THE EFFECT OF CHITOSAN ON GROWTH AND ENZYME PRODUCTION IN GANODERMA SP. IN VITRO AND IN VIVO INDUCTION OF PLANT DEFENSE RESPONSE IN OIL PALM

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This thesis is dedicated to my Appa and my daughter, Davinah for being my greatest inspiration to achieve my goal

ABSTRACT

THE EFFECT OF CHITOSAN ON GROWTH AND ENZYME PRODUCTION IN *GANODERMA* SP. *IN VITRO* AND *IN VIVO* INDUCTION OF PLANT DEFENSE RESPONSE IN OIL PALM

The aim of this study was to evaluate the role of chitosan against *Ganoderma* pathogen of basal stem rot (BSR) disease under *in vitro* and *in vivo* conditions. Therefore, this study was conducted to investigate the effect of chitosan on (1) *in vitro* growth of *Ganoderma* sp. (2) production of lignin degrading enzymes (LDE) by *Ganoderma* sp. under *in vitro* cultivation system (3) disease severity and vegetative growth parameters on oil palm seedlings and (4) elicitation of plant defense response during the interaction between host-pathogen (oil palm-*Ganoderma*).

The identity of four *Ganoderma* sp. isolates (GBB 7, GBB 18, GBLS, and GBU) obtained from oil palm naturally infected with basal stem rot (BSR) disease were determined initially by macro/micro and cultural morphological characteristics. These were further confirmed by using molecular technique which results an expected 167 bp of PCR product by using GbF and GbR primers. The pathogenicity test revealed that oil palm seedlings artificially infected with GBLS isolate at six months post inoculation exhibited yellowing and mottling of leaves, loss of vigour and plant death. These symptoms were not evident in palms inoculated with the other three isolates (GBB 7, GBB 18 and GBU). Thus, GBLS

isolate of *Ganoderma* sp. were used in the subsequent work conducted throughout this project.

Antifungal studies showed fungiststic effect of chitosan against GBLS and the highest percentage of inhibition of radial growth (PIRG) was 88.99 % when mycelium was treated with low viscosity chitosan at 2.0 % (w/v) after 20 days of incubation period. In addition, chitosan demonstrated cessation of basidiospore germination and germ tube emergence. The lowest germination rate was noted in spores treated with 1.0 % (w/v) concentration of chitosan (5.0 %) after 14 days of incubation period. Scanning electron microscope (SEM) observations confirmed alterations in mycelial surface morphology in chitosan treated *Ganoderma* which imputes a possible mode of action of chitosan on fungal growth.

Preliminary screening based on decolourization of remazol brilliant blue R (RBBR) dye by using agar plate test noted that GBLS isolate as a possible lignin degrader. In conjunction with this result, *in vitro* solid-state cultivation system which is close to *in-planta* condition was developed to determine the production of lignin degrading enzymes by GBLS. Rubber wood chips as growth substrate amended with malt extract broth (MEB) under static condition demonstrated optimum stimulation of laccase and manganese peroxidase (MnP) enzyme activities of GBLS. Higher level of laccase compared to MnP production appeared in GBLS cultures suggest that laccase may involve in the process of penetration and infection of *Ganoderma* which leads to BSR disease establishment in oil palm. Lignin peroxidase (LiP) enzyme was not detected in GBLS cultured under these conditions. Laccase and MnP enzyme activities were inhibited when

chitosan at the concentrations of 0.02, 0.04, 0.06, 0.08 and 0.10 % (w/v) were added into the growth medium. Maximum level of inhibition for both enzymes by chitosan was observed at 0.08 and 0.10 % (w/v) concentrations. The percentage of rubber wood chips weight decayed by GBLS was reduced due to chitosan treatment during the culturing time period. The obtained results manifest that chelation properties poses by chitosan may participate in the inhibition or suppression of LDE enzymes secreted by GBLS.

