insensitive to low concentrations of *G. boninense* mycelium present in oil palm petiole tissue. Varying concentrations of mycelium together with 1cm of oil palm petiole were ground in liquid nitrogen. Samples with a concentration of 5mg and lower were carried out in triplicate to provide three independent biological replicates. –ve refers to the negative control containing solely petiole and +ve the positive control with just mycelium. Bands were detected down to 5mg of mycelium. A 100bp ladder marker was used to determine the size of the bands which ran between the 400bp and 300bp marker bands as indicated on the figure.

Having established the minimum detectable level of mycelium, it was equally important to determine how many infecting hyphae this would theoretically constitute. As depicted in Fig.23, the limit for this DNA-based detection method was 5 mg of mycelium per 1 cm (ca. 850 mg) of oil palm petiole. Thus the detectable ratio of the mass of mycelium per mass of petiole was 5:850. Assuming that their densities are equal and uniform, this ratio will be equivalent to the ratio of their cross-sectional areas (CSA). Thus the total space occupied by mycelium in the CSA of a petiole (65 mm^2) was established as 380,117 μm^2.

By knowing the CSA of a single hyphae (12.6 μm^2), measured using an Olympus BH-2 microscope, it was predicted that on average a minimum of 30,240 hyphae per petiole cross-section are required to enable detection. A representation of the space that this number of hyphae would occupy in a petiole can be found in Fig. 24; however in an infected petiole these would exist as single hypha, rather than the area as depicted.

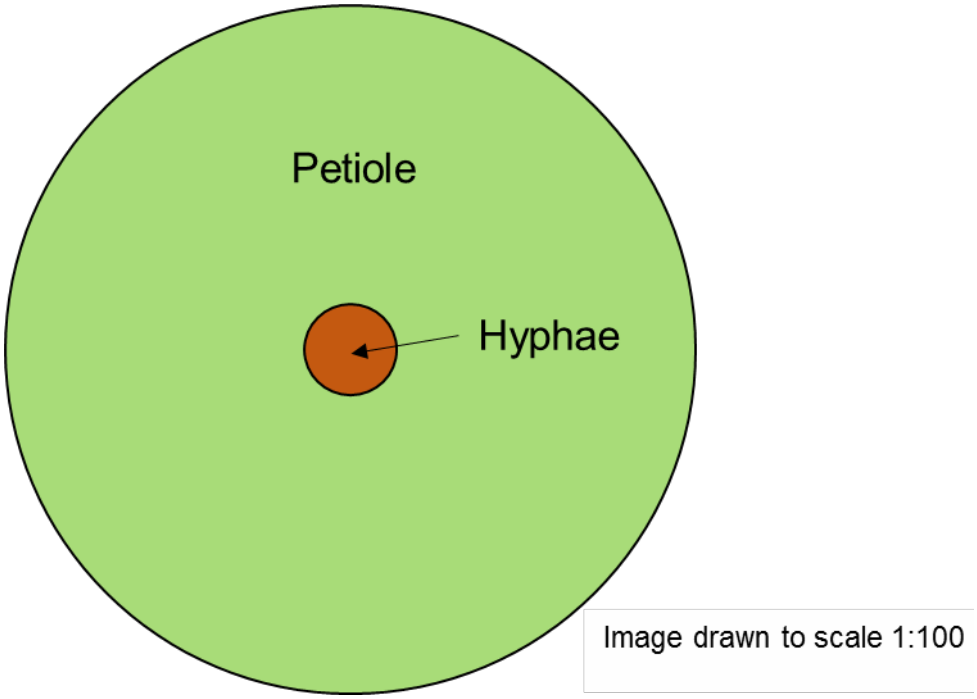

Figure 24. Total area of *G. boninense* hyphae necessary for detection in oil palm petiole. The total CSA that 30,240 hyphae (brown circle) would occupy in the CSA of an oil palm petiole (green circle). The image is calculated based upon the ratio of the minimum detectable mass of mycelium per mass of petiole.

3.3.10.2.3 Detection sensitivity of *G. boninense* basidiospores in the presence of palm tissue

A dilution series of *G. boninense* basidiospores was prepared in order to determine the minimum number of basidiospores that can be detected in a 1 cm palm petiole by the PCR method. *G. boninense* basidiospores from five different basidiocarps from five oil palms were combined and adjusted to $2x10^6$ spore/mL in SDW. Suspensions of spore dilutions down to $2x10^1$ spores/mL were added separately to 1 cm oil palm petiole tissues and subsequently ground in a mortar and pestle with liquid nitrogen. 100 mg of each sample was transferred into 2 mL screw cap tubes in 400 µL of modified CTAB buffer. DNA extraction, DNA clean-up and PCR amplification was done as above.

A minimum concentration of $2x10^5$ spores was necessary to form an amplification product of 321 bp product (Fig.25). No band was produced in any of the samples with lower spore amounts.

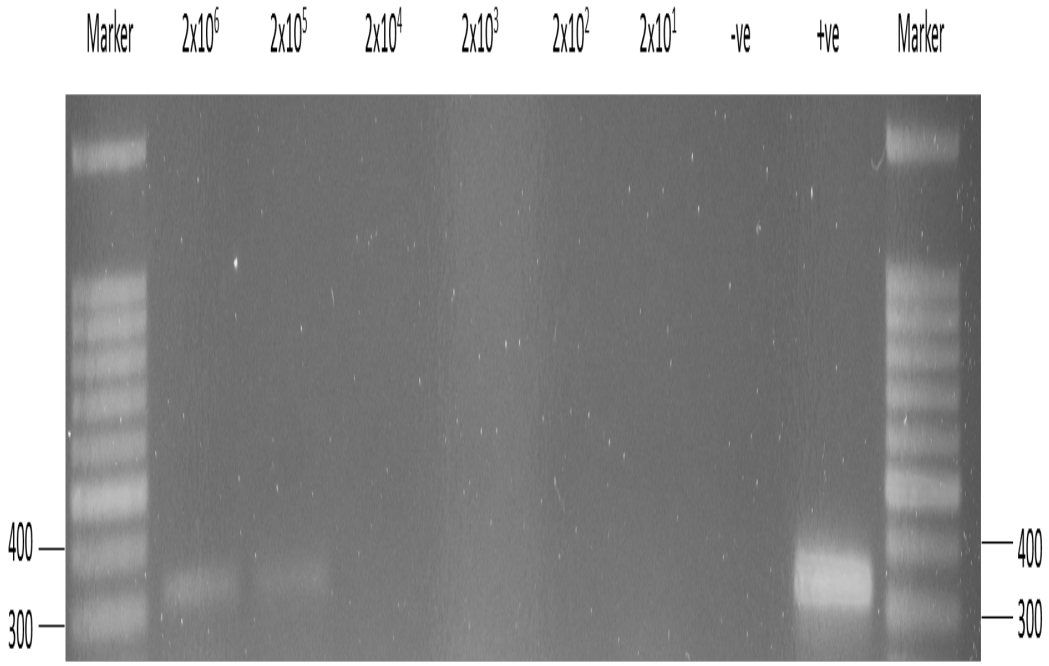

Figure 25. Detection system is relatively insensitive to low concentrations of *G. boninense* basidiospores present in oil palm petiole tissue. Varying concentrations of spores in suspension were ground together with 1cm of oil palm petiole. -ve refers to the negative control containing solely petiole and +ve the positive control with just mycelium. Bands were detected down to $2x10^5$ spores. A 100bp ladder marker was used to determine the size of the bands which ran between the 400bp and 300bp marker bands as indicated on the figure

3.3.11 PCR based DNA detection of *G. boninense* from basidiospores-inoculated petioles

The optimized DNA extraction protocol was used to detect *G. boninense* presence within inoculated petioles samples from experiment in section 3.3.9 Three replicate petioles were harvested at week 3 (7 cm) and week 6 (10 cm) (post inoculation). These samples were divided into 1 cm sections and stored at -80°C until use. Each section was subjected to DNA extraction. These petiole sections were then frozen in liquid nitrogen and ground to powder in a mortar and pestle. Approximately 100 mg of sample (powder) was extracted by the optimized DNA extraction protocol and PCR amplification was done as described above. Caution was taken not to let samples (stored at -80°C) thaw prior to extraction.

The procedure detected *G. boninense* presence in most petiole samples. A band was observed in all of the first to six cm samples of the three petioles sampled (Fig.26). The intensity of the 321 bp amplification band was varied (medium to low intensity) among the three petioles, while a very intense band was observed from DNA of pure *G. boninense* culture. Fig. 27 shows the presence of *G. boninense* in the 10 cm section in the six week inoculated petiole. Low intensity bands were observed from samples 1 cm to 4 cm and 10 cm. No amplification product was obtained in 4 cm to 9 cm sections (Fig.27).

These observations indicate that *G. boninense* spores were able to survive, germinate and colonize down the vessels to cross the vessel barrier towards the bole, where *G. boninense* could initiate BSR. Furthermore this PCR-based DNA detection method is more rapid and sensitive than detection by re-isolation for tracking the movement of *G. boninense* down the petiole.

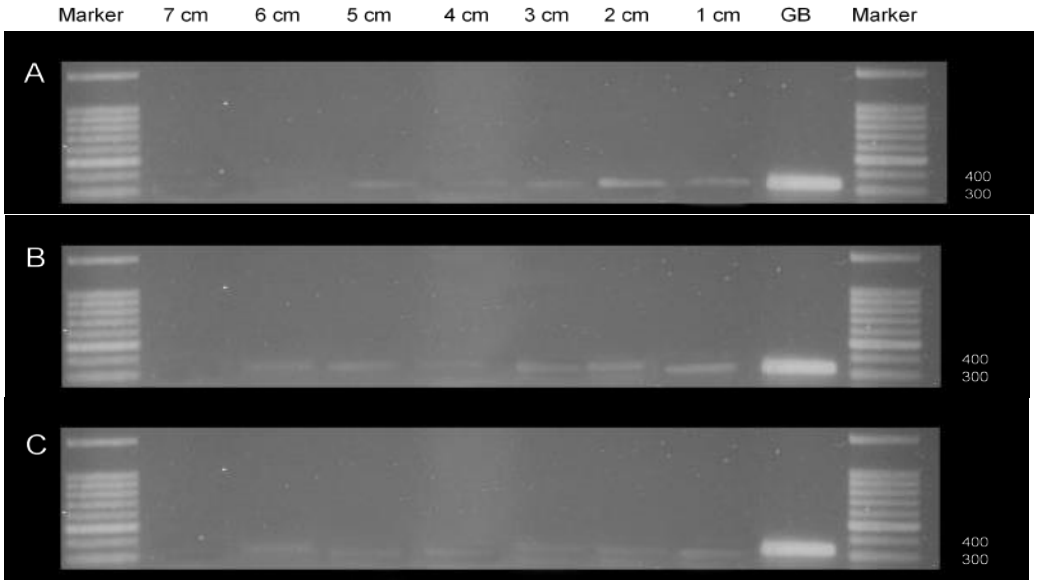

Figure 26. Detection of *G. boninense* colonisation 3 weeks after spore inoculation of oil palm petioles. 1 cm samples taken starting from the point of spore inoculation at the cut oil palm petiole up to 7 cm. Samples were taken from three different inoculated petioles (A, B, C) providing three independent biological replicates. Presence of *G. boninense* was detected from the first cm up to six cm of seven cm petioles. A 100 bp DNA ladder (Promega) was used to determine the size of the band which ran between the 400 bp and 300 bp marker bands as indicated. GB=Positive control of DNA of *G. boninense* mycelium.

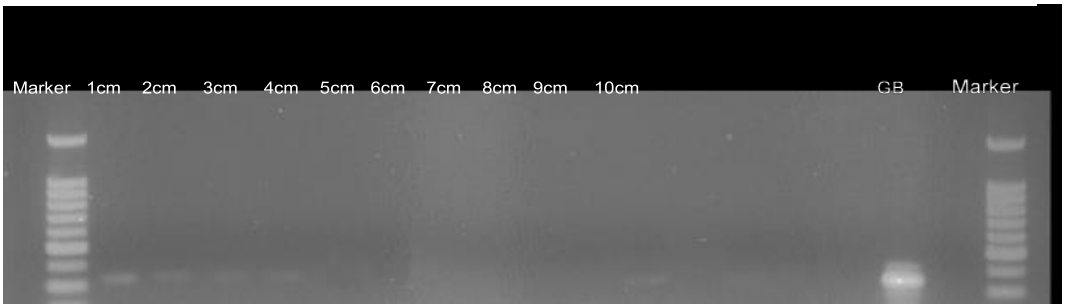

Figure 27. Detection of *G. boninense* colonisation 6 weeks after spore inoculation of oil palm petioles. 1 cm samples taken starting from the point of spore inoculation up tp 10 cm from one inoculated petioles. A 100 bp DNA ladder (Promega) was used to determine the size of the band which ran between the 400 bp and 300 bp marker bands. GB= Positive control of DNA of *G. boninense* mycelium.

3.3.12 RNA detection of viable *G. boninense*

Although PCR-based detection in this study proved to be suitable for detection of Ganoderma DNA in inoculated petioles, a major drawback is its inability to discriminate viable from non-viable fungal cells (Capote *et al.* 2012). Specifically, dormant *G. boninense* basidiospores, which are taken up passively and may not have germinated or are non-viable, could give a positive detection. Identification of *G. boninense* via the extraction of RNA and amplification of fungal RNA (reverse-transcriptase PCR) was attempted with the aim of exclusively detecting the biologically active and transcribing fungal cells.

The RNeasy Plant mini kit (Qiagen, Hilden, Germany), in combination with cell lysis by bead beating with ceramic beads, was used to extract total RNA from various samples. These were designed to test the sensitivity of the method and any possible interference by palm petiole tissues. 1) *G. boninense* mycelium (40 mg) with oil palm petiole (60 mg); 2) Ungerminated spores (40µL of a $5X10^6$/mL suspension) with oil palm petiole (60 mg), 3) *G. boninense* mycelium 100 mg, 4) Germinated *G. boninense* spores (40µL of a $5x10^6$/mL suspension) with oil palm petiole, and 5) Germinated spores ($5x10^6$). All spore suspensions for germinated spore treatments were plated on WA overlay with cellulose membrane for 48 h, average germination percentage was 45%).

ITS cDNA was not detected in non-germinated *G. boninense* spores. Moreover, ITS cDNA was amplified from germinating spore and mycelium samples with or without petiole tissue. None of the RNA samples had been contaminated with DNA prior to cDNA synthesis (Fig 28). These results indicate the technique does discriminate between viable and non-viable *G. boninense* (spore or mycelium), is not affected by palm tissues and should accurately reveal in petioles the presence of actively colonizing *G. boninense.* However this RNA detection method failed to amplify/detect any *G. boninense* from week 3 and week 6 samples of inoculated petioles (refer to inoculated

section 3.3.9). Further optimization of the method and sensitivity of the method need to be determined for it to be effective as a detection tool for *G. boninense* in palm tissues.

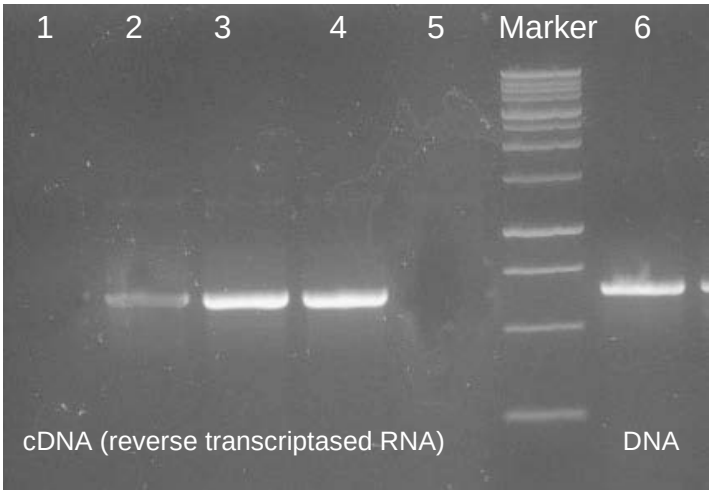

Figure 28. Agarose gel electrophoresis of ITS cDNA from 1. Ungerminated spores with petiole, 2. Germinated spores only, 3. *G. boninense* mycelium with petiole, 4. *G. boninense* mycelium only 5. Germinated spores with petiole 6. Negative control of ungerminated spore by DNA detection. The marker used is the PROMEGA 100 bp DNA Ladder.

3.3.13 Colonisation of petiole (frond) bases in a Malaysian plantation

Mature oil palm fronds were used to further determine the potential of basidiospores to colonize oil palm under field conditions. Basidiospores were collected from four basidiocaps on spatially separated, infected trees within a mature oil palm block. The spores were mixed and suspended in sterile distilled water (pH 5.0) to form a $1x10^8$ spores/ml suspension and were applied to fronds within 1 h. The viability of basidiospores from four basidiocarps ranged 55-73% based on a germination test on water agar (data not shown).

For inoculation of basidiospores, fronds were cut by machete to leave a smooth, horizontal surface onto which 5 ml of the spore suspension was immediately applied. Cut frond bases were then covered with a plastic bag to prevent removal of spores by rainfall and to maintain high RH. After 6 months, treated fronds were cut up to 10 cm. Five trees of the same age and from the same plantation were used and one frond from each tree was assessed.

G. boninense was detected on GSM from cut fronds after 6 months inoculation (Fig. 29a). Presence of *G. boninense* was detected at 2 and 8 cm and confirmed microscopically by the presence of clamp connection (Fig 29c-d). Further study could not be performed because of inadvertent pruning of these palms by UPM ground staff. Further confirmation by PCR DNA detection using *G. boninense* specific primers GanET and ITS3 (Bridge *et al.,* 2000) showed *G. boninense* DNA presence from the amplification product of 321bp in both samples (Fig.30).

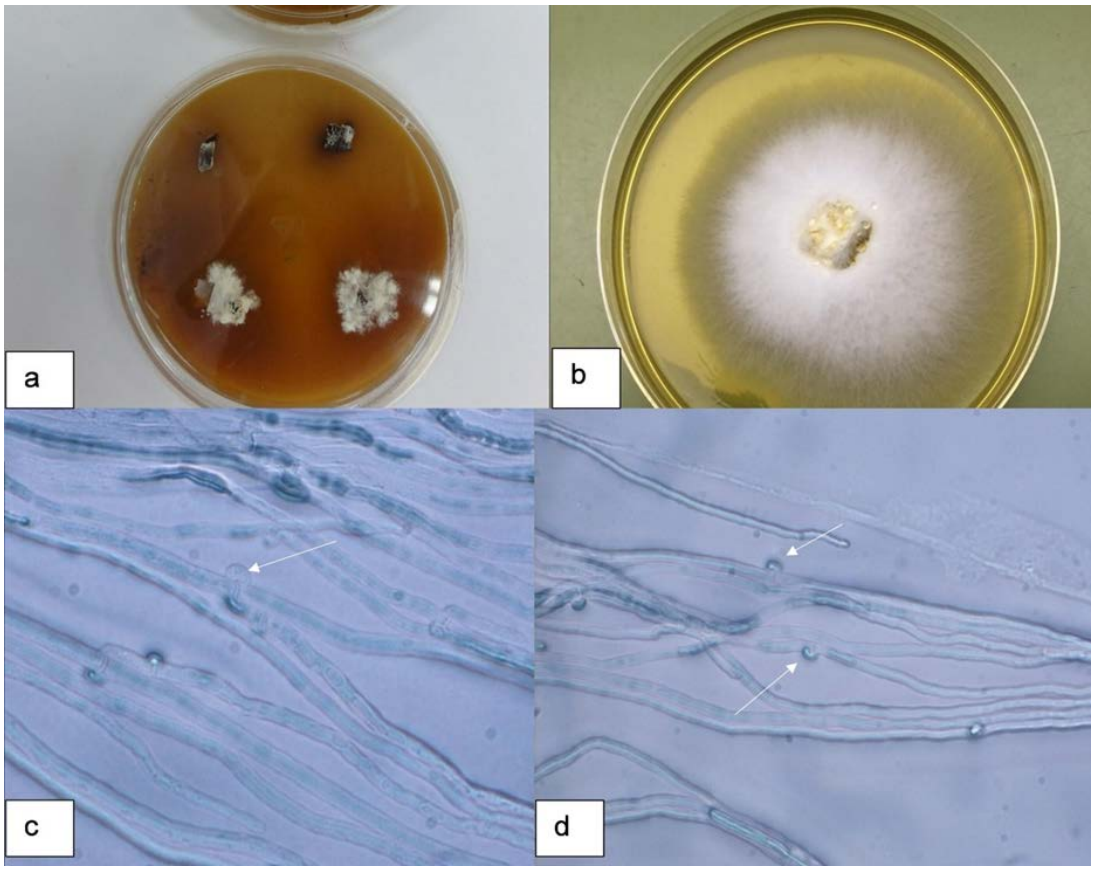

Figure 29. Re-isolation of *Ganoderma boninense* from oil palm frond at 2 cm (c) and 8 cm (d). a) Re-isolation of *G. boninense* on to GSM. b) Following re-isolation from GSM plate, showing *G. boninense* mycelia and cut away cubes used to re-isolate. c) Microscopic confirmation at 200X of clamp connections at 2 cm. d) Microscopic confirmation at 200X of clamp connection at 8 cm from the surface of cut frond.

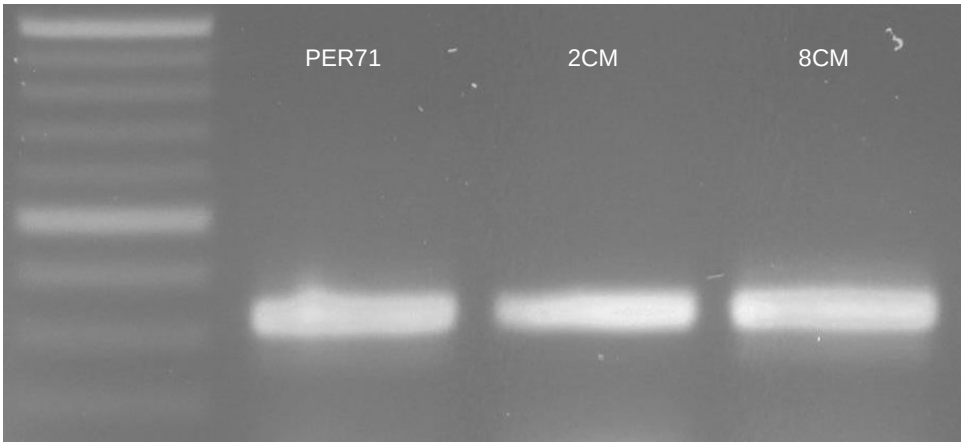

Figure 30. DNA detection of *G. boninense* colonisation at 2 and 8cm, 6 months after spore inoculation of an oil palm frond. A 100 bp DNA ladder (Promega) was used to determine the size of the band which ran between the 400 bp and 300 bp marker bands as indicated on the figure. G. boninense-specific PCR primers ITS3/GanET were used and amplification product of 321bp were observed in *G. boninense* Per71 (+ control) and samples (2 and 8 cm).

3.3.14 Assessment of xylem vessel length in oil palm petioles

To determine how far basidiospore may be drawn into severed xylem vessels, eosin dye and fluorescent particles were used to provide an estimate of xylem vessel lengths in oil palm seedling petioles (15 months old).

Addition of eosin dye to a wounded petiole surface allows visualization of functional xylem vessels (Fig. 31). The dye can be observed progressing into vessels and is not limited by the presence of end walls. End walls serve to stop the progression of microbes within xylem tissue and consist of secondary wall permeated by pits where only primary wall has been laid down (Rees *et al.* 2012). In addition, Table 5 shows that the number of functional vessels decreased over time until none were translocating dye after 48 hours. The dye travelled progressively shorter distances along the vessels over time from 204 mm at 0 hours to 41 mm after 24 hours.

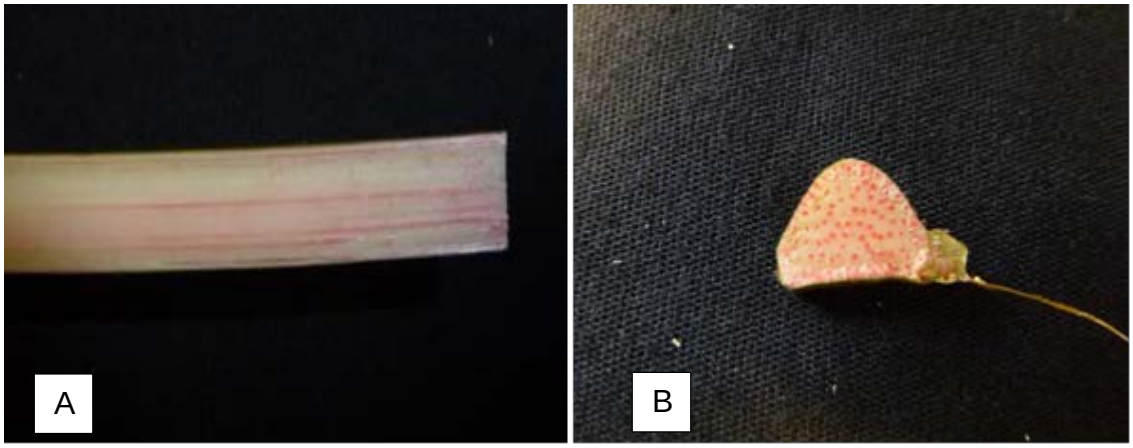

Figure 31. Longitudinal petiole section (A) and cross section (B) showing xylem vessels stained with eosin.

Table 5. Decreasing vessel functionality and distance travelled by eosin dye over time

Time (hours)	Number of Functional Vessels	Maximum distance eosin dye travelled (mm)
0	172	204
4	132	131
8	81	68
24	65	41
48	0	0

To determine how long xylem vessels are and how far basidiospores may be pulled into wounded palm petioles due to negative tension, red, fluorescent particles of the size range of fungal spores were mixed with eosin dye and applied to cut surfaces. Analysis of sections using light microscopy (Olympus) and fluorescence microscopy (AVROS) was carried out to detect presence of the red particles deep within the vascular system; particles blocked by vessel end walls would be unable to progress further (Fig. 32-33).

Red particles were observed within tissue to a depth of 35 mm and frequency of particles was greatest nearest the cut surface (Table 6). This suggests that vessel lengths do not extend far beyond 35 mm and only a few extend to these lengths. *Ganoderma* basidiospores could conceivably be dragged a substantial distance within oil palm tissue and therefore be protected from microbial competition, damaging UV irradiation, dehydration extreme temperature and may gain considerable access eventually to the bole of the palm.

Figure 32. Oil palm vascular bundle under transmitted light. 1 mm section taken at the site of application. Arrows indicate xylem vessels situated in the centre of a vascular bundle. Arrows indicate presence of particles partly obscuring otherwise open vessels Images were taken under an light microscope (Olympus): a) 10X objective lens b) 20X objective lens.

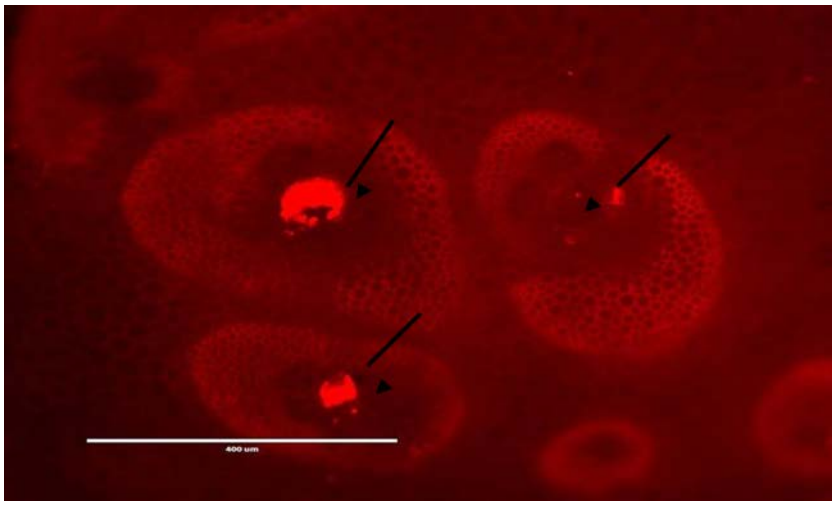

Figure 33. Oil palm vascular bundle under red fluorescence light. 1 mm sample taken at the site of application. Arrows indicate xylem vessels situated in the centre of a vascular bundle. A fluorescent signal within the xylem demonstrates the presence of a fluorescent particle. Images were taken under an EVOS® fluorescence microscope with a 10X objective lens. Scale bar represent 400µM.

Table 6. Xylem vessel length in cut oil palm petioles and percentage occupation with spore-sized fluorescent particles at 5mm intervals

Distance from cut (mm)	No. of vessels with fluorescent particles[a, c]	Total no. of vessels[b, c]	Percentage of vessels with particles (%)[c]
0	75	128	58.59
5	33	142	23.24
10	14	128.5	10.89
15	11.5	130.5	8.81
20	6	137	4.38
25	4.5	140	3.21
30	3	131.5	2.28
35	1	135	0.74
40	0	136	0
45	0	140.5	0
50	0	136	0
55	0	143.5	0
60	0	141.5	0
65	0	139	0
70	0	139	0
75	0	142.5	0

[a] Fluorescent particles were counted with an EVOS® fluorescent microscope under a red fluorescent light in 1 mm sections of petiole.

[b] The total number of vessels were counted with an EVOS® fluorescent microscope under a transmitted light in 1 mm sections of petiole.

[c] Data are representative of two independent biological replicates

3.3.15 Scanning electron microscopy of basidiospore germination and penetration on surfaces of oil palm roots

Oil palm roots in the field could be a potential infection route for *Ganoderma* basidiospores. The roots of oil palm consist not only of underground roots but many aerial roots which are partly exposed above the soil surface. Thousands of primary roots spread from the base, with new primaries replacing dead ones (Corley and Tinker, 2015). Rain, often frequent and substantial in south east asia will inevitably wash myriad spores down to contact these roots. The potential of *Ganoderma* basidiospores to germinate and penetrate oil palm roots remained unknown, although equivalent studies with *Heterobasidion* have revealed the potential of its spores to infect host roots (Asiegbu, 2000). In order to test this possibility, two types of oil palm roots (mature, brown and young, white) were excised and inoculated with basidiospores.

Cut roots (1.5 cm) were washed with sterile distilled water to remove soil and debris and surface sterilised with 3 % sodium hypochlorite for 1 min and washed three times with sterilised distilled water. Basidiospores from four palms were mixed and 2 µL of spore suspensions ($1x10^6$) were inoculated at discrete, marked points on the root surface; they were then placed in a sterile petri dish, elevated with a glass rod in a sealed container containing sterile distilled water to maintain high RH. All samples were incubated at 27°C in the dark. Inoculated roots were fixed after 24, 48, 72 and 96 h (refer section 3.2.12) and observed by scanning electron microscopy (SEM).

SEM Images of basidiospores from the surface of the cut oil palm root (brown) are shown in Fig. 34. Images of basidiospores from the surface of the cut oil palm root (white) are shown in Fig. 35. Images of potential penetration are shown in Fig. 36. *Ganoderma* spores adhered at some stage between 24 and 48 h on oil palm brown and white (Fig 34a and 35a). This conclusion resulted from the absence of any visible spores after 24h fixation (presumably washed off during the fixation process), but abundant spores were present on roots by 48h.

Basidiospore germination was higher (ca. 40-50%) on white roots than on brown roots (ca. 10-15%) based on random visual assessment of approximately 100 basidiospores over five fields of view (Fig 36). On brown roots spores germinated only after 72 h (Fig. 34b) and at 96 h extension of germ tubes occurred (Fig. 34c). While on white roots basidiospores germinated earlier by 48 h (Fig. 35b) and germination was much higher with more rapid germ tube extension was observed from 72 to 96 h than on brown roots (Fig. 35c-e).

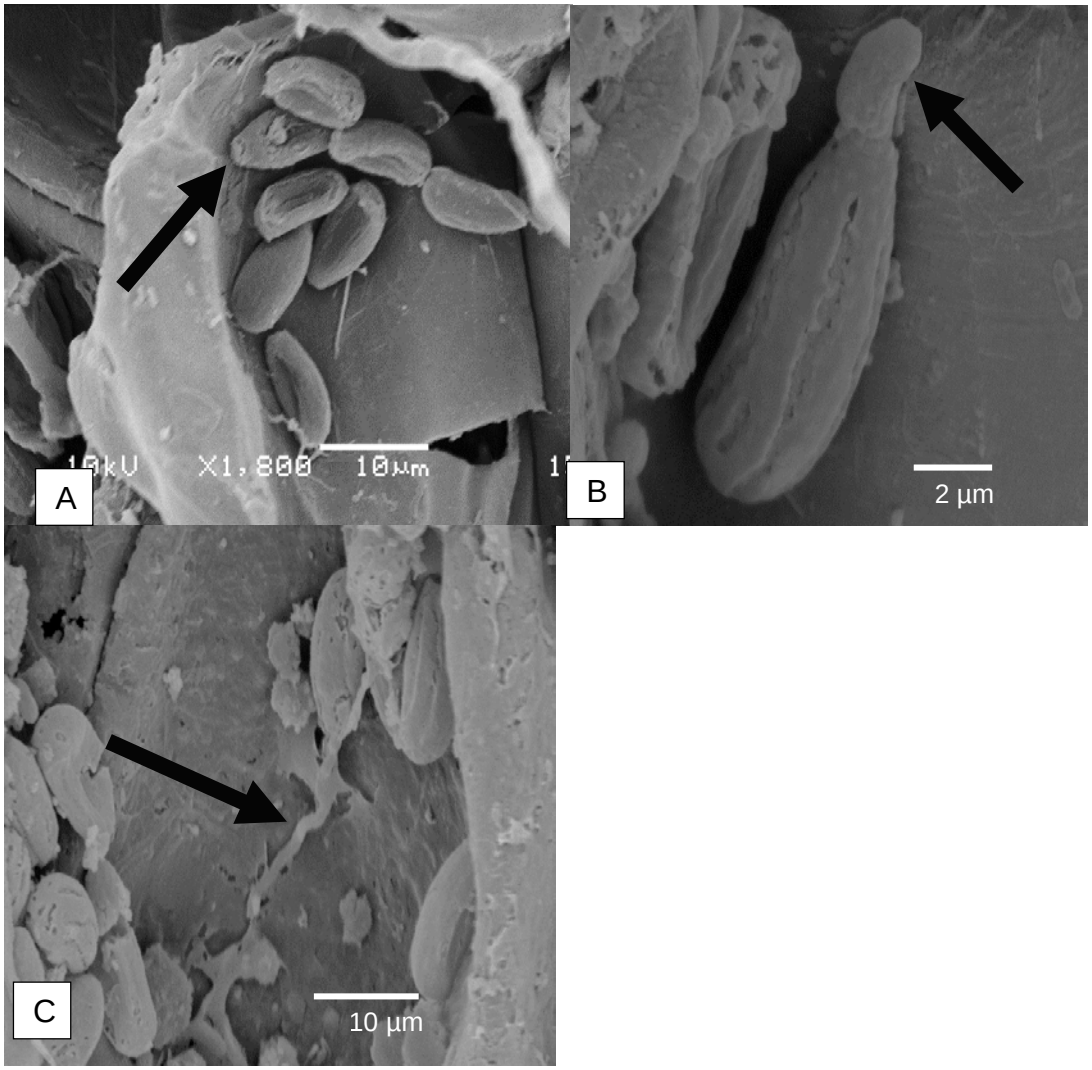

Figure 34. Scanning electron micrographs showing stages in spore adhesion and development on mature (brown) oil palm roots. *Ganoderma* basidiospores adhered (arrow) on the root surface by 48 h (A). Germ tube (arrow) formation by 72 h (B). Extension of germ tube (arrow) by 96 h (C).

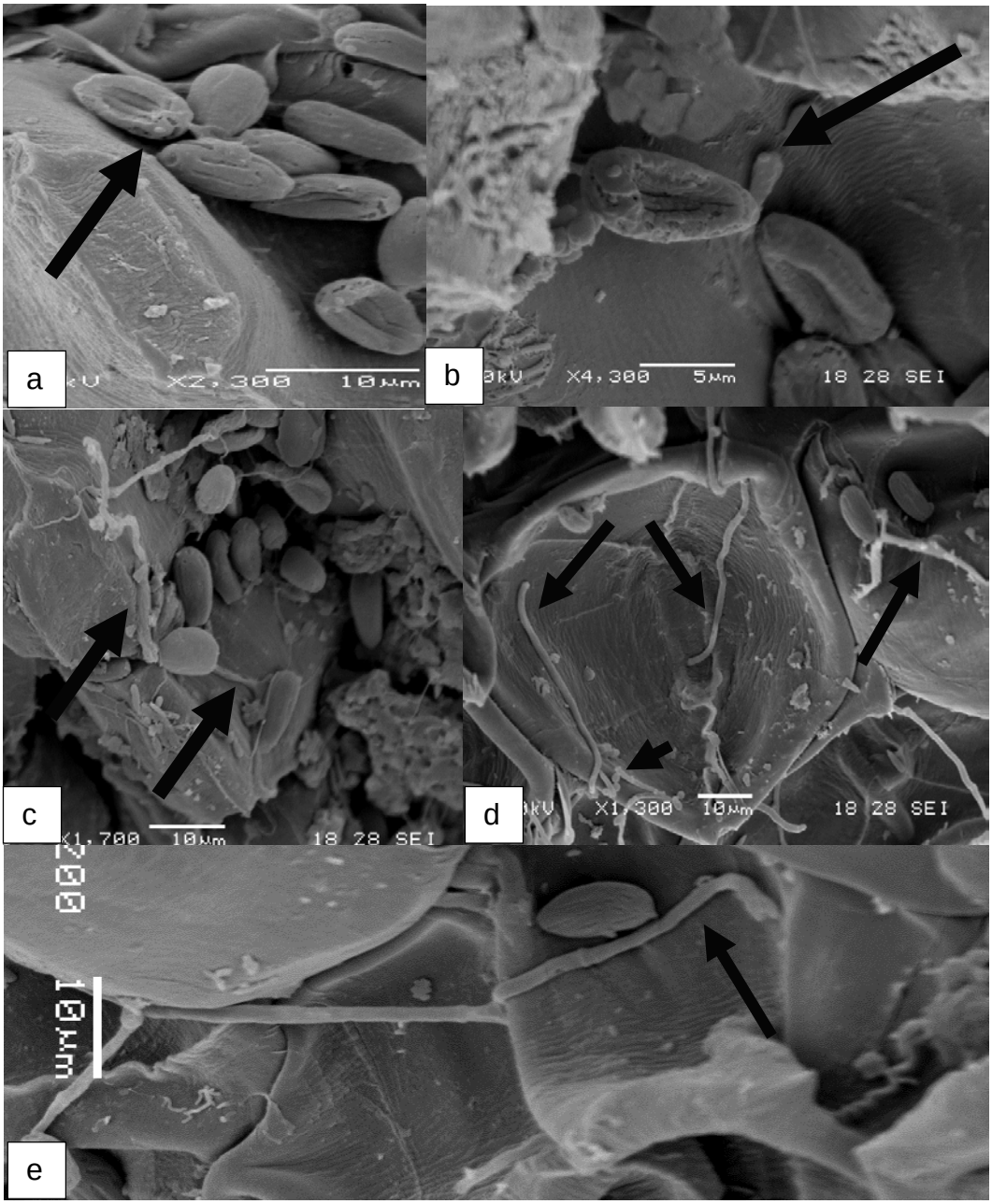

Figure 35. Scanning electron micrographs showing stages in spore adhesion and development on young (white) oil palm roots. *Ganoderm*a spores adhered (arrow) onto root surface by 48 h (A). Germ tube (arrow) formation after 48 h (B). Increased spores germination (arrow) after 72 h (C). Germ tube extension (arrow) and production after 96 h (D). Germ tube extension (arrow) on root surface x2000 (E).

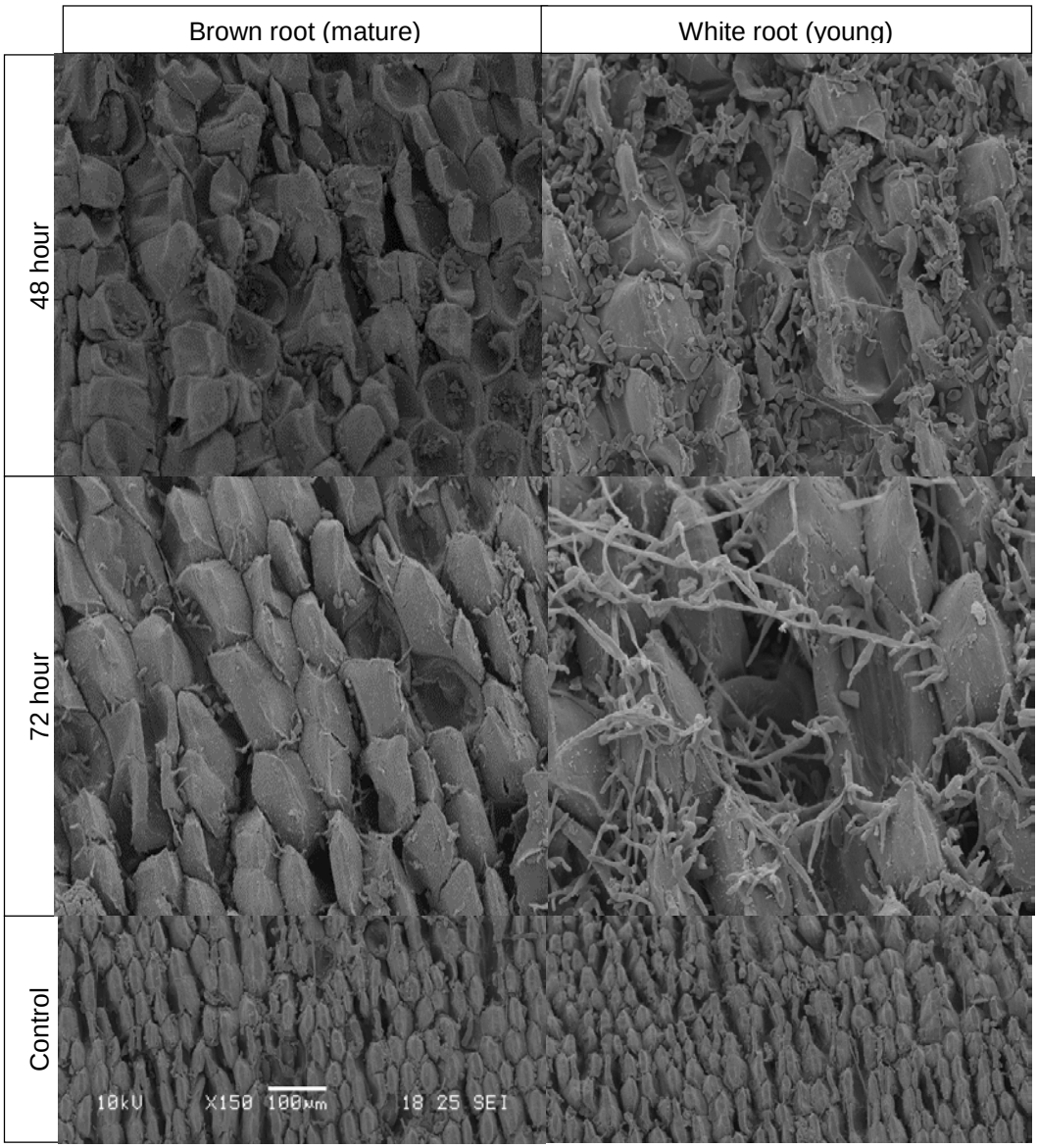

Figure 36. Representative of germination of basidiospores on the oil palm roots mature (brown) and young (white) at 48 h and 72 h. Basidiospore germination was more rapid and with more extensive germ tube elongation on white roots than on brown roots.

Penetration and possible degradation of root epidermis by *G. boninense* was observed only on white oil palm roots after 72 h to 96 h without any apparent infection structures formed (Fig. 37). At 72 h basidiospore germ tubes appeared to penetrate the root epidermis (Fig. 37a). Anastomosis and clamp connections were also evident (Fig. 37b and 37c). Alteration in the outer epidermal wall might suggest mucilage around the root surfaces where in contact in contact with *Ganoderma* hyphae with an apparent penetration (Fig. 37d).

In summary, roots provide a potential site for infection by basidiospores, which eventually adhere, germinate and apparently penetrate. Young (white) roots are more vulnerable because survival and growth was substantially lower on more mature (brown) roots.

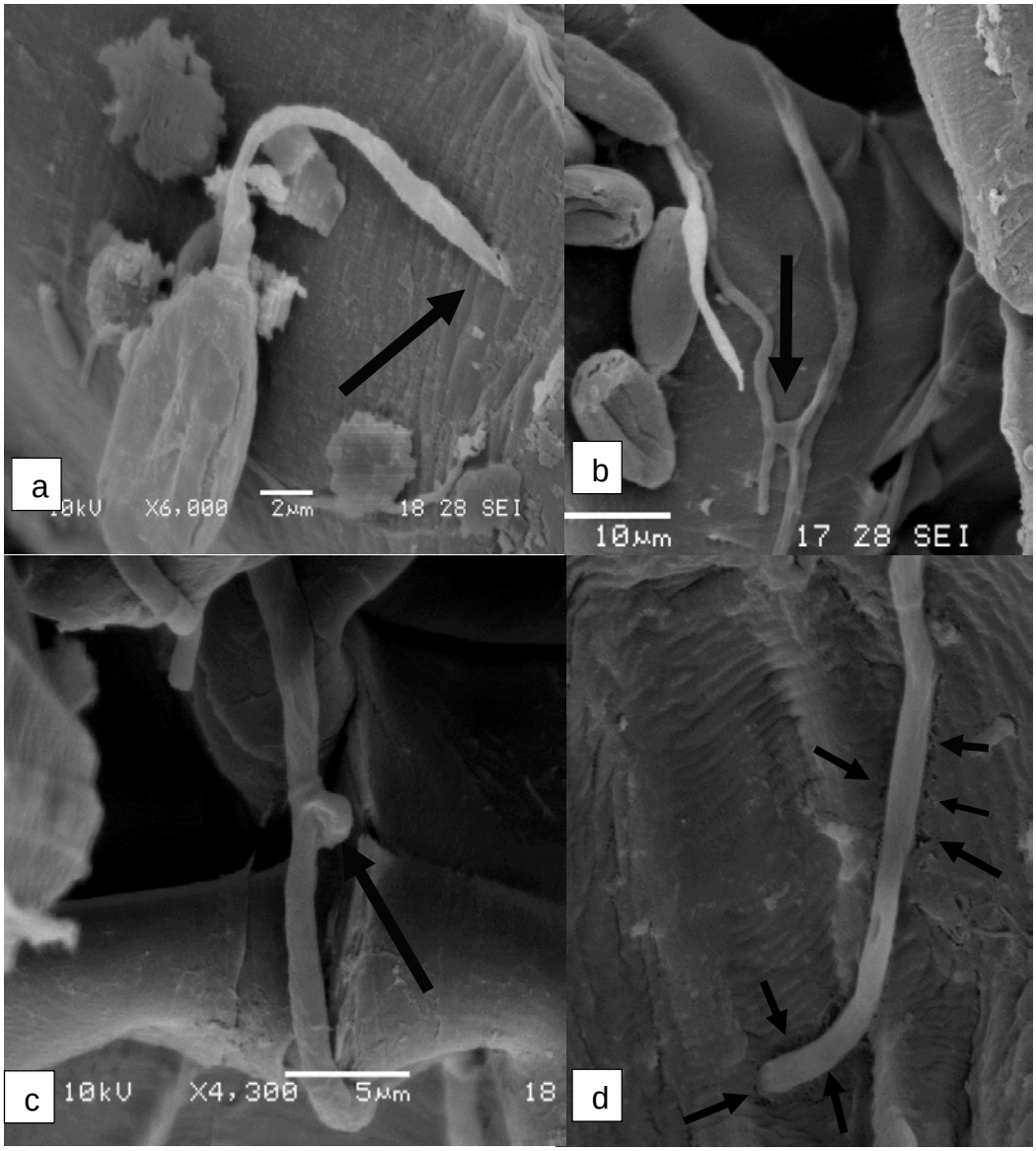

Figure 37. Root epidermis apparent penetration with no appressorium formation on white oil palm roots. *G. boninense* germ tube and hyphae apparent penetration (arrow) into epidermis 72 h and 96h after inoculation (a and d). Anastomosis (arrow) 72 h after inoculation (b) Clamp connection (arrow) formation at 72 h (c).

3.3.16 Assessing the biological control potential of *Trichoderma* on cut oil palm petiole

Trichoderma species are saprotrophic fungi recognised worldwide as potential, often effective, biological control agents (BCA) of many root infecting fungal plant pathogens. Trichoderma grows and survives within plant debris, roots as well as in soils (Howell *et al.* 2003). In BSR, addition of *Trichoderma* as a BCA into the soil has been shown to be partly effective in disease suppression, as it can associate closely with the palm root surface, growing with the expanding root system, persisting in high numbers and inducing resistance in the oil palm against *Ganoderma* infection (Soepena *et al.*, 2000; Rees, 2007; Nur Ain Izzati and Abdullah, 2008; Sundram, 2013).

However, *G. boninense* basidiospore germination and colonization within the xylem vessels of oil palm has been shown in this study to represent a different route of invasion affording a new control strategy for BCA. *Trichoderma* establishment and persistence on wounded surfaces of cut petiole has not been investigated in oil palm. For the successful application of *Trichoderma* as a BCA onto cut petioles, the fungus must be able to germinate and associate initially with the oil palm petiole vessels and survive for a long period of time in petiolar tissues.

The first step towards investigating this was to determine the persistence of *Trichoderma* in inoculated petioles of oil palm seedlings. Successful colonisation of wounded oil palm petioles by *Trichoderma* could out-compete *Ganoderma* and prevent it colonising the xylem vessels and causing infection.

Two *Trichoderma* isolates (TPP4 and T1-203), obtained from CABI Bioscience culture collections and antagonistic to *G. boninense* (unpublished data), were inoculated as spore suspensions onto 16 oil palm petioles (1 year old). A total of 32 petioles were cut horizontally with a sterile scalpel blade and immediately inoculated with 0.5mL of 5x107 spores/mL of both *Trichoderma* isolates; re-isolation onto *Trichoderma*-selective medium (TSM) (Willams *et al.,* 2003) was then attempted weekly. Uninoculated cut petioles were used as a controls.

Both *Trichoderma* isolates germinated and colonised the surface of the cut petioles, and persisted for the duration of 8 week testing after inoculation (Fig.38). Isolate TPP4 colonised the xylem to a maximum mean distance of 65 mm from the cut surface. Isolate T1-203 colonised to a maximum mean distance of 52.5 mm. Moreover Figure 39 shows that the maximum distance of *Trichoderma* colonisation was greatest at one week for both isolates and then generally declined. No *Trichoderma* was re-isolated from the control petioles.

Observations of *Trichoderma* hyphae in vessels by SEM are shown in Appendix 3. Because palms used were at a different stage to those described earlier, evaluation of vessel lengths were made, as described in section 3.3.14.The range of lengths were 45-55 mm (Appendix 4). In summary, *Trichoderma* appears to have the potential to protect newly wounded frond surfaces from basidiospore invasion.

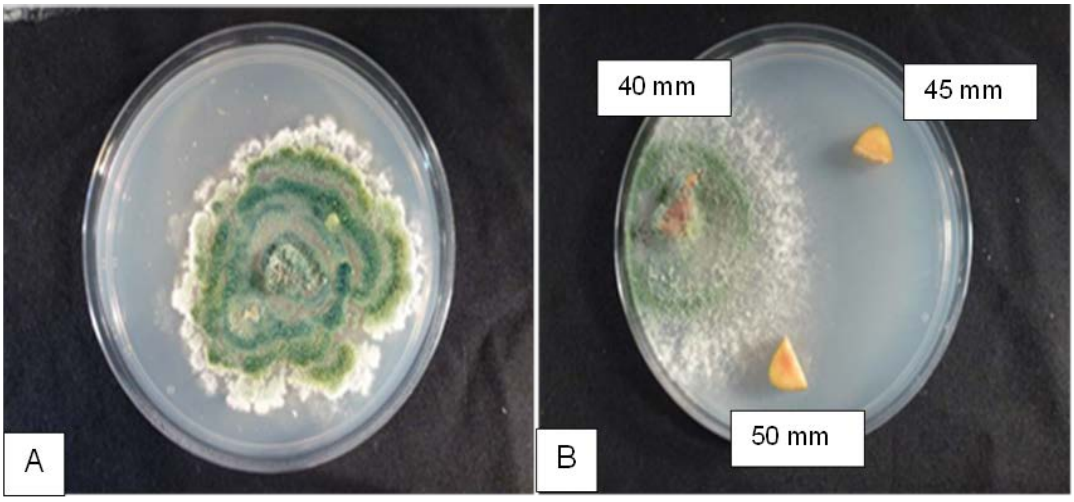

Figure 38. Left: Section of petiole surface inoculated with isolate T1-203, 1 week after inoculation. Right: Sections of isolate T1-203 taken at 40 mm, 45 mm and 50. Only one is positive down the petiole from initial cut, three weeks after inoculation. *Trichoderma* is persisting within the xylem down to 40 mm in this petiole.

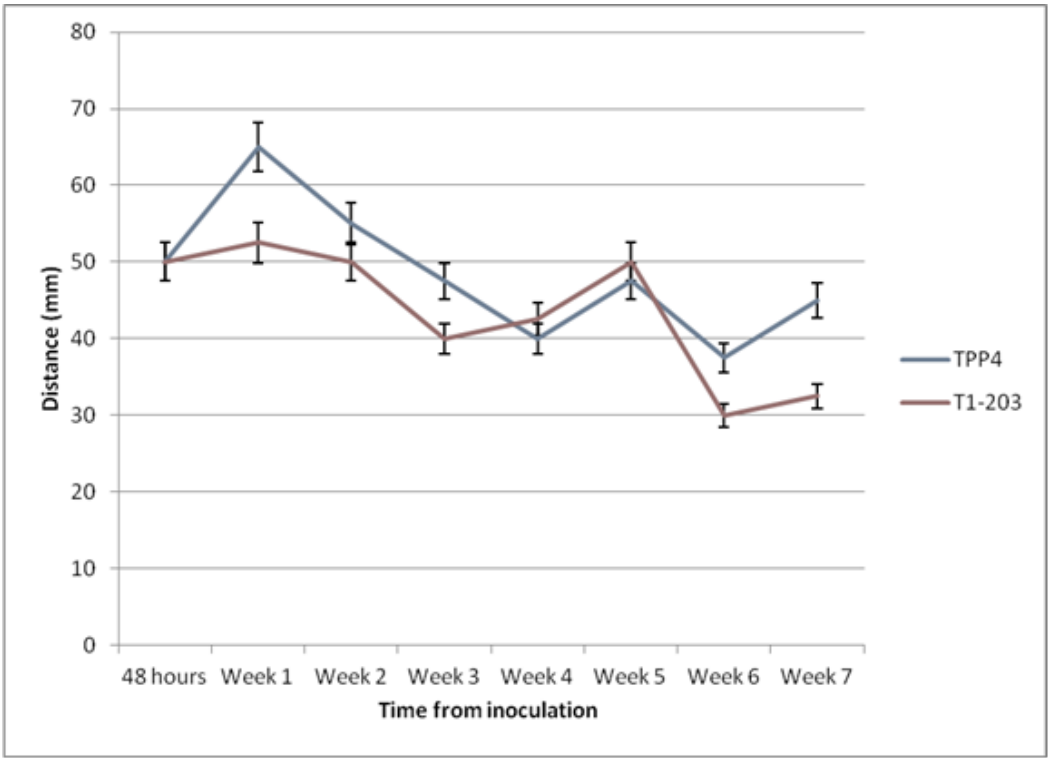

Figure 39. Mean distance (mm) from inoculation site of colonisation of two isolates of *Trichoderma* TPP4 and T1-203 in cut petioles. Error bars show the minimum and maximum distance of colonisation from the cut site in the two repeats for each isolate

3.4 Discussion

3.4.1 Infection and survival of *G. boninense* mycelium

This study confirmed that root infection of oil palm by *G. boninense,* ultimately leads to penetration of basal stem of infected palms and expression of BSR symptoms. Root infection was observed under controlled inoculation under glasshouse conditions in the UK. This finding agrees with other studies described previously (Khairuddin*,* 1990; Lim *et al.,* 1992; Sariah *et al.,* 1994; Breton *et al.,* 2006; Rees *et al.,* 2007; Mohd As'wad *et al.,* 2011; Rees *et al.,* 2012).

Intimate contact between the pathogen and the root is a prerequisite for a smaller inoculum 2.5cm^3 to induce infection in unwounded oil palm roots and potentially increase infection rate. Rees *et al.* (2007) similarly reported that artificial inoculum as small as 3cm^3 could cause infection of seedlings oil palms, while Ariffin *et al.* (2000) demonstrated infection of oil palm had been initiated from artificial inoculum only slightly bigger than an average oil palm root. Therefore with small inoculum able to initiate BSR, it is important to remove as much of the infested palm tissues as possible from previous planting before replanting an area with a new generation of palms. Inoculum left buried in soils, such as infected boles have been shown to severely increase BSR infection in the field (Flood *et al.,* 2002).

The lack of mycelial growth in the natural plantation substrates including soils and organic debris, yet ability to colonize sterilized equivalent substrates, confirmed that *G. boninense* is a poor competitor against soil microflora. This may explain the inability to obtain infection from inoculum sources without intimate contact and that close association between the pathogen and oil palm roots is more important for disease establishment than inoculum size. The basidiomycete pathogen *Armillaria* spp. spread by producing below ground and under the bark rhizomorphs, facilitating survival and infection of intact roots (Travadon *et al.,* 2012). Although *G. boninense* appeared to produce mycelial cords rather than true rhizomorphs in sterilized substrates soils and

organic debris, they were not formed in unsterilized substrates and there are no reports of the structure observed in the field (Pilotti, 2005).

Ganoderma boninense inoculum in oil palm plantation soils has never been determined and it is unclear how and for how long *G. boninense* resting structures remain viable in infected palm tissues and in soil. Darmono (2000) has described the presence of sclerotium-like structures (1-5 cm across) which he called Ganoderma resting bodies in infected oil palm tissue, while Rees *et al.* (2009) observed formation of melanised hyphae around infected oil palm roots, which they called pseudo-sclerotia like structures. These structures were associated with fruiting body formation and might provide a long term survival mechanism for the pathogen in plant tissues. The present study shows formation of *G. boninense* melanised mycelial structures surrounding the mycelium that is directly in contact with the unsterilized soils and organic debris. This melanised mycelium was shown to remain viable after 3 to 4 years in a dehydrated state and strongly suggests this mechanism could be its means of long term survival in the field. However this observation was made under controlled, axenic condition *in vitro*, rather than the much more exacting conditions of wet/dry periods and microbial competition and invasion. Thus, it is necessary to repeat this study in plantations by burying melanised RWB fragments of different sizes with nylon mesh bags in a range of soil types, to determine the likely duration of pathogen survival and how this relates to size of infested palm debris.

3.4.2 Screening for disease resistance

Resistance screening of oil palm may be the only sustainable solution to select resistant palm genotypes to prevent *G. boninense* infection in the field. However, present artificial infection with RWB is slow in terms of symptom formation, requires considerable laboratory space over a period of months to supply the infested blocks, and the inoculum used is relatively enormous compared with any other fungal disease and when compared to the size of oil palm roots.. In the present work, wheat grain inoculum was shown to establish infection when firmly attached onto oil palm roots. Disease establishment by

wheat grains was comparable to RWB, with more than 80% palms infected. Wheat grain is easily obtained and in this study it shows the greater flexibility of wheat grain inoculum to be manipulated than RWB and therefore it is a viable alternative to RWB. This indicate wheat grains is potential inoculum as alternative to RWB. Wheat grain inoculum has been used previously to study pathogenicity of various fungi, for example to confirm pathogenicity of *Roselinia necatrix* on camellia roots (Mansilla *et al.*, 2002) and in biocontrol studies on cacao (Garcia *et al.,* 2003). White root rot induced by *R. necatrix* is one of the most serious diseases affecting camellia and avocado in Spain, but it has wide host range of 170 plant species in 63 genera (Ruano Rosa *et al.,* 2010).

Generally *G. boninense* infection progresses slowly from infected roots and reaches the bole of the stem where extensive infection or degradation results in basal stem rot. The slow invasion and colonization of roots can take months (Rees *et al.,* 2007) so circumvention of this stage could considerably hasten the development of BSR. However direct inoculation of oil palm stem/bole by *G. boninense* did not induce BSR. Also the inoculum was often invaded by *Trichoderma* spp. The small number of replicates and the limitation of numbers of oil palms used in this experiment may have contributed. Therefore in the future it would be worthwhile to test this inoculation procedure using younger palms and under Malaysian conditions. However other pathogens such as *Heterobasidion* can bypass root to initiate infection on the stem for a more rapid infection (Kerio *et al.,* 2014). *Heterobasidion perviporum* was inoculated directly onto the stem of *Picea abies* clones under field conditions and over time necrotic lesions were observed around inoculated areas. *Verticillium dahliae* was inoculated directly into stems of cacao rather than inoculating roots. This required much younger plants, reduced the time for disease development by months and gave the same disease resistance ranking (Resende *et al*., 2003).

3.4.3 Basidiospore production and characteristics

The presence of airborne spores is a prerequisite for primary infection by most fungi (Ingold, 1971; Gonthier *et al.,* 2005), and the abundance of the aerial spores, often referred to as inoculum potential, is directly correlated with inoculum potential of the pathogens (Sinclair, 1964; Stambaugh *et al.,* 1962; Gonthier *et al.,* 2005). Temporal quantification of G. boninense airborne basidiospores within plantations in Sumatra was made by Rees *et al.* (2012) where a centrifugal air sampler (Biotester) was placed at the mid-point of inter-rows and fixed at 2 m height. This revealed over 11 000 spore m^{-3} in the plantation in the early evening, but production continued throughout 24 hours. However, airborne basidiospore levels had not been determined within and between Malaysian plantations. Therefore, aerial basidiospores from five plantations in North, West, East and South of Malaysia, were determined using a Biotester and quantification was made by light microscopy. It was possible to discriminate between *Ganoderma* basidiospores from spores from other fungi as the unique characteristic of a large vacuole present in the basidiospores as well as reddish brown, narrow ellipsoid with a visible hilar appendage (Rees *et al.,* 2012). In this study abundant aerial spores were produced throughout the sampling period within most plantations, which is in agreement with the observation made by Rees *et al.* (2012). This indicates there is constant potential inoculum of *G. boninense* to colonize wounds and palm debris in the plantation.

Airborne spore concentrations clearly will be dependent on frequency of basidiocarps on infected and dead palm stems. This was confirmed by the very low number of basidiospores detected in one of the plantations in spite of claimed high BSR occurrence. Within this plantation, infected or decayed palm had been mounded with soil to prolong tree lifespan (Hushiarian *et al.,* 2013), which might explain why basidiocarps were rarely observed.

Basidiospores have the potential to spread and to infect palm trees directly or indirectly as previously discussed. All of these possible contributions to BSR and USR depend on survival of the dispersed spores in the field. Plantation palms in Malaysia receive ample sunlight 5-7 hours a day in tropical climate and sunlight consists of about 95% UVA radiation (Allam and Zaher, 2014). Solar radiation (UV) can play a significant part in the survival of fungal spores in the field. Mortality of *Peronospora tabacina*, *Uromyces phaseoli* and *Alternaria solani* spores increased following direct exposure of solar radiation (sunlight) in the field (Rotem *et al.,* 1985). Direct exposure of *G. boninense* spore to solar radiation in the field (Malaysia) over time revealed that viability remained after 6-12 hours exposure. This might suggest that basidiospores will survive long enough to enable dissemination by wind within and between plantations. The dark brown pigmentation of *G. boninense* basidiospores is assumed to be melanin. Melanin deposition protects pigmented cells from physical and biological stresses (Nicolaus *et al.,* 1964). The primary (evolutionary) function of melanin in microorganisms is to reduce the detrimental effects of UV radiation, for example, through DNA damage (Henson *et al.,* 1999; Allam and Zaher, 2014). Many fungal spore types and structures designed for long term survival have melanised cell walls (Agrios, 2005).

A dormancy period in fungal spores, particularly pathogens, can be important for long term infection and survival in unfavourable conditions and dormant phase may remain for very considerable periods (Morin *et al.,* 1992; Aime and Miller, 2003; Nara, 2009). Most sexual spores of fungi exhibit constitutive dormancy (Gottlieb, 1950; Allen, 1965; Campbell, 1970; Feofilova *et al.,* 2012), when self-inhibitor(s) prevent germination even when the conditions appear favourable (Deacon, 2005). Furthermore, this type of dormancy required a period of maturation or activation such as heat shock or cold shock. It is evident from this study that *G. boninense* spores do not have a dormancy period and even after exposure to heat-shock treatment there was no significant increase in spore germination. This finding relates to the rapid loss of viability as revealed by temporal decline in spore germination. Freshly

collected *G. boninense* spores were capable of germinating after 3-4 days when visible colonies of the fungus appear on water agar as also found by Pilotti *et al.*, (2003) and Rees *et al.* (2012).

In the plantation it remains unknown where *G. boninense* spores could germinate and establish. Although some researchers have speculated that organic debris or decayed palm may provide suitable substrates, no one has ever provided conclusive evidence. Colonization of dead materials (debris) by basidiospores in the field would ensure sufficient build-up of critical mass of pathogen to allow invasion of living oil palms (Hasan and Flood, 2003). However this indirect infection by basidiospores is difficult to imagine for monokaryons to survive and compete for nutrients with other microbial community already present in the dead materials. The failure of established mycelium with a nutrient base (wheat grain) to establish mycelium has already been made clear. The monokaryotic phase of *G. boninense* is thought to be only a temporary stage before mating with a compatible monokaryon mycelium to form pathogenic dikaryotic mycelium (Flood *et al.,* 2005). Therefore direct infection presumably is a more reasonable option for basidiospores to invade and initiate disease in oil palm. The potential for infection sites in plantation palms is significant, with extensive wounds created by routine harvesting by severing the fruit bunch peduncle and pruning of frond bases to make way for the fruit bunch. Moreover wounding of the trunk is likely to occur during harvesting older palms having considerable height.

3.4.4 Inoculation of petioles and roots with basidiospores

Under controlled glasshouse conditions this study demonstrates for the first time that basidiospores can not only germinate in but also invade cut petiole xylem vessels of oil palm seedlings. Spores on the cut petiole surfaces are drawn up into xylem vessels as a result of negative tension within functional vessels (Cooper, 1981). Vessel lengths were determined microscopically to be 60 mm. This was confirmed by re-isolation of inoculated petioles on GSM

showing the initial maximum distance of basidiospores was c.60 mm. These vessels would provide temporary protection for basidiospores against dehydration, solar radiation and microbial competition. However in at least one instance the natural barrier of vessel end walls were breached where dikaryotic *G. boninense* was re-isolated c. 10 cm from the cut petiole. According to Pilotti, (2005) compatible spores readily mate and hybridise resulting in the formation of a heterokaryon. It would not be possible for spores to travel beyond the natural barrier, therefore it reveals the formation of dikaryotic mycelium in the vessels and that it has penetrated vessel end walls. Anastomosis was apparent *in situ* from Rees *et al.* (2012) cryo-scanning electron microscopy images further supporting dikaryon formation in vessels. Although this might be a rare event with this relatively weak pathogen, yet it shows for the first time the potential for *G. boninense* to continue to invade and eventually reach the bole to cause BSR, or higher up the trunk, USR.

Due to the genetic diversity of *G. boninense* observed within oil palm plantations it has been suggested that the spread of BSR could be achieved via basidiospore colonisation and anastomosis with compatible mating types (Cooper *et al*., 2011). Thus the ability for basidiospores to be internalised at the site of cut petioles and infect the oil palm was examined. Therefore to track the progression of the *G. boninense* colonisation in the petiole, three different method have been evaluated. Re-isolation of the cut petiole onto *Ganoderma* selective medium (GSM) is rather slow as it takes 14 days to grow and suffers from contamination as *G. boninense* is a poor competitor as shown in this study. Hence, DNA based detection was developed to overcome this shortcoming.

Nucleic acid-based diagnostic techniques depending on the variations in the nucleotide sequences of the pathogen DNA have become the preferred method for detection of fungal pathogen in plants. This is because of their greater speed, specificity, sensitivity, reliability, and reproducibility of the results obtained, following the development of polymerase chain reaction (PCR) (Narayanasamy, 2011). Through this method, the presence of *G. boninense* in the petiole was successfully tracked by using specific primer (GanET), suggesting that this alternative detection method proved to be a better than GSM. *G. boninense* DNA was detected up to 10 cm vessel length which indicates the reliability of the method to track the progress of *G. boninense* in the palm petioles. Similar finding by Panchal and Bridge, (2005) reported the presence of *G. boninense* from 0.25-1 cm immediately below the surface of cut fronds 10-15% (200 total) was detected. This result highlights the necessity for caution as a positive signal could be as a result of dead or dormant spores, merely representing spore uptake, instead of colonization by germinating spores or active mycelia (West *et al*., 2008).

In this study the development of RNA detection method through precursor ITS rRNA was able to discriminate between viable and non-viable *G. boninense* basidiospores. This result was supported by Van Der Linde and Haller, (2013) the finding of viable and non-viable spores in various basidiomycete fungi such as *Armillaria mellea*, *Coprinus* sp, *Marasmius oreades* and *Agaricus bisporus*. They also found that the use of precursor ITS rRNA failed to detect RNA before spore germination in any of the tested basidiomycete fungi. Finally, they concluded that the precursor ITS rRNA should be used for the analysis and characterization of active species composition, when contamination of ungerminated basidiospores is needed to be avoided. Hence supporting the work done in the *G. boninense* colonisation on the cut frond of oil palm.

The main challenge and limitation of conducting basidiospore infection in UK is availability of fresh *G. boninense* spores. Therefore it is important to continue this work in the future where basidiospores are readily obtainable. Furthermore it is essential to extend this study to plantations using mature palms with an extensive replication of inoculated fronds. It will be necessary to conduct the experiments over long periods because invasion of petioles may be a rare event and colonization might be slow. Under plantation conditions with palms grown for up to 25 years and with continual pruning of petioles and continual bombardment by spores, rare events could still explain the frequency of BSR. It is possible that previous attempts to infect with spores have not used enough replication or patience.

Thus there have been no reports of successful infection of oil palm with basidiospores inoculum in the field (Thomson, 1931; Miller *et al.,* 1999; Hasan *et al.,* 2005). In plantation palms, Hasan *et al.* (2005) attempted to directly infect oil palm trees with fresh spore suspensions. These were either injected or painted onto fresh cut fronds, however both experiments failed to induce infection throughout 3 years observations.

A primary requirement for the dispersal of pathogenic fungal spores is contact with its host. Following contact, the survival of the pathogen will ultimately depend on its viability and ability to establish on the new host. Viability and development of the spores are in turn influenced by nutrient availability and interaction between the host and pathogen. As shown in this study, *G. boninense*, the most important pathogen of oil palm, is no exception regarding its physiological interaction needs for adaptation to its host. This study considered and showed for the first time that spores of *G. boninense* could adhere to roots, germinate and appeared to penetrate the roots directly.

In this study, *G. boninense* basidiospores easily adhere to the roots of brown or white of oil palm seedling. However subsequent establishment and germination seems to be affected by host surface substances and constituents. This was evident in the variation recorded in the number of germinated spores between brown and white root which may be attributed to the certain components of the host material in brown root that are inhibitory to spore germination. Host substances have been implicated in the inhibition of spore germination of other fungi. For example, fresh leaves of sagebrush inhibit the germination of *Cronartium comandrae* completely (Krebill, 1972) and leaf fragments with abundant glandular hairs (Robinson, 1914) or water-soluble exudates from *Pinus pinaster* inhibit germination in *Puccinia malvacearum* and *M. pinitorqua* (Desprez-Loustau, 1986), respectively (Gold and Mendgen, 1991). Furthermore, proteins on root and spore surfaces and mucilage have been implicated to influence adhesion and spore germination as reported by (Asiegbu, 2000) in *H. annosum* spore study. He showed that the removal of proteinaceous substances on root and spore material by pretreatment with pronase E led to a significant reduction in the number of spores able to germinate on the root. Furthermore, oxidation of carbohydrate constituents on the roots by pretreatment with periodic acid abolished almost completely the germination of spores on the root surface. This could imply that roots and stem which lack the right nutritional constituents and have inhibitory surface substances may escape infection or be poorly colonized.

By using a non-suberized conifer root as an experimental model system, Asiegbu (2000) had characterised the establishment and development of *H. annosum* spores. He showed that *H. annosum* spores readily adhered onto the root, subsequently germinated and formed an appressorium infection structure directly penetrated the root through natural opening as well as direct enzymatic degradation of waxes on root surfaces. However in this study no formation of appressorium and hyphae formed from *G. boninense* spores appears to penetrate root surfaces directly with apparent mucilage around the hyphae observed. A mucilaginous sheath has been implicated in adhesion of the appressorium of *H. annosum* (Asiegbu *et al.,* 2005a), *Colletotrichum* spp.,

(Young and Kauss, 1982), rust fungi (Bushnell and Roelfs, 1984; Mendgen *et al.,* 1988; Gold and Mendgen, 1991).

These observations by SEM may suggest the possibility of a novel route of infection by *G. boninense*. Spore infection of roots would allow direct progress into the basal stem of palms and provide the typical image of root infection, previously always considered to be *via* mycelium. Mature palms in plantations produce abundant superficial roots including some in part above ground. When the palm grows it will produce secondary roots that mostly extending on the periphery of the root-soil plate. These roots often will appear above ground through the trunk bases of mature palms in the field (Jourdan *et al.,* 1997). Furthermore plantation palms receive both heavy and frequent tropical rains which inevitably will wash down millions of aerial *G. boninense* spores present onto above aerial and superficial roots.

For basidiospores to cause direct infection, anastomosis of two compatible spores would be essential to generate an infective dikaryon (Rees *et al.*, 2007; Hasan *et al.,* 2005). Observations of germinated basidiospores on roots of palm seedlings in this study suggests that anastomosis between compatible spores does occur on the roots. The mycelium might be secreting cell wall degrading enzymes (CWDE) by appearance of possible degradation of the epidermal surface layer adjacent to the mycelium; alternatively the material might represent secreted glycoprotein or polysaccharide as are often associated with fungal infection structures (Gold and Mendgen, 1991; Mendgen and Deising, 1993; Asiegbu, 2000; Asiegbu *et al.,* 2005; Rees *et al.,* 2009; Ruiz-Roldan *et al.,* 2011). In order to confirm actual root penetration, transverse sectioning combined with light and electron microscopy as well as GFP tagged strain will be required.

Spores from other fungal tree pathogens are known to infect trees through stumps and roots. With *H. annosum* airborne spores colonise injured roots and establish infection before progressing into the basal of the tree, killing all parts of the tree and progressively spreading to other roots (Rishbeth, 1950; Hodges, 1969). The author, R M Cooper (pers. comm.) and Idris *et al*. (2006) all have observed formation of basidiocarps of *G. boninense* on above ground roots, which may indicate the occurrence of direct root infection by basidiospores in plantation palms. Proof of this putative route of infection along with petiole infection would change the way Malaysian pathologists and planters view the nature of disease epidemiology and such research should be given priority.

4 Genetic diversity of *Ganoderma boninense*

4.1 Introduction

Ganoderma boninense has been the subject of numerous investigations because of its economic importance as the causal agent of BSR of oil palm in South East Asia. Most research on *G. boninense* has been focused on various aspects of early detection, pathology, biological and chemical control (Idris *et al.,* 2006; Susanto *et al.*, 2005; Mohd As'wad *et al.,* 2011). The population structure, sexuality and genetics of *G. boninense* have received little attention and there are serious deficiencies in our knowledge of these aspects in spite of decades of research on BSR and USR. Determination of *G. boninense* diversity has the potential to significantly enhance the effort to control of BSR and USR and provide a better understanding of disease epidemiology in oil palm plantations (Cooper *et al.,* 2011).

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4.1.1 Population structure of *G. boninense*

Genetic studies have showed that the majority of *G. boninense* isolates found in oil palm plantations were genetically distinct within and between neighbouring palms in South East Asia (Miller *et al.,* 1995; Ariffin *et al.*, 1996; Pilotti *et al.,* 2003; Rees *et al.,* 2012). For example Miller *et al.* (1999) tested multiple *G. boninense isolates* from basidiocarps from two plantations for somatic incompatibility (vegetative incompatibility) and mitochondrial DNA RFLP (mtDNA RFLP) was also used to assess relatedness of isolates. Out of the 39 isolates collected from one plot, 34 distinct somatic incompatibility groups were observed. Furthermore, somatic incompatibility suggested more than one isolate of *G. boninense* was present within an individual infected palm in 6 out of 8 palms tested. Presence of multiple somatic incompatibility groups (SIG) within a relatively small area, on adjacent trees and even within individual infected trees suggests spread of *Ganoderma* does not occur vegetatively through the soil, but indicates spatially separated populations

originating from a diverse initial inoculum. MtDNA analysis supported this finding by detecting a similar number of different mtDNA RFLP groups from the same isolates, however the groups were not always in accordance with SIG groups.

Similarly, Pilotti *et al.* (2003) using mating type factors, showed that the majority of *G. boninense* isolates in Papua New Guinea (PNG) were genetically distinct and do not have direct hereditary relationships with each other. Isolates from four planting blocks in PNG were paired and a total of 81 A and 83 B alleles were detected within the population sampled. The majority of *G. boninense* isolates genotypes on neighbouring palms within the population had unique mating type alleles, thus indicating different individuals (Pilotti *et al.,* 2003). They also showed the possibility of *G. boninense* dispersal with some isolates from two distanced planting blocks which shared common alleles, indicating that sexual reproduction is an important aspect of the population dynamics of *G. boninense* (Pilotti *et al.,* 2003).

Genetics of multiple *Ganoderma* isolates obtained from USR and BSR from five planting plots of two plantations (Sungai Bejanker Estate and Bah Lias Estate) in Sumatra, Indonesia involved 57 *G. boninense* isolates from basidiocarps and diseased tissues. These were tested through random amplified microsatellites (RAMs) to determine disease spread and diversity of isolates within and between infected palms (Rees *et al.*, 2012). They reported that most *G. boninense* genotypes in trees did not coincide with those in fallen palms or between infected palms, whether adjacent or from any of the planting locations. Similar genotypes in trees were observed only in Bah Lias Estate, where two infected adjacent palms showed apparently the same genotype. Analysis of the RAMs profiles agree with Pilotti *et al.* (2003) showing that isolates of USR were identical within individual palms but each USR isolate was unique. BSR infection in Sumatra showed multiple isolates of *G. boninense* present within individual palms in 3 out of 7 BSRs, which indicates multiple infections from different *G. boninense* isolates and contradicts the findings of Pilotti *et al.* (2003). Furthermore they also observed high genetic diversity within and between two plantations, strongly suggesting inbreeding

restriction of *G. boninense* within a population, and implies sexual reproduction and sexual recombination of basidiospores are important factors for maximum diversification of *G. boninense* in a populations.

4.1.2 Sexuality of *G. boninense*

Ganoderma boninense is heterothallic with a tetrapolar mating system (bifactorial incompatibility) favouring out-crossing within a population (Miller *et al.,* 1999; Pilotti *et al.,* 2003; Rees *et al.,* 2012). Individual basidiospores of *G. boninense*, will give rise to monokaryotic mycelia that are characterised by simple septa and mycelia are self-sterile. Mating must take place between two sexually compatible monokaryotic mycelia in order to form a mycelium capable of infecting oil palm (Miller *et al.,* 1999; Pilotti *et al.,* 2003; Rees *et al.,* 2012). Mating is initiated by anastomosis (fusion of hyphae) from different monokaryons and results in the formation of dikaryotic mycelium. A dikaryon will have simple septa and clamp connections (Idris *et al.,* 2006; Pilotti *et al.,* 2003; Rees *et al.,* 2012).

Mycelial interactions within *G. boninense* fall under two general categories: sexual and vegetative. Sexual interactions, involve anastomosis, then plasmogamy (cytoplasmic fusion). Formation of basidiocarps, karyogamy, meiosis and formation and discharge of basidiospores are dependent on successful mating (Idris *et al.,* 2006; Pilotti, 2005). Consequently, important evolutionary processes such as genetic recombination and gene flow are also affected. Sexual interactions occur between monokaryons or between dikaryons and monokaryons, with dikaryons acting as nuclear donors (Pilotti *et al.,* 2003; Pilotti, 2005). Vegetative interactions involve only dikaryons. Different dikaryotic genotypes exhibit antagonistic interactions upon confrontation restricting the exchange of genetic material through plasmogamy. Vegetative mycelial interactions thus represent self vs non-self-recognition and consequently may affect intra-specific competition and resource partitioning of individual substrates (Pilotti, 2005). In addition, Pilotti (2005) suggested that new alleles of *G. boninense* genotypes would arise

through sexual recombination of basidiospores from outside planting areas every year. Overall it seems clear that basidiospores must be responsible for the great genetic diversity found with isolates causing BSR and USR. Nevertheless as discussed in chapter 3, attempts to inoculate and infect oil palms with basidiospores have to date not been successful (Hassan and Flood, 2003).

The genetic diversity and sexuality of *G. boninense* from different regions in Peninsular Malaysia has not been evaluated before and little is known about its sexuality and evolutionary background there. Therefore in this study somatic incompatibility and molecular markers are used to determine genetic diversity of *G. boninense* from different geographical locations (south, west, east and north) of Malaysian oil palm plantations with high incidences of BSR disease.

4.1.3 Somatic incompatibility in assessing fungal genetic diversity

Somatic incompatibility (also called heterokaryon or vegetative incompatibility) involves self or non-self-recognition mechanisms in fungi that has been used to describe diversity and fungal population structure (Rayner and Boddy, 1985; Miller *et al*., 1999; Ota *et al*., 2000; Pilotti *et al.,* 2003; Gatto *et al.,* 2009; Garbelotto *et al.,* 2013). Somatic incompatibility testing distinguishes individuals by the inability of two strains to fuse and form one colony as an effective means of identifying intra-specific variations within field populations of plant pathogens (Rayner and Boddy, 1985). Somatic incompatibility is assessed via confrontations of isolates from different fruiting bodies on nutrient media. If isolates produce a zone of somatic rejection at the point of interaction (growth inhibition or barrage zones), this is interpreted as an incompatibility reaction, indicating that the two isolates are genetically distinct. However a compatible reaction (confluent growth between them) has been interpreted as evidence to indicate that the isolates are genetically identical (Rayner and Boddy, 1985). The genetic basis for somatic incompatibility has been relatively unknown in *G. boninense* or any other Basidiomycete. In most

Basidiomycetes, vegetative incompatibility is controlled by allelic interactions in which two individuals are compatible only if they share the same alleles at all mating type loci (Rayner and Boddy, 1985; Pilotti *et al.,* 2003; Garbelotto *et al.,* 2013).

Somatic incompatibility testing has been used widely in wood-rotting and tree-pathogenic Basidiomycetes (e.g. *Suillus bovinus*, *Armillaria spp*., *Heterobasidion spp*.) to delineate different genotypes, which are frequently called individuals or clones within a population (Ota *et al.,* 2000; Dai *et al.,* 2002; Latiffa and Ho*,* 2005; Baumgartner *et al.*, 2011; Garbelotto *et al.,* 2013). Population structure study of *Heterobasidion annosum* in many Norway spruce stands in Northern Europe showed identical individuals were detected in neighbouring trees (Stenlid*,* 1985). The number of trees infected by one clone varied from 1 to 13. Furthermore nine clones were identified within a 60 × 60 m area and the largest area occupied by one single clone was 30 m in diameter (Stenlid*,* 1985). Similarly, a somatic incompatibility study of *Rosellinia necatrix* on avocado plants in southern Spain showed an identical individual isolates detected over neighbouring infected trees up to 150 m. Isolates of *R. necatrix* either from an avocado tree or from nearby trees were somatically compatible, whereas isolates from distant trees were somatically incompatible (Jimenez *et al.,* 2002). Furthermore somatic incompatibility revealed the occurrence of high diversity in the *R. necatrix* population in the study (Jimenez *et al.,* 2002).

Somatic incompatibility has been used to determine genetic diversity of *Ganoderma* in oil palm plantings (Miller *et al.,* 1999; Pilotti *et al.*, 2003; Latiffa and Ho, 2005). In contrast with the *H. annosum* and *R. necatrix* findings above, somatic incompatibility in culture indicated that *Ganoderma* generally occurred as numerous distinct individuals, with compatible individuals usually confined to individual palms. The high polymorphism in somatic incompatibility phenotypes and the relative ease with which they can be assayed have made these phenotypes popular for studying fungal populations (Miller *et al.,* 1999;

Dai *et al.,* 2002; Pilotti *et al.,* 2003; Latiffa and Ho, 2005; Garbelotto *et al.,* 2013).

4.1.4 Molecular markers for genetic analysis of fungi

The recent availability of inexpensive and easy-to-use molecular markers has facilitated research in genetic diversity of fungal pathogen populations. Molecular markers have advantages over other kinds, where they show genetic differences of isolates directly based on the banding patterns on a gel providing good detail without interference from environmental factors, and involving techniques that are robust and provide rapid results (Binneck *et al.,* 2002; Garcia *et al.,* 2004; Saker *et al.,* 2005; Goncalves *et al.,* 2008; Souza *et al.,* 2008; Rees *et al.,* 2012; Garbelotto *et al.,* 2013). Molecular markers have been used widely to study genetic diversity of economically important wood-rotting and tree pathogenic Basidiomycetes populations such as *Armillaria spp*. and *Heterobasidion spp*. (Garbelotto *et al.,* 2013).

The following techniques are those most used in genetic diversity studies: RFLP (restriction fragment length polymorphism) (Botstein *et al.,* 1980), AFLP (amplified fragment length polymorphism) (Vos *et al.,* 1995), RAPD (randomly amplified polymorphic DNA) (Williams *et al.*, 1990), ISSR (inter-simple sequence repeats) (Zietkiewicz *et al.,* 1994) or RAMs (random amplified microsatellites) (Hantula *et al.,* 1996), SSR (simple sequence repeats or just microsatellites) (Tautz, 1989), SNPs (single nucleotide polymorphisms) (Chen and Sullivan, 2003) and other high throughput platforms.

In this study two techniques; RAMs and SSRs were used due to rapidity and ease of use of large samples; also the techniques have been optimised previously: RAMs by Rees *et al.* (2012) and SSRs (Unpublished paper, Wei Chee Wong pers. comm.).

4.1.4.1 Random amplified microsatellites (RAMs)

RAMS technique was originally described by Zietwicz *et al.* (1994) who showed that species-specific fingerprints could be obtained using oligonucleotide primers to amplify random SSR sequences from a range of organisms including humans, dogs and fish (Zietwicz *et al.,* 1994). The method was shown to be applicable to fungi by Hantula *et al.* (1994) who proposed the RAMs acronym and it combines the benefits of random amplified microsatellites with the universality of random amplified polymorphic DNA (RAPDs). RAMs uses primers containing microsatellite sequences and degenerate anchors at the 5' end and should produce fewer bands than RAPDs, as simple sequence repeats are present with relatively low frequency in basidiomycetes, this should make resolution of gels easier. In RAMs analysis the DNA between the distal ends of two closely located microsatellites is amplified and the resulting PCR products are separated electrophoretically in an agarose gel (Zietkiewicz *et al*., 1994; Hantula *et al*., 1996).

RAMs have now been used in a number of studies on fungi including virulence and diversity of the phytopathogen *Colletotrichum lindemuthium* (Mahuku *et al.,* 2004), analysis of genetic variation of pine rusts (*Peridermium pini* and *Cronartium flaccidum*) (Hantula *et al.,* 2002), determining variation between *Phlebiopsis gigantea* isolates from North America and Europe (Hantula *et al.,* 1998) and genetic diversity in isolates of *P. gigantea* from European countries (Hantula and Muller*,* 1997). Furthermore RAMs has been used in determining diversity of *G. boninense* in oil palm plantations in Malaysia (Latiffa and Ho*,* 2005) and Indonesia (Rees *et al.,* 2012) and revealed high genetic diversity among *G. boninense* isolates within and between oil palm plantations. RAMs analysis using four microsatellite primers, 5’BDB(ACA)5 , 5’DD(CCA)5, 5’DHB(CGA)5 and 5’YHY(GT)5 G, produced variable banding patterns among the isolates from infected oil palms and coconut stumps (Latiffa and Ho, 2005) and from isolates from infected neighbouring palms within and between plantations (Rees *et al.,* 2012).

In addition, RAMS amplified fragments can be cut from the gel and cloned or re-amplified and sequenced in order to characterise microsatellite loci within fungi so as to develop multi-locus microsatellite typing (MLMT), an adaptation of the MLST technique (Taylor and Fisher*,* 2003).

4.1.4.2 Single sequence repeats (SSRs)

A different approach to arbitrary PCR amplification such as RAMS, consists of the amplification of target regions of a genome through specific primers. Microsatellites is the commonest term used to describe tandem repeats of short sequence motifs, such as penta-, tetra-, tri-, and di-nucleotide repeats, but are also known as simple sequence repeats (SSRs) (Rees *et al.,* 2012). SSRs are tandemly repeated motifs of one to six bases found in the nuclear genomes of all eukaryotes tested and are often abundant and evenly dispersed (Tautz and Renz, 1984; Lagercrantz *et al.,* 1993). SSRs range from 20-100 bp in length and are useful for evolutionary and genetic studies because of their inherent instability and high polymorphism due to the high mutation rates in microsatellite loci. For example, microsatellite loci mutation rates in yeast, was estimated to be 10^{-4} to 10^{-5} compared with 10^{-9} for point mutations (Taylor and Fisher*,* 2003).

With the advent of high-throughput sequencing technology, abundant information on DNA sequences for the genomes of many plant and fungal species has been generated (Mondini *et al.,* 2009). Expressed Sequence Tags (EST) of many fungal pathogen species have been generated and thousands of sequences have been annotated as putative functional genes using powerful bioinformatics tools. ESTs are single-read sequences produced from partial sequencing of a bulk mRNA pool that has been reverse transcribed into cDNA. EST libraries provide a snapshot of the genes expressed in the tissue at the time of, and under the conditions in which, they were sampled (Mondini *et al.,* 2009).

SSRs have now been used in a number of population studies on fungi including diversity of isolates of *Zymoseptoria tritici* causal pathogen of leaf blotch of wheat in Northern Europe (Gautier *et al.,* 2014), populations of *Phytophthora infestans* isolates in the UK, across Europe and worldwide (Lees *et al.,* 2006), diversity and population study of *Armillaria sp*. a soil-borne Basidiomycete (Xing *et al.,* 2015) and analysis of *Heterobasidion irregular,* a conifer pathogen in Italy (Garbelotto *et al.,* 2013). The advantage of SSRs markers fingerprinting is that they are selectively neutral and are more variable than random microsatellite markers such as RAPD or RAMS, they amplify single loci, and more importantly are co-dominant, so heterozygotic individuals in a diploid organism can be easily determined. These SSRs advantages are of particular value for *G. boninense* population studies in oil palm plantations in Malaysia.

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4.2 Materials and methods

4.2.1 Site selection, and re-isolation of *G. boninense* isolates

Five oil palm plantations from different geographical locations of Peninsular Malaysia and planting blocks within the plantations were randomly selected. All were known to be affected by high disease incidence of BSR (20-35%) (Fig.40). Isolates were derived from *G. boninense* basidiocarp tissue and re-isolations of *G. boninense* were done on *Ganoderma*-selective medium (GSM). Growth and storage of isolates is described in section 2.1. A total of 86 *G. boninense* isolates from infected oil palms (BSR or USR) from different estates and areas in Malaysia were used (Table 7).

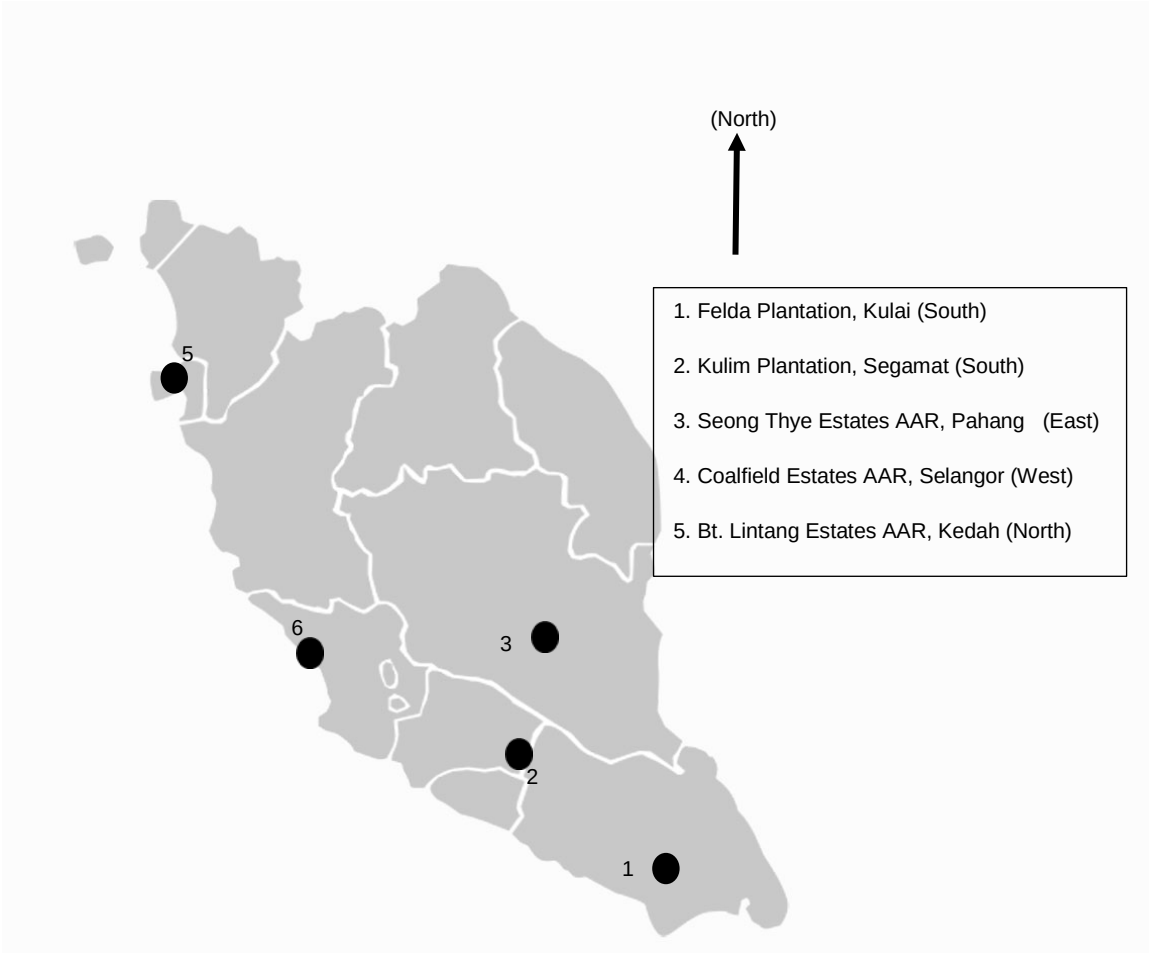


Figure 40. Geographical location of *G. boninense* collection sites in Peninsular Malaysia, from five different oil palm plantations with high incidence of basal stem rot (BSR) disease (pers. comm. plantation managers).

Table 7. Isolates of *G. boninense*, geographical location, oil palm plantation blocks and planting year.

Isolate codes	Type	Site	Location	Region (Malaysia)	Planting Year
FT1B1 S2	BSR	S2	FELDA Taib Andak	South	1994
FT1B2 S2	BSR	S2	FELDA Taib Andak	South	1994
FT1B3 S2	BSR	S2	FELDA Taib Andak	South	1994
FT1B4 S2	BSR	S2	FELDA Taib Andak	South	1994
FT2B1 S2	BSR	S2	FELDA Taib Andak	South	1994
FT2B2 S2	BSR	S2	FELDA Taib Andak	South	1994
FT2B3 S2	BSR	S2	FELDA Taib Andak	South	1994
FT3B1 S2	BSR	S2	FELDA Taib Andak	South	1994
FT3B2 S2	BSR	S2	FELDA Taib Andak	South	1994
FT3B3 S2	BSR	S2	FELDA Taib Andak	South	1994
FT4B1 S2	BSR	S2	FELDA Taib Andak	South	1994
FT4B2 S2	BSR	S2	FELDA Taib Andak	South	1994
FT4B3 S2	BSR	S2	FELDA Taib Andak	South	1994
FT5 S2	BSR	S2	FELDA Taib Andak	South	1994
FT6 S2	BSR	S2	FELDA Taib Andak	South	1994
FT1 S1	BSR	S1	FELDA Taib Andak	South	1994
FT2 S1	BSR	S1	FELDA Taib Andak	South	1994
FT3 S1	BSR	S1	FELDA Taib Andak	South	1994
FT4 S1	BSR	S1	FELDA Taib Andak	South	1994
FT5 S1	BSR	S1	FELDA Taib Andak	South	1994
FT6 S1	BSR	S1	FELDA Taib Andak	South	1994
FT7 S1	BSR	S1	FELDA Taib Andak	South	1994
FT1 S3	BSR	S3	FELDA Taib Andak	South	1994
FT2 S3	BSR	S3	FELDA Taib Andak	South	1994
FT3 S3	BSR	S3	FELDA Taib Andak	South	1994
FT4 S3	BSR	S3	FELDA Taib Andak	South	1994
FT5 S3	BSR	S3	FELDA Taib Andak	South	1994
FT6 S3	BSR	S3	FELDA Taib Andak	South	1994
FT1 S4	BSR	S4	FELDA Taib Andak	South	1994
FT2 S4	BSR	S4	FELDA Taib Andak	South	1994
FT3 S4	BSR	S4	FELDA Taib Andak	South	1994
FT4 S4	BSR	S4	FELDA Taib Andak	South	1994
FT5 S4	BSR	S4	FELDA Taib Andak	South	1994
FT6 S4	BSR	S4	FELDA Taib Andak	South	1994
FT1 S5	BSR	S5	FELDA Taib Andak	South	1994
FT2 S5	BSR	S5	FELDA Taib Andak	South	1994
FT3 S5	BSR	S5	FELDA Taib Andak	South	1994
PT1 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT2 S1	BSR	S1	Sg. Jerneh Estate	East	1994

PT3 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT4 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT5 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT6 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT7 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT8 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT9 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT10 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT11 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT12 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT13 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT14 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT15 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT16 S1	BSR	S1	Sg. Jerneh Estate	East	1994
PT1 S2	BSR	S2	Sg. Jerneh Estate	East	1994
PT2 S2	BSR	S2	Sg. Jerneh Estate	East	1994
PT1 S3	BSR	S3	Sg. Jerneh Estate	East	1994
JT1 S1	BSR	S1	Coal Fields Estate	West	1994
JT2 S1	BSR	S1	Coal Fields Estate	West	1994
JT3 S1	BSR	S1	Coal Fields Estate	West	1994
JT4 S1	BSR	S1	Coal Fields Estate	West	1994
JT5 S1	BSR	S1	Coal Fields Estate	West	1994
JT6 S1	BSR	S1	Coal Fields Estate	West	1994
JT6 S1	BSR	S1	Coal Fields Estate	West	1994
JT1 S2	BSR	S2	Coal Fields Estate	West	1994
JT2 S2	BSR	S2	Coal Fields Estate	West	1994
JT3 S2	BSR	S2	Coal Fields Estate	West	1994
JT1 S3	BSR	S3	Coal Fields Estate	West	1994
JT2 S3	BSR	S3	Coal Fields Estate	West	1994
JT3 S3	BSR	S3	Coal Fields Estate	West	1994
JT4 S3	USR	S3	Coal Fields Estate	West	1994
JT5 S3	USR	S3	Coal Fields Estate	West	1994
KT1 S1	BSR	S1	Bt Lintang Estate	North	1994
KT2 S1	BSR	S1	Bt Lintang Estate	North	1994
KT3 S1	BSR	S1	Bt Lintang Estate	North	1994
KT4 S1	BSR	S1	Bt Lintang Estate	North	1994
KT1 S2	BSR	S2	Bt Lintang Estate	North	1995
KT2 S2	BSR	S2	Bt Lintang Estate	North	1995
KT3 S2	BSR	S2	Bt Lintang Estate	North	1995
KT4 S2	BSR	S2	Bt Lintang Estate	North	1995
KT1 S3	BSR	S3	Bt Lintang Estate	North	1995
KT2 S3	BSR	S3	Bt Lintang Estate	North	1995
KT3 S3	BSR	S3	Bt Lintang Estate	North	1995
KT4 S3	BSR	S3	Bt Lintang Estate	North	1995
KT5 S3	BSR	S3	Bt Lintang Estate	North	1995
KT6 S3	BSR	S3	Bt Lintang Estate	North	1995

KT7 S3	BSR	S3	Bt Lintang Estate	North	1995
KT8 S3	BSR	S3	Bt Lintang Estate	North	1995
LT1 S1	BSR	S1	Kulim Estate	South	1997
LT2 S1	BSR	S2	Kulim Estate	South	1997
LT3 S1	BSR	S3	Kulim Estate	South	1997
LT4 S1	BSR	S4	Kulim Estate	South	1997
LT5 S1	BSR	S5	Kulim Estate	South	1997
LT6 S1	BSR	S6	Kulim Estate	South	1997
LT7 S1	BSR	S7	Kulim Estate	South	1997
LT8 S1	BSR	S1	Kulim Estate	South	1997
LT9 S1	BSR	S2	Kulim Estate	South	1997
LT10 S1	BSR	S3	Kulim Estate	South	1997

4.2.2 Vegetative compatibility testing

Isolates were examined under the microscope to confirm the presence of clamp connections, and each somatic incompatibility group (SIG) determined by pairing heterokaryotic isolates in agar culture, using procedures similar to those described previously (Adams, 1974). Inocula ($1mm^3$), taken from actively growing cultures, were paired in all combinations in a 10-mm-square grid pattern arrangement in 120 mm-square plastic Petri plates containing 1% MEA. Plates were then incubated inverted in darkness at 25°C for up to 28 days and reactions observed every 7 days. All combinations were evaluated in triplicate. Incompatibility reactions between paired isolates, evident as either a zone of sparse mycelial growth or barrage formation with or without dark pigment deposition, indicated different genetic constitution, whilst a compatible reaction, evident as a coalescence of paired isolates, indicated identical genetic constitution (Rayner and Todd, 1979; Rayner *et al*., 1984).

4.2.3 Random amplified microsatellites (RAMS)

The primers used to amplify microsatellite DNA were adapted from Hantula *et al*. (1996). The degenerate primers were 5'DHB$(CGA)_5$'3 (RAMS1), and 5'HBH$(GAG)_5$ 3' (RAMS2) in which D, H, and B were used as degenerate sites and D = (G, A or T); H = (A,T or C); B = (C, G or T). The protocol for RAMS amplification was based on that of Rees *et al*. (2011). PCR amplification was performed on an MJ Research Cycling (PTC-100^{TM}) using a total volume of 50µl consisting of 0.4 µM of each primer, 50 mM KCL, 10 mM Tris-HCl (pH 9.0 at 25ºC), 1.5 mM $MgCl_2$, 0.1% Triton® X-100, 0.1 mM of dNTP mix (10mM, Promega), 0.02 u/µl GoTaq® DNA polymerase (5u/µl, Promega), 20 ng of genomic DNA, and sterile Milli-Q water. Reactions were performed with an initial denaturation of 10 min at 95ºC followed by 37 cycles of 30 seconds denaturation at 95ºC, 45 sec annealing at 61ºC and 2 min extension at 72ºC. A final extension of 10 min at 72ºC was performed after the cycles ended. Amplification products were assessed on a 2% w/v agarose gel stained with ethidium bromide run for 5 hours at 50 volts in a large gel tank to obtain clear band separation and visualized under UV illumination. Comparisons of the banding patterns were made using the 1kb marker as a molecular size standard.

4.2.4 Simple sequence repeat markers (SSRs)

The selected cDNA-SSR markers were converted into IRDye®-detection markers, in which a fusion of the forward cDNA-SSR primer with a M13 (-29) tail, 5'-CACGACGTTGTAAAACGAC-3' (19bp), at the 5'end was synthesised for PCR amplification (Schuelke, 2000; Wong *et al*., 2015). During the PCR amplification, the universal IRDye®-labeled M13 (-29) primer was incorporated into the PCR reaction for labelling the PCR products with the fusion primer and standard reverse primer. Higher resolution of genetic profile for *G. boninense* was achieved when DNA fingerprinting was performed on NEN 4300 DNA Analyzer (Li-COR Biosciences, USA). PCR mixture consisting of 5µl of TopTaq Master Mix Kit (Qiagen, Germany), approximately 40 ng of

DNA template, 0.05µM of M13 Forward (-29) IRDye® 700 primer, 0.5µM of each primer and 1.5µl of nuclease free water to make up a volume of 10µl reaction. The PCR programme used was one cycle of 94°C for 3 minutes; 94°C for 30s; 55°C for 30s; 72°C for 60s followed by 34 cycles of 94°C for 30s; 55°C for 30s; 72°C for 60s and an additional extension of 10 minutes at 72°C before cooling to 10°C. The thermocycling was performed with VeritiTM Thermal Cycler (Applied Biosystem, USA). Binary and codominant scoring was carried out manually to avoid misinterpretation of mixed intensity DNA fragment patterns. Cluster analysis was carried out using the multivariate statistical package MVSP (Kovach Computing Services, Anglesey, Wales) to genetically distinguish the *G. boninense* isolates collected from different disease severity areas.

4.2.5 Basidiospore collection

Sporulating basidiocarps (fruit bodies) were identified within COALFIELD Estates oil palm plantation (refer Fig. 20 in section 4.2.1). Spores were collected from single fresh basidiocarps by suspending a plastic plate covered with clean paper 5–10 mm below the surface of the pores. The entire fruiting body and suspended plate was then covered with aluminium foil to prevent airborne contamination. After 30– 60 min, the spore prints were collected along with the basidiocarps. To determine the uniformity of mycelia in a single palm, three spore prints were collected from 10 palms displaying multiple basidiocarps.

4.2.6 Isolation of monokaryons

Spores were suspended in a drop of sterile distilled water and streaked onto plates containing distilled water agar. Plates were sealed with parafilm and incubated in the dark at 27°C. After germination (1-2 d) single spores were transferred with a scalpel to malt extract agar (MEA) using a stereomicroscope at 50 x under dark field illumination. All monokaryons and dikaryons were maintained on MEA at 27°C in the dark, and all isolates used are stored in 2°C in water and MEA slant.

4.2.7 Mating experiments

Agar discs of cultures of sibling monokaryons (a minimum of nine) were placed 1 cm apart on PDA in all combinations and incubated at 30°C for 3–4 days. A small section of the area at the confluence of the two cultures was then transferred to PDA plates and incubated at 30°C for a further 2–3 days. This period was found to be sufficient for clamp formation if sub-culturing was done. In the absence of sub-culturing, clamp formation was seen 5–7 days after mating. Mycelia were checked *in vitro* for the presence of clamps by direct observation at 320×magnification. Tester strains representing the four mating types from each basidiocarp were retained and crossed with the four tester isolates of the other basidiocarps in all combinations as described (Pilotti *et al.,* 2003).

4.2.8 Genetic variation and *pairwise* relatedness analysis

At least three RAMS and SSR amplifications were performed for each *Ganoderma* isolate and only amplicons that reproduced consistently were scored for presence (1) or absence (0). Identical banding patterns were regarded as genetically identical and were only introduced to the matrix once for statistical analysis. The reduced matrix was analysed using cluster analysis based on standardised Euclidean distance (Rees *et al.,* 2012) and using the unweighted pair-group method with arithmetic means (UPGMA) (Gherbi *et al.,* 1999) to investigate genetic relationships using MVSP package (Kovach Computing, Anglesey).

The mycelia of single spore culture of *G. boninense* only has one allele at each locus, whereas pure mycelia culture isolated from fruiting body (or diseased trunk tissue) showed either one or two alleles per locus. This confirmed that *G. boninense* has a dikaryon phase (n+n) on the course of invasion in oil palm. For the purpose of measuring the *G. boninense* genetic diversity in the plantations, we assumed each pure mycelial culture represents at least two alleles per locus. We calculated the allele frequencies by population and analysed the heterozygosity (Ho and He), polymorphism, Shannon's information index and Fixation index by locus and pairwise relatedness of the *Ganoderma boninense* isolates from the plantations according to frequency based statistical procedures in GenAlEx 6.5 (Peakall and Smouse, 2012).

4.3 RESULTS

4.3.1 Determination of genetic relationship of *G. boninense* isolates using somatic incompatibility, RAMS and SSRs

In this study, determination of genetic diversity of *G. boninense* isolates was conducted to address these key issues: i) Are neighbouring palms infected by the same isolate of *G. boninense* ii) Is the same isolate of *G. boninense* found in individual living palms and iii) How diverse are *G. boninense* isolates from different geographical locations within Peninsular Malaysia.

4.3.1.1 Somatic incompatibility test

This technique has been used widely in genetic variation studies of many fungal pathogens, notably the related conifer rot caused by *Heterobasidion annosum* (Garbelotto *et al.,* 2013) and root rots by *Armillaria* spp. (Gatto *et al.,* 2009). Also it has found application with *Ganoderma* in Malaysia and Papua New Guinea (Miller *et al.,* 1995, Ariffin *et al*., 1996, Pilotti *et al.,* 2003).

Basidiocarp tissue was cut and surface sterilised in sodium hypochlorite, washed three times with sterilised distilled water, plated onto GSM and incubated at 27°C in the dark for 7 days. A total of 86 *G. boninense* isolates were collected (Table 7 in section 4.2.1), and confirmed microscopically by the presence of clamp connections and PCR DNA detection (Fig. 41 and section 3.3 respectively). The somatic incompatibility group (SIG) test was carried out as described in section 4.2.2. Different *Ganoderma* isolates from within and between different plantations were paired by placing a 1 mm^3 agar disc of each dikaryon isolate 1 cm apart on malt extract agar plates. Plates were observed and recorded macroscopically after 14 d incubation.

All isolates of *Ganoderma* possessed clamp connections, indicating that they were heterokaryons (Fig. 41). Reactions between incompatible strains, macroscopically visible after 7 days, became progressively more distinct over 14 days. All isolates completely intermingled when paired against themselves, indicating self-compatibility. This phenotype was used as a standard; when pairs of isolates from different origins gave a similar reaction, they were judged to be compatible. Evidence of incompatible reactions (zone of sparse mycelia, barrage and/or pigment deposition) was interpreted as representing different genetic constitutions. All three replicates of pairings of isolates consistently revealed the same distinctions between compatible and incompatible groups.

In all self-pairings, the mycelia merged with no presence of a demarcation line on MEA forming a single colony indicating somatic compatibility (Fig 42a). On the other hand, pairing of all isolates from different plantations showed incompatibility reactions with the presence of demarcation lines with or without barrage formation (Fig. 42b-c).

Interactions of all combinations in the somatic incompatibility test are shown in Table 8. The majority >90% of the pairings were somatically incompatible within and between plantations, indicating these isolates were of different genetic origin.

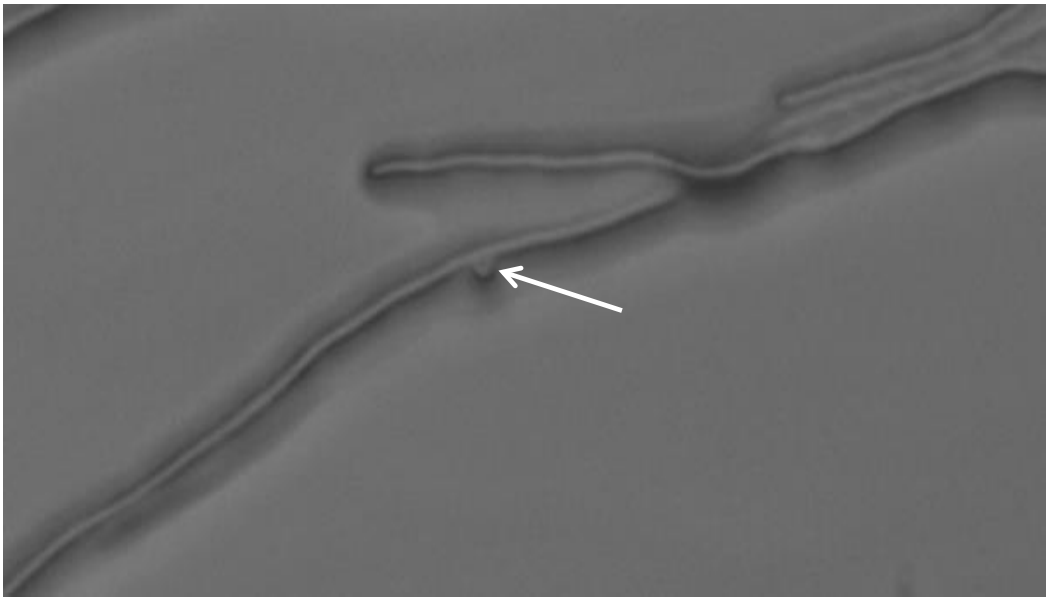


Figure 41. Microscopic confirmation of *Ganoderma* based on clamp connection (arrow) formation. Images were taken under a light microscope (Olympus) at 200x magnification.

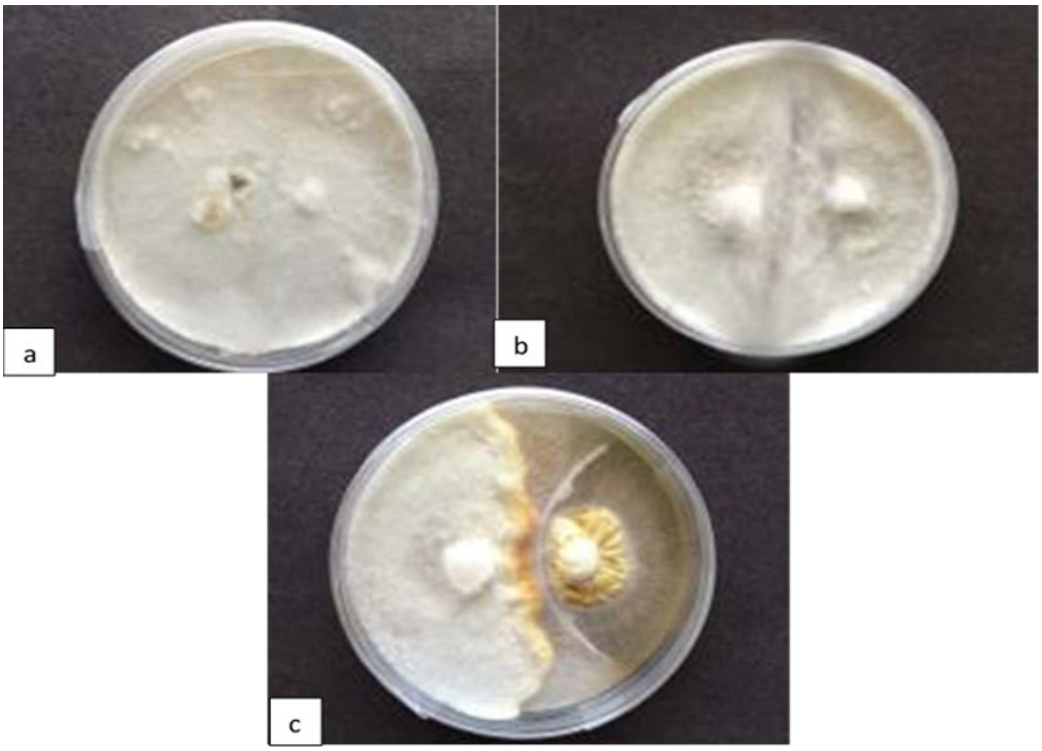


Figure 42. Somatic incompatibility reactions of *G. boninense*: different antagonisms reaction observed in this study. a: Self-pairing (control) showing somatic compatibility, no antagonism. b: Weak somatic incompatibility reaction, no pigmentation. c: Strong somatic incompatibility reaction, with raising of the mycelial mat, usually with pigmentation.

Table 8. Somatic incompatibility tests of *G. boninense* from four oil palm plantations in South, West, East and North of Peninsular Malaysia.

	FT1 S2 BSR	FT2 S2 BSR	FT3 S2 BSR	FT4 S2 BSR	FT1 S1 BSR	FT2 S1 BSR	FT3 S1 BSR	FT4 S1 BSR	FT5 S1 BSR	FT2 S3 BSR	FT3 S3 BSR	FT4 S3 BSR	FT1 S4 BSR	FT2 S4 BSR	FT1 S5 BSR	FT2 S5 BSR	PT1 S1 BSR	PT2 S1 BSR	PT3 S1 BSR	PT4 S1 BSR	PT5 S1 BSR	PT6 S1 BSR	PT1 S2 BSR	PT2 S2 BSR	PT1 S3 BSR	JT1 S1 BSR	JT2 S1 BSR	JT3 S1 BSR	JT4 S1 BSR	JT1 S2 BSR	JT2 S2 BSR	JT3 S2 BSR	KT1 S1 BSR	KT2 S1 BSR	KT3 S1 BSR
FT1 S2BSR	0																																		
FT2 S2BSR	+	0																																	
FT3 S2 BSR	++	+	0																																
FT4 S2BSR	+++	++	+	0																															
FT1 S1 BSR	++	++	++	++	0																														
FT2 S1 BSR	++	++	++	++	+	0																													
FT3 S1 BSR	++	++	++	++	++	+	0																												
FT4 S1 BSR	++	++	++	++	++	++	++	0																											
FT5 S1 BSR	++	++	++	++	++	++	++	++	0																										
FT2 S3 BSR	++	++	++	++	++	++	++	++	++	0																									
FT3 S3 BSR	++	++	++	++	++	++	++	++	++	++	0																								
FT4 S3 BSR	++	++	++	++	+++	++	++	++	++	++	++	0																							
FT1 S4 BSR	++	++	++	++	++	++	++	++	++	++	++	++	0																						
FT2 S4 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	0																					
FT1 S5 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0																				
FT2 S5 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0																			
PT1 S1 BSR	+++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0																		
PT2 S1 BSR	+++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0																	
PT3 S1 BSR	+++	++	++	++	++	++	++	++	++	++	++	++	+++	++	++	++	+	++	0																
PT4 S1 BSR	+++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0															
PT5 S1 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0														
PT6 S1 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	0													
PT1 S2 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0												
PT2 S2 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	0											
PT1 S3 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	0										
JT1 S1 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0									
JT2 S1 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0								
JT3 S1 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0							
JT4 S1 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0						
JT1 S2 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0					
JT2 S2 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0				
JT3 S2 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	0			
KT1 S1 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	0		
KT2 S1 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	0	
KT3 S1 BSR	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	0

Somatic incompatibility: 0= compatible, +=weak incompatibility, ++=strong incompatibility
Key represents 4 different plantations: FT= Felda plantation, PT= Seong Thye estate, JT= Coalfieds estate, KT= Kulim plantation

4.3.1.2 Determination of genetic relationship of *G. boninense* isolates using RAMS

RAMS developed by Zietkiewicz *et al.* (1994) combines the universality of the RAPD analysis and the advantages of microsatellite analysis. Microsatellites represent optimal markers because they are polymorphic within populations, highly abundant, and evenly dispersed throughout eukaryotic genomes, giving RAMs analysis a high discriminatory power (Hearne *et al.*, 1992; Wu and Tanksley, 1993; Guarro *et al.*, 2005)

Cultures of 86 *G. boninense* isolates from five different oil palm plantations of different geographical locations within Peninsular Malaysia (see Table 7) were grown from stocks and DNA extraction was performed as described in materials and methods. All DNA was subsequently quantified by Nanodrop and all samples used in this investigation were above the necessary DNA concentration for RAMs amplification, >20ng. Three separate RAMs amplifications were conducted.

The RAMS amplification provided 2-14 clear bands per amplification, ranging from 200-2250 base pairs for each *G. boninense* isolate. Gel images were scored manually and identical banding patterns were regarded as probably clonal; a binary matrix was compiled from the different profiles and is shown in Table 9. This technique was reproducible and Fig. 43 shows PCR reactions from multiple isolates from a single BSR infected palm revealing the same RAMS profile. The procedure discriminated between *G. boninense* isolates within and between planting blocks of different plantations from different geographical locations that were inseparable by vegetative incompatibility testing.

Examination of the RAMS profiles mostly did not reveal identical genets from BSR infected trees within planting blocks from any of the oil palm plantation blocks (Fig. 44-47). Therefore there was no evidence to indicate vegetative spread of the disease from infected palms to adjacent palms. Only one plantation block contained adjacent BSR palms that appeared to have identical fingerprints. KT2S1 BSR had an identical band pattern to KT3S1 BSR in Bt. Lintang Estates, block 3 (situated in the north of Peninsular Malaysia) (Fig.46; Lane 1 and lane 2). In all other cases RAMS profiles from isolates obtained from neighbouring and separate trees were genetically distinct. For two identical banding patterns to occur on adjacent palms, infection of the trees is almost certain to have occurred through vegetative mycelial growth.

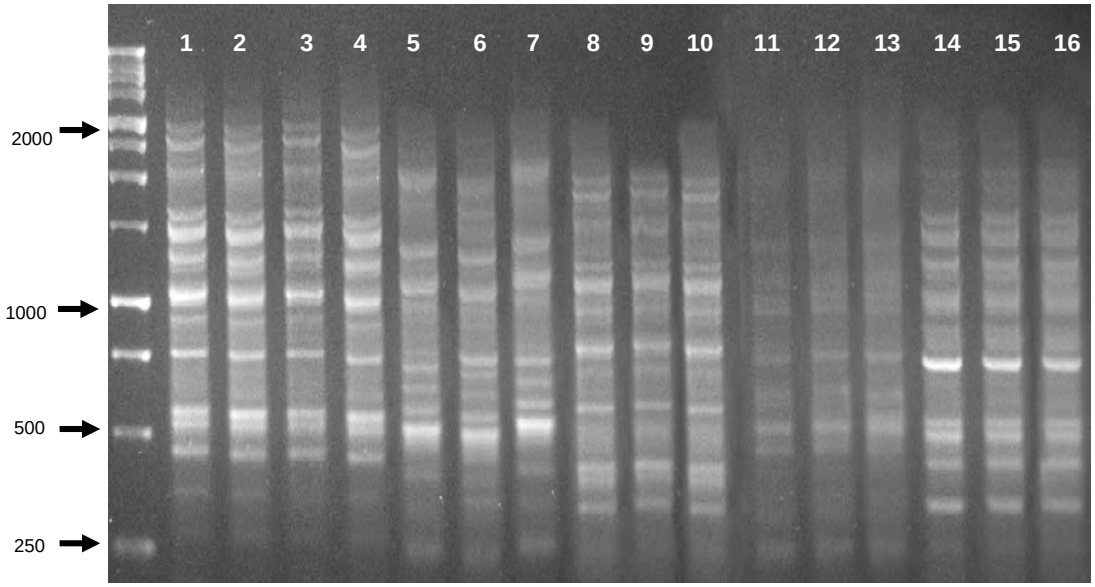


Figure 43. RAMS fingerprints from five individual BSR infected trees with multiple basidocarps within one block of a FELDA plantation. Lanes represent PCR reactions of a *G. boninense* isolate from different basidiocarps within the same palm. Lanes with similar banding patterns represent G. boninense isolate within the same tree. Lane 1-4: FT1S2 B1, FT1S2 B2, FT1S2 B3, FT1 S2 B4. Lane 5-7: FT2S2 B1, FT2S2 B2, FT2S2 B3. Lane 8-10: FT3S2 B1, FT3S2 B2, FT3S2 B3. Lane 11-13: FT4S2 B1, FT4S2 B2, FT4S2 B3. Lane 14-16: FT5S2 B1, FT5S2 B2, FT5S2 B3.
F= Felda plantation; T1= Tree number; S2= Plantation block; B1= Bracket no.

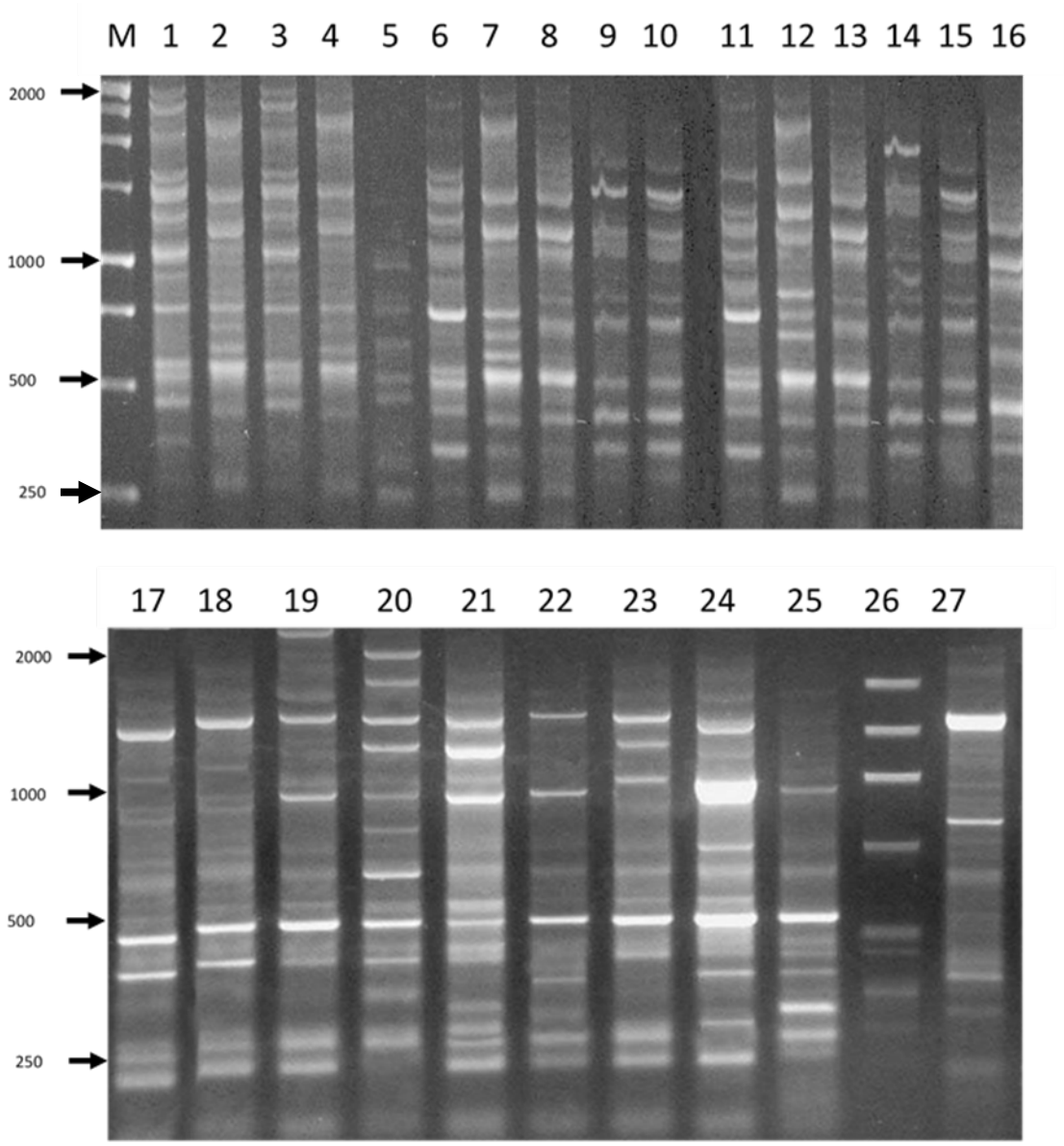


Figure 44. RAMS fingerprints of *G. boninense* isolates from BSR infected trees in FELDA plantation from different planting blocks. Each lane represent PCR amplification. Lane1-6: FT1S2, FT2S2, FT3S2, FT4S2,FT5S2, FT6S2. Lane 7-13: FT1S1, FT2S1, FT3S1, FT4S1,FT5S1, FT6S1, FT7S1. Lane 14-20: FT1S3, FT2S3, FT3,S3, FT4S3, FT4S3, FT5S3, FT6S3. Lane 20: FT1S4,FT2S4, FT3S4, FT4S4, FT5S4, FT6S4. Lane 24-27: FT1S5, FT2S5,FT3S5. Adjacent palm= lane 6, 7 and 8; 12, 13, 14; 20, 21 and 22.

F= Felda plantation; T1= Tree number; S2= Plantation block.

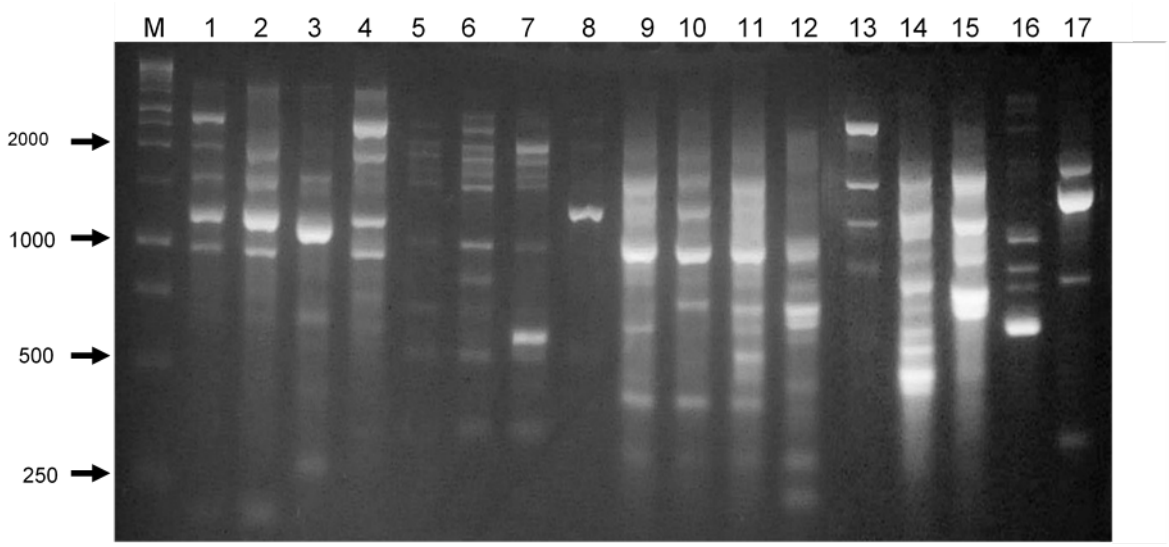


Figure 45. RAMS fingerprints of *G. boninense* isolates from BSR infected trees in Seong Thye Estates, Pahang from different planting blocks. Each lane represent PCR amplification. Lane 1-16:PT1S1, PT2S1, PT3S1, PT4S1, PT5S1, PT6S1, PT7S1, PT8S1, PT9S1, PT10S1, PT11S1, PT12S1, PT13S1, PT14S1, PT15S1, PT16S1. Lane 17: PT1 S2.
Adjacent palm: lane 2 and3; 12, 13, 14 and 15.

P= Seong Thye estates; T1= Tree number; S= Plantation block no.

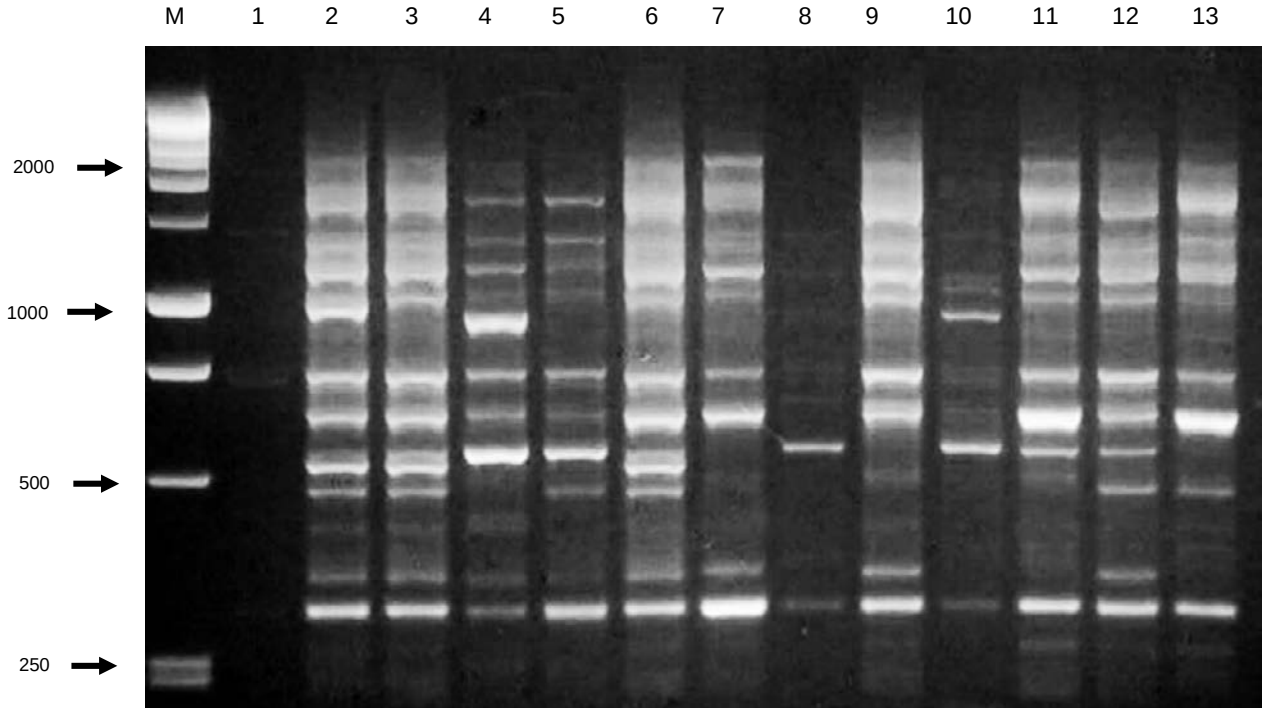


Figure 46. RAMS fingerprints of *G. boninense* isolates from BSR infected trees in Bt. Lintang Estates, Kedah from different planting blocks. Each lane represent PCR amplification. Lane 1-4: KT1S1, KT2S1, KT3S1, KT4S1. Lane 5-8: KT1S2, KT2S2, KT3S2, KT4S2. Lane 9-13: KT1S3, KT2S3, KT3S3, KT4S3, KT5S3.
Adjacent palm: Lane 2 and 3, 7, 8 and 9 label.
K= Bt. Lintang estates; T1= Tree number; S= Plantation block no.

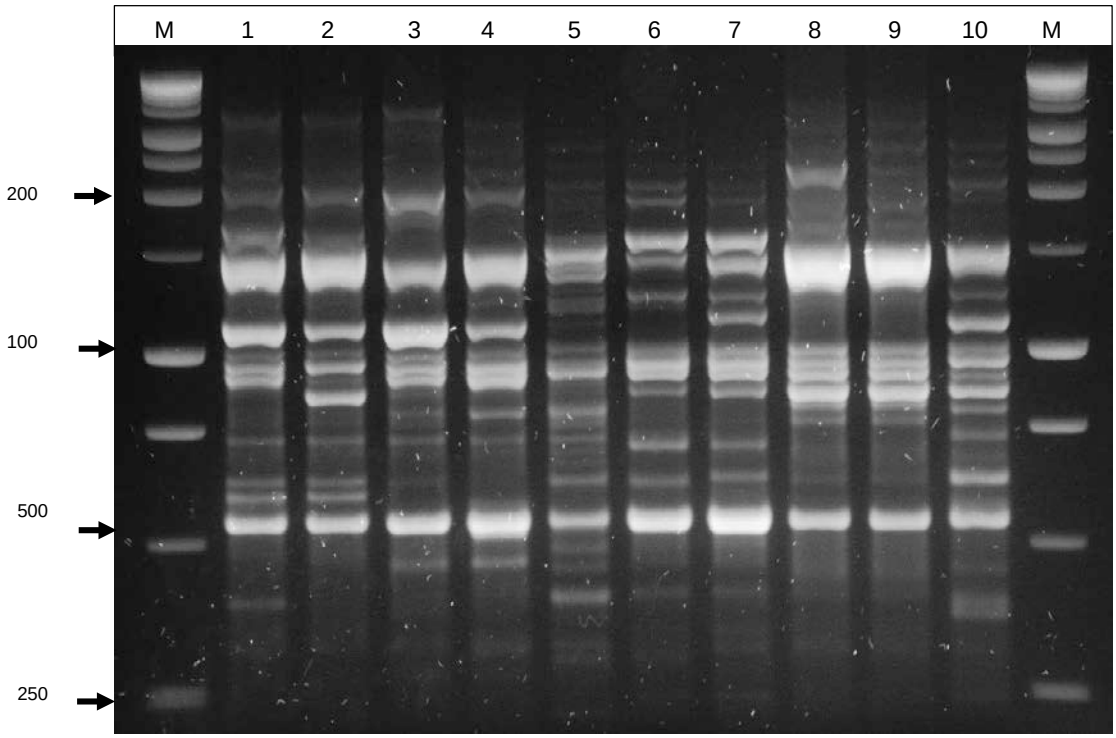


Figure 47. RAMS fingerprints of *G. boninense* isolates from BSR infected trees in Kulim Plantation, Johor from different planting blocks. Each lane represent PCR amplification. Lane 1:-10 = LT1S1– LT10S1.Adjacent palm= lane 2 and 3, 5, 6, 9, 10. L= Kulim plantations; T1= Tree number; S= Plantation block no.

Table 9. RAMs profiles of GB isolates from five different plantations of different geographical locations within Peninsular Malaysia. Matrix of presence (1) or absence (0) of the 27 RAMs amplified fragments showed highly polymorphic bands with the primers ACG and CCA. Column numbers 250-2250 represent molecular weight of observed bands. Note the same profile from isolates KT3S1 BSR and KT2S1 BSR obtained from neighbouring palms in plot 1-BT Lintang Estates,Kedah (North of Peninsular Malaysia). Different profiles were obtained from isolates within the several BSR infected palms from several different oil palm plantations from different geographical location: BSR trees from North: Bt. Lintang Estate, Kedah, West: COALFIELD Estates, Selangor, East: Seong Thye Estates, Pahang, South: Felda Plantation, Kulai, South: Kulim Plantation.

Isolates	250	300	350	400	450	470	500	550	600	650	670	700	750	800	900	1000	1100	1200	1250	1300	1500	1700	1900	1950	2000	2250
FT1S2BSR	0	0	1	0	1	0	1	1	0	0	0	0	1	0	1	1	0	0	1	0	1	0	0	0	1	0
FT2S2BSR	1	0	0	1	0	0	0	1	1	0	0	1	1	0	0	0	1	0	0	0	1	0	0	0	0	1
FT3S2BSR	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0	1	0	0	1	0	1	0	0	0	0	0
FT4S2BSR	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1
FT5S2BSR	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
FT6S2BSR	1	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0
FT1S1BSR	0	1	0	0	0	0	1	1	0	1	0	1	0	0	0	0	1	0	1	0	0	0	0	0	1	0
FT2S1BSR	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0
FT3S1BSR	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FT4S1BSR	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
FT5S1BSR	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0
FT6S1BSR	0	1	0	1	1	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0
FT7S1BSR	1	0	0	1	1	0	0	0	0	1	0	0	0	0	1	1	1	0	1	0	0	0	0	0	0	0
FT1S3BSR	1	1	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0
FT2S3BSR	0	0	0	1	1	0	0	0	1	0	0	1	0	0	1	1	1	0	1	0	1	0	0	0	0	0
FT3S3BSR	0	1	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0

FT4S3BSR	1	1	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	0	1	0	0	1	0	0	0	0
FT5S3BSR	1	1	0	0	0	0	0	0	1	1	0	0	1	1	0	0	1	0	0	0	0	0	1	0	0	1
FT6S3BSR	0	1	0	1	0	0	1	1	1	1	0	0	1	0	1	0	1	0	0	0	1	0	1	0	0	0
FT7S3BSR	1	1	1	1	0	0	1	1	1	1	0	0	1	0	0	0	1	0	0	0	1	1	0	0	0	0
FT1S4BSR	1	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	0	1	0	0	0
FT2S4BSR	1	1	0	0	1	0	1	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0
FT3S4BSR	1	1	0	0	0	0	1	0	1	1	0	1	1	1	0	0	1	0	0	0	0	1	0	0	0	1
FT4S4BSR	0	0	1	0	1	0	1	1	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
FT5S4BSR	0	1	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0
FT6S4BSR	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	0	0	0
FT1S5BSR	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
PT1S1BSR	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
PT2S1BSR	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1
PT3S1BSR	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0
PT4S1BSR	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	1
PT5S1BSR	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
PT6S1BSR	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0
PT7S1BSR	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
PT8S1BSR	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PT9S1BSR	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
PT10S1BSR	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
PT11S1BSR	0	0	1	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
PT12S1BSR	1	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
PT13S1BSR	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
PT14S1BSR	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
PT15S1BSR	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
PT16S1BSR	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	0	0	1	0	0	0	0	0	1	0
PT1S2BSR	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
PT2S2BSR	0	0	0	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0
JT1S1BSR	0	0	0	0	1	0	1	1	0	1	1	0	0	1	1	0	1	0	1	0	0	0	0	0	1	0

JT2S1BSR	0	0	0	0	0	1	0	0	0	0	0	1	0	1	1	0	1	0	1	0	0	0	1	0	1	0
JT3S1BSR	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	1	0	0	0	0	0	0	0
JT4S1BSR	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1
JT5S1BSR	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	1	0	0	0	0	1	1	0	0	0
JT6S1BSR	0	0	0	0	0	1	0	1	0	1	0	0	1	1	0	1	0	0	0	0	1	1	1	0	0	1
JT6S1USR	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	1	0	0	0	0	1	1	0	1	0	0
JT1S2BSR	0	0	0	1	1	1	0	0	1	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	1
JT2S2BSR	0	1	0	0	0	1	0	1	1	1	0	1	1	0	0	0	1	0	0	0	0	1	0	1	0	0
JT3S2BSR	0	0	1	0	1	1	0	1	1	1	0	1	0	1	0	1	1	0	0	0	1	0	0	0	0	0
JT1S3BSR	0	0	1	0	0	1	0	1	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0	0	1
JT2S3BSR	1	0	1	0	0	1	0	0	1	0	0	1	1	1	1	1	1	0	0	0	0	1	0	0	0	1
JT3S3BSR	0	0	0	0	0	0	0	1	0	0	0	0	1	0	1	1	1	1	0	0	0	1	1	0	0	1
JT4S3BSR	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	1	1	0	0	1
JT5S3USR	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	1	0	0	1	0	1	0	1	0	1	1
KT1S1BSR	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
KT2S1BSR	0	0	1	1	1	0	1	1	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1
KT3S1BSR	0	0	1	1	1	0	1	1	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1
KT4S1BSR	0	0	1	1	1	0	0	1	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0
KT1S2BSR	0	0	1	1	0	0	1	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
KT2S2BSR	1	1	1	1	0	0	1	1	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1
KT3S2BSR	0	0	1	1	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1
KT4S2BSR	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
KT1S3BSR	1	1	1	1	1	0	1	0	0	0	0	1	1	0	0	1	0	0	1	0	0	0	0	0	0	1
KT2S3BSR	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0
KT3S3BSR	1	1	1	0	0	0	1	0	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1
KT4S3BSR	0	0	1	1	0	0	1	0	1	1	0	0	1	0	0	1	0	0	1	0	0	0	0	0	0	1
KT5S3BSR	0	1	1	0	0	0	1	0	1	1	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1
KT6S3BSR	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
LT1S1BSR	0	0	1	1	1	0	0	0	1	0	0	1	1	0	0	0	1	0	1	0	1	0	1	0	1	0
LT2S1BSR	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0	0

LT3S1BSR	0	1	0	1	0	0	0	0	1	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0
LT4S1BSR	0	0	0	1	0	0	0	0	1	0	0	0	1	0	1	1	1	0	1	0	0	1	1	0	0	0
LT5S1BSR	0	0	0	0	0	0	1	1	1	0	0	0	1	0	1	1	1	0	1	0	0	1	1	0	0	0
LT6S1BSR	0	1	1	0	0	0	0	0	0	1	0	0	1	1	1	1	1	0	1	0	0	0	0	0	0	1
LT7S1BSR	0	1	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	0	1	0	0	0	0	0	0	1
LT8S1BSR	0	1	1	0	0	0	1	0	0	1	0	0	1	1	1	1	1	0	1	0	1	0	0	0	0	0
LT9S1USR	0	0	1	1	0	0	1	0	1	0	0	1	0	0	0	1	1	0	0	0	0	1	0	0	0	0
LT10S1USR	1	0	1	1	0	0	1	0	1	0	0	1	0	0	0	1	1	0	1	0	1	0	0	0	0	1

The dendrogram based on UPGMA cluster analysis of RAMS bands is shown in Fig. 48. Examination of the cluster analysis grouped the isolates into two clusters at a similarity level of <10% based on similarity coefficient (DICE) (Rees *et al.,* 2012). One group contained only eleven isolates (JT1S1 to FT5S2) collected at the East and South of Peninsular Malaysia (Seon Thye Estate and Felda Plantation) with a similarity level 20% to 40%. In addition the degree of clustering within the major group of the *G. boninense* isolates investigated ranged from between 20% (KT6S3 BSR) to 97% (isolates KT3S1 BSR and KT2S1 BSR), indicating a high intra-specific genetic variability in the regions amplified by the primers.

The majority of the isolates did not group together based on their locality. However some isolates from the same plantation were observed to form a sub group. For example, there are two subgroups: the Bt Lintang Estates (sub group) isolates KT5S3 BSR to KT2S1 BSR and Felda Plantation (sub group) FT1S4 BSR to FT1S3 BSR. These suggest that the possible *G. boninense* inoculum e.g. KT2S1 of Bt Lintang Estates had spread within Bt. Lintang palm planting blocks through spore dispersal between Block S1, S2 and S3. Furthermore, cluster analysis shows that the only identical isolates from neighbouring trees are KT2S1 BSR and KT3S1 BSR isolates in Bt. Lintang plantation, North of Peninsular Malaysia and Kulim Plantations BSR isolates LT6S1 and LT7S1, with 100% and 82% genetic similarity respectively. These may indicate that here *G. boninense* is spreading vegetatively by means of root to root contact in this oil palm plantation.

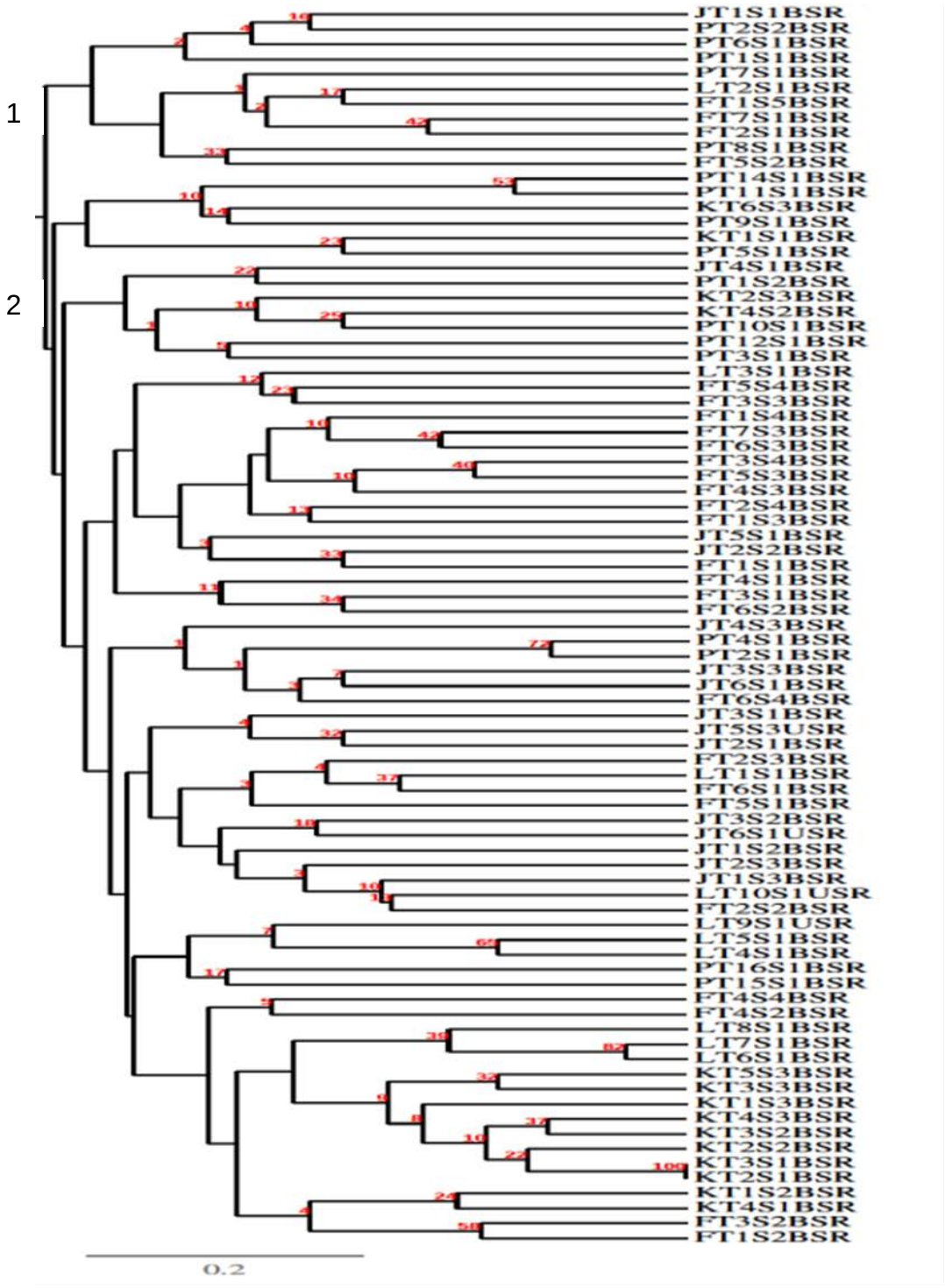


Figure 48. Dendrogram illustrating high genetic diversity of *G. boninense* isolates from different geographical areas of Peninsular Malaysia (South, West, East, North). Clonal isolates were detected between neighbouring palm (KT3S1 and KT2S1). FT= Felda plantation, PT=Seon Thye estates, KT= Bt. Lintang estate, LT= Kulim plantation. T= Tree no. S= Plantation block no.

4.3.1.3 *Ganoderma* genetic profiling using Simple Sequence PCR based markers (EST-SSRs)

This SSR fingerprinting of *G. boninense* was done in collaboration with the oil palm company, Applied Agriculture Resources (AAR), Malaysia. Eight SSR *G. boninense* markers developed by AAR, Malaysia (unpublished data; pers. comm. Wei Chee Wong) were used to fingerprint *G. boninense* isolates as listed in Table 7 (section 4.2.1). All the cDNA-SSR markers are associated with *Ganoderma* functional genes (*Ganoderma* expression sequence tags (EST) transcriptomes), which are known to be well conserved between different isolates. These markers were claimed to be useful for distinguishing *Ganoderma* isolates and did not amplify any other microorganism's genomic DNA of bacteria and fungi (Unpublished data, Wei Chee Wong).

Preparation of *G. boninense* isolates and DNA extraction were done as described (2.5.1). Due to contamination and low DNA yield obtained from *G. boninense* isolates in this study, out of 64 only 39 *G. boninense* isolates from four different oil palm plantations (Bt. Lintang Estates, Coalfield Estates, Seong Thye Estates and Felda Plantations) were used for SSR fingerprinting (refer Table 7 for list of isolates). The SSR PCR amplification procedure is described in section 4.2.4. The PCR-resulting electrophoretic patterns were analysed and the total number of amplified and polymorphic fragments was calculated. Only the most intense and highly reproducible fragments were analysed thereafter.

Eight polymorphic cDNA-SSR markers (Table 10) were used for fingerprinting the 39 *G. boninense* isolates collected from different oil palm plantations. Allelic diversity by locus was estimated using heterozygosity (H_o and H_e) and polymorphism information content value (PIC). In this study, cDNA-SSR markers with PIC more than 0.5 were used as a standard criterion to identify the informative and high polymorphic marker for distinguishing the *G. boninense* isolates. Results showed that 8 cDNA-SSR markers were each successful in genotyping at least 39 individual isolates from different oil palm

plantations and exceeded 0.5 for the value of He and PIC (Table 10). The mean expected heterozygosity for the population of 39 isolates was 0.7755 and the combined non-exclusion probability (identity) for this set of 8 SSR markers (Table 10) was 1.16^{E-09}. High heterozygosity implies that out-crossing, which causes diversification, is favoured within the population of *G. boninense* isolates. This is agreeable with the findings of Pilotti *et al.* (2002; 2003) although they did not report the heterozygosity of *G. boninense*.

Table 10. A total of 8 cDNA-SSR markers show high genetic diversity of 39 *G. boninense* isolates. High heterozygosity (He) indicates outcrossing within *G. boninense* population. K: Number of alleles at the SSR locus; N: Number of individuals genotyped; Ho: Observed heterozygosity; He: Expected heterozygosity; PIC: Polymorphic information content.

No.	Locus	K	N	HO	HE	PIC
1	P4_1	12	37	0.568	0.879	0.855
2	P4_10	10	39	0.41	0.858	0.829
3	P4_24	9	39	0.418	0.817	0.78
4	P4_62	11	39	0.41	0.711	0.682
5	P4_64	8	39	0.333	0.837	0.594
6	P5_4	4	39	0.385	0.706	0.64
7	P5_13	11	39	0.538	0.832	0.807
8	P6_11	7	36	0.333	0.749	0.697

Table 11 and 12 show a genetic similarity matrix of *G. boninense* isolates within and between different palm plantations. Isolates PT2 to PT13 within the Song Thye Estates showed a genetic similarity from 0.087 to 0.364, (< 40% similarity) (Table 11). Similarly, genetic similarity of isolates between Seon Thye Estates and Felda Plantation (situated in North and South of Peninsular Malaysia respectively) showed genetic similarity from 0 to 0.400 (< 40% similarity) (Table 12). The low genetic similarity of *G. boninense* isolates within and between plantations indicate high genetic diversity which possibly result from out-crossing of *G. boninense* basidiospores. However in Bt. Lintang Estates, *G. boninense* isolates between neighbouring trees (KT3S1 and KT2S1) and (KT4S2 and KT3S2) showed high genetic similarities, 0.974 and 0.929 (> 90% similarity) indicating the possibility of vegetative spread of *G. boninense* from root to root contact in the plantation.

Following a measure of UPGMA cluster analysis with Nei and Li's coefficient, the 39 *G. boninense* isolates also were clustered into two clusters at 0.239 similarity level (Fig. 49). Cluster analysis showed that majority of the individuals within a plantation did not cluster together based on locality (as with RAMS fingerprinting). In addition, most of the isolates from different geographical areas were sub-grouped together. For example, the Bt. Lintang isolates KT3S3, KT2S2 and KT4S1 in North of Peninsular Malaysia form a close subgroup with isolates FT5S2 and FT4S2 from Felda plantation in South of Peninsular Malaysia. These suggest that the possible *G. boninese* inoculum spread from different geographical locations through spore dispersal over a significant distance between Bt Lintang Estates and Felda Plantations (see Fig. 40).

Table 11. Genetic similarity matrix of *G. boninense* isolates within Seong Thye Estates and between different Felda plantations situated in the East and South of Peninsular Malaysia respectively. All isolates showed high genetic diversity based on the SSRs marker amplification

	PT2	PT6	PT8	PT10	PT13
PT2	1				
PT6	0.333	1			
PT8	0.182	0.364	1		
PT10	0.087	0.261	0.19	1	
PT13	0.083	0.333	0.182	0.261	1
FT1S2	0.364	0.182	0.2	0.095	0
FT3S2	0.333	0.25	0.091	0.174	0.333
FT4S2	0.381	0.19	0.316	0.1	0
FT5S2	0.286	0.19	0.316	0.2	0.095
FT6S2	0	0.25	0.273	0.348	0.45
FT1S1	0.24	0.4	0.174	0.083	0.16
FT2S1	0.154	0.231	0.25	0.16	0.231
FT3S1	0.364	0.273	0.1	0.095	0.273
FT4S1	0.174	0.261	0.381	0.182	0.261
FT5S1	0.16	0.24	0.261	0.167	0.24
FT6S1	0.24	0.08	0	0.167	0.24
FT7S1	0.333	0.333	0.182	0.174	0.25
FT1S3	0.167	0.167	0.364	0.261	0.167
FT3S3	0.167	0.167	0.364	0.261	0.167

Table 12. Genetic similarity matrix of *G. boninense* isolates within two planting blocks of Bt. Lintang Estates, (North Peninsular Malaysia) based on the SSRs marker amplification. All isolates showed high genetic diversity within and between planting blocks. However two adjacent palms from both block (S1 and S2) are genetically similar (KT3S1 and KT2S1) and (KT4S2 and KT3S2) indicating vegetative spread.

	KT1S	KT2S	KT3S	KT4S	KT1S	KT2S	KT3S	KT4S	KT1S	KT2S	KT3S
KT1S	1										
KT2S	0.214	1									
KT3S	0	0.974	1								
KT4S	0.16	0.174	0.4	1							
KT1S	0.083	0.364	0.211	0.421	1						
KT2S	0.087	0.286	0.556	0.778	0.235	1					
KT3S	0.133	0.486	0.24	0.32	0.5	0.261	1				
KT4S	0.143	0.546	0.174	0.174	0.455	0.19	0.929	1			
KT1S	0.148	0.32	0.455	0.545	0.19	0.6	0.296	0.24	1		
KT2S	0.214	0.308	0.435	0.522	0.455	0.476	0.357	0.308	0.48	1	
KT3S	0.08	0.261	0.6	0.7	0.526	0.667	0.56	0.435	0.455	0.522	1

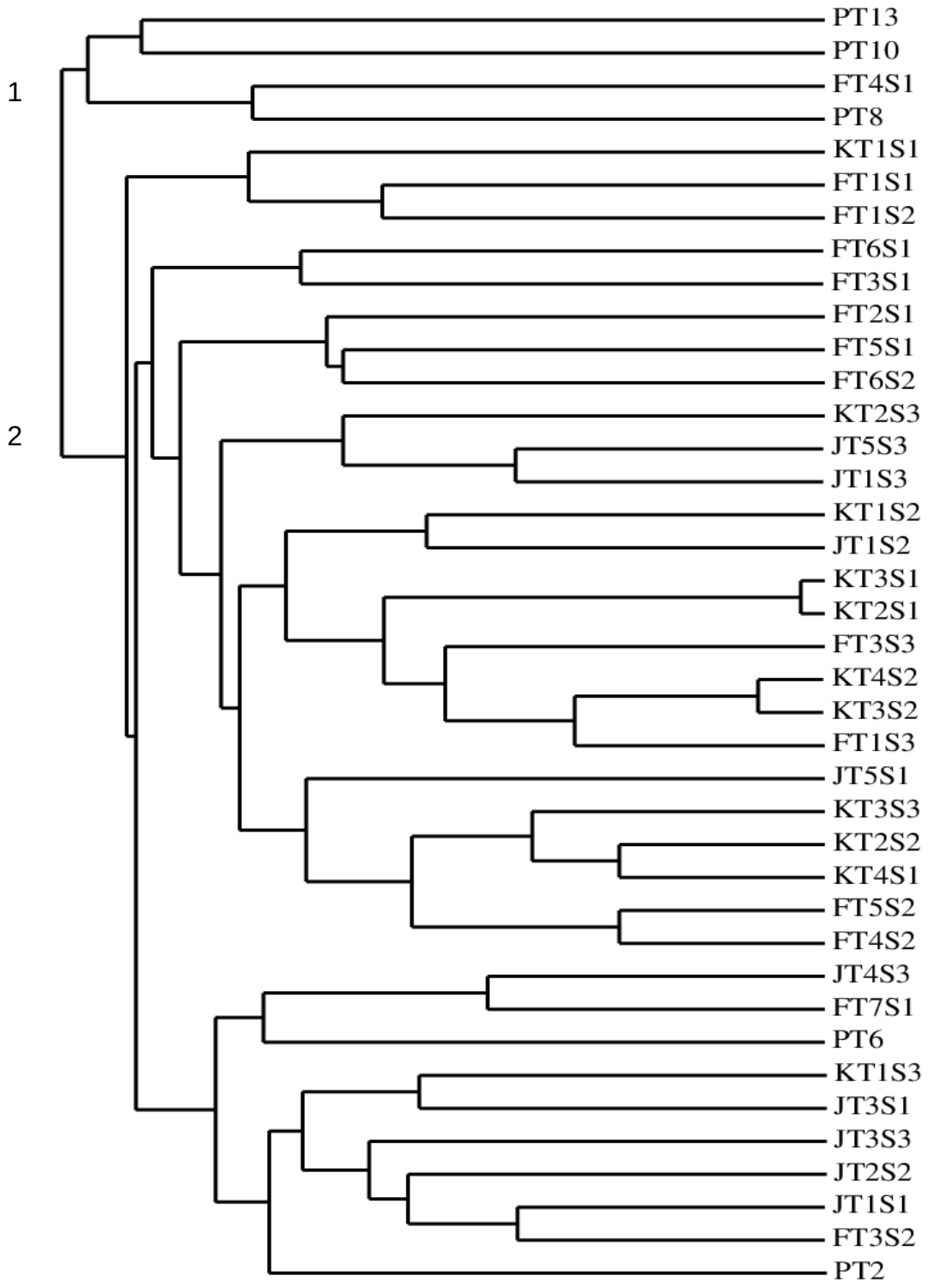


Figure 49. Dendrogram illustrating high genetic diversity of *G. boninense* isolates from different geographical areas of Peninsular Malaysia (South, West, East, North) based on SSRs markers amplification. Clonal isolates were detected between neighbouring palms in two block of Bt. Lintang Estate (KT3S1 and KT2S1) and (KT4S2 and KT3S2).

4.3.2 Mating compatibility study of *Ganoderma boninense*

In this study, the sexuality of *G. boninense* and the somatic interaction between monokaryotic and dikaryotic mycelia were determined through genetically defined field material. These experiments were to confirm the incompatibility mechanisms which may operate to delimit individuals of *G. boninense* in a population. Also dikaryon-monokaryon (di-mon) matings might contribute, along with production of meiotically produced basidiospores, to the extensive genetic diversity which is found in plantations.

Ganoderma boninense spores were collected from basidiocarps of different oil palm trees in Malaysian oil palm plantations and were transported to University of Bath. Spores from each bracket were suspended in a drop of sterile distilled water and streaked onto water agar plates separately as described in section 4.2.7. Spores were grouped into two categories: sibling (spores from the same basidiocarp) and non-sibling (spores from basidiocarps from different trees).

In order to determine mating types, reciprocal crosses between a minimum of nine sibling monokaryons were carried out as described by Pilotti *et al.* (2002). One agar plug of each monokaryotic culture were placed 1 cm apart on MEA and incubated in the dark at 30°C. After 3-4 d growth, a small area at the junction of the two cultures was sub-cultured onto MEA and incubated for a further 3-4 day. Cultures were then examined directly for presence of clamps. Each cross was scored as positive (clamp formation) or negative (clamps not formed). Mating types were then assigned arbitrarily to each monokaryon.

Dikaryons were generated from siblings (sibcom- posed dikaryons) and from out-cross of monokaryons from different basidioma (non-sibcomposed dikaryons). Dikaryotic-monokaryotic (di-mon) mating was carried out using both types of dikaryons with either sib- or non-sib monokaryons, e.g. $(A_1B_1+A_2B_2)x\ A_3B_3$ or $(A_1B_1+A_2B_2)\ x\ A_1B_1$.

The di-mon mating was carried out as above but the sample was taken from the periphery of the monokaryotic culture after 14 d growth at 27°C in the dark.

Sub-cultured samples were examined microscopically (x400) directly for presence of clamp connections.

The presence of clamp connections after mating between siblings (from the same basidiocarp) indicated compatible mating types (where A and B alleles are different). It was necessary to sub-culture the area at the confluence of the two cultures for rapid results. Clamps were formed within 2 d after sub-culturing. Interaction of monokaryotic mating amongst siblings showed different antagonism reactions (Fig.50). All fully sexually compatible crosses which formed dikaryons showed an intermingling of hyphae with no antagonism at the interaction zone (Fig.50a). In contrast, incompatible mating between siblng monokaryons showed weak or medium antagonism reactions (Fig. 50b-c).

The results of crosses between siblings from a single basidiocarp are shown in Table 13. The ratio of compatible to incompatible crosses (1:3) obtained, indicated bifactorial incompatibility with a tetrapolar mechanism. Monokaryons were self-sterile indicating heterothallism. Crosses from two other basidiocarps showed similar results.

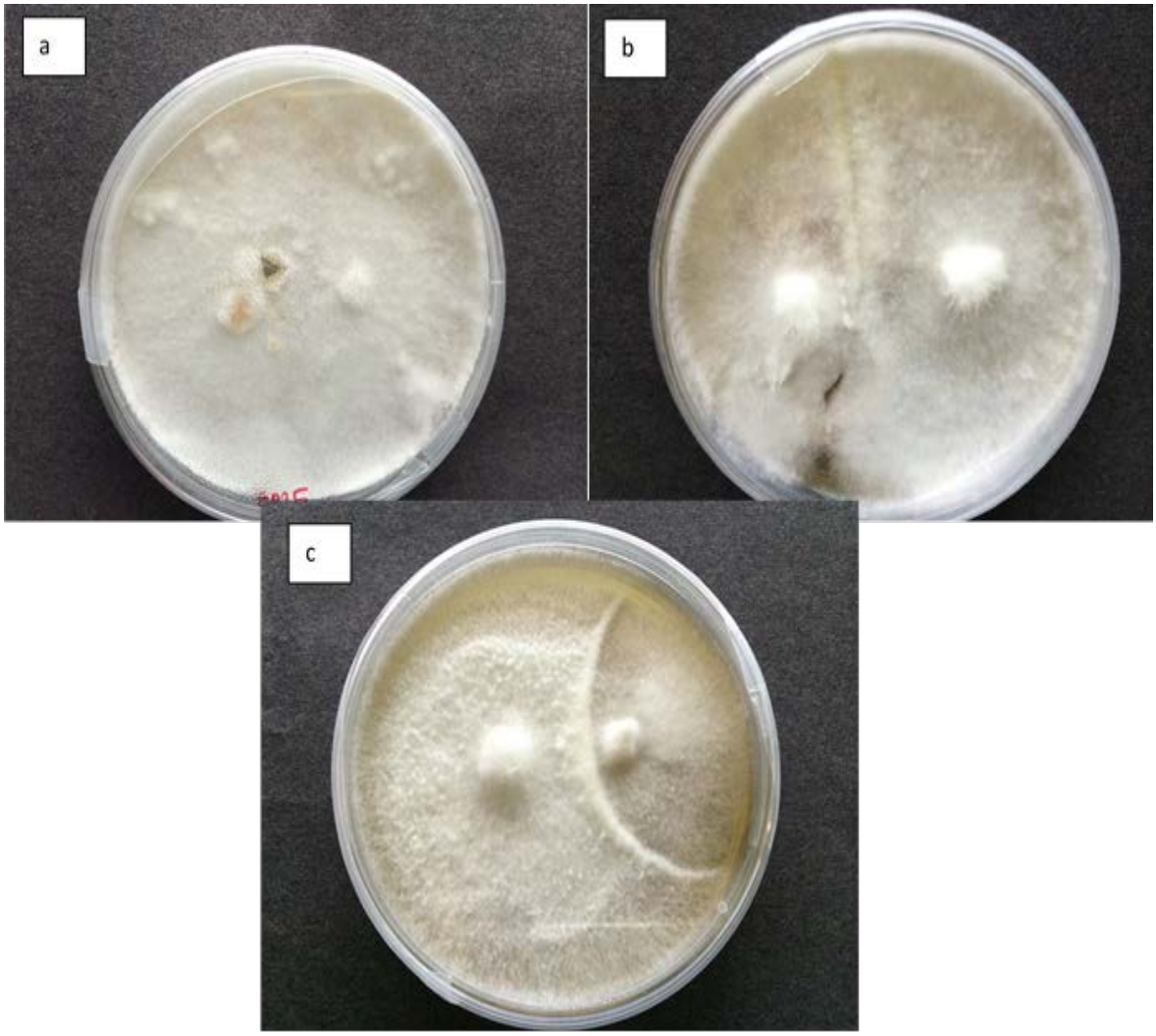


Figure 50. Interactions between monokaryotic mycelia showing three types of antagonism. (a).Fully compatible monokaryon, intermingling of hyphae with no antagonism, Incompatible monokaryon mating showed (b) weak antagonism or (c) medium antagonism (raising of mycelia).

Table 13. Matings amongst siblings showing that each mating type is sexually compatible with only one other haploid sibling. Alleles (e.g. A1B1) have been assigned arbitrarily. Separate mating test of sibling monokaryon from three basidiocarps. Isolates code (e.g 311B1 A to Q) indicate *G. boninense* monokaryon from a single basidiocarp assigned arbitrarily.

BASIDIOCARP 1

ISOLATES		311B1 A	311B1 B	311B1 C	311B1 D	311B1 Q	311B1 R	311B1 S
	ALLELES	A1B1	A1B2	A2B2	A2B1	A1B1	A2B2	A1B2
311B1 A	A1B1	-	-	+	-	-	-	-
311B1 B	A1B2			-	+	-	-	-
311B1 C	A2B2				-	+	-	-
311B1 D	A2B1					-	-	+
311B1 Q	A1B1						+	-
311B1 R	A2B2						-	-
311B1 S	A1B2							-

BASIDIOCARP 2

ISOLATES		467A2 A	467A2 B	467A2 C	467A2 D	467A2 E	467A2 F	467A2 G
	ALLELES	A3B3	A3B4	A4B4	A4B3	A4B4	A4B3	A3B4
467A2 A	A3B3	-	-	+	-	-	-	-
467A2 B	A3B4		-	-	+	-	-	-
467A2 C	A4B4			-	-	-	-	-
467A2 D	A4B3				-	-	-	+
467A2 E	A4B4					-	-	-
467A2 F	A4B3						-	+
467A2 G	A3B4							-

BASIDIOCARP 3

ISOLATES	ALLELES	17B10 M A15B15	17B10 P A15B16	17B10 Q A16B16	17B10 R A16B15	17B10 T A15B15	17B10 U A16B16	17B10 V A15B16
17B10 M	A15B15	-	-	+	-	-	-	-
17B10 P	A15B16			-	+	-	-	-
17B10 Q	A16B16				-	+	-	-
17B10 R	A16B15					-	-	+
17B10 T	A15B15						+	-
17B10 U	A16B16						-	-
17B10 V	A15B16							-

+, clamp formation: -, clamps not formed

When mating of monokaryons representing each mating type from each of the three basidiocarps were crossed in all combinations, full sexual compatibility was observed as indicated by the formation of clamp connection in culture (Table 14). These indicate multiple alleles exist for both mating type loci. Mating type alleles were arbitrarily assigned to each monokaryon. All outcrosses were fully sexually compatible, showed an intermingling of hyphae with no antagonism at the interaction zone, as above.

Table 14. Out-crosses between monokaryons from three different basidiocarps showing sexual compatibility of all mating types.

ISOLATES		467A2 A	467A2 B	467A2 C	467A2 D	467A2 E	467A2 F	467A2 G	282A3 A	463B9 A	182B10 A	17B10 M	17B10 P	17B10 Q	17B10 R	17B10 T	17B10 U	17B10 V
	ALLELES	A3B3	A3B4	A4B4	A4B3	A4B4	A4B3	A3B4	A5B5	A6B7	A10B11	A15B15	A15B16	A16B16	A16B15	A15B15	A16B16	A15B16
311B1 A	A1B1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
311B1 B	A1B2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
311B1 C	A2B2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
311B1 D	A2B1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
311B1 Q	A1B1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
311B1 R	A2B2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
311B1 S	A1B2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

4.3.3 Di-mon mating

Dikaryotization of monokaryons in sibling-sibling mating (semi-compatible) proceeded as expected, presumably with only the compatible nucleus being transferred to the recipient monokaryon (Table 15). Transfer of nuclei from the dikaryon to the periphery of the monokaryotic mycelial zone occurred after 16-22 days (Fig. 51). Illegitimate combinations in which the monokaryon contains common mating alleles within each nucleus in the dikaryon (e.g. A1B1 +A2B2x A2B1) did not result in dikaryon formation (Table 15).

In fully compatible di-mon crosses using non-sib monokaryons (e.g. A1B1 + A2B2 x A3B3) selective transfer of one of the nuclei in the dikaryon was observed (Table 16). Duplicates of each mating were carried out and the results were consistent (Fig. 52). The nuclear constitution of each resultant dikaryon was determined by back-pairing of all dikaryons formed to the original (donor) dikaryons as well as to synthesized dikaryons containing the alternative nuclear pair.

Table 15. Dikaryotization of monokaryons with sib-composed dikaryons showing nuclear composition of each dikaryon. Only legitimate matings produced dikaryons. Mating type designations as in Table 13.

Original dikaryon	Sibling monokaryon	Resultant dikaryon	Nucleus transferred
311B1 A + 311B1 C (A1B1+A2B2)	311B1 Q (A1B1)	311B1 Q + 311B1 C(A1B1+A2B2)	311B1 C(A2B2)
311B1 A + 311B1 C (A1B1+A2B2)	311B1 R (A2B2)	311B1 A + 311B1 R (A1B1+A2B2)	311B1 A(A1B1)
311B1 A + 311B1 C (A1B1+A2B2)	311B1 B (A1B2)	NOT FORMED	NONE
311B1 A + 311B1 C (A1B1+A2B2)	311B1 D (A2B2)	NOT FORMED	NONE
311B1 B + 311B1 D (A1B2+ A2B1)	311B1 Q (A1B1)	NOT FORMED	NONE
311B1 B + 311B1 D (A1B2+ A2B1)	311B1 R (A2B2)	NOT FORMED	NONE
311B1 B + 311B1 D (A1B2+ A2B1)	311B1 B (A1B2)	311B1 D + 311B1 B (A1B2+A2B1)	311B1 D (A2B1)
311B1 B + 311B1 D (A1B2+ A2B1)	311B1 D (A2B1)	311B1 B + 311B1 D (A1B2+A2B1)	311B1 B (A1B2)
467A2 A + 467A2 D (A3B3+A4B4)	467A2 A (A3B3)	467A2 A + 467A2 D (A3B3+A4B4)	467A2 D (A4B4)
467A2 A + 467A2 D (A3B3+A4B4)	467A2 D (A4B4)	467A2 A + 467A2 D (A3B3+A4B4)	467A2 A (A3B3)
467A2 A + 467A2 D (A3B3+A4B4)	467A2 B (A3B4)	NOT FORMED	NONE
17B10 P + 17B10 R (A15B16+ A16B15)	17B10 P (A15B16)	17B10 R + 17B10 P (A16B15+ A15B16)	17B10 R (A16B15)
17B10 P + 17B10 R (A15B16+ A16B15)	17B10 R (A16B15)	17B10P+17B10R (A15B16+A16B15)	17B10 P (A15B16)
17B10 P + 17B10 R (A15B16+ A16B15)	17B10 M (A15B15)	NOT FORMED	NONE
17B10 P + 17B10 R (A15B16+ A16B15)	17B10 U (A16B16)	NOT FORMED	NONE
17B10M+17B10Q(A15B15+A16B16)	17B10 M (A15B15)	17B10M+17B10Q(A15B15+A16B16)	17B10 Q (A16B16)
17B10M+17B10Q(A15B15+A16B16)	17B10 Q (A16B16)	17B10M+17B10Q(A15B15+A16B16)	17B10 M (A15B15)

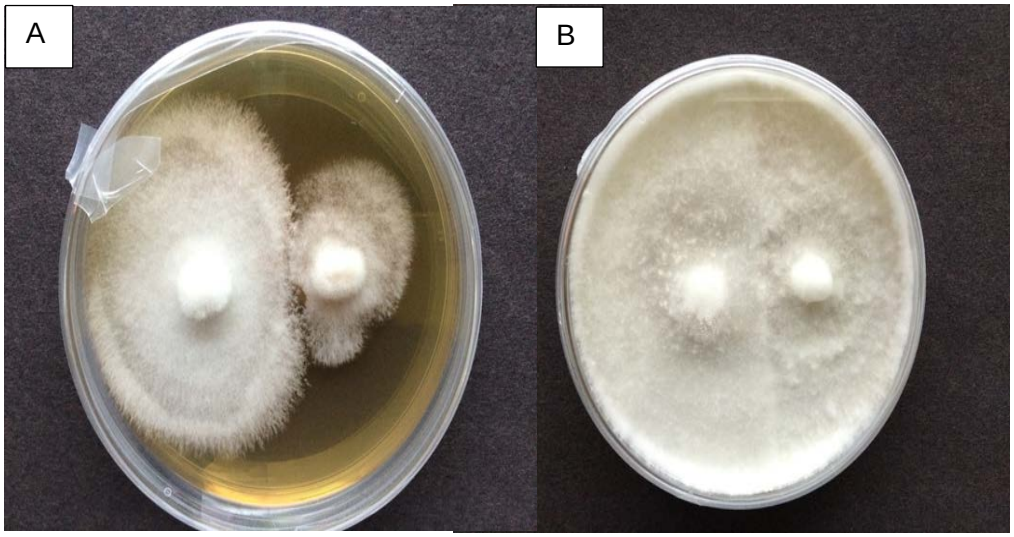


Figure 51. Di-mon mating of monokaryon with sib-composed dikaryon showing no antagonism. A, 7 day old mycelial interaction and (B) 21 day old mycelial interaction between monokaryon and sib-composed dikaryon.

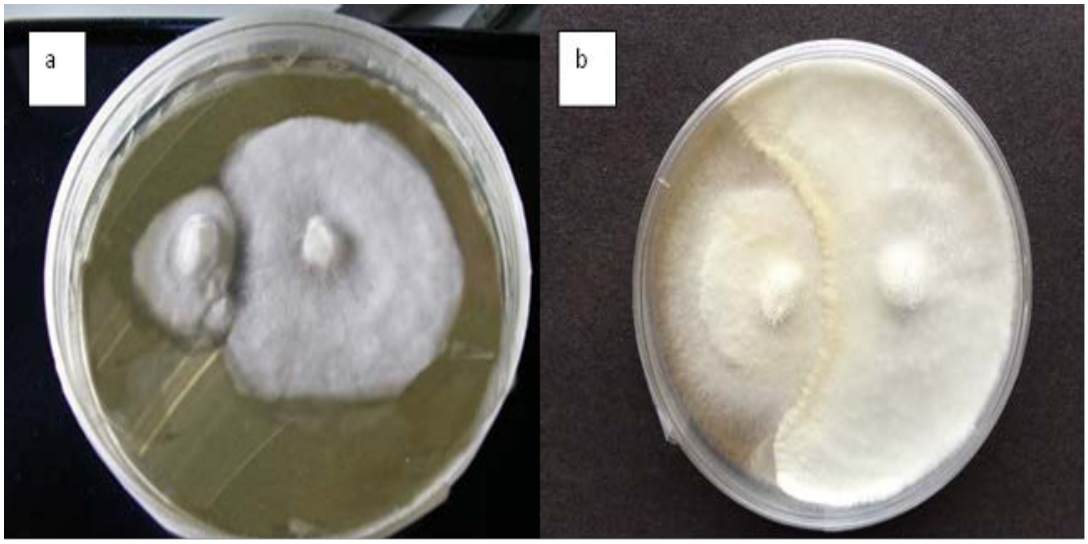


Figure 52. Di-mon mating of non-sib monokaryons and dikaryon (sib-composed) showing antagonism after the formation of new dikaryon. (a).7 day old mycelial interaction and (b) 21 day old mycelial interaction between non-sib monokaryon and sib-composed dikaryon.

Table 16. Dikaryotization of non-sib monokaryons by sibcomposed dikaryons showing the selective transfer of one nucleus in the majority of matings. Letters refer to each nucleus.

Original dikaryon	Non-sib monokaryon	Resultant dikaryon	Nucleus transferred
311B1 A + 311B1 C (A1B1+A2B2)	467A2 A (A3B3)	467A2 A + 311B1 A (A3B3+A1B1)	311B1 A (A1B1)
311B1 A + 311B1 C (A1B1+A2B2)	467A2 B (A3B4)	467A2 B +311B1 (A3B4+A1B1)	311B1 A (A1B1)
311B1 A + 311B1 C (A1B1+A2B2)	467A2 C (A4B4)	467A2 C + 311B1 A (A4B4+A1B1)	311B1 A (A1B1)
311B1 A + 311B1 C (A1B1+A2B2)	467A2 D (A4B3)	467A2 D + 311B1 A (A4B3+ A1B1)	311B1 A (A1B1)
311B1 A + 311B1 C (A1B1+A2B2)	467A2 E (A4B4)	467A2 E + 311B1 C (A4B4+A2B2)	311B1 C (A2B2)
311B1 A + 311B1 C (A1B1+A2B2)	282A3 A (A5B5)	282A3 A + 311B1 C (A5B5+ A2B2)	311B1 C (A2B2)
311B1 A + 311B1 C (A1B1+A2B2)	463B9 A (A6B7)	463B9 A + 311B1 C (A6B7+ A2B2)	311B1 C (A2B2)
311B1 A + 311B1 C (A1B1+A2B2)	182B10 A (A10B11)	182B10 A + 311B1 C (A10B11+A2B2)	311B1 C (A2B2)
467A2 A + 467A2 D (A3B3+A4B4)	311B1 A (A1B1)	311B1 A + 467A2 D (A1B1+A4B4)	467A2 D (A4B4)
467A2 A + 467A2 D (A3B3+A4B4)	311B1 B (A1B2)	311B1 B + 467A2 D (A1B2+A4B4)	467A2 D (A4B4)
467A2 A + 467A2 D (A3B3+A4B4)	311B1 C (A2B2)	311B1 C + 467A2 D (A2B2+A4B4)	467A2 D (A4B4)
467A2 A + 467A2 D (A3B3+A4B4)	311B1 D (A2B1)	311B1 D + 467A2 D (A2B1+A4B4)	467A2 D (A4B4)
467A2 A + 467A2 D (A3B3+A4B4)	311B1 Q (A1B1)	311B1 Q + 467A2 A (A1B1+A3B3)	467A2 A (A3B3)
467A2 A + 467A2 D (A3B3+A4B4)	311B1 R (A2B2)	311B1 R + 467A2 A (A2B2+A3B3)	467A2 A (A3B3)
467A2 A + 467A2 D (A3B3+A4B4)	311B1 S (A1B2)	311B1 S + 467A2 A (A1B2+A3B3)	467A2 A (A3B3)
17B10 M + 17B10 Q (A15B15+A16B16)	282A3 A (A5B5)	282A3 A + 17B10 M (A5B5+A15B15)	17B10 M (A15B15)
17B10 M + 17B10 Q (A15B15+A16B16)	463B9 A (A6B7)	463B9 A + 17B10 M (A6B7+A15B15)	17B10 M (A15B15)
17B10 M + 17B10 Q (A15B15+A16B16)	182B10 A (A10B11)	182B10 A + 17B10 M (A10B11+A15B15)	17B10 M (A15B15)
17B10 M + 17B10 Q (A15B15+A16B16)	467A2 A (A3B3)	467A2 A + 17B10 Q (A3B3+A16B16)	17B10 Q (A16B16)
17B10 M + 17B10 Q (A15B15+A16B16)	467A2 B (A3B4)	467A2 B + 17B10 Q (A3B4+A16B16)	17B10 Q (A16B16)
17B10 M + 17B10 Q (A15B15+A16B16)	467A2 C (A4B4)	467A2 C + 17B10 Q (A4B4+A16B16)	17B10 Q (A16B16)
17B10 M + 17B10 Q (A15B15+A16B16)	467A2 G (A3B4)	467A2 E + 17B10 Q (A4B4+A16B16)	17B10 Q (A16B16)

4.3.4 Summary

- Somatic incompatibility and molecular DNA fingerprinting using RAMS and SSRs all showed similar results indicating high genetic diversity of *G. boninense* isolates within and between plantations. Even isolates between adjacent trees are genetically different as showed in RAMS band profiles and SSRs genetic similarities.
- Mating compatibility studies indicate that *G. boninense* isolates from Malaysia are heterothallic with a tetrapolar mating system and multiple alleles at both mating type loci which favours out-breeding and results in high genetic diversity within a population.

4.4 Discussion

The importance of basidiospores in basal stem rot (BSR) infection may be inferred from a knowledge of *G. boninense* genetic diversity. *G. boninense* releases abundant sexual spores (basidiospores) from basidiocarps, day and night in field palms (Rees *et al.,* 2012). Furthermore *G. boninense* has a tetrapolar mating system, which favours outcrossing and anastomosis with compatible mating type is required to form the potentially invasive and faster growing heterokaryon for infection. Therefore *G. boninense* field isolates from plantations in different locations of Malaysia were examined using somatic incompatibility, random amplified microsatellites (RAMS) and simple sequence repeat (SSR) markers to determine genetic variation of the pathogen from BSR disease palms.

In this study high genetic diversity of *G. boninense* isolates was observed within and between the plantation blocks in all of the plantations. Different *G. boninense* isolates were also observed even from adjacent infected palms in most of the plantation blocks. This high level of diversity must have arisen through sexual recombination and subsequent dispersal via basidiospores throughout the plantation. The use of somatic incompatibility testing in the study of disease development in populations is well documented within basidiomycete tree pathogens (Rayner and Boddy, 1985; Coates and Rayner, 1985; Stenlid, 1985; Abomo-Ndongo *et al.,* 1997; Miller *et al.,* 1999; Qu *et al.,* 2013). It is a simple and useful technique that distinguishes individuals by the inability of two strains to fuse and form one colony and is an effective means of identifying intraspecific variations within field populations of plant pathogens.

Somatic incompatibility reactions between all paired isolates (except self-pairs) observed in this study indicate that *G. boninense* isolates in each palm within each plantation are genotypically distinct individuals and are not clones.. This contrasts with some other basidiomycete pathogens such as *Armillaria mellea* and *Heterobasidion annosum*, with typical clonal distribution patterns. Incompatibility between paired isolates of *G. boninense* was observed over distances that could theoretically permit root to root contact, and hence mycelial spread, between neighbouring palms (9m apart), between isolates, or between non-adjacent palms (up to 36m apart). Although Miller *et al*. (1999) observed incompatibility reactions for isolates from within the same palms, in this study all isolates from the same palm showed compatible reaction.

The RAMS technique combines most of the benefits of RAPD and microsatellite analyses. The efficiency of the RAMS approach depends on the abundance and type of simple sequence repeats (SSRs) or microsatellites in the target species. The primers used in these studies were 5' and 3' anchored repeat primers which generate inter simple sequence repeats (ISSRs) or RAMS marker. Based on RAMs banding patterns, there was no evidence to link BSR infected trees to neighbouring palms, as no identical RAMS profiles were observed between isolates from adjacent infected palms indicating genetically diverse population and importance of basidiospore which concurs in previous finding in Sumatra (Rees *et al.,* 2012), PNG (Pilotti *et al.,* 2003) and Malaysia (Miller *et al.,* 1999). However analysis of isolates from within two adjacent BSR infected palms in Bt. Lintang Estates, did show identical profiles, indicating vegetative spread of the pathogen. Miller *et al.* (1999) obtained a similar result with mtDNA RFLP, where adjacent BSR palms 102SB and 103SB, contained similar banding pattern. Similarly Pilotti *et al.* (2003) reported that the interfertility test of *G. boninense* isolates on neighbouring palms planted 10 m apart also displayed unique mating type alleles. These studies show that genetically identical individuals were present in adjacent palm trees and this is extremely unlikely to occur if infection of the palms had been by basidiospores as the mating system in *Ganoderma* favours outcrossing (Pilotti and Bridge*,* 2002; Rees *et al*., 2012). Even though some of the findings indicate vegetative infection, Miller *et al.* (1999), Pilloti *et al.*

(2003) and Rees *et al.* (2012) argue that the general high genetic variability within plantations is evidence against vegetative spread of the pathogen.

Analysis by RAMS of isolates present within BSR infected palms showed that all trees tested contained the same isolate of *G. boninense.* BSR infections may therefore contain single individual within an infected tree. This concurs with a previous study by Pilotti *et al.* (2003) who used interfertility test as a marker in PNG. They showed that nine BSR infected palms contained a single isolate, as shown by the lack of antagonism in pairings of isolates from these palms, suggesting infection by one isolate. Expressed sequence tag (EST)-based SSR markers have been widely used to estimate genetic diversity and population structures in recent studies (Yeh, 1997). The use of SSR markers is superior to RAMS because it based on expressed real sequences from *G. boninense* and not on random markers and other advantages: co-dominance inheritance, locus specificity and hyper-variability, which is of considerable importance for evolutionary, genetics and population studies (Szabo and Kolmer, 2007; Jia *et al.,* 2015).

In this study, we used eight pairs of SSR markers to examine the genetic diversity of 39 *Ganoderma boninense* isolates collected from different oil palm plantations. The data revealed that the genetic diversity of *G. boninense* is higher (HE ¼ 0.7755 at population level) than that of the other basidiomycete fungi such as *Armillaria* spp. and *H. annosum* based on analysis using different molecular data (Xing *et al.,* 2015). This result suggests that EST-SSRs can be successfully used for estimation of genetic diversity and population structure. Furthermore, a similarity matrix of all *G. boninense* isolates showed low similarity distance within and between plantations. However in Bt. Lintang plantation genetic similarity of 2 neighbouring palms showed more than 90% similaritiy. This indicates some vegetative spread of *G. boninense* occurs, presumably through root to root contact. This finding agrees with the similarity matrix resulting from RAMS which showing a similar trend.

Overall, the importance of basidiospore dispersal in BSR disease establishment is shown when clustering analyses was conducted on the binary matrix produced from both RAMS and SSR markers. i.e. isolates from a single plantation blocks often do not cluster together, which concurs with previous findings where a high variability was observed within oil palm plantation blocks in Sumatra (Rees *et al.,* 2012), PNG (Pilotti *et al.,* 2003) and Malaysia (Miller *et al.,* 1999). This high diversity of *G. boninense* isolates in populations within oil palm plantations makes clear that basidiospores play a crucial role in disease epidemiology and genetic diversity in BSR and USR infections. Isolates from USR were not tested in this study because of the infrequency of USR in the plantations surveyed.

This study confirmed the bifactorial sexual incompatibility of *G. boninense* as previously reported by Pilotti *et al.* (2003) and is consistent with the sexuality of other *Ganoderma* species (Do, 1980; Adaskaveg and Gilbertson, 1986; Triratana and Chaiprasat, 1991). Multiple alleles were observed at two unlinked mating type loci designated as A and B. This multi-allelic mating system is also found in other basidiomycetes including *Armillaria* spp., *Schizophyllum commune* and *Coprinus cinerus* (Buller, 1931; Rayner and Todd, 1977; Worrall, 1997). This type of mating system is the most complex with a restriction of inbreeding of 25% which favours out-crossing within a population (Pilotti and Bridge, 2002).

Dikaryotisation of monokaryons readily occurs in fully compatible mating between monokaryons and occurs non-randomly with the selective transfer of nuclei from the dikaryon. All duplicate cultures in this study gave identical reactions and thus the results obtained are not attributed to chance. This concurs with the finding by Pilotti and Bridge (2002) and Kope (1992) on *G. boninense* and the ectomycorrhizal fungus *Pisolithus arhizus* respectively, which showed similar results. This suggests that recognition mechanism could be activated by the recipient monokaryons to select for one nucleus. Pilotti *et al.* (2003) assumed that the recognition of heterozygosity at some loci is

possibly linked to the mating type loci, where a series of recognition events leads to selection after plasmogamy.

Dikaryon and monokaryon mating of *G. boninense* was consistent with the findings of Pilotti and Bridge (2002) and with those generally reported in basidiomycetes where only the compatible nucleus was transferred from the dikaryon to the recipient monokaryon (Coates and Rayner, 1985; May, 1988). This migration of nuclei from the dikaryon into the monokaryotic colony to form a dikaryon was first reported by Buller (1930). Furthermore, illegitimate combination matings between dikaryons and monokaryons (A1B1 + A2B2) x A1B2) were not detected in this study. Confirmation of the resultant dikaryons is done by observing the vegetative interactions with synthesised dikaryons of known nuclear composition (Coates and Rayner, 1985; May, 1988). This type of dikaryon formation contrasts with the hyphal fusion mechanism of *Trametes versicolor* where the recipient cell nucleus degenerate to accommodate both the dikaryon nuclei (Aylemore and Todd, 1984).

The high genetic diversity of *G. boninense* populations could thus arise through mating between dikaryons and monokaryons of the pathogen. In plantations natural di-mon matings could provide genetic alternatives to the genotypes of dikaryotic mycelium (long established in decayed palm stem or roots) through anastomosis with monokaryotic hyphae (bearing new traits) that subsequently infect the palm.

Basidiospores have been shown to germinate and possibly penetrate palm roots in this study. Thus monokaryotic mycelium would highly likely encounter any established dikaryotic mycelium present in infected palm roots or lower stem, with potential opportunity for mating. If newly established monokaryons become dikaryotized then proliferate and form basidiocarps, then the resulting propagules could perpetuate the genetic diversity in the population. Di-mon mating compatibility may provide an alternative means of introducing new genotypes into a population via infective *G. boninense* dikaryons long established in infected stems or roots. This would allow genetic changes to occur without sexual reproduction, and thus it could serve as a survival

advantage to a fungus such as *G. boninense* that has a reproductive cycle requiring an extensive decay of the palm before basidiocarps can form and sexual recombination to occur.

However it remains unknown when and where could *G. boninense* monokaryons actively colonise palm. Basidiospores are abundant in plantations, and germination and direct penetration could occur in great numbers on petioles, stem and roots of palms to establish monokaryotic mycelium. Monokaryons are generally considered short-lived and dikaryons assumed to be the dominant phase, although there is little evidence for this. Indeed, some homokaryons, for example those of the wood-rotting species *Trametes versicolor* and *Heterobasidion annosum* do actively colonise and can persist in the field for several years, and possibly much longer (Coates & Rayner, 1985; Garbelotto *et al.,* 1997; Redfern *et al.,* 2001). Redfern *et al.* (2001) found that the homokaryon of *H. annosum* colonised and penetrated the lower surface of sitka spruce stump to a depth of 9.5 cm and was able to survive for up to 2 years in the colonised stumps.

An ongoing experiment has been conducted with *G. boninense* monokaryon wood block inoculum on the possibility of asymptomatic colonisation of oil palm seedling roots, which yet to be analysed. Potential colonization of monokaryotic mycelium will be determined through re-isolation and by light microscopic analysis, observing any possible monokaryotic mycelium presence in the roots.

Basiospores of many rust species infect directly on the plant surface such as *Puccinia graminis* on the alternate host barberry (Wang *et al*., 2015), White pine blister rust disease caused by *Cronartium ribicola* (McDonald *et al.,* 2006), European pear rust induced by the fungus *Gymnosporangium sabinae* (Kalisz *et al.,* 2015). Rust fungi basidiospores penetrate into epidermal cells directly via appressoria that generally occur close to the anticlinal walls of the epidermis where penetration of the cuticle and the epidermis occur. The ultrastructure of the early basidiospore infection process have been described clearly in the rust fungi for example, *Melampsora pulcherrima* (Longe *et al.*,

1994), *Cronartium quercuum* f. sp. fusiforme (Gray *et al.*, 1986), and *Uromyces appendiculatus* var. appendiculatus (Gold and Mendgen, 1984). It is clear that at least with rust fungi, basidiospores are adapted for host penetration and initial colonisation. The evidence from this study showing high airborne basidiospore numbers, enormous genetic diversity for BSR isolates, and at least two sites for infection (cut petioles and superficial roots) indicates the strong likelihood of basidiospores initiating infection. Subsequent pathogenic heterokaryons might be formed at the infection stage or later within host tissues.

Potentially, monokaryons probably could colonize stem and root of palms, but asymptomatically. Furthermore, mating between compatible monokaryons or nucleus transfer from established dikaryotic mycelium could occur to generate a new dikaryotic invasive mycelium of *G. boninense*.

5 *Ganoderma* Pathogenicity

5.1 Introduction

Little is known about *G. boninense* pathogenicity other than its ability to extensively degrade oil palm cell walls (Rees *et al.,* 2009; Rees, 2006). This paucity of information partly reflects the difficult host-pathogen system of oil palm and *G. boninense*, but also the lack of application or expertise in host-pathogen interactions in labs in South East Asia. In addition there has been an excessively long delay in obtaining and releasing the genome of *G. boninense*. In parallel, attempts to study the transcriptome of *G. boninense* during infection have not used controlled inoculations, but rather field material and therefore have largely focussed on host genes and revealed little information about the full potential of the pathogen's arsenal.

Experience with other fungal pathogens of diverse hosts has rarely shown that in-depth understanding of components of pathogenicity or virulence has led to improved disease control (R Cooper, pers. comm.). Nevertheless, in some cases pathogen toxins have been used for screening for disease resistance (Sneller *et al.,* 2012) and some pathogen effectors are currently being exploited to find and best use resistance genes by so-called effectoromics (Ellie *et al.,* 2009; de Jong *et al*., 2011; Vleeshouwers and Oliver*,* 2014). There follows a brief coverage of: cell wall polymers and their degradation; cell wall degrading enzymes in *Ganoderma* and related tree pathogens; a consideration of strategies used by other necrotrophic fungi; approaches to studying infection and gene expression during *Ganoderma* infection.

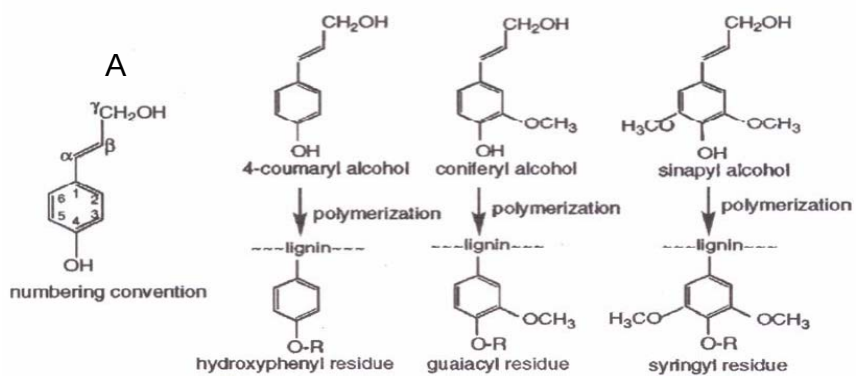
5.1.1 Plant cell wall polymers and their degradation

Lignin is a branched polymer of substituted phenyl propane units joined by carbon-carbon and ether linkages. It is abundant in secondary cell walls, especially in wood, and provides compressive strength, resistance to degradation by microbial enzymatic attack and water impermeability to the polysaccharide-protein matrix of the cell wall. The chemical structure of the polymeric lignin is complex consisting of at least three monomeric building units (Fig. 53). Biosynthesis of lignin progresses from the phenolic precursors, *p*-coumaryl, coniferyl, and synapyl alcohol. Twelve different types of linkages at least could be generated, for example aryl-ether and carbon-carbon bonds connecting the aromatic nuclei which make its biodegradability very restricted (Paterson, 2007). In vitro studies have shown that white rot fungi cannot utilise lignin alone as a carbon source for nutrients, which indicates that lignin degradation requires the input of energy predominantly from cellulose breakdown (Penttila and Saloheima, 1999; Paterson, 2007).

There are three major families of fungal enzymes that affect lignin structures either directly or indirectly; laccases with O_2 as the electron acceptor; lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) with H_2O_2 as electron acceptor (Tien and Kirk, 1983; Tien and Kirk, 1984; Kuwahara *et al.*, 1984; Mtui and Nakamura, 2002). These enzymes can act with low-molecular-weight aromatic mediator compound to bring about lignin oxidation (D'Souza *et al.,* 2006; Elisashvili *et al.,* 2010). These lignin degrading enzymes have been extensively studied due to their ability to degrade recalcitrant pollutants such as industrial dye effluents and bio-pulping and bleaching of pulps (D'Souza *et al.,* 1999; Songulashvilli *et al.,* 2007). LiP and MnP oxidize non-phenolic aromatic compounds with high oxidation-reduction potentials, which are the major components of the lignin polymer. Laccase can oxidize phenolic compounds thereby creating phenoxy radicals, while non-phenolic compounds are oxidized via cation radicals. (D'Souza *et al.,* 1999). Investigations by Hammel (1997) established the basis for lignin

enzymes oxidation: susceptible aromatic nuclei are oxidised by one electron generating unstable aryl cation free radical, which are unstable and undergo a variety of spontaneous cleavage reactions. These free radicals can either induce polymerization by forming new linkages with other lignin subunits, or turn into ring cleavage products. Nevertheless, no lignin polymerisation has been observed during delignification process in vivo (Kirk and Farrell, 1987; Kurek *et al.,* 1998).

Hydrogen peroxide-generating systems are important for the fungal delignification mechanisms, as peroxides are required for peroxidase function. Hydrogen peroxide may be also utilised by cellobiose dehydrogenase (CDH) together with Fe(III) and Mn(IV) reduction (Roy *et al.,* 1994) for the generation of oxyradicals that are able to cause bond cleavages in lignin and cellulose. Several fungal enzymes have been characterised as H_2O_2 producers, but most of them are intracellular, and only few extracellular enzymes have gained more attention. Enzymes generating H_20_2 include glyoxal oxidase (GLOX, a copper radical protein found in *P. chrysosporium* (Urek *et al.,* 2004) and aryl-alcohol oxidase (AAO) found in fungus *P. eryngii* (Kersten, 1990).



B

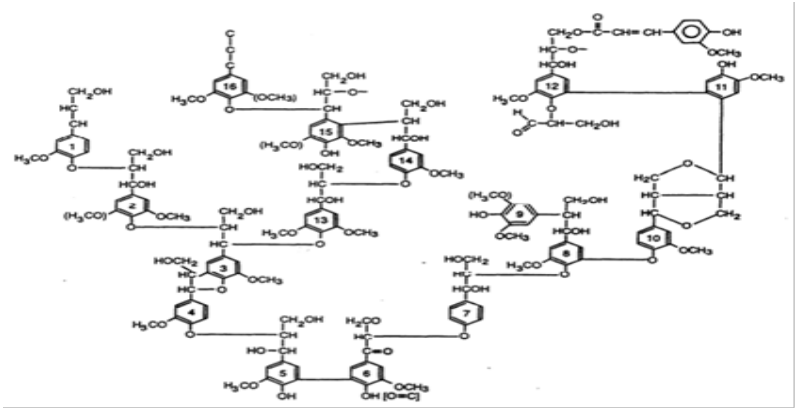


Figure 53. Structure of the three lignin precursors (A) and the residues derived from them and Chemical structure of lignin (B) (Pentilla and Soleheimo, 1999).

Cellulose is the primary structural component of plant cell walls and is the most abundant polysaccharide on earth (Fig. 54). The morphology and physical structure of cellulose is complex, and fine structural details are only visible by electron microscopy as microfibrils of *ca.* 10 nm diameter (Cooper, 1984). It consists of a collection of β-1,4-linked glucan chains that interact with each other via hydrogen bonds which form crystalline microfibrils (Somerville, 2006), which give the strength to the cell wall (Cosgrove, 2005). Although relatively linear in compound composition, the extensive inter- and intra-molecular bonding pattern of cellulose creates a crystalline structure that can exclude water and makes it relatively resistant to enzymatic degradation.

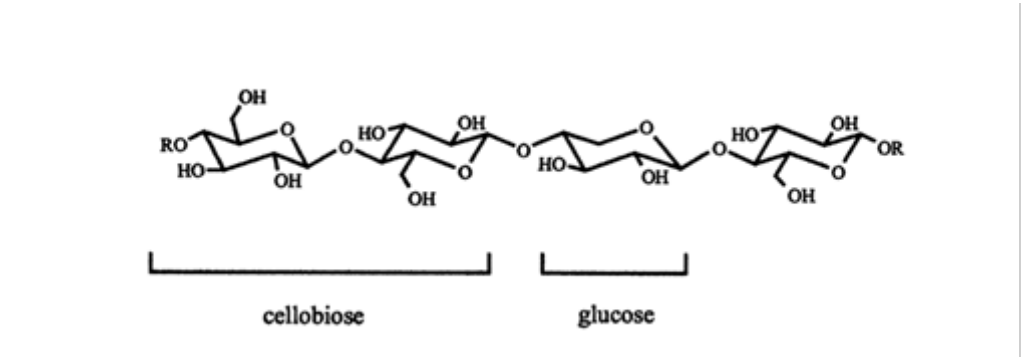


Figure 54. A schematic structure of the cellulose chain (Miller *et al.*, 1960).

Cellulose degradation can involve a complex of cellulase enzymes including endoglucanase (endo-1,4-β-D-glucanase), cellobiohydrolase (exo-1,4-β-D-glucanase) and β-glucosidase (1,4-β-D-glucosidase), which open up the cellulose chain by breaking bonds between glucose units in non-crystalline regions to form cellobiose to glucose (Bhat and Bhat, 1997).

Endoglucanases (EG, E.C. 3.2.1.4) belong to endo-type enzymes attack by hydrolysing β-linked bonds within cellulose microfibrils in the amorphic parts of the fibril, thus opening up cellulose chains in middle part of the cellulose fibre (Divne *et al.,* 1994). Endoglucanase enzyme production and activity probably exists in all wood-degrading fungi (Highley, 1988). Cellobiohydrolases (CBHs, E.C. 3.2.1.91) are exo-type enzymes that binds edges of crystalline cellulose fibres, cleaving cellobiose units from both reducing and non-reducing ends. β-glucosidase hydrolyse cellobiose and short chain cellodextrins to glucose (Teeri, 1997). Multiple versions of EG and CBH have been identified; genes encoding several isoforms of EG and CBH were identified in *Trichoderma reesei* (Teeri *et al.,* 1987), *Phanerochaete chrysosporium* (Igarashi *et al.,* 2008), *Coprinus cinerea* (Yoshida *et al.,* 2009) and *Auricularia fuscosuccinea*, *Pleurotus giganteus*, *P. eryngii*, *P. ostreatus*, and *P. sajor-caju* (Chukeatirote *et al.,* 2012).

Generally, the enzymes involved in cellulose degradation are stimulated by the presence of cellulose breakdown products, mainly cellobiose, and are repressed in the presence of low molecular weight compounds that are easily metabolised, notably monomeric glucose (Cooper, 1984; Longoni *et al.*, 2012).

Hemicellulose in general represent 15-35% of plant cell wall biomass, and these polymers, unlike cellulose can be solubilised in dilute alkali. Hemicelluloses consist of backbones of 1,4-β-D-xylans, 1,4-β-mannans or 1,4-β-D-galactans. In hardwoods and softwoods, xylan consisting of D-xylose units, and glucomannans, consisting of D-glucose and D-mannose

units respectively are the main hemicelluloses in the plant cell wall. These side groups render hemicelluloses poorly crystalline or non-crystalline, so that they exist more like a gel than as oriented fibres (Kuhad and Singh, 1993).

Some of hemicellulose function includes the formation of complexes with cellulose which form a matrix together with pectins and proteins in primary plant cell walls and with lignin in secondary cell walls. (Girio *et al.,* 2010). Covalent hemicellulose-lignin bonds involving ester or ether linkages form lignin-carbohydrate-complexes in the S2 cell layer and degradation of these polysaccharides is postulated to be required for initiation of lignin degradation (Watanabe *et al.,* 1989).

Complex set of enzymes are required for degradation of hemicellulose reflecting the variability of the structure composition (Fig. 55). Hydrolysis of the polysaccharide requires 1,4-β-D-xylanases (E.C. 3.2.1.8), 1,4-β-D-mannanases (E.C. 3.2.1.78), 1,4-β-D-galactanases (E.C. 3.2.1.90) that hydrolyse the glycosidic linkages in the backbone of the polymer (Eaton and Hale, 1993). Side chains are hydrolysed by diverse enzymes including α-L- arabinofuranosidases, acetyl xylan esterases, ferulic and *p*-coumaric acid esterases (Jeffries, 1991). Mechanisms of hemicellulase gene regulation have been studied in filamentous fungi, mainly in *Trichoderma* (Stricker *et al.,* 2008) and *Aspergillus* (Noguchi *et al.,* 2009), and regulation appears to be less strictly regulated than cellulase, with reports of both constitutive and inductive enzyme production (Eriksson *et al.,* 1990). However, the production of these extracellular enzymes (cellulase and hemicellulose) is an energy-consuming process, so the enzymes are produced only under conditions in which the fungus needs to use plant polymers as an energy and carbon source (Amore *et al.,* 2013).

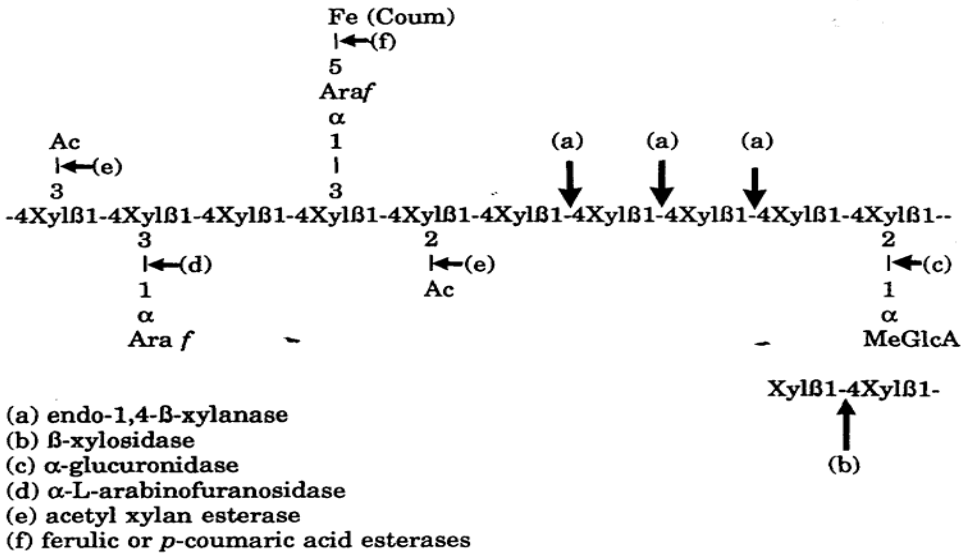


Figure 55. Xylan hemicellulose and sites of attack by xylan degrading enzymes. Ac, acetyl group; Ara *f*, L-arabinofuranose; MeGlc, 4-O-methyl-D-glucuronic acid; Xyl, D-xylose.

Pectin is a generic name for a group of amorphous polymers that exist in plant cell walls, especially in the middle lamella. They are rich in galacturonic acid which can exist in methyl esterified or in a free acidic form. Polygalacturonic acid polymers also contain neutral sugars, D-galactose, L-arabinose, L-rhamnose, D-xylose, L-fucose and D-apiose. In hardwoods and softwoods pectin contributes to less than 1% of the cell wall components which are often replaced by lignin in the middle lamella (Eaton and Hale, 1993). Pectin polymer form hydrated gels and have several functions, including determination of cell wall porosity and thickness and formation of the middle lamella which glues cells together (Cosgrove, 2005).

Degradation of the α-1,4-galacturonide backbone of pectin involves the action of four major enzymes groups: pectin lyase, pectate lyases, pectin methylesterases and polygalacturonases, all of which have been identified in *Fusarium* and most plant pathogenic fungi (Cooper, 1984; de Vries and Visser, 2001; Babalola, 2010) and are unique in causing indirect cell killing (Perombelon, 2002; Babalola, 2010). Analysis of transmission electron

microscope (TEM) of B*otrytis cinerea* showed induced wall swelling and death of adjacent cell (indirect killing) indicative of the degradation of pectin in the matrix of the epidermal wall, resulting in water absorption (Mansfield and Richardson, 1981). Although pectin only contributes around 1% of lignified wood cell components, it is likely that *G. boninense* also produces pectin degrading enzymes since it can readily be grown on pectin substrate (Ho and Nawawi, 1991). Pectinases could play a role in infection of oil palm tissue involving primary walls, especially in juvenile plants where the proportion of these polymers is significantly greater. Three major enzymes are involved in its degradation and are shown in Fig. 56.

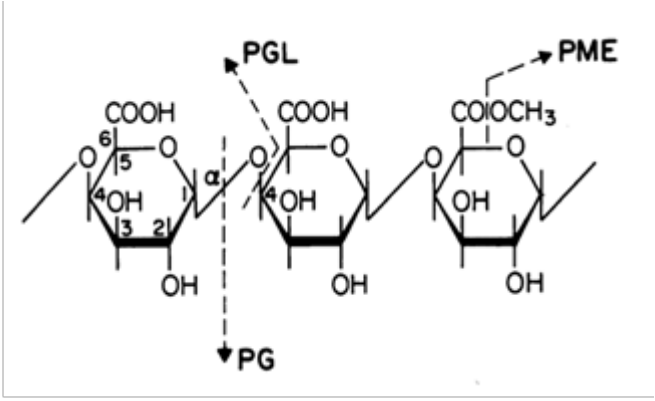


Figure 56. Polygalacturonide and its enzymatic cleavage. Endo-polygalacturonase (PG) and endo-polygalacturonide lyase (PGL) cleave the α-1,4-galacturonide chain and de-methoxylation is by pectin methylesterase (PME) (Cooper,1984).

5.1.2 Cell wall degrading enzymes from *Ganoderma* spp.

Basidiomycete white rot fungi (WRF) are efficient wood degraders and play an important role in the carbon cycle (Paterson, 2007). Most WRF are saprotrophic living on dead wood but some are parasitic colonizing living trees and subsequently causing tree rot. For example, *Armillaria* root rot, *Heterobasidion* butt rot and *Ganoderma* basal stem rot, which all cause significant losses of forest trees and plantation crops (Khairuddin, 1990; Rao, 1990, Cooper *et al.,* 2011). WRF possess a wide range of cell wall degrading enzymes (CWDE) for the degradation of lignin (resulting in the characteristic white appearance of degraded wood), hemicellulose and cellulose components of trees (Hatakka, 1994; Paterson, 2007).

Extracellular lignin-modifying enzymes (LME), comprise manganese peroxidases (MnP), E.C. 1.11.1.13; lignin peroxidases (LiP), E.C. 1.11.1.14 and laccases (Lac), E.C. 1.10.3.2, and are directly involved in degradation of lignin in natural substrates (Hatakka, 1994). Some white rot fungi produce all three LMEs, while others produce only one or two of them (Hatakka, 1994). It has been reported that synthesis and secretion of these enzymes is often induced by limited carbon and nitrogen levels (Keyser *et al.,* 1978, Raghukumar *et al.,* 2004), in other words they are controlled by C and N metabolite repression (Fenn and Kirk, 1981; Faison and Kirk, 1985; Perez and Jeffries, 1993; Rothschild *et al.*, 1995; Kachlishvilli *et al.,* 2006; Levin *et al.,* 2010).

Ganoderma spp. as a white rot possess a wide array of CWDE that can degrade lignin and other plant cell wall polysaccharides into monosaccharides, such as arabinose, xylose, fructose and glucose for energy sources (Yeoh *et al.,* 2012; Manavalan *et al.,* 2012; Jo *et al.,* 2011; Elissetche *et al.,* 2007; Elisashvili *et al.,* 2009). However different *Ganoderma* spp. may only possess one or two of the enzymes. In addition the regulation of these enzymes among *Ganoderma* strains have been reported to be different (D'Souza *et al.,* 1996; Zhou *et al.,* 2013). Several

factors have been determined that affect the type of enzymes produced by *Ganoderma* such as medium composition, substrates and culture conditions (D'Souza *et al.,* 1996; D'Souza *et al.,* 1999; De Souza Silva *et al.,* 2005; Varela *et al.,* 2000; Elissetche *et al.,* 2006; Zhou *et al.,* 2013).

Adaskaveg *et al.* (2000) isolated six species of *Ganoderma* including *G. colossum*, *G. meredithiae*, *G. oregonense*, *G. zonatum* and *Ganoderma* spp. from different hosts, and observed that different strains have varying abilities to secrete/produce lignin-degrading enzymes (Adaskaveg *et al.,* 1999). *G. australe* A272 only produced MnP activity while *G. australe* A464 produced laccase activity on the wood chips substrate. Similarly D'Souza-ticlo *et al.* (2005) observed different lignin-degrading enzymes from four different *Ganoderma* spp. strains (CB364, GASI4.4, CCB209 and GASI2) grown in liquid culture containing wheat bran. Two of the strains CB364 and GASI2 secreted only laccase and the other two strains GASI3.4 and CBB209 produced laccase, LiP and MnP simultaneously. Furthermore, Zhang *et al.* (2005) reported ten out of 16 *Ganoderma* strains produced laccase and peroxidase and no lignin enzymes were detected on the other 6, which indicate that different *Ganoderma* strains may have a different sets of lignin degrading enzymes (Zhou *et al.,* 2013).

It is evident that substrates and medium composition have a profound effect on ligninase productions by *Ganoderma* spp. Ashger *et al.* (2010) investigated enzyme production by *G. lucidum* on different waste materials using solid state fermentation. All substrates stimulated production of LiP, MnP and laccase but substantial differences in activities were detected. The maximum enzyme levels were found with rice straw as a substrate: laccase, 338 IU/ml; LiP, 2185 IU/ml and MnP, 1972 IU/ml (Ashger *et al.,* 2010). In contrast Murugesan *et al.* (2007) showed that only laccase activity was produced when *G. lucidum* was grown on wheat bran.

Inducers such as low-molecular-weight aromatic compounds and host substrate have been reported to induce lignases in *Ganoderma* spp (Elisashvili *et al.,* 2009; Elissetche *et al.,* 2007). Laccase production in *G. lucidum* was enhanced by high nitrogen conditions in shaken culture supplemented with syringic acid, a substitute for wood lignin syringyl residues (D'Souza *et al.,* 1999). In line with metabolite repression already described, laccase levels observed in high-nitrogen (24 mM N) shaken cultures were much greater than those seen in low-nitrogen (2.4 mM N), malt extract, or wood-grown cultures (D'Souza *et al.,* 1999).

Other cell wall degrading enzymes (CWDE) are also produced by *Ganoderma* spp. (Elissetche *et al.,* 2006; Ling *et al.,* 2009). Endo-cellulase and xylanase were detected from *G. australe,* while only xylanase was detected from *G. lucidum* on wood substrates (Elissetche *et al.,* 2006). Ling *et al.* (2009) claims that *G. applanatum* WP1 has the highest ability to degrade cellulose than *G. australe* and *Ganoderma* spp. Endo-glucanases (EGs), Cellobiohydrolases (CBHs) and β-glucosidase (βGLs) were isolated from *Ganoderma* spp., namely, *G. lucidum*, *G. applanatum*, *Ganoderma boninense*, *Ganoderma neo-japonicum*, *G. australe* (Yeoh *et al.,* 2012; Manavalan *et al.,* 2012; Jo *et al.,* 2011; Elissetche *et al.,* 2007; Elisashvili *et al.,* 2009). In addition, Manavalan *et al.* (2012) reported that characterised EGs from *G. lucidum* has a molecular weight of 42.67 kDa and a pI of 4.67.

Recently the whole complete genome of saprotrophic *G. lucidum* have been made public (Chen *et al.*, 2012). The 43.3-MB G. lucidum genome sequence revealed an array of 16, 113 predicted genes. The genome encodes one of the richest sets of wood degradation enzymes among all of the sequenced basidiomycetes. In particular, the genome encodes 417 carbohydrate active enzymes (CAZymes) enzymes for the digestion of the three major classes of plant cell wall polysaccharides: cellulose, hemicelluloses and pectin. *G. lucidum* possesses a large and complete set of ligninolytic peroxidases along with laccases and a cellobiose dehydrogenase of 36 ligninolytic oxidoreductases genes.

5.1.3 Pathogenicity of other necrotrophic fungi

Necrotrophic fungi secrete pathogenicity factors such as phytotoxins, barrier-degrading enzymes, proteases and effectors into host tissue both prior to and during colonization, with primary infection involving the formation of expanding necrotic lesions (Alfano and Collmer, 1996; Walton, 1996). To cause disease, a pathogen must overcome the physical and chemical defence barriers of the plant. Plants have developed effective response mechanisms to defend against pathogen attack. The earliest response to pathogen attack is the generation of an oxidative burst that in some circumstances can contribute to effecting host cell hypersensitive cell death (Govrin and Levine, 2000). The HR is thought to deprive the pathogens of a supply of nutrients, create an antimicrobial environment through oxidative bursts and thereby confine them to initial infection site. However necrotrophic pathogens, such as *Sclerotinia sclerotium, Botrytis cinerea* and *Heterobasidion annosum* are believed to utilise the dead tissue as nutrient sources, aiding colonisation process of the pathogens (Govrin and Levine, 2000; Asiegbu *et al.,* 2005b).

Govrin *et al.* (2000) demonstrated that hypersensitive cell death, does not protect plants against infection by *B. cinerea* and *S. sclerotiorum*, which both trigger ROS and HR in Arabidopsis. They reported that the generation and accumulation of superoxide or hydrogen peroxide contribute directly to their pathogenicity. They also showed that growth of *B. cinerea* was suppressed in HR-deficient mutant *dnd1* and enhanced by HR caused by simultaneous infection with an avirulent strain of the bacterium *Pseudomonas syringae*. Moreover, H_2O_2 levels during HR correlated positively with *B. cinerea* growth but negatively with growth of virulent *P. syringae* (Govrin *et al.,* 2000).

Many necrotrophic fungi produce various low-molecular weight phytotoxic metabolites, ranging from host-selective toxins to those having adverse effects on many diverse species (van Kan, 2006). Others secrete phytotoxic

proteins known to induce necrosis, with the vast majority of broad host necrotrophs producing multiples of both (Pemberton and Salmond, 2004; Gijzen and Nurnberger, 2006; Choquer *et al.,* 2007). Throughout infection, these fungi also actively manipulate host cellular machinery in order to suppress defenses and/or aid in disease progression. Some necrotrophs have adapted mechanisms to detoxify host metabolites that interfere with virulence (Morrissey and Osbourn, 1999).

Oxalic acid (OA) can perform many functions to contribute to pathogenicity. *Sclerotinia sclerotiorum* secretes OA during infection. OA-deficient mutants were non-pathogenic to bean tissues while the oxalic acid-producing wild type and revertant were pathogenic. (Godoy *et al.,* 1990). Furthermore Cessna *et al.* (2000) and Guimaraes and Stotz (2004) suggested that the fungus utilizes oxalate to actively suppress host-induced oxidative bursts as well ABA-induced stomatal closure (Cessna *et al.,* 2000; Guimaraes and Stotz, 2004).

Heterobasidion annosum secretes several low molecular weight toxins including fomannoxin, fomannosin, fomannoxin acid, oosponol and oospoglycol (Basset *et al.,* 1967; Sonnenbichler *et al* 1989; Asiegbu *et al.,* 2005b). Direct application of fomannosin to stem wounds provoked systemic response leading to accumulation of pinosylvin (Basset *et al.,* 1967). Fommanoxin toxin has also been isolated from *H. annosum* s.l. infected Sitka spruce stem wood (Heslin *et al.*, 1983) and had a 100-fold greater toxicity than fommannosin according to Hirotani *et al.* (1977). Uptake of fomannoxin by Sitka spruce seedlings resulted in rapid browning of the roots accompanied by chlorosis and progressive browning of needles. This, and the production of fomannoxin by actively growing hyphae, suggests a role for fomannoxin during pathogenesis (Asiegbu *et al.,* 2005b).

Necrotrophic fungi such as *Fusarium*, *Armillaria* and *Heterobasidion* possess wide arrays of enzymes including amylase, catalase, cellulase, esterase, glucosidase, hemicellulase, manganese peroxidase, laccase, pectinase, phosphatase and proteases. However their role in pathogenesis is not completely understood. Studies to elucidate virulence determinants from pathogenic fungi have often found that targeted mutation of suspected pathogenicity factors such as cell wall degrading enzymes have little impact on virulence. This problem of analysis has been attributed to functional redundancy of key pathogenicity genes within fungi. This has been demonstrated by Karlsson *et al.* (2003), where a cDNA library was constructed from total RNA extracted from *H. annosum* mycelium challenging scots pine seedlings. Analysis of 923 expressed sequence tags (EST) produced by sequencing a random sample of cDNA showed that many of the genes expressed during early infection were involved in metabolism and protein synthesis only, however numerous potential pathogenicity factors including glucanase and superoxide dismutase (SOD) were present in multiple isoforms. Redundancy of genes within fungi is also clearly shown from the genome of the basidiomycete *Phanerochaete chrysosporium*, which has ten genes encoding LiP, five encoding MnP and over two hundred and forty carbohydrate active enzymes (Janse *et al.,* 1998).

Analysing gene expression patterns under different conditions is an additional way to elucidate the role of putative pathogenicity factors. In this context, fusing the promoter of a gene of interest to a marker gene, such as the gene encoding Green Fluorescent Protein (GFP), is one way to study gene regulation in basidiomycetes (Ma *et al.* 2001). Therefore development of a successful gene delivery system for *G. boninense* would have a great benefit by enabling gene knock-out studies and make analysis of promoters of genes with unknown functions possible. Additionally GFP-tagging of G. *boninense* strains would make it possible to visualize the tagged pathogen during hyphal-host interactions microscopically. Microscopic observations of

strawberry plant colonization by a GFP-labelled strain of *Fusarium oxysporum* f. sp. *Fragariae* revealed attachment of spores and the formation of germ tube and with hyphae produced suction cup-like structures for infection on the root surfaces. Colonization of hyphae was observed mainly in the epidermal and cortical tissues with only a few hyphae detected in the vascular tissues after colonization (Yuan *et al.*, 2014). The most important gain from an optimised transformation system, however, is for future studies when the total *G. boninense* genome or transcriptome sequences are available.

Transcriptomic analysis provide powerful insights towards identifying specific genes with potential roles associated with pathogenesis and other metabolic functions during pathogen-host interaction. For example transcriptome analyses of *Colletotrichum higginsianum* infecting *Arabidopsis thaliana* showed that preinvasion perception substantially reprograms fungal gene expression. *Colletotrichum*-genes transcribed in waves linked to pathogenic transitions: Effectors & secondary metabolism before penetration & during biotrophy; hydrolases & transporters at switch to necrotrophy (O'connell *et al.,* 2011). In addition transcriptome analysis of basidiomycetes *H. irregulare* showed transcribed genes linked to saprotrophic and pathogenic phases. A wide range of cellulose-degrading enzymes are expressed during wood decay. By contrast, pathogenic interaction between *H. irregulare* and pine engages fewer carbohydrate-active enzymes, but involves an increase in pectinolytic enzymes, transcription modules for oxidative stress and secondary metabolite production (Olson *et al.,* 2012). In addition a growing number of studies have examined genes expressed in conifer pathogens, including *Armillaria spp.* (Collins *et al.,* 2013), *Phellinus sulphurascens,* that causes laminar root rot of Douglas fir, *Grosmannia clavigera*, a mountain pine beetle-associated pathogen of lodgepole pine (Hesse-Orce U *et al.,* 2010; Williams *et al.,* 2014), and *Heterobasidion annosum sensu lato*, a complex of closely related white-rot basidiomycetes that cause wood-decay in pine, spruce and fir trees (Karlsson *et al*., 2003; Yakovlev *et al.,* 2008; Olson *et al.,* 2012).

While significant progress has been made on transcriptomic aspects of the response by oil palms to *G. boninense* infection (Ho and Tan, 2015), the fungus itself is hardly characterized at the molecular level. Transcriptomic analysis of *G. boninense* should provide in-depth understanding of pathogenicity factors involved during infection and *G. boninense* interaction with oil palm.

This section is aimed at investigating from *G. boninense* its spectrum and regulation of CWDE, the only pathogenicity factors for which there is any current evidence. Also studied are its ability to generate and modify reactive oxygen species as a possible contribution to necrotrophy, but conversely, as a means of detoxifying host ROS. Toxicity to plant tissues from extracellular fluids from de-repressed cultures is also examined. Stages of the life cycle of *G. boninense* representing saprotrophic and parasitic phases are established to then generate RNA for subsequent RNA seq and transcriptomic analysis.

5.2 Materials and methods

5.2.1 Chemicals

All chemicals were acquired from Sigma chemicals plc, unless otherwise stated.

5.2.2 Liquid culture conditions

G. boninense isolates were grown in static liquid culture in the dark at 28ºC in 250 ml Nunclon tissue culture flasks (Fisher) fitted with cap filters for aeration. Growth medium comprised 40 ml modified basal medium (see below) and 2g/10 ml dry weight of perlite to create semi-solid culture conditions. Basal medium was further supplemented with 0.05 g/l glucose as an initial energy source and 1.5% w/v oil palm sawdust (see below) to induce production of cell wall degrading enzymes.

5.2.3 Modified basal medium

Basal medium modified was made up in 9.76g/l MES 2-N-morpholino ethanesulphonic acid 50 mM pH to 5.5 with NaOH. Basal medium consisted of 0.9g $(NH_4)H_2PO_4$, 2.0 g $(NH_4)2H\ PO_4$, 1.0 g KH_2PO_4, and 0.5 g $MgSO_4.(7H_2O)$. Trace elements were made into a 100 x stock solution and consisted of 0.2 ppm $FeSO_4.7H_2O$, 1.0 ppm $ZnSO_4.7H_2O$, 0.02 ppm $NaMoO_4.2H_2O$, 0.02 ppm $CuSO_4.5H_2O$, 0.02 ppm $MnCl_2.4H_2O$ (Fisher).

5.2.4 Oil palm saw dust preparation

Oil palm wood was reduced to small fragments in the UK and placed into a Waring blender to produce a fine sawdust-like sample. Resultant cell wall material was then left to dry overnight at 70ºC in a drying oven before aseptic

transfer to sterile glass jars. Jars were stored desiccated at room temperature with silica to prevent rehydration.

5.2.5 Set up of *G. boninense* model systems

5.2.5.1 Oil palm wood block sampling

After 12 days of incubation, *G. boninense*-infected wood blocks were placed at 15°C to halt mycelial growth and processed for enzyme extraction over the next 5 days. At this stage the blocks were completely colonised externally by the fungus. Wood blocks were split open and internal zones of infection were identified: darker coloured wood near the top surface of the block was designated the established mycelium zone, whereas lighter coloured wood towards the bottom surface of the block was designated the invading mycelium/wood interface. Four of the infected wood blocks were subsequently sectioned into established and invading mycelium zones preceding enzyme extraction.

5.2.5.2 Oil palm root inoculation

The roots from two young oil palms were removed and rinsed to remove soil particles. Primary roots and thick secondary roots were used for this model system. The roots were cut to similar lengths to fit into 140 mm petri dishes and particularly twisted or necrotic sections were discarded. The roots were surface sterilised in 2% sodium hypochlorite solution for 3 min then rinsed in sterile distilled water. The open root ends were cut to remove any residual sodium hypochlorite that may have moved into the vessels. The roots were rinsed twice more in sterile distilled water and laid onto *G. boninense* infested wheat grain, making sure that were contacting mycelium along their entire length. In total there were 9 dishes of inoculated roots, with each plate containing 7-9 roots in similar proportions of colour varieties (white, light brown and dark brown). In addition, 3 dishes were used as un-inoculated controls with roots placed in a dish containing a piece of sterile filter paper soaked in sterile distilled water.

5.2.6 Enzyme extraction from wood and root systems

5.2.6.1 Enzyme extraction buffer

Enzyme extraction buffer was prepared as previously described (Cooper *et al.* 1988; Williams *et al.* 2003b). It is designed to allow the maximum desorption of enzymes from wood and root substrate, and prevents enzymes from denaturing (Rees, 2006). The buffer contains dithiothreitol to reduce oxidation, potassium chloride to desorb proteins from cell walls and insoluble polyvinyl polypyrrolidone (PVPP) to adsorb phenols. The final volume of extraction buffer contained 5mM dithiothreitol, 0.2M KCl and 5% PVPP in 50mM sodium phosphate buffer (pH 6.0) and was kept at 4°C.

5.2.6.2 Processing of wood and root materials

Prior to enzyme extraction, *G. boninense* colonised wood and control/infected root samples with associated mycelium were weighed. Wood samples weighed approximately 10 g per zone per block whereas root samples weighed 7-8 g per dish. The wood and root samples were frozen in liquid nitrogen and ground up in a pre-cooled pestle and mortar. Wood samples were ground up roughly due to the presence of tough fibres, whereas roots were ground into a fine powder. Chilled enzyme extraction buffer was then added to each sample at a ratio of 5 ml buffer: 1 g material (Williams *et al.,* 2003b). The samples were briefly vortexed and allowed to undergo enzyme extraction for 25 minutes on ice. All samples were centrifuged at 5000rpm for 10 minutes at 4°C and filtered through sterilised muslin to remove fine debris.

5.2.6.3 Supernatant dialysis and concentration

Enzyme extract supernatants were dialysed against 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer in 10mm dialysis tubing with a pore size of 12-14,000 Daltons in a 2 L measuring cylinder at 4°C. Each dialysis session included an overnight period with stirring followed by several further hours of dialysis during which the buffer was changed twice in an attempt to remove low molecular weight sugars in the enzyme extracts. Whilst remaining in the dialysis tubing, the extracts were re-concentrated for

8 minutes (roots) or 10 minutes (wood) with solid 35,000 mw polyethylene glycol (PEG) in a large tray. 5-10 ml of each resultant extract was kept as working stock on ice at 4 °C while the rest was frozen at -20 °C.

5.2.7 Enzyme analysis

Enzyme activities were recorded from culture supernatant from liquid cultures or from enzyme solutions extracted from solid oil palm blocks.

5.2.7.1 Extraction of Cell Wall Degrading Enzymes (CWDE) from wood blocks

Oil palm wood blocks (3x3x3cm) were autoclaved at 121ºC for 1 h and then inoculated with three 1 cm2 discs of *Ganoderma* grown on PDA. *G. boninense* was then grown in the dark at 28ºC for periods of 3 and 6 wks before enzyme extraction. Enzymes were retrieved from the wood blocks in an extraction buffer designed to prevent denaturation of the enzymes and to allow maximum desorption from the wood substrate (Williams *et al.,* 2003a). The buffer consisted of 5 mM dithiothreitol (to prevent oxidation), 0.2 M KCL (allowing desorption of proteins from cell walls) and 5% polyvinyl polypyrrolidone (to adsorb phenols) in 50 mM sodium phosphate (pH 6.0) as described by Williams *et al.* (2003b) (all chemicals, Sigma, UK).

5.2.7.2 Dialysis of culture supernatant

Twenty millilitres of supernatant was filtered through sterilised muslin into dialysis tubing. Tubes were then sealed and placed in 10 mM MES buffer in a 5 l vessel in the cold room (4ºC) overnight with stirring. The buffer was then changed and left for several more hours to remove low molecular weight sugars that contaminate reducing sugar enzyme assays. Supernatant was then re-concentrated to 20 ml using 35,000mw polyethyleneglycol (PEG) (FULKA). Half of the supernatant was then frozen and stored at –70ºC, and a 10 ml working stock was used for immediate reactions and was stored on ice at 4ºC.

5.2.7.3 Lignin degrading enzymes assays

5.2.7.3.1 Azure B for Lignin Peroxidase assay

Lignin peroxidase activity was measured at room temperature using the method described by Archibald (1992). The reaction mixture contained 32 µM Azure B and 100 µM H_2O_2 in 50 mM Na tartrate buffer (pH 4.5, 25ºC). The dye and H2O2 were made up in x100 stock solutions and 10 µl of each was added to a 1 ml final reaction volume, 500 µl of culture supernatant (or enzyme extraction buffer) was used for each reaction and assays begun with addition of H_2O_2. Decrease at A651 was determined over a 15 min period using a chart recorder. Activity was determined by referring to a concentration standard for Azure B at A651nm (Appendix 5). Reactions were performed in duplicate with boiled controls.

5.2.7.3.2 ABTS for Laccase assay

Laccase activity was measured using the protocol adapted from Yaropolov *et al.* (1994), which measures the oxidation of 2,2-azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS). Reaction mixtures contained 14 µM ABTS in 50 mM glycine-HCL buffer (pH 3.0, 30˚C) in a 1 ml reaction mixture containing 0.5 ml culture supernatant. Reactions began with mixing the culture supernatant with the buffer containing ABTS in a cuvette. The cuvette was immediately placed in a spectrophotometer and read at A405nm for 5 min. It was determined that the linear rate of reaction could be accurately calculated from absorbance readings recorded over 200 seconds.

5.2.7.3.3 MBTH/DMAB assay for Manganese-dependent Peroxidase

The MBTH/DMAB assay detects manganese-dependent peroxidase activity by measuring the oxidative coupling of the two compounds 3-Methyl-2-benzothiazolinone hydrazone (MBTH) and 3-(dimethylamino)benzoic Acid (DMAB), causing a blue-purple coloration (Castillo *et al.,* 1994; Castillo *et al.,* 1997). The final 1 ml reaction mixtures contained 500 µl enzyme extract, 0.07 mM MBTH, 0.99 mM DMAB, 0.3 mM MnSO4 and 0.05 mM H_2O_2 in 100 mM citrate buffer (pH 4.5). Enzyme extracts and reaction mixtures were initially incubated separately at 37°C for 5-10 min before combining in a disposable cuvette, and H_2O_2 was added to start the reaction. Change in A590nm was measured every 10 seconds for 5 min. Manganese-dependent peroxidase activity was represented by the increase in A590nm over the first minute.

5.2.7.4 Assays to detect cellulose/hemicellulose degrading enzymes

5.2.7.4.1 DNS assay for cellulase

The DNS assay detects cellulase activity by measuring the reduction of 3,5-dinitrosalycylic acid (DNS) through release of reducing sugars from cellulose. This protocol was adapted from previously described cellulase-detecting methods utilising DNS and cellulose substrate (Miller, 1960; Eveleigh *et al.,* 2009). The DNS reagent was made by dissolving 12 g sodium potassium tartrate in 8 ml of 2M sodium hydroxide solution by heating on a hot plate, before adding to 20 ml 96 mM DNS solution and diluting to 40 ml with distilled water. Final 200 µl reaction mixtures contained 100 µl enzyme extract and 2 mg homogenised sterilised Whatman filter paper with 100 µl of 50 mM citrate buffer (pH 4.8). Reaction mixtures were incubated in 1.5 ml tubes at 35°C for 30 and 60 min at which point 100 µl DNS reagent was added to stop the reaction. The tubes were boiled for exactly 5 min, cooled to room temperature in a water bath then centrifuged at 11,600 x g for 2 min. The resulting supernatant was added to 700 µl distilled water in a disposable

cuvette and A540nm was read after 30 and 60 min against a glucose concentration standard (Appendix 6) to determine the amount of reducing sugar released, which was considered to be proportional to cellulase activity.

5.2.7.4.2 RBB assay for xylan-degrading enzymes

The RBB assay detects xylan-degrading enzymes through measurement of the endoxylanase/β-xylosidase-mediated release of dye fragments from Remazol Brilliant Blue (RBB) dyed xylan, which is precipitated upon addition of an organic solvent (Biely *et al.,* 1985). The proportions of the reaction components were optimised for a spectrophometric protocol adapted from a microtitre plater reader method (Rees, 2006). Final 200 µl reaction mixtures contained 50 µl enzyme extract and 150 µl of 0.1% RBB dyed xylan in 50 mM sodium citrate buffer (pH 5.0). Enzyme extracts and buffered RBB dyed xylan were incubated together at 37°C for 30 min, at which point the reaction was stopped by addition of 700 µl of 96% ethanol. Tubes were inverted and left to stand for 20 min, allowing for precipitation of remaining RBB dyed xylan substrate. Tubes were centrifuged at 11,600 x g for 2 min. 700 µl of the resulting supernatant was pipetted into a disposable cuvette and the absorbance was read at 595nm. Xylanase activity was considered to be directly correlated with increased A595nm, compared to blank solutions containing citrate buffer instead of RBB xylan.

5.2.7.4.3 P-nitrophenol-linked glycosides assay for glycosidases

The glycosides assay detects glycosidase activity by measuring the release of *P*-nitrophenol from linked glycosides (β-D-glucoside and β-D-galactoside), causing a yellow coloration (Hayano, 1973). Final 200 µl reaction mixtures contained 20 µl enzyme extract and 180 µl of 5 mM glycoside in 50 mM citrate buffer (pH5.0). Upon addition of enzyme extract to the buffered glycoside in disposable cuvettes, the reaction mixtures were incubated at 37°C for 30 min. At this point, 700 µl of 1 M sodium bicarbonate solution was added to stop the reaction and the absorbance was read at 410 nm against blanks containing 50 mM citrate buffer instead of buffered glycoside. This

was slightly adapted from Rees (2006) who used 500 µl of 1 M sodium bicarbonate solution. However, it was noted that absorbance readings at 410nm were more stable upon addition of 700 µl of sodium bicarbonate. The increase in A410 nm was read against a *p*-nitrophenol concentration standard (Appendix 7) to determine glycosidase activity.

5.2.7.5 Assays to detect pectin-degrading enzymes

5.2.7.5.1 Pectin Lyase assay

Pectin lyase (PL) activity was measured by the production of 4,5-unsaturated galacturonides through enzyme-mediated breakdown of pectin (Ayres *et al.* 1966). The final 1 ml reaction mixture contained 300 µl of enzyme extract and 700 µl of 0.25% pectin (Sunkist) and 1 mM calcium chloride in 50 mM Tris-HCl buffer (pH 9.0). After addition of enzyme extract to the other components in disposable cuvettes, the reaction mixtures were incubated at 35°C. At zero, 15 and 30 min A240nm was measured by transferring the reaction mixture to a quartz cuvette. Increased absorbance over 30 min was used to infer PL activity by transformation using the molar extinction coefficient for pectin (ε_{240nm} = 5500 M^{-1} cm^{-1}) (Rees, 2006).

5.2.7.5.2 NaPP assay for endo-polygalacturonase

The NaPP assay detects endo-polygalacturonase (PG) activity by measuring the breakdown of sodium polypectate (NaPP) causing a relative decrease in viscosity (Durrands and Cooper, 1988). The final reaction mixtures contained 2 ml enzyme extract and 8 ml of 1.75% NaPP (Sunkist) in distilled water (pH 5.17). The assay was carried out using U shaped viscometers. The flow-time for water (T_w) was measured for each viscometer, followed by the flow-time for the control substrate (8 ml NaPP solution and 2 ml water) (T_c). Finally the reaction mixture was set up and flow-time was monitored. The time taken for viscosity to be reduced by 25% was measured in most cases by extrapolating the linear trend line observed over the first hour of reaction. PG

activity was expressed as 1000 multiplied by the reciprocal of the time taken to reach 75% of original viscosity.

5.2.8 Isoelectricfocusing (IEF) detection methods

IEF was performed over the pH range 3-6, using Servalyt Precotes 3-6 polyacrylamide gels from Serva. After applying the protein samples (0-5-1.0 pg protein per well in analytical gels, 200400 pg in preparative gels), gels were run for 30 min at 200 V, 15 min at 400 V, 5 min at 800 V and then the voltage was raised to 1200 V until the current reached 2 mA. MnP bands were developed by soaking the gel in a solution prepared as follows : 15 mg 4-chloro-1-naphthol was dissolved in 5 ml methanol; 1 ml of this solution was mixed with 6.3 ml 20 mM Tris/HCl pH 7-5, 0-5 M NaC1. In turn, 4ml of the resulting solution was mixed with 25 ml50 mM sodium tartrate pH 5.0 containing 0.2 mM MnSO,. Hydrogen peroxide was added to a final concentration of 50 mM. The solution for developing the bands corresponding to laccase was the same but lacked MnSO, and hydrogen peroxide. Occasionally, both enzymes were developed with ABTS or phenol red as substrates, which were present at final concentrations of 5 mM and 480 pM respectively.

5.2.9 Reactive oxygen species and related enzymes determination

5.2.9.1 Modified Basal Medium (MBM) Liquid Culture of *G. boninense ROS*

Modified Basal Medium (MBM) contained (in one litre) 0.09g of ammonium phosphate dibasic ((NH4)H2PO4), 0.2g of ammonium hydrogen phosphate ($(NH_4)2HPO_4$), 1.0g of monopotassium phosphate (KH2PO4), magnesium sulphate ($MgSO_4$ ($7H_2O$)) and either 1% (w/v) of D-Glucose or 0.05% (w/v) of D-Glucose and 1.5% (w/v) palm wood extract. The palm wood extract was produced by using a Black & Decker KA292E 350W sander to sand a block of palm wood, creating a sawdust-like powder. Added to this was 1% (v/v) of

a solution of trace elements containing (in one litre) 0.2mg of FeSO4.7H2O, 1.0mg of $ZnSO_4.7H_2O$, 0.02mg of $NaMoO_4.2H_2O$, 0.02mg of $CuSO_4.5H_2O$ and 0.02mg of $MnCl_2.4H_2O$. To this, 9.76g of 2-(N-morpholino) ethane-sulphonic acid (MES) (adjusted to pH 5.5 with NaOH) was added. This was all then made up to one litre with distilled water.

The cultures were set up using sterile 75cm2 Easyflask filter tissue flasks (Fisher Scientific). 12 flasks were used per MBM type, representing 3 repetitions of 3 sampling times and one medium control per each of the 3 sampling times. To inoculate each flask, 500µL of homogenised mycelium was added to MEB, and these were incubated at 28°C ± 2°C for 7 days until the mycelia fully covered the base of the flask. Following this, the MEB was replaced with 30mL of either type of MBM. To confirm that there was no contamination from the surrounding incubation area, or that there was no contamination from the method used, flasks of MEB were also incubated in this way. Collection of the culture fluids sacrificed the culture for every sampling day. The sampling days were at day 2, 4 and 6 after initial inoculation. Sampling consisted of the removal of the culture fluid from the mycelia, by careful pipetting in to a 50mL falcon tube, until around 25mL of liquid had been obtained. This was then centrifuged for 10 minutes at 5000 × g, and the supernatant transferred to a new falcon tube to be used for future assays. This was repeated for the medium control for each sampling day.

5.2.9.2 Determination of hydrogen peroxide content

Following the method of Velikova *et al*. (2000), with some modifications, H_2O_2 contents of the two different *G. boninense* liquid culture samples were determined. Reaction mixture contained 0.25mL of culture fluid, 0.25mL of 10mM potassium phosphate buffer (pH 7.0) and 0.5mL of 1M potassium iodide. This was then left to incubate at room temperature for 10 minutes and was then transferred to a quartz cuvette. H_2O_2 content was observed at 390nm using a spectrophotometer and the content per sample was determined using a standard curve based on 10 – 100µM H_2O_2.

5.2.9.3 Peroxidase (POD)

To identify POD activity in the two different *G. boninense* liquid cultures and across different sampling days, the assay followed the method of Venisse *et al*. (2001) with some modifications, using guaiacol as the hydrogen donor. Each replicate from each sample day was assayed. Reaction mixture contained 1.2mL of 0.0.25M guaiacol, 0.2mL of 0.25M H2O2 and 1.5mL of the culture fluid. POD activity was measured by observing an increase in absorbance at 470nm using a Thermo Spectronic Helios Gamma spectrophotometer (Thermo Scientific). POD activity units were ΔA470/sec. A reaction mixture replacing the culture fluid with distilled water was used as the blank.

5.2.9.4 Superoxide Dismutase (SOD)

To identify SOD activity in the two different *G. boninense* liquid cultures and across different sampling days, the assay followed the method of Rao *et al*. (1996) with some modifications. Each replicate from each sample day was assayed. Reaction mixture contained 0.9mL of 50mM sodium phosphate buffer (pH7.8), 0.3mL of 130mM methionine (MET), 0.3mL of 100μM EDTA-Na2, 0.3mL of 750μM nitro blue tetrazolium (NBT), 1mL of sample and 0.3mL of 20μM riboflavin. To encourage the development of a blue formazan, samples were done in duplicate with one incubated in a light box and incubated in the dark for 10 minutes. After this, SOD activity was measured by observing absorbance at 560nm using a Thermo Spectronic Helios Gamma spectrophotometer (Thermo Scientific). SOD activity units were ΔA560/sec. A reaction mixture replacing the culture fluid with distilled water was used as the blank.

5.2.10 Toxicity Bioassays

5.2.10.1 Infiltration of palm leaves

To identify the potential toxicity of the *G. boninense* palm wood culture fluids, a preliminary bioassay was performed. Three young leaves from the same palm frond were inoculated with all three of the replicates from day 2, 4 and 6 samplings of palm wood culture fluids. The two controls were distilled water and control palm wood medium and were used to show whether the method itself caused damage or whether the medium itself was toxic. Application of approximately 1mL of each sample was performed using a plastic 3mL sterile needleless syringe held against the underside of the leaf. As the plunger was depressed, the liquid in the barrel entered the stoma. This was done around midday to ensure the stomata were open. The liquid cultures were applied in three different orientations across the leaves to allow for positional bias.

5.2.10.2 Cell viability

To evaluate the toxicity of the culture fluids in an alternative way, the cell viability of potato discs after immersion in different reaction mixtures was assessed. Potato discs of 8mm diameter and 0.5mm thickness were soaked in distilled water for a maximum of two hours to remove excess starch, and then 20 discs were placed in each small petri dish filled with 2mL of 0.2M citrate buffer (pH 4.5), 4mL of distilled water and 6mL of either sterile distilled water (control), palm wood medium (medium control) or replicate 1, 2 or 3 from that sampling day. From these five reaction mixtures, 5 discs were removed at 10, 20, 30 and 40 minutes after immersion. The petri dishes were swirled around every few minutes to disperse the mixture evenly. After removing the discs from the mixtures, they were transferred to staining blocks and soaked in 2mL of 0.02% buffered neutral red dye (10mL of 0.2% neutral red: 0.1g in 50mL distilled water, added to 90mL of plasmolysing buffer) for 10 minutes and then washed in a de-staining solution for a further few minutes. The plasmolysing buffer/destaining solution comprised 0.9M potassium nitrate (KNO_3) and 0.1M phosphate buffer (pH 7.5).

5.2.11 *Agrobacterium tumefaciens*-mediated transformation

Transformation of *G. boninense* was performed as described by Burns *et al.* (2006), with some modifications. An *Agrobacterium tumefaciens* LBA1126 containing pGR4-GFP or pGR4-4iGM3′eGFP as used in Burns *et al.* (2006) were supplied by Andy Bailey from School of Biological Sciences, Bristol University and A. tumefaciens and AGL-1 containing pBGgHg-GFP as used in Eckert *et al*. (2005) were supplied by Adrian Newton from Scottish Crop Research Institute, Dundee, UK. The plasmid was originally produced at Rothamsted Research, Harpenden, UK. *Agrobacterium* was grown in 5 ml Minimal Medium containing kanamycin (50 µg/ml) for 2 d at 28°C. Then, the bacterial cells were harvested by centrifugation at 16 000 g for 2 min, re-suspended and grown in Induction Medium containing kanamycin (50 µg/ml) at 28°C until the OD 600 reached 0.15 after 6 h incubation at 200 rpm.

Homogenised mycelium (100µl) were mixed with 100µl of A. *tumefaciens* culture and spread onto nitrocellulose membrane (Whatman Cat. # 7141 104; 47 mm diam; 0.45 µm pore size) placed on the co-cultivation medium. This mix (200µl per plate) was plated on a 0.45-µm pore, 45-mm diameter nitrocellulose filter (Whatman, Hillsboro, OR) and placed on co-cultivation medium (same as IM except that it contains 5 mM glucose instead of 10 mM glucose) in the presence and absence of 200 µM acetosyringone (AS). Following incubation at 25°C for 2 d, the filter was transferred to MM containing hygromycin B (75 µg/ml) as a selection agent for transformants and cefotaxime (200 µM) to kill the *A. tumefaciens* cells. Individual transformants were transferred into CDA amended with hygromycin B (75 µg/ml). Incubation at 28ºC was prolonged 3-5 days until the transformed colonies became clearly visible.

5.2.12 Transcriptome of *G. boninense*

5.2.12.1 Sample preparation

Preparation of *G. boninense* inoculation of RWB, Cut roots, and oil palm seedling root inoculation as described in section 5.2.5 and 3.3.1.

5.2.12.2 RNA extraction and PCR reaction

RNA extraction and PCR reaction as described in section 3.2.10.

5.2.13 Statistical analysis

Data sets from enzyme assays were analysed statistically using appropriate tests in SPSS statistics software version 21 (IBM, Armonk NY). All datasets were checked for normal distributions and equality of variances before application of parametric or non-parametric analyses. Results were considered to be significant at $p < 0.05$.

5.3 Results

5.3.1 Cell wall degrading enzymes (CWDE) production in palm wood and roots

This study was conducted to determine the secretion and the activity of CWDE by *G. boninense* during saprotrophic and parasitic stages. Model systems were developed by using palm wood and roots to mimic different phases of lifestyle by the pathogen. Palm wood blocks were used as a substrate for the saprotrophic phase and oil palm roots were used to represent a parasitic phase. In palm wood, enzyme activities were compared between invading mycelium within wood and established mycelium of the pathogen on the outside of wood blocks. CWDE in roots were detected over a time course of 6 days and compared with non-inoculated roots

5.3.1.1 Colonization and appearance of palm wood blocks and palm roots

After 12 days of incubation at 30°C, *G. boninense* infested wood blocks were completely colonised externally by the fungus (Fig. 57C). Palm wood blocks were easily colonised by *G. boninense* from agar plugs inoculum placed on their surface. An advancing white mycelial mat occurred on the blocks' surfaces. Wood blocks were split open and internal zones of infection were identified: darker coloured wood near the top surface of the block was designated the established mycelium zone, whereas lighter coloured wood towards the bottom surface of the block was designated the invading mycelium/wood interface (Fig 57D). Four of the infected wood blocks were subsequently sectioned into established and invading mycelium zones before processing for enzyme extraction.

For infected roots, wheat grain inoculum was used for infection and was placed beneath the roots. The grain inoculum showed a uniform and rapid colonisation by pathogen mycelium over the roots' surface. At 2 days post-inoculation, *G. boninense* mycelium had adhered to oil palm roots. Roots

became covered, either in part or fully, with *G. boninense* mycelium on the underside (Fig. 58). There was no obvious difference in mycelium coverage between roots of different colours (mature root (Brown), young root (white). Control roots displayed no evidence of contamination (Fig. 58C).

At 6 days post-inoculation, roots were fully covered in mycelium and there was indication of necrosis within roots and at cut ends (Fig. 58). Since full invasion of roots had occurred, and because excised roots had a limited life, no further time point was considered. Also, some control roots at 6 days showed evidence of microbial contamination and necrosis at cut ends (Fig. 58H-58I). Therefore, only uncontaminated sections of roots were utilised.

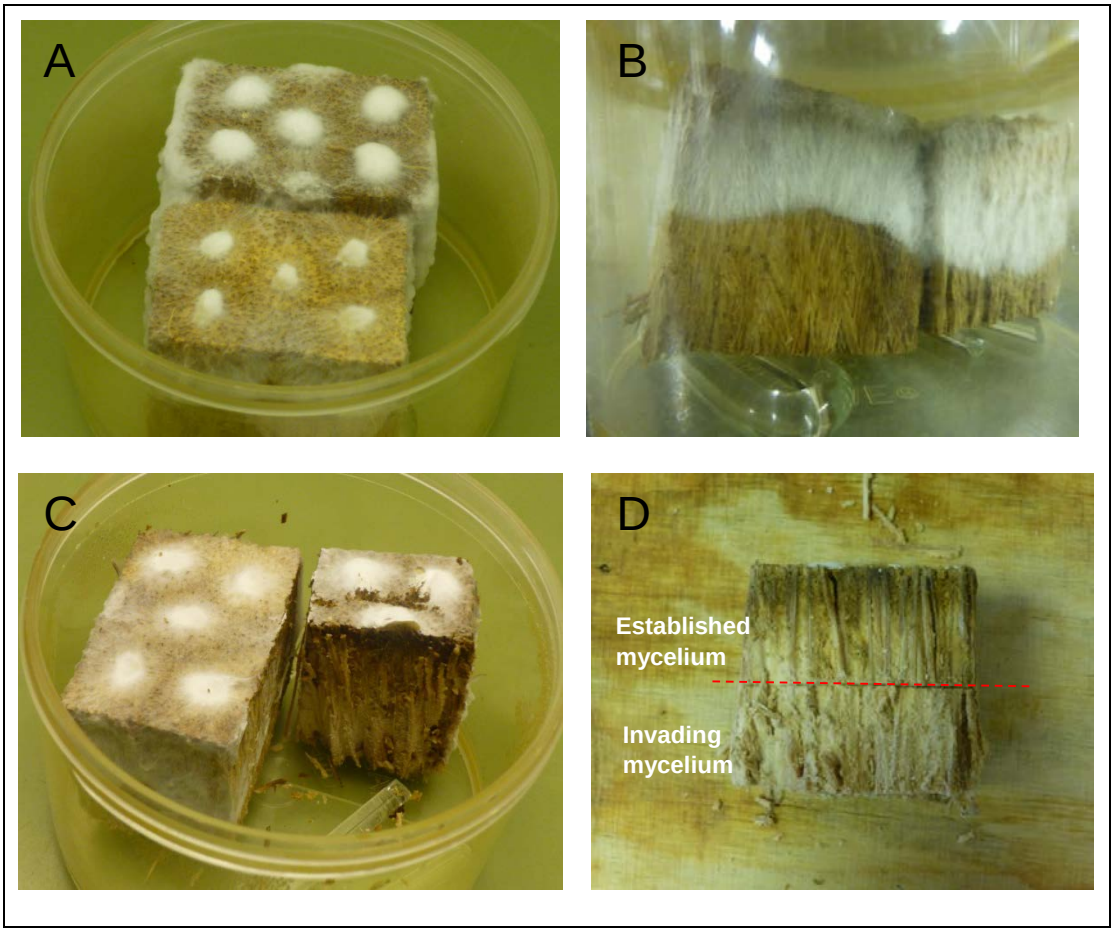


Figure 57. Images demonstrating oil palm wood block preparation and inoculation. A. 6mm diameter discs of *G. boninense* were applied evenly upon the malt agar-coated top surface. B. *G. boninense* mycelium at 8 days post-inoculation had grown downwards along the outside edges of each block. C. Wood block surfaces were extensively covered with mycelium after 12 days incubation. D. Wood blocks were sectioned into established mycelium (dark brown, degraded) and invading mycelium (lighter brown, still intact) zones.

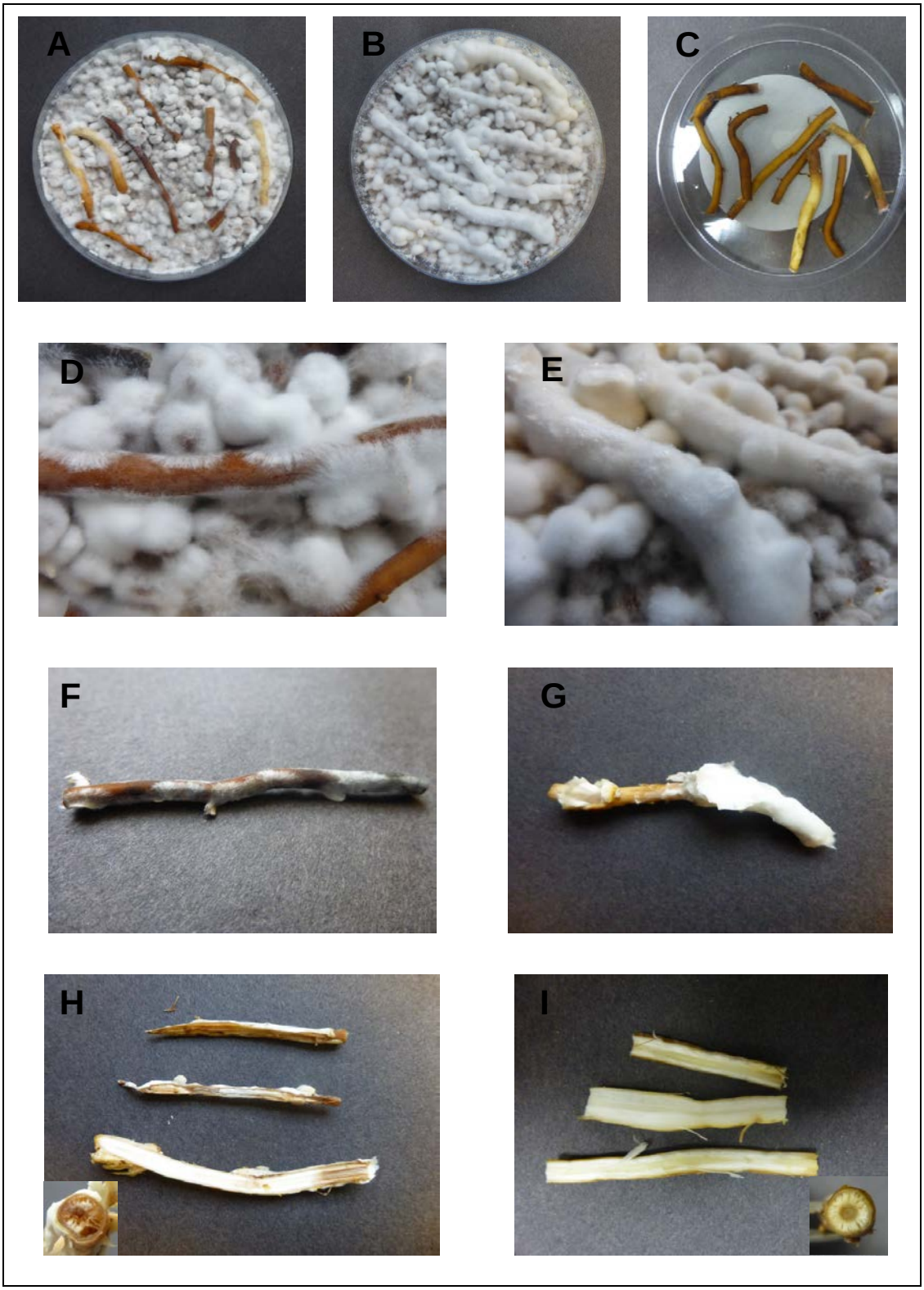


Figure 58. *G.boninense*-inoculated and control roots. At 2 days, inoculated roots were partially covered in *G.boninense* mycelium (A) while after 6 days they had been completely colonised by mycelium (B). Control roots at 6 days were free of mycelium, although a small amount of contamination had occurred (C). At 2 days post-inoculation, *G.boninense* mycelium adhesion to roots had taken place (D) while after 6 days, root tissue was no longer visible (E). Removed roots at 2 and 6 days retained much of the associated mycelium (F and G, respectively). At 6 days post-inoculation, roots were necrotic both at cut ends and within the centre, shown by lateral view of cut open roots (H; inset shows medial view). Control roots displayed some necrosis at cut ends, but considerably less than in inoculated roots (I; inset shows medial view).

5.3.1.2 Lignin-degrading enzymes in *G. boninense*-infected colonized wood and infected roots

In wood samples, LiP activity was detected in both invading mycelium and established mycelium after 12 days post-inoculation (Fig. 59A). LiP activity was 15-fold higher in invading mycelium, reaching 35 nkats and in established mycelium activity was 20 nkats (t-test, p=0.012).

LiP activity was detected in infected roots at 4 and 6 days post-inoculation and was not detected in control roots (Fig. 59D). Maximal activity of LiP (4.3 nkats) occurred at 4 days and by day 6 it had declined 3-fold (t-test, p=0.005) (Fig. 59D). LiP activity was detected in both infected palm wood and roots.

Laccase activity was detected in advancing and established mycelium of infected palm wood, but not in control wood (Fig. 59B). Comparing the two zone data, there was a significant difference of 2-fold increase in laccase activity between established and advancing mycelium after 12 day post-inoculation (t-test, p=0.005).

In root extracts, laccase activity was detected from 2 to 6 days but not in the control roots (Fig. 59E). The activity increased gradually with a significant 6-fold increase between 2 to 6 days (t-test, p=0.000) (Fig. 59E).

Laccase was present in all extracts from infected wood and roots. Activity was high from day 6 infected root extracts and established mycelium on wood. Because of the high activities, the assay was optimised by increasing the concentration of ABTS in the final volume to 42 µM in order to obtain reliable initial reaction rates.

MnP activity in wood showed that activity in invading mycelium was twice that in established mycelium (Fig. 59C) There was a significant difference between the activity in the invading mycelium and the established mycelium (t-test, p=0.04)

In root extracts MnP activity was highest however enzyme activity appeared to be similar between control and infected roots, and was significantly different between 2 to 6 days post-inoculation (t-test, p=0.176) (Fig. 59F). MnP activity was detected in both root and wood samples.

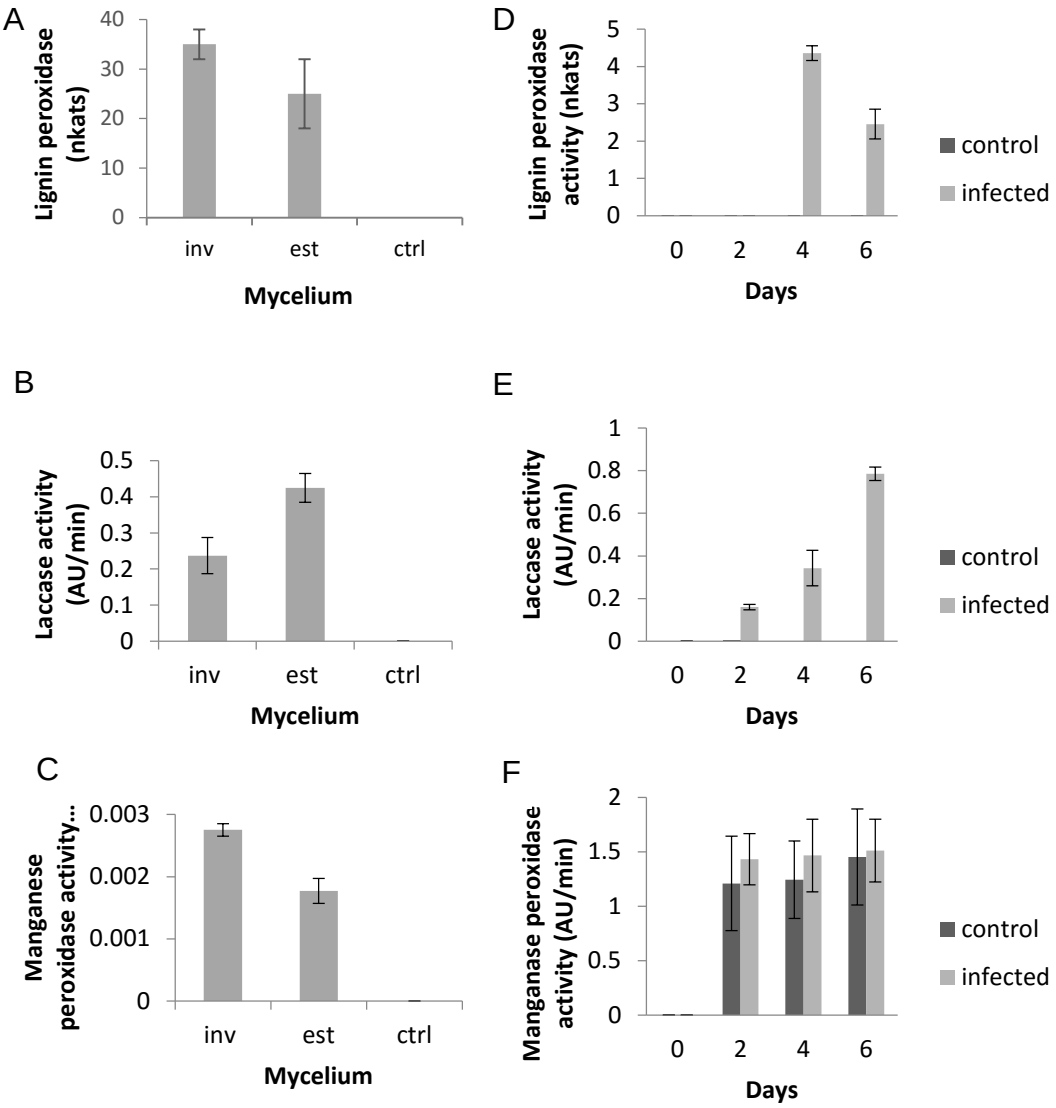


Figure 59. Lignin degrading enzymes activities by *G. boninense* in palm wood block and roots. Palm wood blocks: Lignin peroxidase activity in palm wood measured by azure B assay (A). Laccase activity in palm wood measured by ABTS assay (B). Manganese peroxidase activity in palm wood measured by MBTH/DMAB assay (C). Oil palm roots Lignin peroxidase (D). Laccase activity measured by ABTS assay (E). Manganese peroxidase (F). Error bars indicate means of standard deviation of three replicates samples per time point for roots and four replicates samples per treatment for palm wood blocks.

5.3.1.3 Cellulase and hemicellulase activity in *G. boninense* wood and roots

In wood extracts, cellulase activity was found in invading and established mycelial zones. The enzyme level significantly increased 7-fold in established mycelium/wood compared to invading mycelium wood extract (t-test, $p<0.05$) (Fig. 60A). In root extracts, maximal cellulase activity (27nkats) was observed after days 2 and subsequently activity decreased gradually to 23 nkats at day 6, for 4-fold significantly (t-test, $p=0.03$) (Fig. 61A). Comparison between the two systems showed cellulase activity was generally higher in infected roots than in wood extracts (Fig.60A and 61A).

Xylanase activity in wood increased steadily in invading and established mycelium. However there was no significant difference observed between the activities with time (t-test, $p=0.067$) (Fig.60B). In root extracts, xylanase was found in control and infected roots at 2, 4 and 6 days. Activity was much lower in control roots than in infected roots at all time points, and was highest in infected roots at 6 days (Fig. 61B). Comparison between the two systems showed that xylanase activity is much higher in roots than in wood (Fig. 60B and 61B).

Glucosidase and galactosidase activities were present in invading and established mycelium wood extracts. There was no significant difference between glucosidase activity and galactosidase activity in established mycelium and invading mycelium ($p=1.000$) (Fig. 61D). In infected roots, glucosidase activity was higher at day 6 than day 2 (Fig. 61C). Similarly galactosidase activity at day 6 was higher than at day 2. At 2 days, glucosidase activity was significantly higher than galactosidase activity ($p<0.05$). Similarly at 6 days, glucosidase activity was significantly higher than galactosidase activity ($p<0.05$). Glucosidase and galactosidase activities were present in both wood and roots extracts and in general enzymes activities was higher than galactosidase activity (Fig. 60C).

Both PG and PL were detected in infected wood and roots (Fig. 60D and 61E). In wood, pectin lyase (PL) activity was 2-fold higher in established mycelium than in invading mycelium (significant difference, $p<0.05$) (Fig. 60D). Polygalacturonase (PG) activity in wood extracts was significantly higher (2-fold) in invading mycelium than in established mycelium extracts ($p=0.034$) (Fig. 60E).

In roots, PL was detected at day 4 and was three-fold greater by day 6 (Fig. 61E). PG activity was highest in infected root extracts at day 6 than in (Fig. 61F). Although PG activity was detected in control root extracts, activity appeared to remain constant between 2 to 6 days with no significant difference (t-test, $p=1.000$), while the distribution of activity in infected roots increased 9-fold between day 2 to day 6 ($p<0.05$). Moreover, PG activity was apparently higher in root extracts than in wood extracts (Fig 60E and 61F). Although Rees (2006) reported extensive loss of starch in oil palm stems degraded by *G. boninense* no amylase activity was detected in either roots or wood. However, in both wood and root extracts there were high background reducing sugar levels, indicated by tests with boiled wood samples and control root samples. This prevented accurate determination of amylase(s) because the assay is based on release of reducing sugars.

Overall, it is apparent that *G. boninense* deploys a wide range of polysaccharide-degrading, potential CWDE, during its saprotrophic and parasitic phases

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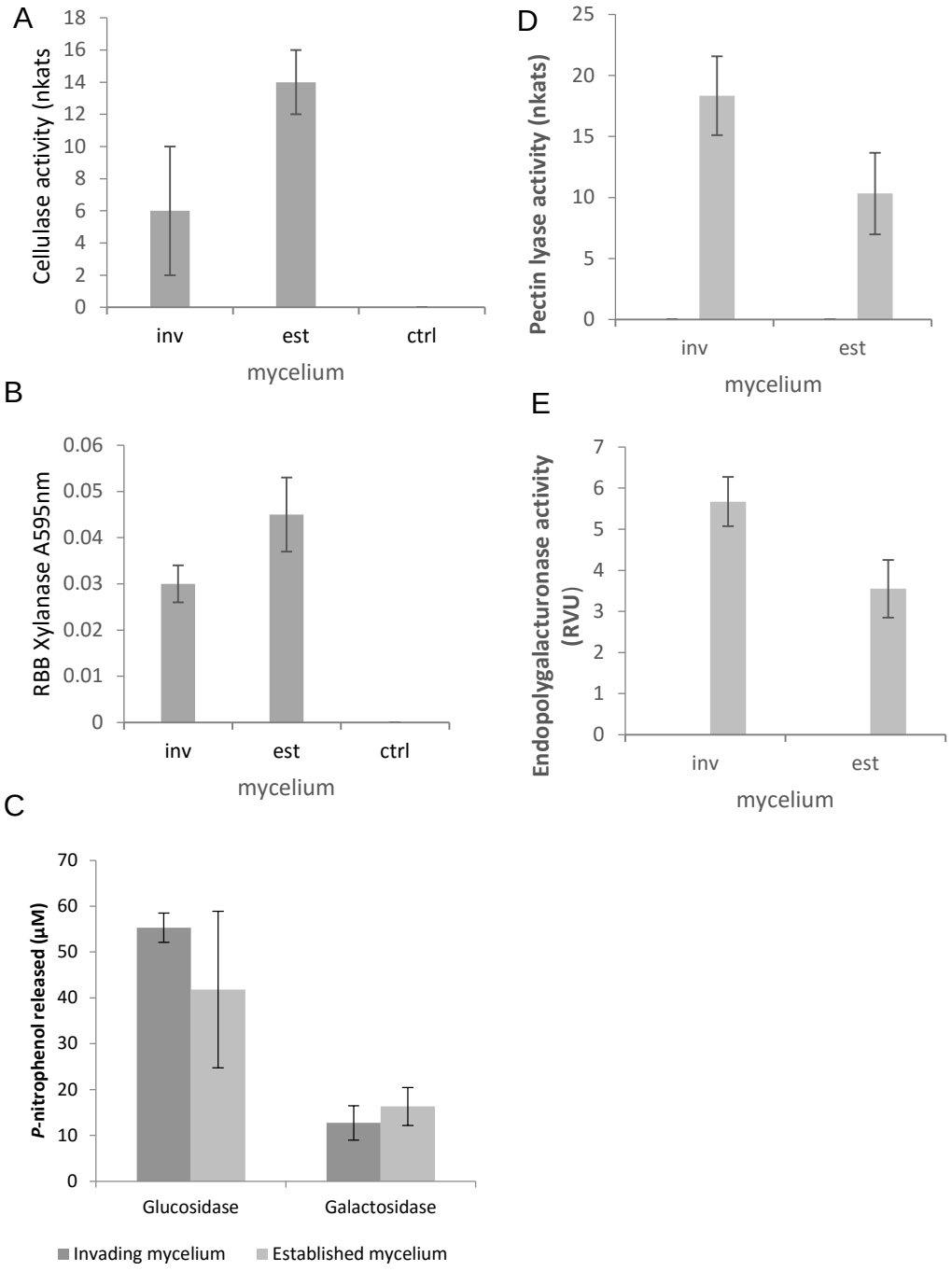

Figure 60. Polysaccharidase activities by *G. boninense* in palm wood. Cellulase (A), xylanase (B), glucosidase and galactosidase (C), pectin lyase (D) endo-polygalacturonase (E) of invading (inv) and established (est) mycelium on palm wood blockc and control block (uninoculated). Error bars indicate means of standard deviation of four replicates samples per treatment.

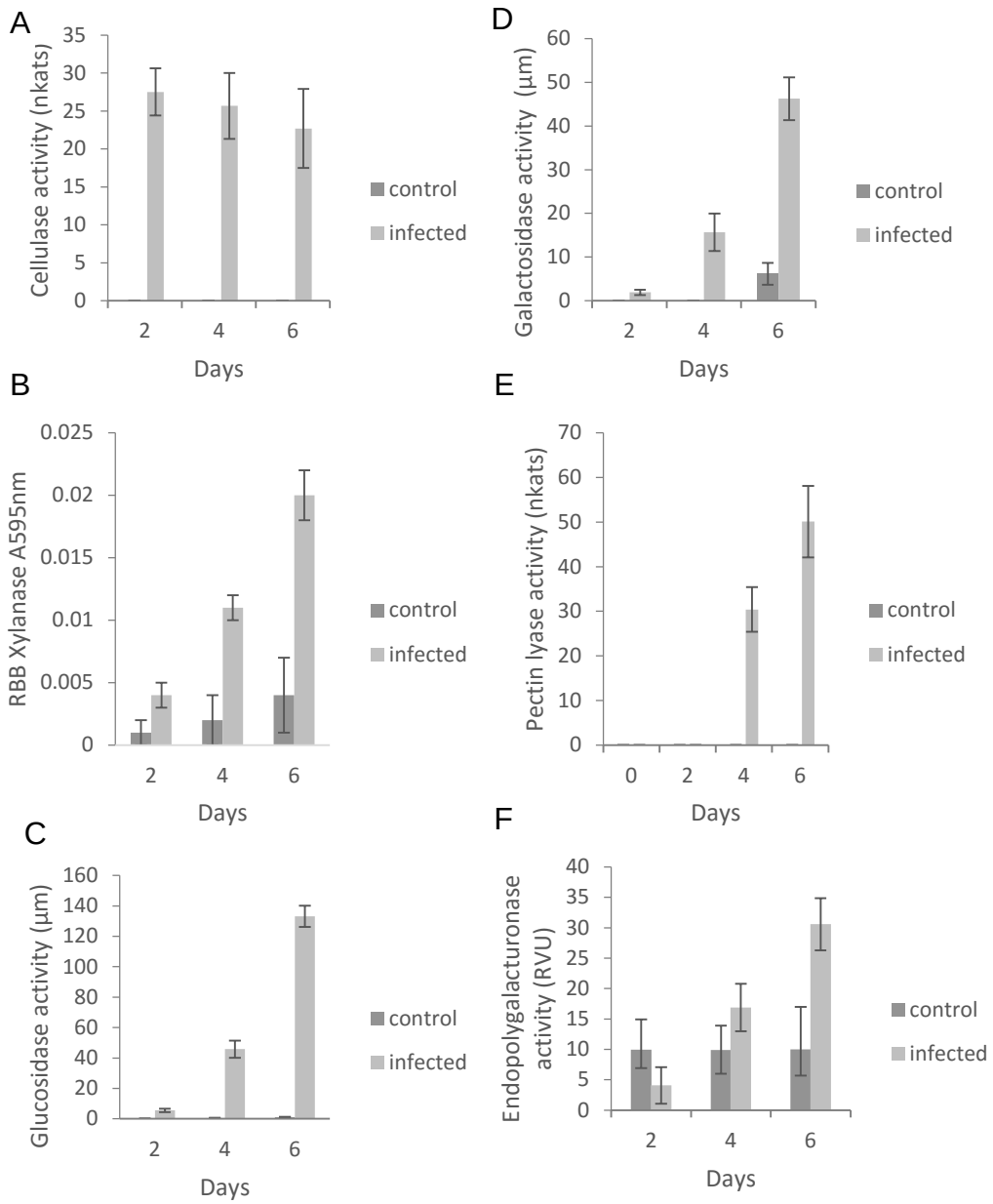

Figure 61. Polysaccharidase activities in roots. Cellulase activity (A), xylanase activity (B), glucosidase activity (C) galactosidase activity (D) pectin lyase activity (E) endo-polygalacturonase activity (E). Enzymes activities were detected mostly in *G. boninense* infected roots and were absent in uninoculated roots (control). Error bars indicate means of standard deviation of three replicated samples per time point.

5.3.1.4 Ligninase isoforms from infected roots and colonized wood

Detection of lignin degrading enzymes in section 5.3.1 in this study showed the presence of peroxidase activity in healthy oil palm roots. Plants are known to produce an array of peroxidase enzymes involved in diverse physiological processes and are abundant, especially in roots (Lee *et al*., 2007; Gill and Tuteja, 2010). Some defence-related genes are peroxidases (Govrin and Levine, 2000; Olson *et al.,* 2012) and might be expected to be induced following *Ganoderma* inoculation. This study was conducted to differentiate peroxidase enzymes produced by *G. boninense* and by palm roots by identifying isoenzymes.

Extracts of infected palm wood and roots from experiment in section 5.3.3.1 were prepared as described in section 5.2.6 and subjected for IEF electrophoresis as described in section 5.3.1.3. The gel was cut into several sections, which were developed under different conditions. For detection of manganese peroxidase (MnP) isoenzymes the developing solution included 4-chloro-1–naphthol as substrate, with hydrogen peroxide and Mn(II) were added simultaneously. While for detection of laccase, only 4-chloro-1–naphthol was used as the substrate.

Multiple strong bands were developed on the IEF gel (Fig 62 and 63), which correspond to peroxidase and laccase enzymes. Fig 62 shows potential isoenzymes of MnP separated into different pI values by IEF. Identical MnP isoenzymes patterns were observed in infected roots and palm wood blocks but not in the control root, which indicates some isoenzymes were produced by *G. boninense*. Three MnP isoenzymes were detected ranging from 4.2-4.5 pI values. In contrast, oil palm roots produce multiple peroxidase isoenzymes with pI values differing from the activities from *G. boninense*. Approximately five peroxidase isoenzymes were detected ranging from pI 3.5-6.6.

Detection of laccase isoenzymes showed the presence of three bands ranging from pI 3.45 to 3.65 both in infected roots and palm wood colonized by *G. boninense*. Overall it is apparent that *G. boninense* is deploying potential ligninases during its saprotrophic and parasitic phases.

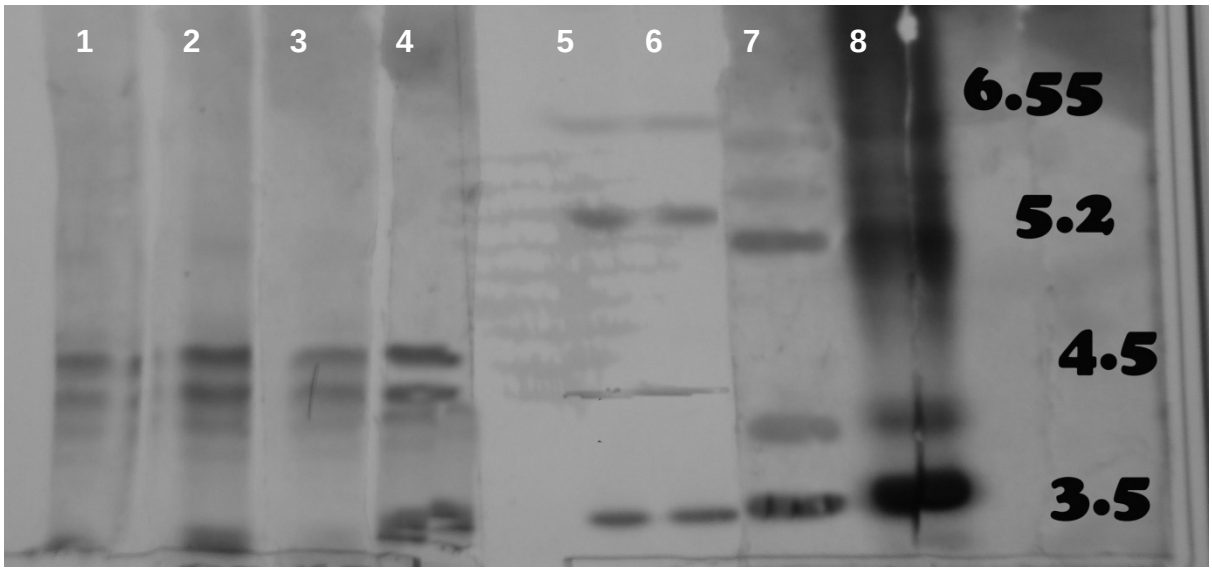

Figure 62. IEF of MnPs from *G. boninense*. Lanes 1-2: MnP isoenzymes produced in infected roots of oil palm seedlings; lanes 3-4: MnPs produced in infected wood blocks; lanes 5-6: IEF marker; the numbers on the right are the pI values. Lanes 7-8: peroxidases present in uninoculated oil palm roots (control)

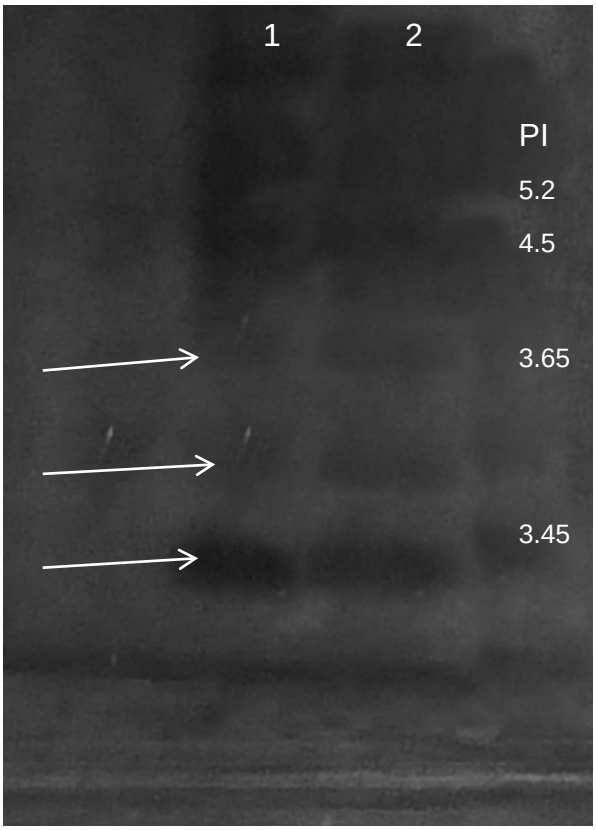

Figure 63.Laccase isoenzymes produced by *G. boninense* separated by IEF. Lane 1: Multiple isoenzymes produced in infected palm wood block; lane 2: Multiple isoenzymes produced in infected oil palm roots. The number on the right are the pI values

5.3.2 *Ganoderma* cell wall degrading enzymes (CWDE)

Lignin degrading enzymes are thought to generally occur at the onset of the secondary growth phase of the fungus, as early utilisable nutrients are depleted and primary fungal growth ceases (Tien and Kirk, 1984; Mikiashvilli *et al.,* 2004). Nitrogen (Keyser *et al.*, 1978), carbon or sulphur (Jeffries, 1991) depletion triggers the production of ligninolytic enzymes and lignin degradation for example in *P. chrysosporium* (Tien and Kirk, 1984; Kirk *et al.,* 1990) and *H. annosum* (Maijala *et al.,* 2003). While *in vitro*, production of polysaccharides enzymes such as pectolytic enzymes by non-pathogenic and pathogenic fungi e.g. *Colletotrichum* spp, *Verticillium albo-atrum* and *Fusarium oxysporum*, requires substrate and is repressed by preferred carbon sources such as glucose (Cooper, 1983). Catabolite repression has

been proposed to explain the diphasic growth pattern exhibited by some hemibiotrophs and necrotrophs and thereby the way in which these pathogens cause disease. However, there is no information available on the factors regulating production of these potential pathogenicity factors. Therefore various forms of culture were examined, along with influence on CWDE production on carbon sources and nitrogen de-repression and possible inducers.

5.3.2.1 Regulation of *G. boninense* CWDE production *in vitro*

Liquid cultures contained in Nunclon™ cell culture flask containing minimal medium with different nitrogen concentrations (low 4.0 mM and high 40 mM) and different carbon sources (glucose and oil palm wood) were established to try to stimulate production of cell wall degrading enzymes. This was in order to determine the array of enzymes produced and to quantify enzymatic activity. Individual enzymes were not isolated or characterised at this stage, as the aim was to determine the enzymic potential of *G. boninense*.

Obtaining enzyme activity from liquid cultures was more difficult than anticipated as the minimal medium did not support sufficient growth of the pathogen. Therefore, malt extract medium was used to obtain sufficient growth approximately 5 days and subsequently was replaced with minimal medium. Caution was taken to ensure no malt extract medium residue was left by washing the mycelium, still in the original vessels Nunclon™ culture flask multiple times with sterile distilled water. Furthermore, it was necessary to adapt the liquid culture by addition of perlite to create semi-solid conditions, shown to enhance growth and enzyme production by other basidiomycetes (D'souza *et al.,* 1999; Fernandes *et al.,* 2008; Rees, 2006).

5.3.2.2 Effect of different carbon sources and nitrogen concentrations on enzyme productions

Lignin-degrading enzymes were detected in most of the culture treatments tested as shown in Fig. 64. Carbon sources and nitrogen concentration influenced production of ligninases. Generally ligninase production was favoured by low nitrogen and by palm wood as a sole carbon source. Lignin peroxidase (LiP) (Azure B assay) activity was induced by low nitrogen and palm wood extract. The enzyme was not detected in other treatments (Fig. 64A).The enzyme was detected after 2 days, then activity peaked at day 4. Subsequently activity declined by 6 days and beyond and completely ceased by day 13.

Laccase production (ABTS assay) was stimulated by low nitrogen with palm wood or glucose as the sole carbon sources (Fig.64B). Levels on low N were approx. 5-fold greater than on high N. Palm wood with low N resulted in approx. 40% greater activity than on glucose. Laccase was detected after 1 day and increased to a maximum after 5 days before declining. Similarly MnP activity (phenol red oxidation assay) was detected in all liquid culture fluids (Fig. 64C). Low nitrogen stimulated the production of the enzyme with either palm wood or glucose (Fig. 64C), but levels were higher with palm wood. However both treatments showed a similar activity profile. Low N with palm wood increased production over high N by approx. 50%, yet activity was of similar order with low N and glucose. Activity was detected after 2 days and continued to increase throughout (Fig.64C).

It is clear that all ligninases are repressed by high N and that one (LiP) requires the presence of inducer(s) provided by palm wood extract; whereas laccase and MnP are influenced by both means of regulation.

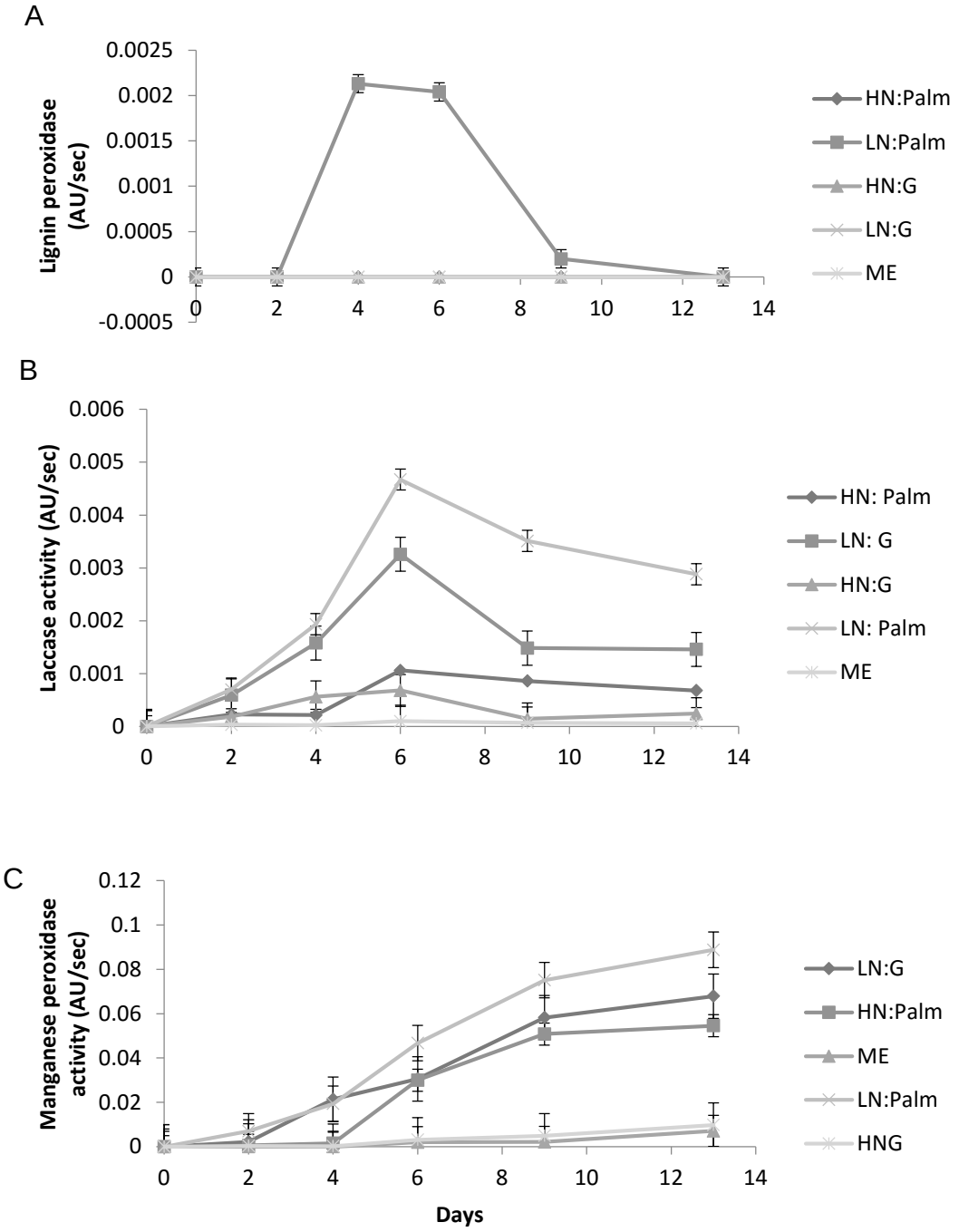

Figure 64. Effect of different carbon sources and nitrogen concentrations on lignin degrading enzymes production by *G. boninense*. Lignin peroxidase (LiP) (A). Laccase (B). Manganese peroxidase (MnP) (C). Low nitrogen with glucose carbon source (LN:G), low nitrogen with palm wood carbon source (LN:Palm), high nitrogen with glucose carbon source (HN:G), high nitrogen with palm wood carbon source (HN:Palm), malt extract medium (ME). Error bars indicate standard deviation of mean of three replicates per time point.

Polysaccharide-degrading, potential CWDEs were also detected in the liquid cultures. Cellulase, xylanase, pectinases (Endo-polygalacturonase (PG) and Polygalacturonide lyase (PL)), glucosidase and galactosidase activities were detected if cultures were supplemented with palm wood as a sole carbon source (Fig. 65). No enzyme activities were detected in any medium containing glucose. This indicates glucose represses production of these enzymes.

Polysaccharidases are typically carbon repressed but repression by nitrogen has generally not been reported (Cooper, 1983). Because N levels were already being studied for lignase regulation, it was extended for the production of polysaccharidases, and in a few cases appeared to influence production. It should be borne in mind that although an established mycelium is being used here, further growth might have been stimulated by high N, which could enhance levels.

Greatest enzyme activities were produced when *G. boninense* was grown on high N (40Mm) for cellulase, xylanase, glucosidase and galactosidase (Fig. 65). For example, cellulase activity was 2.11 to 9.11 nkats in high nitrogen while in low nitrogen it was 2.11 to 7.88 nkats during the time course. Similarly xylanase, glucosidase and galactosidase activities were also favoured by high N (Fig. 65). In contrast, pectinase activities were optimal in the presence of low N (Fig. 66).

From the palm wood liquid cultures cellulase activity (DNS assay, filter paper substrate) was first observed after 2 days and continued to increase throughout the time course (Fig. 65a). The assay quantifies the amount of reducing sugar produced from cellulose microfibres in filter paper and relates predominantly to the combined action of EG, CBH and β-glucosidase enzymes. Specific β-glucosidase activity, *p*-nitrophenyl glucoside substrate), showed higher activity than overall cellulase activity. This probably reflects

the solubility of the model substrate compared with relatively inaccessible crystalline cellulose.

Xylanase (RBB xylan assay) was also readily obtained from palm wood liquid cultures and activity showed a similar time course as the cellulase. As with cellulose degradation, hemicellulose is degraded by the combined action of several enzymes such as 1,4-β-D-xylanases, arabinosidases and acetyl xylan esterases. Therefore activity does not correspond to a particular enzyme but rather to the synergistic action of numerous xylan-degrading enzymes. Cellulase, xylanase, glucosidase and galactosidase had similar activity profiles (Fig. 65). with activities present after 2 days and continuing to increase throughout.

Production of pectin-degrading enzymes endo-polygalacturonase (PG; viscometric assay) and polygalacturonide lyase (pectin lyase, PL; 4,5-unsaturated galacturonides production assay) was both stimulated by 2-4 fold by low N. PL appeared sooner (2 days) than PG (4 days).

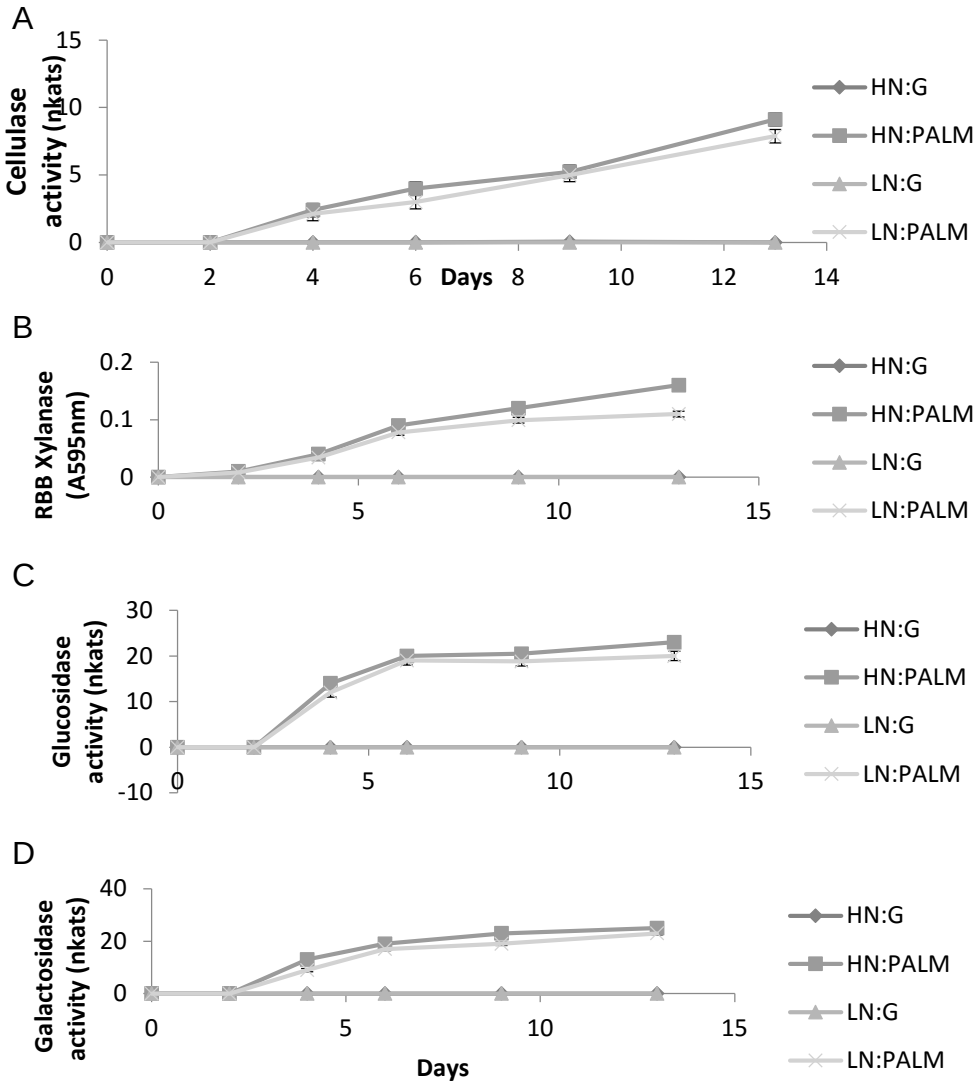

Figure 65. Effect of different carbon sources and nitrogen concentration on cellulose, xylan and hemicellulose -degrading enzymes production by *G. boninense* in liquid medium. Cellulase activity (A). Xylanase activity (B). Glucosidase activity (C). Galactosidase activity (D). Low nitrogen with glucose carbon source (LN:G), low nitrogen with palm wood carbon source (LN:Palm), high nitrogen with glucose carbon source (HN:G), high nitrogen with palm wood carbon source (HN:Palm). Error bars indicate standard deviation of mean of three replicates per time point.

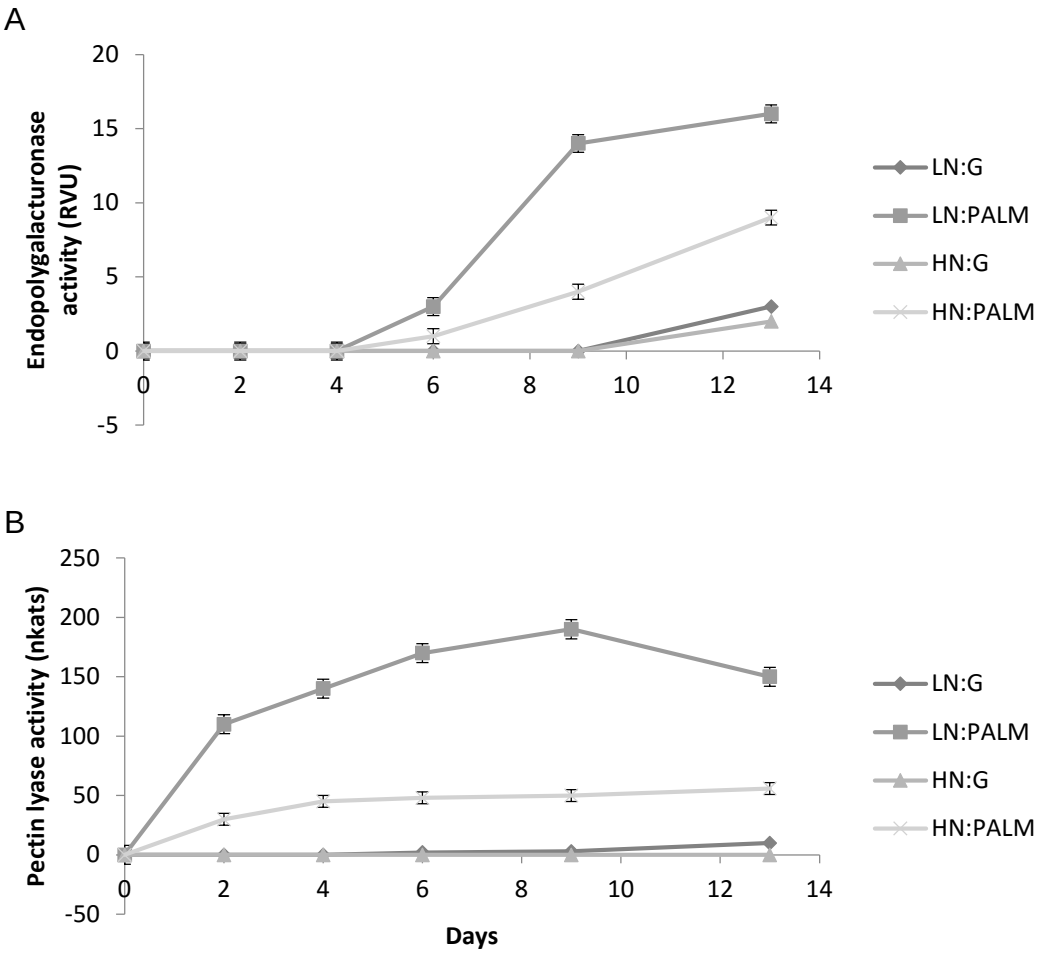

Figure 66. Effect of different carbon and nitrogen concentrations on pectic enzymes production by *G. boninense*. Endopolygalacturonase activity (A). Pectin lyase activity (B). Low nitrogen with glucose carbon source (LN:G), low nitrogen with palm wood carbon source (LN:Palm), high nitrogen with glucose carbon source (HN:G), high nitrogen with palm wood carbon source (HN:Palm). Error bars indicate standard deviation of mean of three replicates per time point.

5.3.2.3 Effect of aromatic compounds on enzyme production

Ligninases have been reported to be induced by the presence of aromatic compounds (Leisola *et al.*, 1985; Castillo *et al.,* 1997; Rees, 2006; Srivastava *et al.,* 2013). For example Palmieri *et al*. (2000) reported a fifty-fold increase in laccase activity in *Pleurotus ostreatus*, a white-rot basidiomycete after the addition of veratryl alcohol. Therefore the effect of several aromatic compounds on lignin-degrading enzymes production were tested. A medium with low nitrogen concentration and glucose as sole carbon source was chosen as it showed low production of two lignases in the previous experiment.

Figure 67 shows the mean activity of lignin-degrading enzymes in the presence of veratryl alcohol, tryptophan and syringic acid. Most of the aromatic compounds used had a stimulatory effect on laccase and manganese peroxidase (MnP) production. However no Lignin peroxidase activity was detected in this study. Previously it was shown that the presence of palm wood was required for its production.

Laccase activity was maximal after 6 days when it had increased 300-fold in the presence of veratryl alcohol, 120-fold with tryptophan and 130-fold with syringic acid. (Fig. 67A). While manganese peroxidase activity was strongly stimulated with tryptophan and syringic acid, no effect was observed with veratryl alcohol. MnP activity was stimulated 8-fold and 5-fold in the presence of tryptophan and syringic acid respectively (Fig. 67B).

Effect of tryptophan, veratryl alcohol and syringic acids was extended to production of other CWDEs. However production of cellulase, xylanase and galactosidase was not significantly affected (Appendix 8).

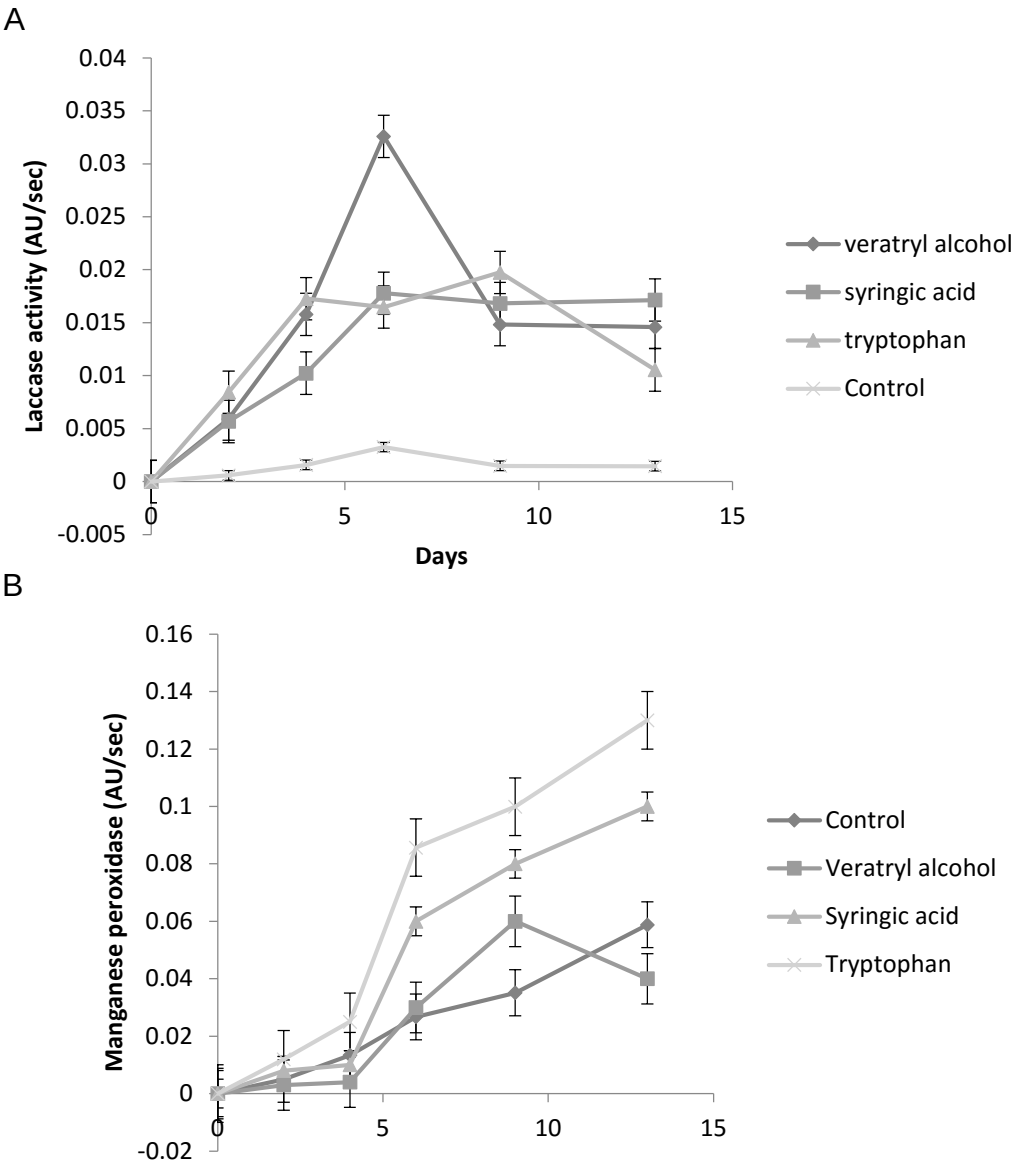

Figure 67. Effect of aromatic compounds on lignin-degrading enzymes production by *G. boninense* in liquid medium. A) Laccase activity is maximal after 6 days, increase 300-fold in the presence of veratryl alcohol, 120-fold with tryptophan and 130-fold with syringic acid. B) MnP activity increase 8-fold with tryptophan and 5-fold with syringic acid. Error bars indicate standard deviation of mean of three replicates per time point.

5.3.3 Reactive oxygen species (ROS) and associated enzymes formation by *G. boninense*

Many plant species produce ROS as part of microbial defence systems, in which oxidative burst allows the plants to use the ROS in cell lignification and in the regulation of resistance genes (Wojtaszek, 1997; Mucha *et al.,* 2015). The hypersensitive response also involves ROS as a component of rapid cell death (Wojtaszek, 1997; Cessna *et al.,* 2000; Zurbriggen *et al.,* 2010; Mucha *et al.,* 2015). To cope with this mechanism, microbes produce enzymes including peroxidase, catalase and superoxide dismutase to avoid or tolerate these responses (Karlsson *et al.,* 2005; Heller and Tudzynski, 2011), and in some cases necrotrophs such as *Botrytis cinerea* even produce their own ROS to target host tissues (Govrin and Levine, 2000). In order to identify whether any of these enzymatic mechanisms or ROS are produced by *G. boninense* a static liquid cultures containing minimal medium were used.

Two types of modified basal medium (MBM) were used, one acting as a nutrient-rich medium containing 1% glucose and the other acting as carbon-deficient, starvation medium containing 1.5% palm wood extract and 0.05% glucose. Palm wood was used potentially to stimulate the ROS or related enzymes and glucose was used as a negative control. All media were supplied with low N (4mM) such that both carbon and nitrogen repression are avoided. One millilitre *Ganoderma boninense* homogenized mycelium with Ultraturrax homogenizer was used to create a uniform and more rapid growth than using agar plugs as inoculum for the static liquid cultures in Nunclon cell culture flask. Sufficient growth of *G. boninense* was first obtained on malt extract medium for 7 days and then changed into minimal medium when sufficient growth was obtained. Crude culture fluids from 2 to 6 days old of *G. boninense* growth were used for enzyme assays.

Figures 68-70 show the production of peroxidase and superoxide dismutase by *G. boninense* in static liquid cultures. No catalase activity was detected in any of the cultures. Accumulation of hydrogen peroxide (H_20_2) was also observed during the sampling time from 2-6 days. Peroxidase (POD) activity (assayed vs guiacol) was detected in both culture containing glucose and palm wood as major carbon sources, but activity was 27-fold higher in palm wood culture (Fig. 68). Activity was detected after day 1 and rapidly increased to reach a maximum at day 4 then declined by day 6.

When assessing peroxidase activity, it was observed that the palm wood culture fluids themselves produced a reaction without addition of H_2O_2 to the assay, indicating presence of hydrogen peroxide (H_2O_2) in the culture fluids. Using culture fluids obtained from both types of MBM, the method of Velikova *et al*. (2000) was followed to detect peroxide, using an assay including potassium iodide (Fig. 69). *G. boninense* palm wood cultures contained higher H_2O_2 content than in glucose cultures. H_2O_2 content increased rapidly to day 2 reaching 98µM then remained constant from 2 to 4 days before declining to 75µM by 6 days. In glucose culture H_2O_2 content was detected at 3-4 days at a concentrations of 10-15 µM.

SOD (photoreduction of NBT assay) was produced from 1-6 days growth in palm wood culture fluids (Fig. 70). SOD activity peaked at 4 days with of 7-fold activity increasing from day 2 from 3.4 -23.4 U/mg protein. (Fig. 70). Activities were 40-fold greater than in glucose grown cultures. Clearly this shows *G. boninense* has the capacity to both produce and to detoxify ROS.

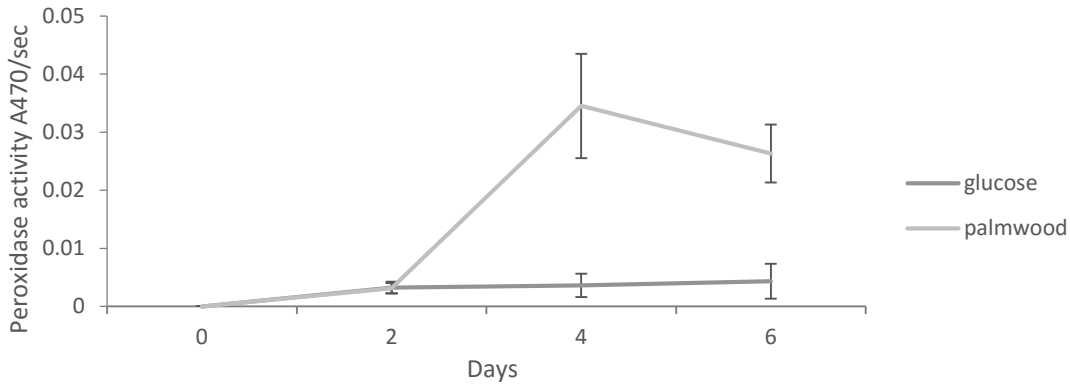

Figure 68. Peroxidase activity by *G. boninense* in minimal medium with different carbon sources, palm wood or glucose. Error bars indicate mean activities of three replicates samples per time point.

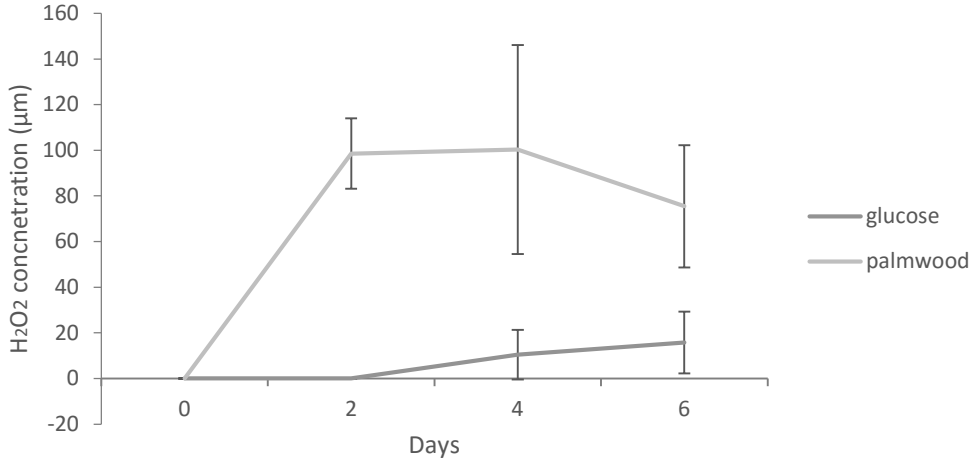

Figure 69. Hydrogen peroxide concentrations in minimal medium with carbon sources palm wood or glucose. Error bars indicate mean concentrations of three replicates samples per time point.

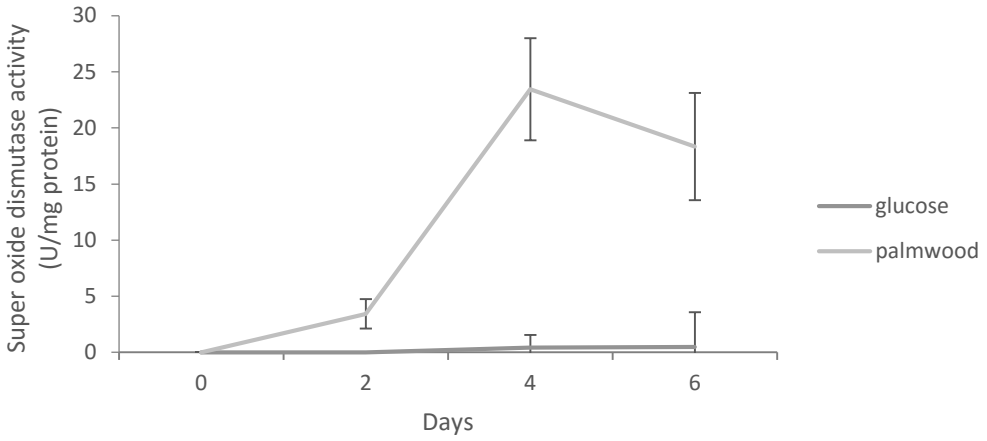

Figure 70. Superoxide dismutase activity by *G. boninense* in minimal medium with carbon sources palm wood or glucose. Error bars indicate mean activities of three replicates samples per time point.

5.3.4 Bioassay of potential toxin production by *G. boninense in vitro* infiltration of palm leaves

To determine *G. boninense* potential extracellular toxins production in the palmwood culture fluids, a crude preliminary bioassay was performed on oil palm leaves with palmwood culture fluids in section (5.2.11.1). Leaves were inoculated from their underside in order to infiltrate the stomata. Controls of sterile distilled water (C1) and the palm wood medium (C2) were used for the assessment of the method. Fig. 71 shows varied results in the position of the leaf and sample day. Small chlorotic lesions resulted in some treatments. However, symptoms were induced in some water controls and from the lack of consistency between treatments and position, it was concluded that the method itself was causing damage to the leaves and that marks related to the liquid cultures could not be defined as due to toxicity.

Figure 71. Infiltration of Oil Palm leaves using 1mL of sample to assess potential toxicity of *Ganoderma boninense* MBM palmwood culture fluids. C1: Sterile distilled water, C2: MBM palmwood Medium, 1: Day 2 Replicate 1, 2: Day 2 Replicate2, 3: Day 2 Replicate 3, 4: Day 4 Replicate 1, 5: Day 4 Replicate 2, 6: Day 4 Replicate 3, 7: Day 6 Replicate 1, 8: Day 6 Replicate 2, 9: Day 6 Replicate 3.

To further assess toxicity of the culture fluids, cell viability bioassays were performed by using Neutral Red assay to estimate cell death of potato disks. Tissue disks (8mm diam x 0.4 mm thick) were treated with the palmwood (day 4 and 6) and control (glucose) culture fluid. Triplicate tissue disks were removed at intervals after the addition of culture fluids and placed in 1.0 ml of a buffered neutral dye solution. After 10, 20, 30 and 40 mins, disks were rinsed in buffer solution without the neutral dye as described in section (MM). Cell viability was estimated visually with a stereomicroscope, based on loss of ability of cells to accumulate neutral red dye (Fig. 73). Fig. 72 shows that Day 4 inoculated medium and Day 6 inoculated medium resulted in a cell viability decrease of 20% and 44%, respectively, over the 40-minute incubation period. The day 6 result in particular had a rapid drop in viability of 30%, between 20 and 40 minutes indicating that Day 6 could be peak of toxicity production by *G. boninense* in the palmwood culture fluids. There was no continual reduction in mean cell viability over time in the medium controls, indicating that the medium was not toxic to the potato discs.

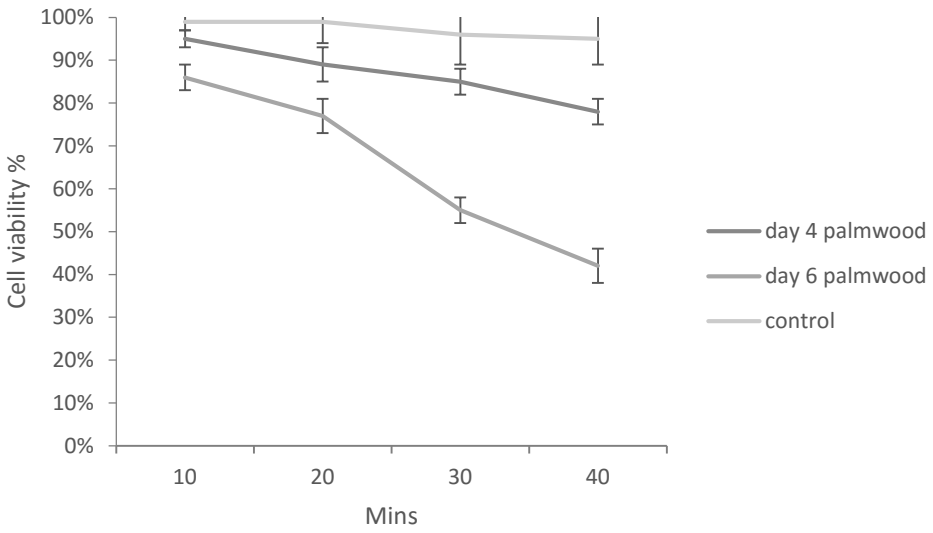

Figure 72. Mean cell viability (±SE) of potato discs following immersion in differing reaction mixtures. Results are shown as mean percentage viability across three replicate discs. Day 4 and day 6 indicate Day 4 and 6 sampling times from palmwood medium and control indicates glucose medium. Both media were inoculated with *G. boninense*.

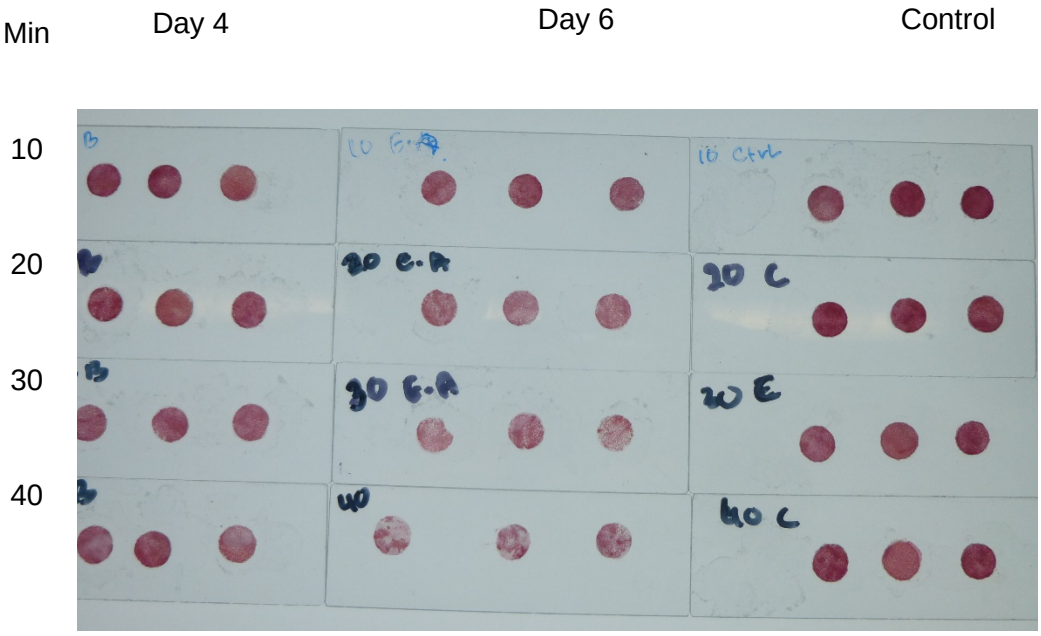

Figure 73. Representative of cell viability of potato discs after treatments with palmwood and control culture fluids. Day 4 indicates potato discs treated with palmwood culture from 4 days, with the mean viability of 95% (10 minutes), 89% (20 minutes), 85% (30 minutes) and 78% (40 minutes) b) Day 6 palmwood culture fluid with the mean viability of 86% (10 minute), 77% (20 minutes), 55% (30 minutes) and 42% (40 minutes).

5.3.5 Development of methods for *Agrobacterium tumefaciens*-mediated transformation of *Ganoderma boninense*

Agrobacterium tumefaciens-mediated transformation (ATMT) has been reported in various filamentous fungal species (Chen *et al.*, 2000; Mikosch *et al.*, 2001; Mullins *et al.*, 2001; Hanif *et al.*, 2002; Pardo *et al.*, 2002; Combier *et al.*, 2003; Rolland *et al.*, 2003; Burns *et al.*, 2005). This technique can be used to transform protoplasts as well as hyphae, spores and even blocks of mycelial tissue from fruiting bodies (de Groot *et al.*, 1998; Mullins *et al.*, 2001).

Despite the significant economic impact caused by *G. boninense*, the mode of infection and the molecular interactions with its hosts are still poorly understood (Cooper *et al.,* 2011; Rees *et al.,* 2012). Therefore this study was conducted to develop a transformation system for *G. boninense* to express *GFP* as a tool to facilitate interaction studies on *G. boninense* infection on oil palm roots by observing point of penetration. Two *A. tumefaciens* strains LBA 1126 and AGL-1 with different vectors were used. Strain LBA 1126 containing vector pGR4-GFP or pGR4-4iGM3′eGFP were obtained from Burns *et al.* (2006) and AGL-1 contain vector pCAMBgfp (Eckert *et al.,* 2005) obtained from Rothamsted Research center, UK.

Selection systems are used widely in fungal transformations to enable the selection of transformed cells. Hence, several selectable markers which are suitable in fungi were tested such as antibiotics (hygromycin B, kanamycin, rifampicin, ampicillin and gentamycin). Among these antibiotics tested, hygromycin B at 150 µg ml-1 showed complete inhibition of *G. boninense* wild type, whereas it gave restricted growth at 125 µg ml-1 (Fig. 74) whereas kanamycin, rifampicin, ampicillin, gentamycin, did not show any significant growth inhibition even at 125 µg ml-1 (data not shown). The hygromycin B is represented by *hph* (hygromycin phosphotransferase) gene, which will be introduced into *G. boninense* all allow for selection of transformed cells.

Hygromycin-resistant putative transformant colonies appeared after 4-7 days. Three rounds of selection were made by transferring margins of the colonies to fresh selection plates, resulting in mycelia, with 12—16 days needed for full growth on the plates of the selection medium. This is in contrast to wild type which grew to the same diameter within 7 days on MEA without resistance pressure.

To verify the expression of the introduced *gfp* reporter gene, mycelium of separate transformants grown either with or without antibiotic were inspected by fluorescence microscopy. However fig.76 revealed expression of GFP in the individual transformants was not stable whereas most of the transformed mycelium was unable to express the *gfp* gene. The transformation was attempted five times and showed similar results.

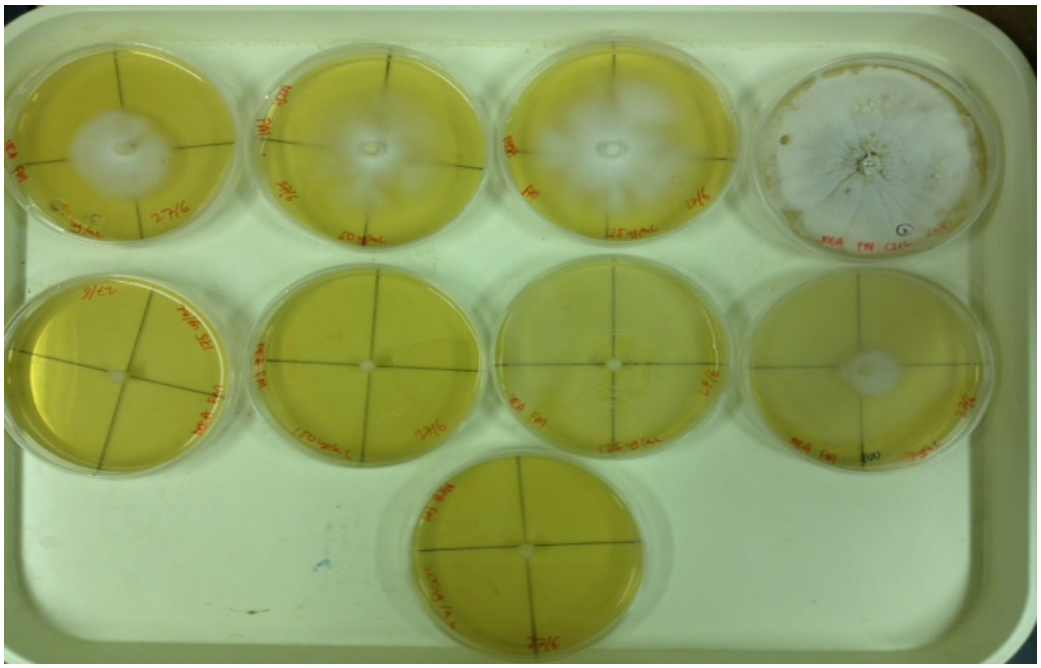

Figure 74. Complete inhibition of *G. boninense* growth at 125 µg/ml by Hygromycin in PDA for the selection of transformant. Hygromycin B concentration ranging from 25-200 µg/ml.

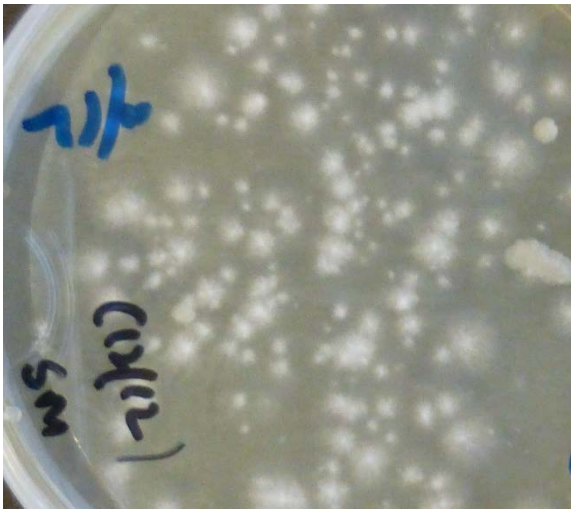

Figure 75. *G. boninense* transformant colonies on selective medium.

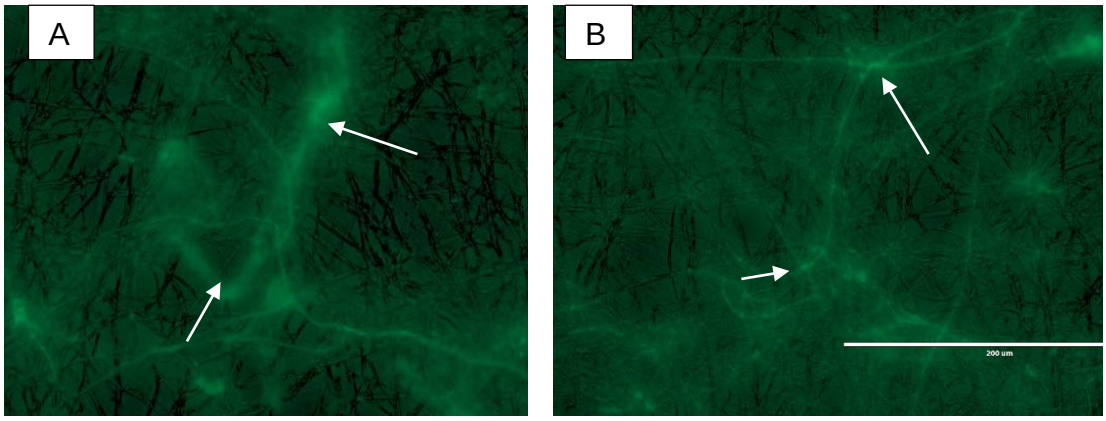

Figure 76. Expression of GFP in separate *G. boninense* transformants without (A) or with (B) hygromycin. Detection of gfp transformants were observed with digital inverted fluorescence microscope (Evos fl). Most of the mycelium was unable to express *gfp* gene (arrows). Scale bar= 200 µM.

5.3.6 Transcriptomic analysis of *G. boninense* pathogenicity

In spite of decades of research, very little is understood about the pathogenicity of *Ganoderma boninense*, causal agent of basal stem rot of oil palm. This deficiency is in great contrast to advanced knowledge of a wide range of biotrophic and necrotrophic, fungal, and plant pathogens, such as *Heterobasidion annosum* (Olson *et al.,* 2012), *Rhizoctonia solani* (Gkamiri *et al.,* 2015), *Moniliophthora roreri* (Meinhardt *et al.,* 2014), *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Heard *et al.,* 2015).

The analysis of the transcriptome using next generation sequencing offers a holistic approach to study *G. boninense* pathogenicity by looking at patterns of gene expression at different stages of the life/infection cycle. This approach is similar to the one for *Heterobasidion annosum* and pine by Olson *et al.* (2012) which followed the EST analysis by Karlson *et al.* (2003) and resembles that of Hori *et al.* (2014) on *Cerioporiopsis subvermispora*.

Therefore in this study to regulate differential gene expression of *G. boninense*, the pathogen was growth in different representative phases of saprotrophic growth on palm wood block (refer section 5.3.1.1), initial host contact and surface colonisation on cut oil palm roots with wheat grain inoculum (refer section 5.3.1.1), then colonization of oil palm seedling roots by rubber wood block inoculum (refer section 3.3.1). Controls comprised mycelium grown on minimal medium with low nitrogen and low carbon to provide conditions without metabolite repression (refer section 5.3.2.2) and in malt extract medium as a rich medium control (refer section 5.3.2.2). These stages were designed to provide sufficient amounts of RNA with high purity for subsequent RNA-seq analysis.

5.3.6.1 CTAB RNA extraction and RNA quality assessment

RNA quality and integrity are critical for many studies in molecular biology, such as northern blotting, microarray hybridisation, RT-PCR analysis and cDNA library construction (Gambino *et al.,* 2008). The rapid-CTAB RNA extraction developed by Gambino *et al* (2008), in combination with cell lysis by bead beating with ceramic beads, was used to extract total RNA from samples above for subsequent RNA-seq analysis.

Table 17 shows RNA concentration and qualities from *G. boninense* mycelium of various samples. The standard CTAB method was efficient for *G. boninense* mycelium RNA extraction as indicated by the A_{260}:A_{280} and A_{260}:A_{230} ratios (Table 16). The RNA was high purity and relatively free of protein, polysaccharide and phenolic compounds as indicated by these ratios. Also the intact 28S and 18S rRNA bands on formaldehyde agarose gel, with little smearing (Fig. 77) indicated that little or no RNA degradation occurred during extraction.

Table 17. Purity and yield (mean ±SD) of total RNA extracted from *G. boninense* mycelium samples on various substrates. For each tissue 3 samples extracted during three independent experiments were measured.

Mycelium samples	A260:A280	A260:A230	RNA yield (µg/µl)
Palm wood blocks	1.95 ± 0.02	2.32 ± 0.15	10.05 ± 3.17
Cut oil palm roots	1.89 ± 0.03	2.29 ± 0.12	9.98 ± 2.15
Malt extract medium	1.93 ± 0.04	2.42 ± 0.12	11.75 ± 1.13
Minimal medium Low nitrogen	1.92 ± 0.09	2.22 ± 0.11	15.86 ± 1.45

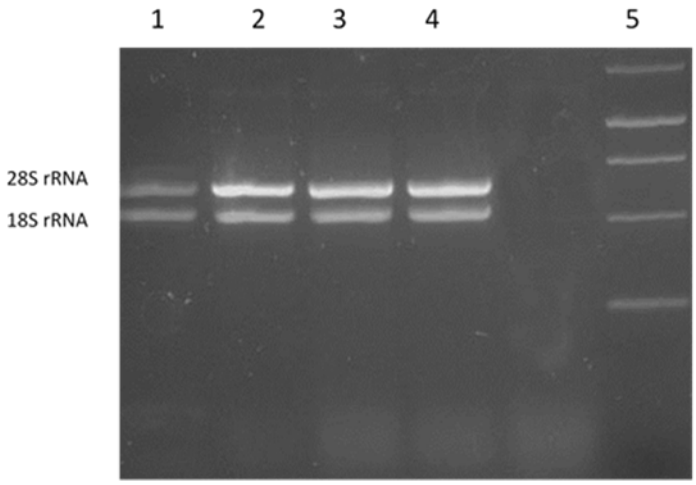

Figure 77. Agarose gel electrophoresis of total RNA extracted by rapid-CTAB protocol from *G. boninense* mycelium on: Oil palm roots (1), Palm wood blocks (2), Malt extract medium (3), Minimal medium (4).

It is proposed that mRNA from these samples will reflect the key stages of *G. boninense* survival and infection. Gene expression should provide much needed novel information about the *modus operandi* of this pathogen. This work is still ongoing and the next stages will involve delivery of mRNAs Rene Smulders, Wageningen UR Plant Breeding for RNA seq analysis and subsequent bioinformatics. The latter will also involve personnel from FELDA (FELDA plantation Oil palm Research center, Malaysia) and AAR (Applied Agriculture Services, Malaysia) and University of Bath.

5.4 Discussion

5.4.1 Cell wall degrading enzymes (CWDE)

The contribution that CWDE make to the pathogenic nature of many fungi has been demonstrated in numerous studies. As white rot fungi *Ganoderma* spp. are known to produce wide array of CWDE in order to facilitate wood degradation. Most of them are saprotrophs, however some have evolved to invade and kill living plants. *G. boninense* is a necrotrophic parasite capable of infecting and degrading palm stems (Rees *et al.,* 2009) and may subsequently survive as a saprotroph in the dead palm (Cooper *et al.,* 2011). This present study, shows *G. boninense* produces a wide array of CWDE including lignin- and polysaccharide- degrading enzymes during both saprotrophic and parasitic phases. These CWDE appear to be produced simultaneously in both phases by *G. boninense*. This concurs with TEM analysis by Rees *et al.* (2009) on oil palm infection by *G. boninense*. They observed that cell wall degradation involved simultaneous attack of all cell wall layers, including middle lamella regions high in lignin content, resulting in development of holes through all wall layers. The areas of cell wall attack were sometimes not directly adjacent to fungal hyphae; Degradation of lignin at distance in secondary walls of mature tissue indicates the necessarily oxidative nature of lignin breakdown, which produces phenoxy radicals and reactive oxygen species that may also be damaging to the fungal cell wall (Kirk, 1987a). Moreover, biodegradation of lignin is a co-metabolic process that requires access to additional carbon sources to provide the needed energy. Thus the white-rot fungal conversion of lignin in wood coincides with hydrolysis of the storage and structural cell wall polysaccharides such as by cellulases and hemicellulases (Hatakka and Hammel, 2010; Lundell *et al.,* 2010).

Lignin degradation occurs via a synergistic action of oxido-reductive metalloenzymes encoded by many fungal gene families (Paterson, 2007; Lundell *et al.,* 2010). In white-rots, the generation of organic radicals and oxidation of lignin are catalyzed by extracellular lignin-modifying enzymes (LMEs) including high redox-potential class II heme peroxidases (Hofrichter *et al.,* 2010), peroxide-generating oxidases such as glyoxal oxidases and aryl-alcohol oxidases (Kersten and Cullen, 2007; Ferreira *et al.,* 2009), and multi-copper oxidases (MCOs) such as laccases. This study confirms that *G. boninense* produces a battery of lignin-degrading enzymes of all the three major families of oxidative lignin-degrading enzymes, including laccases, manganese-dependent peroxidases (MnPs), and lignin peroxidases (LiPs). These activities are present in both saprotrophic and parasitic phases.

Activities of LiP, MnP and laccases are very similar; both catalyse one electron oxidation, creating radicals. The main difference between laccases and peroxidases are that they utilise different prosthetic groups (LiPs contain heme whilst laccases contain copper as part of their active site) and that laccase generally has a lower oxidation potential than peroxidases (Jong *et al.,* 1994). However LiP and MnP have been described as the true ligninases because of the high redox potential. LiP degrades non-phenolic lignin units (up to 90% of the polymer), whereas MnP generates Mn3+, which acts as a diffusible oxidizer on phenolic or non-phenolic lignin units *via* lipid peroxidation reactions (Paterson *et al.,* 2008).

Available genome information of white rot fungi such as *H. irregulare* (pathogenic) (Yakovlev *et al*., 2013) and *G. lucidum* (saprotrophic) (Chen *et al.,* 2012) show a wide range of ligninases and associated enzymes or co factors. *G. lucidum* genome sequences have revealed the fungus possesses a large and complete set of ligninolytic peroxidases along with laccases and a cellobiose dehydrogenase. Annotation of the candidate ligninolytic enzymes encoded in the *G. lucidum* genome revealed a set of 36 ligninolytic oxidoreductases. In addition, several peroxide generating oxidases were also

identified in the *G. lucidum* genome, particularly in the copper-radical oxidase family. (Chen *et al.,* 2012).

The genome sequences of pathogenic *H. irregulare* showed a lack of lignin peroxidase gene and eight manganese peroxidase but generated a higher phenol oxidases and laccases genes 18 and 5 respectively. *H. irregulare* also showed capable to generate H_20_2 with sequences encoding 17 quinone oxidoreductases, four glyoxal oxidases, 34 glucose-methanol-choline oxidoreductases and four manganese superoxide dismutases genes. These show the ligninases system of white rot fungi which is important for delignification process (Yakovlev *et al*., 2013).

Mayer (2002) has suggested a role for laccases in pathogenicity and reported that laccases may be involved in detoxification of phenolic phytoanticipins and phytoalexins. Cucumber fruits, *Cucumis prophetarium,* contain a family of cyclic triterpenoids, cucurbitacins, and resistance to *B. cinerea* is correlated with the ability of extracts from the fruit to repress laccase secretion (Viterbo *et al.,* 1994). Also the phytoalexin, resveratrol, is a phenolic that arises in grapevine in response to attack by *B. cinerea* and a fungal laccase has been shown to detoxify these (Adrian *et al.,* 1998). Additionally, Johansson *et al.,* (1999) determined that aggressiveness of the pine rot pathogen, *H. annosum,* was related to the presence of laccases Furthermore Bermek *et al.* (1998), reported that a laccase-deficient mutant of the white-rot fungus *P. cinnabarinus* lost its ability to degrade lignin.

Laccases also contribute to pigmentation and production of melanin during infection helping to stabilise cell walls and constrain cell turgor pressure and also mediates against adverse environmental conditions (Hoegger *et al.,* 2004). Formation of melanised, thick cell walled hyphae has been shown to be associated with colonised oil palm tissues in this study. Solid media containing tannic acid have been used to screen for laccase production by microbes (Kiiskinen *et al.,* 2004) and under stress conditions on GSM, laccase secretion is evident by oxidation of tannic acid, resulting in brown

discoloration of the agar. Although production of laccase is characteristic of pathogenic *Ganoderma* spp., so far production of laccase has not been shown to be required for infection of oil palm. A screen to detect isolates deficient in laccase secretion and subsequent infection trials could help elucidate this. More specifically, targeted gene disruption of laccase in aggressive *G. boninense* isolates could prove a useful tool for determining its role in pathogenicity.

Phytopathogenic fungi produce arrays of extracellular cell wall degrading enzymes capable of degrading the complex polysaccharides of the cell wall. The three main classes of CWDE were shown to be produced by *G. boninense* in parasitic and saprotrophic phases. A vast array of CWDE/polysaccharides is evident from genome information of white rot fungi *H. irregulare* and *G. lucidum* (Olson *et al.,* 2012; Chen *et al.,* 2012). Genome sequence of *G. lucidum* showed a total of 417 genes found related to carbohydrate-active enzyme (CAZymes) families as defined in the CAZy database (Canteral *et al.,* 2009) making this fungus one of the richest basidiomycetes examined so far in terms of the number of CAZymes. These sequences in particular, encode the candidate enzymes for the digestion of the three major classes of plant cell wall polysaccharides: cellulose, hemicelluloses and pectin. Genome sequence of *H. irregulare* also showed a broad spectrum of carbohydrate- active enzymes with a total of 282 carbohydrate-active enzyme genes. *H. irregulare* is well equipped with regard to the gene families involved specifically in plant cell wall degradation, encoding the enzymes required to digest cellulose, hemicellulose (xyloglucan and its side chains) and pectin and its side chains. In addition, *H. irregulare* has a limited potential for the degradation of a second hemicellulose structure, xylan, with only two xylanases from family GH10 and none from family GH11. Other necrotrophic fungi including *Botrytis cinerea* and *S. sclerotorium* also showed arsenal of genes associated in plant cell wall degradation respectively 346 and 367 genes encoding putative CAZymes, with GH= (Amselem *et al.,* 2011). However, the number of cellulose and

hemicellulose degrading enzymes encoded in *S. sclerotiorum* (220 putative genes) and *B. cinerea* (229 putative genes) genomes is smaller than in all the other PCW-degrading pathogens. These indicate white rot fungi and other necrotrophic fungi likely to use different approaches to completely decompose plant tissues and highlight the strong capability of white rot fungi to degrade the plant cell wall.

The most studied of these enzymes in relation to pathogenicity are the pectinases. There is extensive circumstantial evidence linking them to invasion, but studies have been hampered by functional redundancy, whereby null mutants have an unchanged phenotype. However, loss of function in some cases has resulted in attenuation of virulence. For example, in *Claviceps purpurea*, a biotrophic organ-specific pathogen of grasses and cereals, disruption of two polygalacturonase genes (*cppg1* and *cppg2*) resulted in almost total loss of pathogenicity (Oeser *et al.,* 2002). Similarly, disruption of a PG in aggressive isolates of *Aspergillus flavus* caused a significant drop in the ability of the fungus to damage and spread within cotton bolls. When this gene was introduced into isolates lacking the gene, the transformants became more aggressive (Shieh *et al.,* 1997). However, the role of pectinases in pathogenicity of woody plants is less well established and virulence of the chestnut blight pathogen, *Cryphonectria parasitica,* did not change with disruption of the enpg-1 gene, which was the major form of PG produced *in vitro* (Gao *et al.,* 1996).

Production of polysaccharide-degrading enzymes appears to be dominant during the parasitic phase than lignin enzymes (palm wood colonization) while production of lignin-degrading enzymes are more evident in the saprotrophic phase (infection of roots). These may reflect differences in the composition of polymers of palm roots and wood. Polymer analysis of oil palm stems from established but not mature palms by Rees *et al.* (2009) revealed that the major components of the stem tissue comprised cellulose (56%), lignin (18%) and hemicellulose (11%), with pectin (4%) and starch

(2%) as minor components. Furthermore, Rees *et al.* (2009) showed that there was substantial loss of lignin from oil palm wood blocks when inoculated with *G. boninense*, which was approximately proportionate to overall dry weight loss. Olson *et al.* (2012) reported differential regulation of CWDE genes between saprotropic and parasitic phases of *H. irregulare*. Generally, genes involved in oxidative lignocellulose degradation showed a lower expression level than carbohydrate hydrolysing enzymes during wood degradation.

5.4.2 Enzyme regulations

Regulation of production of polysaccharides-degrading enzymes by *G. boninense* in culture is similar to that observed with other fungi (Cooper and Wood, 1973; Giltrap and Lewis, 1982; Dean and Timberlake, 1989). Polysaccharidase induction specifically requires the substrate and is subject to catabolite repression. e.g. xylan will induce xylanases, cellulose induce cellulases and so on. The actual inducers are mono- or di-saccharides, but only under carbon-limited conditions (Cooper and Wood, 1983). The induction and repression of the hydrolytic enzymes were studied by growing *G. boninense* on palm wood or glucose as carbon sources. All enzyme activities were low or absent on glucose whereas all activities increased with palm wood, but high glucose levels repressed this effect. Earlier studies with various pathogenic fungi such as *Verticillium, Fusarium* and *Colletotrichum* showed the same pattern but extracted host cell walls were used as sources of carbon and inducers (Cooper, 1984).

Regulation of extracellular lignin degrading enzymes activities (Fig.64) was most effective in culture with low nitrogen and palm wood as the carbon source. This concurs with the findings with other wood-rotting basidiomycete including *P. chrysosporium* (Kirk *et al.,* 1990), *G. lucidum* (D'Souza *et al.,* 1999) and *Heterobasidion annosum* (Maijala *et al.,* 1991). Metabolite repression by nitrogen appears to be the critical factor for ligninases

production (Mikhaisvilli *et al.,* 2006). Coincidentally most woody plants typically have a very low content of nitrogen (usually less than 0.1% by weight) (Deacon, 2005a), which potentially will trigger the production of lignin degrading enzymes. Metabolite repression by nitrogen has been especially well described for fungal proteases (Screen *et al.,* 1998).

Lignin degrading enzymes have been considered to be inducible in the presence of lignin (Faison and Kirk, 1985). Activities of LiP, laccase and MnP all markedly increased in the presence of palm wood sawdust (Fig.64). This result is consistent with earlier studies which showed that several white rot fungi only produce all lignin degrading enzymes in the presence of wood (Horvath *et al.,* 1993; Lobos *et al.,* 1994; Schlosser *et al.,* 1997; D'Souza *et al.,* 1999). D'Souza *et al.* (1999) showed that *G. lucidum* produces both laccase and MnP activities only in wood-containing media whereas only laccase was produced in a defined medium. Similarly, Faison and Kirk (1985) observed the induction of lignin degrading enzymes with birch wood lignin in the culture medium of *P. chrysosporium*.

Various low-molecular-weight aromatic compounds have been reported to enhance lignin degrading enzyme production (Boomithan and Reddy, 1992; Kirk and Farrell, 1987; Schlosser *et al.,* 1997; D'Souza *et al.,* 1999). This study showed that in a low N medium all aromatic compounds (veratryl alcohol, syringic acid and tryptophan), enhanced the production of laccase and MnP. Veratryl alcohol (VA) has been shown to enhance laccase production in several white rot fungi (Eggert *et al.,* 1996; Schlosser *et al.,* 1997) and with *G. lucidum* (D'Souza *et al.,* 1999). Furthermore, veratryl alcohol has been ascribed to play a specific role as a cofactor of LiP (Mester *et al.,* 1995), however production of LiP was not detected in this study. Veratryl alcohol is a secondary metabolite that is synthesed by several white rot fungi (De Jong *et al.,* 1994; Hammel, 1997). Comprehensive studies in *P. chrysosporium* showed that both veratryl alcohol synthesis and lignin degradation are secondary metabolic events and that the onset secondary

metabolism is triggered by nitrogen starvation (Shimada *et al.,* 1991; Kirk, 1981; Keyser *et al.,* 1978).

Low-molecular-weight aromatic acids, such as syringic acid that are structurally related to individual phenolic moieties in lignin serve as good inducers of lignases (Yaver *et al.,* 1996). Although the structure of syringic acid is different from that of syringyl moieties of lignin, the ring structures of both are identical. D'Souza *et al.* (1999) reported that addition of syringic acid to defined medium of *G. lucidum* resulted in increased laccase. Similarly in this study addition of syringic acid into glucose medium resulted in higher laccase activity compared to VA and tryptophan inducers. Presumably palm wood, which stimulated ligninases, contains aromatic inducers but their identity remains unknown.Tryptophan is an aromatic amino acid, produced *de novo* by basidiomycetes and it functions as a precursor in the synthesis of N-substituted aromatic secondary metabolites of fungi (Turner & Aldridge, 1983), many of which act as substrates of manganese peroxidase and laccase (Bollag and Leonowicz, 1984; Arora and Gill, 2001). Eggert *et al.* (1996) have recently identified 3-hydroxy-2-aminobenzoate, a tryptophan-derived metabolite, as a mediator in laccase-catalyzed oxidation reactions in the white rot fungus *Pycnoporus cinnabarinus* (Collins *et al.,* 1996). Laccases have also been found to react with the tryptophan derivative 4-hydroxyindole (Collins *et al.,* 1997; Fonseca *et al.,* 2013). Furthermore, direct oxidation of N-substituted aromatic compounds by the white rot fungal enzyme manganese dependent peroxidase (Urzua *et al.,* 1995) and by horseradish peroxidase (Candelas *et al.,* 1996) have been demonstrated.

The addition of tryptophan also enhanced the production of MnP and laccase activity by *G. boninense.* Thus, like VA and syringic acid, tryptophan acts across a range of white rot fungi as a stimulant of ligninase production. Moreover Collins *et al.* (1997) demonstrated that tryptophan is involved in the process of enzyme recycling by protecting the enzyme from inactivation by excess H_2O_2, thus providing a possible explanation for the higher MnP and

laccase activity levels observed in its presence. Production of lignin- and polysaccharide- degrading enzymes seems to be linked as they are produced relatively concurrently. The *in vitro*, semi-solid culture system developed in the present study proved to be very useful as a model system for studying for the range and regulation of CWDE *G. boninense* and could be used to study characterisation of the enzymes in the future.

5.4.3 Reactive oxygen species (ROS) and detoxification

In many plant–pathogen interactions, host defence responses include the hypersensitive response (HR), whereby an oxidative burst by the plant generates reactive oxygen species including superoxide (O_2^-), H_2O_2 and hydroxyl radicals (OH-) that have been associated with the programmed cell death (PCD) of host cells. (Heller and Tudzynski, 2011; Mucha *et al.,* 2015). These responses arrest pathogen growth, particularly of that of biotrophic fungi. It is becoming apparent that some necrotrophic fungi can take advantage of this plant defence process to derive nutrition from the dead host cells facilitating colonization (Hammel *et al.,* 2002; Karlsson *et al*., 2005; Heller and Tudzynski, 2011; Mucha *et al*., 2015). *Botrytis cinerea* and *Sclerotinia sclerotiorum* pathogenicity have been reported to require PCD for successful penetration and invasion. Govrin and Levine (2000) showed that both fungi utilize HR for rapid host colonization. The degree of infection of those pathogens was directly related to the amount of superoxide or hydrogen peroxide generated during the HR. Govrin and Levine *(*2000) also showed that an *A. thaliana* mutant (*dnd1*) that is defective in hypersensitive cell death was resistant to both fungi.

In this study *G. boninense* was shown to produce hydrogen peroxide (H_2O_2) in liquid culture especially in palm wood culture fluids. Production of extracellular H_2O_2 by white rot fungi has been associated with peroxidase production as the co-substrate in lignin degradation (Eichlerova *et al.,* 2006). H_2O_2 presence in cultures was suspected because peroxidase activity was evident without any added peroxide. H_2O_2 production occurred throughout

growth in culture and could aid in the colonization of oil palm involving ligninases. Lignin depolymerization is achieved primarily by one-electron oxidation reactions catalyzed by extracellular oxidases and peroxidases in the presence of extracellular hydrogen peroxide (H_2O_2) (Belinky *et al.,* 2003; Paterson, 2007). Faison and Kirk (1983) have determined the relationship between the production of reactive oxygen species, hydrogen peroxide and the oxidation of synthetic lignin to CO_2 in liquid cultures of the white-rot fungus *P. chrysosporium.* They showed that the kinetics of the synthesis of H_2O_2 coincided with the appearance of the lignin degrading enzyme system; also, H_2O_2 production was markedly enhanced by growth under 100% O_2, mimicking the increase in ligninolytic activity characteristic of cultures grown under elevated oxygen tension. Furthermore they found that lignin degradation in liquid cultures was inhibited by a specific H_2O_2 scavenger, catalase, implying a role for H_2O_2 in the degradative process.

Protection against the deleterious effects of ROS has been analyzed in depth in yeasts, including *Saccharomyces cerevisiae* (Jamieson, 1995), *Schizosaccharomyces pombe* (Lee *et al.,* 1995), and *Candida albicans* (Jamieson *et al.*, 1996) as well as *Aspergillus nidulans* (Aguirre *et al.,* 2006). The mechanisms behind the ability of necrotrophs to tolerate HR are not known, but it is likely that the mechanisms involve enzymes capable of inactivating ROS, such as superoxide dismutase (SOD), catalase, and peroxidase (Mayer *et al.,* 2001). SODs are enzymes that protect cells against oxidative stress by dismuting superoxide ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) (Mucha *et al.*, 2015). *G. boninense* shows the potential to protect itself from ROS by the secretion of ROS-scavenging enzymes.The activity of extracellular peroxidase and SOD were produced in cultures throughout the growth period, suggesting an involvement of those enzymes in H_2O_2 detoxification or regulation. Investigations have shown increased SOD activity in response to hydrogen peroxide regulation/detoxification in a number of different saprotrophic and pathogenic fungi, as well as providing evidence for the need for protein synthesis for this induction (Xu *et al.,* 2014).

When oxidative stress occurs, cells attempt to counteract the oxidant effects and maintain redox balance by activation of antioxidants. SOD appears to be crucial for protection of fungi with elevated levels of ROS as well as an increased tolerance, which has been deemed to be the first defense mechanism against ROS, catalyzes the disproportionation of O_2^- to O_2 and H_2O_2. However, the up-regulation of SOD is implicated in combating oxidative stress, due to the findings that induction of SOD also results in the activation of H_2O_2 in the case of the O_2^- elimination. Such a situation inevitably leads to the formation of H_2O_2 (Scott *et al.,* 1987). Thereafter, promoted activation of CAT occurred, contributing to H_2O_2 scavenging. Catalase, existing in the peroxisomes in nearly all aerobic cells, serves to protect the cell by catalyzing H_2O_2 without the production of free radicals. Catalase activity was examined in different strains of *P. chrysosporium* under various growth conditions (Kwon and Anderson, 2001; Martin *et al.,* 1987). In low-nitrogen cultures but with excess glucose, production of catalase preceded that of LIP by approximately 3 days, whereas in low-carbon cultures, catalase was produced at an even higher level, but LIP activity was not detected (Kwon and Anderson, 2001). However in this study no catalase activity was detected in *G. boninense* cultures.

5.4.4 Toxin metabolites

Toxins are important virulence factors for many necrotrophic fungi. Production of). Fungal toxins are classified into; host-selective or host non-selective. They comprise low-molecular-weight compounds with a diverse range of structures, ranging from simple organic acids, to polyketides and complex heterocyclic structures but most are secondary metabolites (van Kan, 2006; Baby *et al.,* 2015). Their target sites are equally diverse from host membranes, to chloroplasts and mitochondria to inhibition of biosynthetic enzymes.

Production of secondary metabolites has been reported from Basidiomycete fungi including *Heterobasidion* spp and *G. lucidum* (Hansson *et al.,* 2014; Baby *et al.,* 2015). Several phytotoxic secondary metabolites, including fomajorins (Donnelly *et al.*, 1982; Sonnenbichler *et al*., 1983), fomannosin (Bassett *et al*., 1967; Kepler *et al.*, 1967) and fomannoxin (Hirotani *et al.*, 1977; Heslin *et al*., 1983) have been reported in *H. annosum*. Due to their phytotoxic properties, these compounds have been suggested to be important for the plant pathogenic activity of *H. annosum* infection on conifer tree such as pine (Sonnenbichler *et al.,* 1989).

Ganoderma lucidum produces over 240 secondary metabolites all of which have been investigated for medicinal purposes. Terpenoids and steroids are the major groups which show significant biological activities (Baby *et al.,* 2015). Paterson (2007) described *Ganoderma* spp. as the profilic producers of mycochemical secondary metabolites. In India, *G. lucidum* pathogenic to coconut tree has been reported to produce an extracellular, hydrophilic, thermostable glycoprotein phytotoxin (Karthikeyan *et al.,* 2006). This toxin was shown to cause lesions on a detached coconut leaflets bioassay and was also reported to produce symptoms on root, stem and petiole tissues of coconut seedlings (Karthikeyan *et al.,* 2006).

Production of phytotoxic secondary metabolites by *G. boninense* remains unknown and has never been considered before. Therefore in this study the phytotoxicity of culture filtrates of *G. boninense*, was tested using two contrasting bioassays. No necrotic lesions were caused by infiltration of culture fluids into palm leaves. Bioassay sensitivities can vary enormously and it is therefore important that a range of assays and toxin concentrations are attempted (Wheeler, 1976). Gilchrist and Grogan (1975) reported that direct inoculation of *Alternaria* spp culture filtrates on tomato leaves failed to induce necrosis. An adaptation using a developed detached-leaf bioassay *in vitro* induced necrotic symptoms which resembled those of the disease.

Cell viability assays have the potential to evaluate fungal phytotoxins, and have been used extensively with cultured plant cells. The MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay has been used to evaluate host-toxin responses in cultured tomato (Kodama *et al*., 1991), strawberry (Lee *et al*., 1991), and coffee cells (Nyange *et al.,* 1997). Gray *et al.* (1986) used TTC (2,3,5-triphenyltetrazolium chloride) as a viability assay for calli exposed to fungal culture filtrates of *Phialophora gregata,* and tetrazolium dye reduction was determined based on the 485nm absorbance of an ethanol extract from the callus homogenate, but viability of individual cells was not quantified. The neutral red uptake assay provides a visual, quantitative estimate of plant cell viability. It is one of the most used cytotoxicity and phytotoxicity tests with many biomedical and environmental applications. It is based on the ability of viable cells to incorporate and bind the vital dye neutral red in the lysosomes or vacuoles, as in the case with the potato cells bioassay (Gonzalez-Fernandez *et al.,* 2010).

Potato discs were used as an alternative for oil palm cells because of the technical difficulty of obtaining uniform tissue pieces from any palm tissue. Cells exposed to *G. boninense* fluids showed temporal decreased viability that can be caused by toxins or pectinolytic lysis by the fungus. Further work need to be done to conclusively determine the potential of *G. boninense* to produce extracellular phytotoxin(s) that may have an important role during infection and colonisation of BSR disease of oil palm. Identification and characterisation of any putative toxins produce by *G. boninense* will require fractionation of culture fluids, a wider range of bioassays, preferably involving palm cells, chemical characterization and ultimately an investigation of the genome as encouraged by the data emanating from *G. lucidum* and *H. annosum.*

Toxin production has sometimes been shown to be regulated by signals from the host plant (Howlett, 2006). Amylopectin induces fumonison B_1 production by *Fusarium verticillioides* during the colonisation of maize kernels (Waskiewicz *et al.,* 2012). Oka *et al.* (2005) observed that *Alternaria brassicola* AB toxin production was induced by the leaves of Brassica plants, which releases 1.3 kDa oligosaccharides as stimuli. The mechanism for this induction is unknown. It has been suggested that there exists a complex 'back and forth' interplay between plant and fungus in which the fungus metabolises a plant polysaccharide to produce molecules that trigger fumonison B_1 production (Howlett, 2006). Toxicity from *G. boninense* was only detected when oil palm wood was included in the cultures. This may be attributed to toxins production of *G. boninense* is regulated only in the presence of host substrate and nitrogen limitation which appeared to be an essential stimulus for the activation of virulence functions in phytopathogenic fungi (Pusztahelyi *et al.,* 2015). Nitrogen regulators importance for the development of pathogenicity was shown for *M. grisea* (Talbot *et al.,* 1997) and many other fungal plant pathogens, e.g.,*Colletotrichum lindemuthianum, C. acutatum*, and *F. oxysporum* (Kroll *et al.,* 2014). In plant pathogenic *F. graminearum*, nitrogen starvation activated the trichothecene pathway and induced the biosynthesis of the DON toxin, that was identified as a virulence factor (Desjardins *et al.,* 1993; Audenaert *et al.,* 2014),similar to the host selective T-toxin from *Cochliobolus heterostrophus* (*Bipolaris maydis*) (Turgeon and Baker, 2007).

Mutational strategies can determine the role of toxins in virulence, as shown for HC toxin from *Cochliobolus* (Pannacione, 1993). Determination of potential virulence factors in *Ganoderma* might be achieved through targeted gene disruption. This would involve development of an efficient transformation system for *G. boninense*.

5.4.5 *Agrobacterium tumefacien* mediated transformation (ATMT)

Here we show that *G. boninense* mycelium can be transformed by *Agrobacterium*-mediated transformation. However the protocol still needs optimization and molecular analysis (i.e. southern blot and Q-PCR) of *G. boninense* transformant needs to be done to confirm the integration of the gene *hph* into the genome of the fungus. It is assumed that the T-DNA is integrated predominantly as a single copy into the genome as previously shown for a wide range of fungi such as *Agaricus bisporus* (Chen *et al.,* 2000). Although GFP was not observed throughout the transformants generation of hygromycin-resistant transformants were strictly dependent on the presence of (Pgr4GFP and Pgr4-4iGM3), the plasmids carrying the construct between its T-DNA borders, and acetosyringone which induces the *vir*- in *Agrobacterium* for efficient T-DNA transfer. This is consistent with earlier reports that demonstrate the essential role of acetosyringone for *vir* gene expression (de Groot *et al.,* 1998; dos Reis *et al.,* 2004; Michielse *et al.,* 2005). Taken together, these data suggest that the *Agrobacterium vir* genes play a key role in ATMT (Combier *et al.,* 2003).

In this study, we tested successfully ATMT on *G. boninense* with two plasmids. The primary difference between the constructs was the source of the promoter used to drive expression of the antibiotic resistance marker and reporter marker GFP. Of all the plasmids tested, the construct carrying the promoter of *A. bisporus gpdII* and *P. chrysosporium gpd* (Burns *et al*., 2006), resulted in reasonable transformation efficiency. These promoters, which are from the basidiomycetes *A. bisporus* and *P. chrysosporium*, were able to drive the antibiotic resistance gene in *G. boninense*. This might be attributed to the fairly substantial genetic differences between the basidiomycetes and ascomycetes fungi (Berbee and Taylor, 1993). Our results demonstrate that promoter sequences belonging to homologous species are more active, and highlight the importance of using homologous promoters to drive gene expression (Chen *et al.,* 2000; Godio *et al.,* 2004). Furthermore this study confirmed that the gpd promoter from the Ascomycete *A. nidulans* which is one of the most common regulatory sequences used for gene expression in

fungi, was not effective and functional, as was reported in *G. lucidum* (Shi *et al.,* 2012).

Failure to observe the GFP expression in all of the mycelium was probably because the reporter gene is not stably expressed in the transformant. The GFP could be degraded or inactivated by preferential methylation post-transfomation after several rounds of sub-culturing (Mooibroek *et al.,* 1990; Schuren and Wessels, 1998). Therefore optimisation of the transformation system and molecular analysis of the transformant is essential in order to determine and acquire persistence expression of the reporter gene. Optimisation including base alterations for preferential codon usage (Chiu *et al.,* 1996) and addition of an intron in the plasmid construct as described by Burns *et al.* (2005) has been shown to provide a stable reporter genes expression in a transformant.Furthermore the use of *Ganoderma* gpd promoter e.g. gpd promoter from *G. lucidum,* also should be determined to develop an effective transformation system for *G. boninense*. Nevertheless, the successful transformation of *G. boninense via* co-cultivation indicates that this system may prove useful for molecular genetic studies investigatinghost-pathogen interaction and pathogenicity genes for *G. boninense*. On a more basic level, the availability of GFP *G. boninense* could be invaluable for tracking the infection route through petioles or roots.

5.4.6 Model systems and *Ganoderma* transcriptome

A model system to reflect each stage of the life cycle of *G. boninense,* i.e. saprotrophic, hemibiotrophic and parasitic phases was developed in this study for the first time. Palm wood blocks should simulate saprotrophic colonization of palm tissue by; initial colonization of oil palm cut roots *in vitro* may reveal the earlyhemibiotrophic phase of the pathogen and actual colonization of oil palm roots represents the parasitic phase. This validity of this system has been acknowledged and has led to an international collaboration to investigate the transcriptome of *G. boninense* aimed to reveal putative potential pathogenicity gene expression, during infection and

colonization. For all samples, RNA extracted was of sufficient quantity and suitable for Next-genenaration sequencing, as indicated by *A*260:*A*280 and *A*260:*A*230 ratios and the intact 28S and 18S rRNA bands on agarose gel. Furthermore, recent advances in comparative genomics (Soanes *et al.,* 2008; Fernandez-Fueyo *et al.,* 2012; Suzuki *et al.,* 2012), transcriptomics (Sato *et al.,* 2009; Wymelenberg *et al*., 2005, 2009; MacDonald *et al*., 2011; Yu *et al.,* 2012), proteomics (Mahajan and Master 2010; Ryu *et al.,* 2011) and secretomics (Wymelenberg *et al.,* 2005, 2009; Ravalason *et al.,* 2008; Martınez *et al.,* 2009) allow important information about pathogenicity factors such as effectors, toxin metabolites, CWDE etc, to be determined during host-pathogen interactions.

Only a small proportion of known fungal species are pathogenic (Hawksworth, 2012) and these fungal pathogens are most often not closely related (Hibbett *et al.,* 2007; Humber, 2008), suggesting that genes associated with the pathogenic lifestyle have evolved independently and repeatedly over several lineages. Such genes include those involved in the formation of infection structures, cell wall degradation, overcoming or avoiding host defences, responding to the host environment, production of toxins and signal cascades (Idnurm and Howlett, 2001; Yoder and Turgeon, 2001). There have been numerous transcriptomic studies to determine pathogenicity factors, of host-pathogen interactions including the rice blast pathogen *Magnaporthe grisea*, the potato blight pathogen *Phytopthora infestans*, *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Heard *et al.,* 2015) and the related conifer root pathogen *H. annosum*. Analysis of the expressed sequences have revealed many effector genes associated with early infection as well as genes involved during colonization and pathogenesis. Therefore this transcriptomic study of *G. boninense* has been initiated in an attempt to compensate for the paucity of information on the pathogenicity of this major crop pathogen.

6 Conclusions and Future Work

6.1 Epidemiology, infection and resistance screening

Basidiospores clearly play a major role in the spread and infection of *G. boninense* in the field, according to the great genetic diversity as discussed later. Based on field quantification in this study, basidiospores are produced in vast numbers in oil palm plantations and their durability suggests they could spread BSR at distance.

Furthermore *G. boninense* was shown to be a weak competitor and was unable to grow in non-sterilised soil or frond debris. Therefore it is unlikely that *Ganoderma* stem rot infections occur directly from isolates living saprophytically in the soil or from colonised organic matter that collects behind the frond axils. However root infection of *G. boninense* can occur as confirmed in this study, and from field observations remains at least one of the means of establishment of BSR.

Although frequently proposed as the main route of infection in oil palm, root infection under natural conditions has still not been conclusively proven. However, Hasan and Turner (1998) using bait seedlings placed around infected mature palms, and by Hasan and Flood (2003) with seedlings placed around buried palm trunks, showed rapid infection by *G. boninense*, indicative of root infection. Hasan and Flood (2003) showed that felled trunks were only infectious for approximately two years. In the absence of any scientific study, the period of infectivity of *G. boninense*-infested palm material remains unknown but needs investigation.

Survival as melanised mycelium in previously infected palm roots or stem could explain root to root infection over a period of years. Here it was shown that *G. boninense* melanized mycelium can remain viable for at least four years. This putative survival structure is produced when *G. boninense* counters unfavorable condition, such as lack of nutrients, dehydration and presence of microbial competitors in soil and natural organic substrates.

Therefore it is important to determine how well this mycelial structure could survive in the field where the conditions would be much more challenging than the *in vitro* conditions deployed here. Rubber wood blocks with melanised colonizing mycelium could be used and pieces cut of different sizes to be then partially buried in the plantation soils within nylon mesh bags, to observe viability and survival over time. Some plantations pulverize infected palms and distribute the material onto plantation soils for rapid biodegradation, mulching and nutrient recycling. However this material could well contain melanised *Ganoderma* mycelium and provide inoculum awaiting contact by roots of newly planted oil palms.

Although there is clear evidence highlighting the important of spores in BSR, no-one has ever succeeded in establishing infection with basidiospores (e.g. Hasan *et al.,* 2005). However recently basidiospores have been shown capable of germination in palm trunk wounds and in petiolar xylem by Rees *et al.* (2012). Preliminary data from this study suggest that *G. boninense* spores anastomose to form dikaryons and can colonise oil palm cut petioles beyond the natural barrier presented by vessel end walls. This was only detected twice (with somatic incompatibility and DNA detection) and may be a rare event in the field, combined with the relatively low aggressiveness of *G. boninense*. However under plantation conditions, the opportunity for infection is enormous considering the number of airborne spores (with some UV protection to survive beyond 12 hours) and size of petiole wounds (which take up particles into xylem for > 24 hours). Even rare infection events might explain the frequency of BSR over decades. The potential for infection sites in plantation palms is considerable, with extensive wounds created by routine harvesting (severing the fruit bunch peduncle) and pruning of frond base to free the fruit bunch and trunk (Cooper *et al.,* 2011; Rees *et al.,* 2012). Petiole infection would encourage application of antagonistic *Trichoderma* isolates, which we showed colonizes cut petioles and persists. Therefore it is necessary to repeat these trials under field conditions with extensive replication. A combination of DNA detection with *G. boninense* specific primer GanET and *Ganoderma* selective medium as optimised in this study is capable of accurately tracking the progress of *G. boninense* basidiospores

establishment in the cut fronds. Hence, this will avoid false positive detection of dead/ dormant spore in the cut frond.

Additionally basidiospores can adhere to and germinate on detached root sections. SEM images also suggested early penetration as shown in this study. This is the first time roots have been shown to be a potential entry site for *G. boninense*. Future work should repeat this inoculation and extend the analysis by light and transmission electron microscopy of transverse sections in order to confirm actual penetration. Such inoculations also need to be performed on roots of seedlings and root infection evaluated over time, as infection could be chronic. Greater awareness of basidiocarp formation on aerial roots should be given in plantation surveys as this information might lend weight to the hypothesis of direct infection of roots by spores..

The weak competitive ability of *G. boninense* in all other natural plantation niches (soil, organic debris between frond bases and trunk, frond debris left on soil) must question the ability of spores to establish and create a potential inoculum. In other words, our focus should be on investigating direct infection.

In view of the inevitable role of spores, removal of as many basidiocarps as possible from plantations should be a key part of disease management. Suppression of basidiocarps of infected palms within estates has been practiced in Sime Darby plantations by mounding soil around infected stems and in PNG they manually removed the basidiocarp from the infected stem as a disease management practice (Sanderson *et al.,* 2000).

Resistance remains the only sustainable means of control, but we need to locate it from the many genetic crosses that have been created (Cooper *et al.,* 2011; Hushiarian *et al,* 2013; Ho and Tan, 2015). The test needs to be efficient and reproducible but must not suppress any potential resistance/tolerance. This study has demonstrated that smaller RWB 2.5 x 2.5x 2.5 cm and wheat grain approx. 30 g are able to induce infection on oil palm as long it is applied in intimate contact with the palm roots. The wheat

grain inoculum has potential to be used in resistance screening due to its ease of handling compared with RWB. Therefore it is necessary to conduct further trials with seedlings in Malaysia. Palms used in UK in this work were too large to allow easy manipulation and sufficient replication.

6.2 Genetic variation of *G. boninense*

Field surveys of *Ganoderma* genetic diversity in Peninsular Malaysia using RAMS and SSRs clearly indicate high genetic diversity of *G. boninense* populations not just between five different plantations but even between within plantations as shown in this study. This variation must be derived from sexually produce basidiospores. Whether they infect directly or indirectly remained to be established.

Mating type analysis in this study confirmed that *G. boninense* is heterothallic with multiple alleles at both mating type loci, which favours out-crossing. Furthermore, dikaryotisation of *G. boninense* monokaryotic mycelium occurred readily in *in vitro* di-mon matings. These monokaryotic isolates reflected the high genetic diversity of *G. boninense* populations in Peninsular Malaysia. Notably, genetic variation could also arise from di-mon mating within colonised tissues in field palm. For example, a dikaryon causing BSR or USR might be invaded by monokaryotic mycelium resulting from a basidiospore infection. However the ability of *G. boninense* monokaryons to infect and colonise oil palm remains an ongoing investigation. This study might need developing further in Malaysia with a greater availability of seedling palms, conducive climate and ready supply of basidiospores.

The high level of genetic diversity of *G. boninense* has implications for host resistance, assuming one day it will be found. Polygenic resistance will be desirable whereas resistance based on major, single genes is likely to be vulnerable with such a diverse pathogen. Resistance to *Fusarium* wilt of oil palm in Africa appears to be polygenic and has remained stable for decades (Cooper, 2011).

6.3 *G. boninense* pathogenicity

Pathogenicity factors are likely to include multiple CWDE as evidenced here by detection of ligninases and polysaccharidases during saprotrophic and parasitic stages. Likewise, ROS-detoxifying enzymes which are produced by *G. boninense* might play a role. Toxin production remain speculative and requires genomic information, chemical analysis of culture fluids and suitable bioassays. It is predicted that the transcriptome information discussed below, will answer many of the questions relating to factors involved in infection and aggressiveness.

It has been shown that *G. boninense* appears well adapted to colonise and obtain nutrients from oil palm with several pathogenicity factors. *In vitro* detection of CWDE, ROS, ROS enzymes, toxin and *in vivo* detection for CWDE are in line with many other necrotrophs (Asiegbu *et al.,* 2005b; Heller and Tudzynski, 2011; Chung, 2011). Existence of virulence effectors awaits genome sequence information which is now long overdue for a pathogen of such major significance. However this only provides circumstantial evidence for any role in pathogenicity. Transcriptomic analysis has provided invaluable information for biotrophic, hemibiotrophic and necrotrophic fungal pathogens (Govrin and Levine, 2000; Koeck *et al.,* 2011; Lunden *et al.,* 2015). Notable progress with related basidiomycete *Heterobasidion* spp transcriptome studies showing a switch from saprotrophic mode (CWDE) to parasitic mode (pectinases, oxidative stress e.g. SOD, secondary metabolites). Putative effectors were detected by a large number of orphan genes, and many repetitive elements linked with virulence, comparable to effector regions in *Phytophthora infestans* (Lunden *et al*., 2015; Karlsson *et al*., 2005; Karlsson *et al*., 2003).

This study has provided controlled inoculation protocols and subsequent quality RNA to initiate an important international collaboration. It is hoped that ongoing work by several groups in Malaysia will provide concurrently

the genome of *G. boninense*, which ideally needs to be used in parallel in order to ascribe functions to those genes expressed at critical stages of infection. In particular, any discovery of virulence effectors could speed up the search for disease resistance, as these agents can be tools for exploring the nature of plant immunity or revealing the existence of resistance genes and their durability (Heard *et al.,* 2015). Hence probing germplasm for new resistance traits is poised to improve breeding and deployment of R genes (Vleeshouwers and Oliver, 2014). For example genome-wide catalogues of *P. infestans* potato late blight effectors are available, enabling effectoromics approaches that accelerate R gene cloning and specificity profiling. This information may provide essential, novel information for the control of *Ganoderma* basal stem rot of oil palm.

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APPENDICES

Appendix 1 (CHAPTER 2, section 2.3.1, p. 38)

Malt extract agar (MEA)

1. 30 g Malt extract agar
2. 1000 ml of distilled water

Appendix 2 (CHAPTER 2, section 2.3.2, p. 38)

Malt extract broth (MEB)

1. 15 g Malt extract
2. 1000 ml of distilled water

Appendix 3 (CHAPTER 3, section 3.3.16, p. 121)

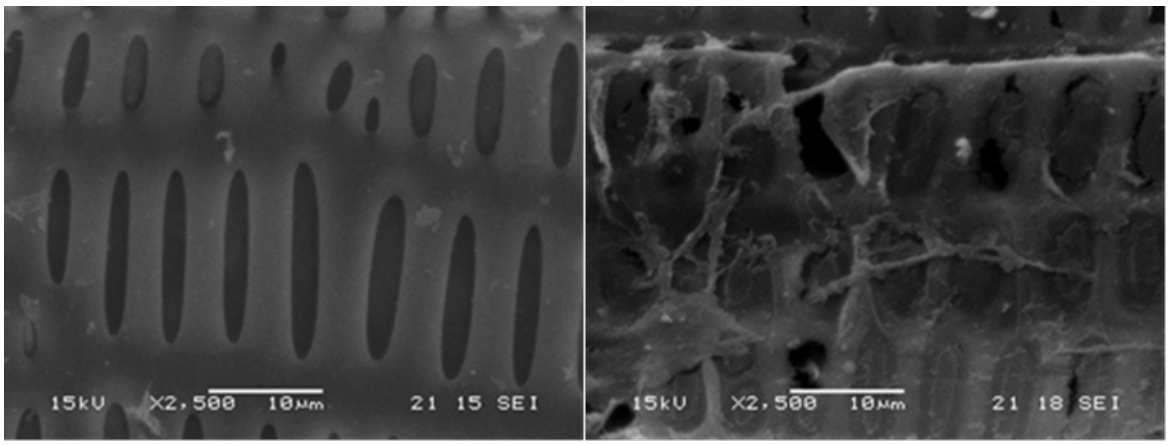

Figure 1. Scanning electron microscopy of intact xylem in control petiole (left). Oil palm xylem, five weeks after inoculation with *Trichoderma* (right). A few hyphae are visible (H) but not in great numbers. There is some evidence of destruction of the vessel wall.

Appendix 4 (CHAPTER 3, section 3.3.16, p. 121)

Table 1: The proportion of vessels with vinyl particles present.

Distance (mm)	Proportion of vessels with particles	Percentage of vessels with particles (%)
0	127/151	84.1
5	43/165	26.1
10	14/126	11.1
15	19/121	15.7
20	17/134	12.7
25	13/103	12.6
30	4/122	3.3
35	6/117	5.1
40	1/130	0.8
45	0/126	0
50	1/119	0.8
55	2/124	1.6
60	0/107	0
65	0/115	0
70	0/117	0
75	0/124	0

Appendix 5 (CHAPTER 5, section 5.2.7.3.1, p. 210)

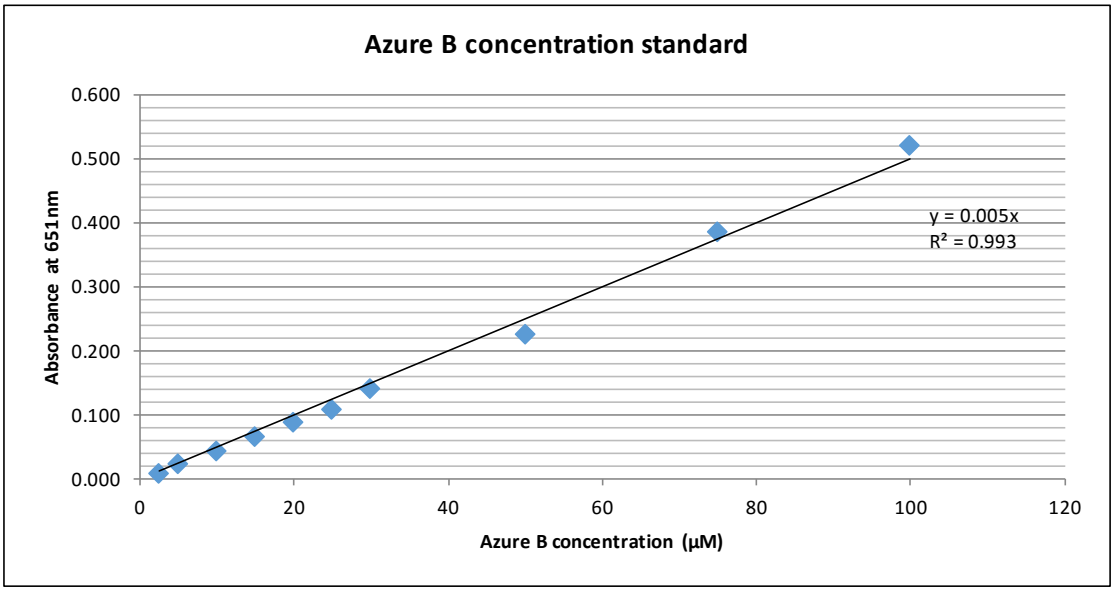

Figure 2: Azure B concentration standard for Azure B assay.

Appendix 6 (CHAPTER 5, section 5.2.7.4.1, p. 212)

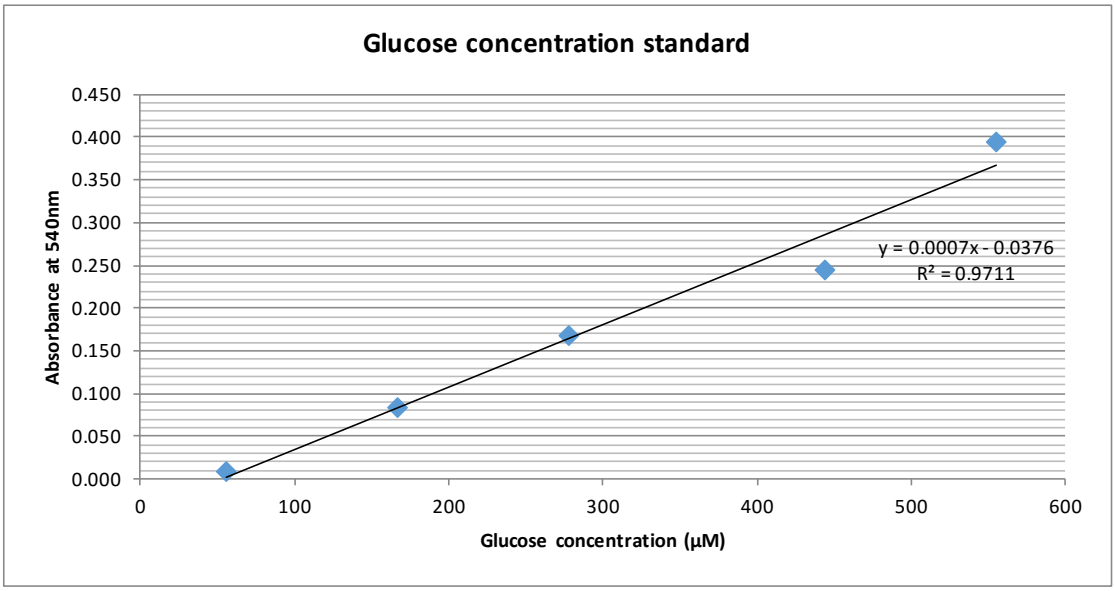

Figure 3: Glucose concentration standard for DNS (cellulase) assay.

Appendix 7 (CHAPTER 5, section 5.2.7.4.3, p. 213)

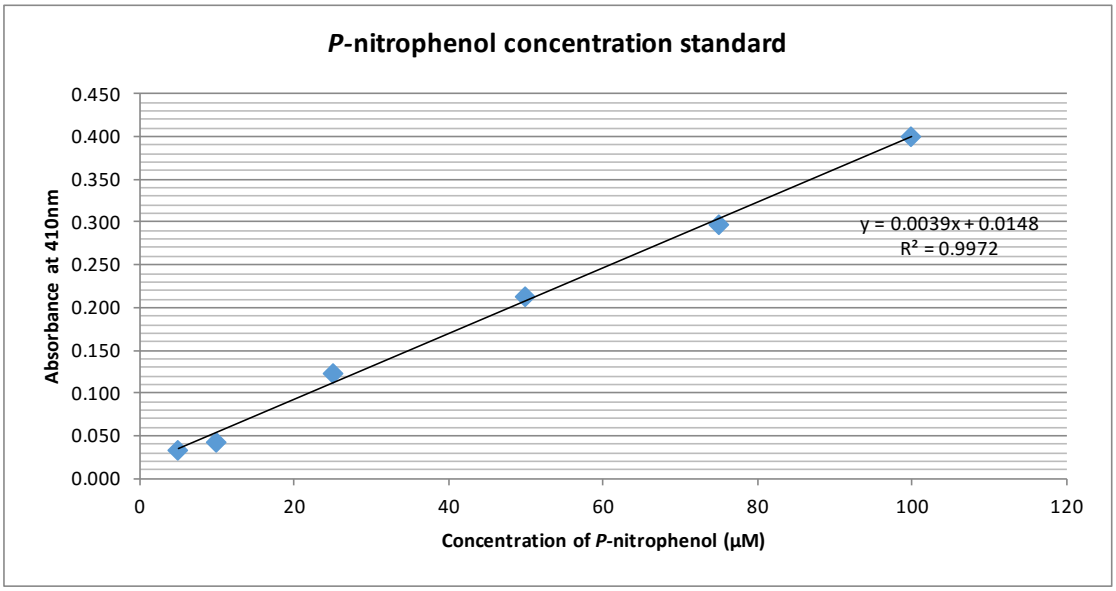

Figure 4: *P*-nitrophenol concentration standard for glycosidase assay.

Appendix 8 (CHAPTER 5, section 5.3.2.3, p. 239)

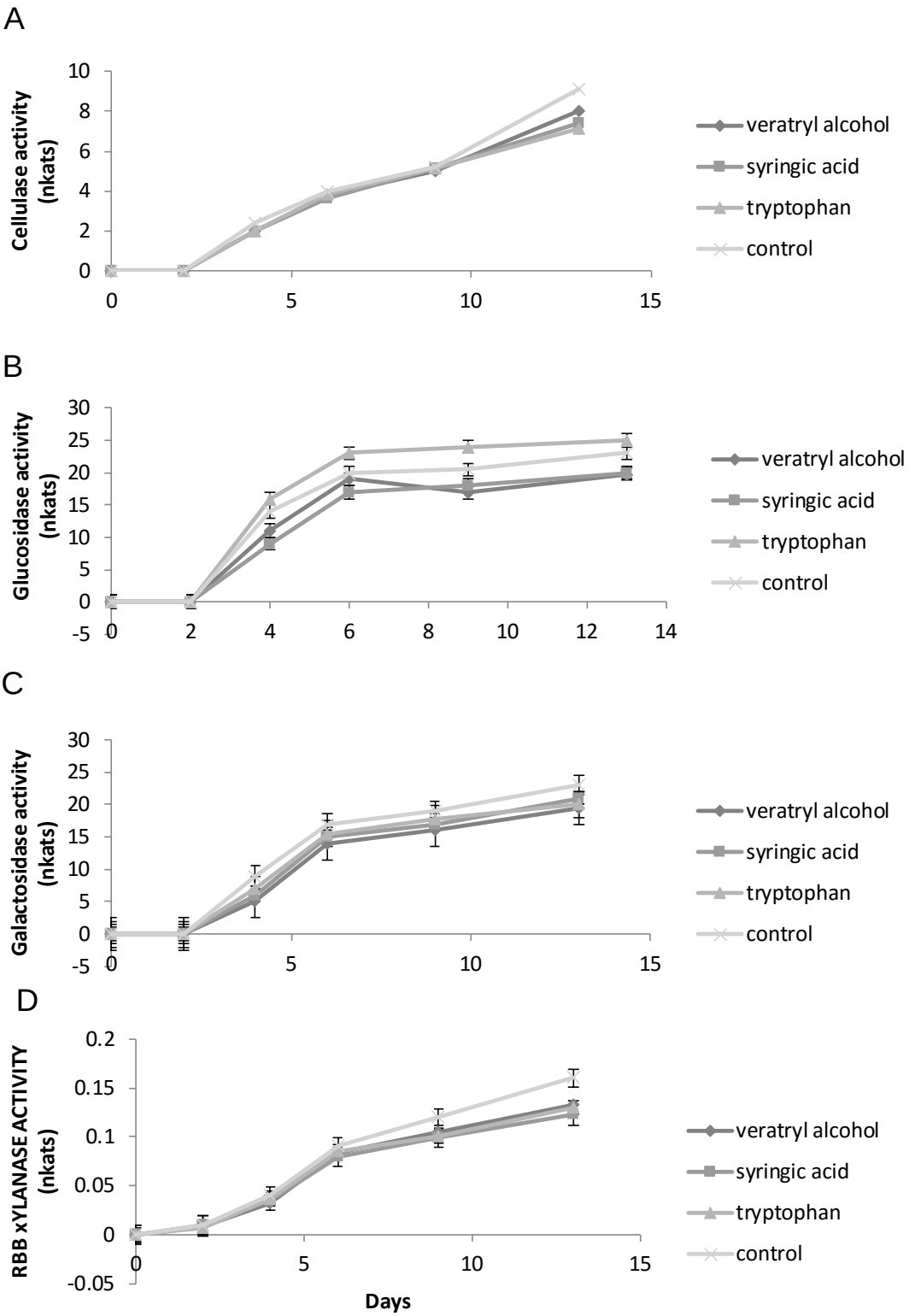

Figure 5: Effect of aromatic compounds on cellulase (A), glucosidase (B), galactosidase (C), and xylanase (D) production by *G. boninense* in liquid medium. Error bars indicate standard deviation of three replicates per time point.