Optimization of shadehouse studies were designed to determine the efficacy of chitosan in term of concentration of chitosan (0.5, 1.0, 1.5 and 2.0 % w/v), application method (root dipping, soil amendment and soil drenching) and effect on the increased multiple addition of chitosan throughout the evaluation period. The results for this work revealed 0.5 % (w/v) concentration of chitosan (100 ml/ polyethylene bag) *via* soil drench method and increased addition of chitosan at the intervals for nine months of evaluation period significantly reduced *Ganoderma* BSR disease severity of disease in oil palm seedlings. Disease severity was assessed based on the foliar symptoms typical of *Ganoderma* infection and internal bole infection through destructive sampling of the plants at the end of experiment. The seedlings treated with chitosan also promote vegetative growth which displayed an increase in plant height, stem diameter, fresh weight and root mass. The results from SEM observations showed cross section of oil palm seedlings roots treated with chitosan had rough surface which may due to coating of chitosan material and the thickening of pit membrane were noted.

In addition, chitosan at 0.5 % (w/v) concentration *via* soil drenching application induced a significant accumulation of phenolic content and plant defense related enzyme activities which are the phenylalanine ammonia-lyase (PAL), peroxidase (PO) and chitinase in the roots of oil palm seedlings artificially inoculated with GBLS isolate of *Ganoderma*. Increased additions of chitosan application have greater influence on the stimulation of these defense responses. Control seedlings (inoculated/untreated) were noted with these defense activities but at lower levels. Thus, these findings suggest that efficacy of chitosan in minimizing BSR disease severity of oil palm seedlings was accompanied by its potential to elicit defense reactions. Phenolic content and the defense enzymes may involve in restricting entry of *Ganoderma* in oil palm roots and consequently reduced disease infection progress. This present study would be a novel model to commercialize the application of chitosan and explore its role against *Ganoderma* pathogen in oil palm under field condition.

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CHAPTER 1

INTRODUCTION AND OVERVIEW OF RESEARCH

Oil palm in economic terms is the most significant industrial crop in Malaysia and its yield surpasses any other oil producing crop in the world. Malaysia has played a central role in making sure that palm oil quality and quantity remains competitive in the global market by increasing the land area under cultivation and introducing superior clones for better yield of oil products. Currently, the oil palm industry's contribution to Malaysia is about RM 52.7 billion and it has been forecasted to grow to RM 178 billion by 2020 under the Economic Transformation Programme and National Key Economic Area (PEMANDU, 2012). However, oil palm plantations in Malaysia face a significant challenge to maintain the maturity and vigour the palms due to threats of pest and diseases. The major constraint to sustainable oil palm production which has gained great attention is a disease known as the basal stem rot (BSR) caused by the fungal pathogen *Ganoderma* (Pilotti *et al*., 2003; Breton *et al*., 2006; Idris, 2009). *Ganoderma* BSR is a disease that has remained incurable for the past 50 years and which continues to increase and causes severe economic losses in oil palm plantations. *Ganoderma* is a white-rot fungus that infects oil palm through root to root contact and dispersal of basidiospores (Rees *et al*., 2009; 2012). It degrades stem tissues by utilizing oxidative and hydrolytic enzymes (Sanderson, 2005; Paterson, 2007 Paterson *et al*., 2008; 2009) that act on lignin components of wood

and expose white cellulose which is utilized as a nutrient source (Mazliham *et al*., 2007; Paterson, 2007).

All stages of oil palm, from seedlings to old palms, may be infected although, previously, infections had only been noted on old and weak palms (Flood *et al*., 2000). BSR disease progression is initiated by decay of the root system and lower stems which eventually leads to disruption of translocation process of water and nutrients to the upper part of palms (Turner, 1981; Singh, 1991). There is no evidence of early infection of *Ganoderma* as the plant remains without visible symptoms for several years. Earliest symptoms appear in the foliage, such as the appearance of abundant and unexpanded spear leaves and the mature leaves exhibit wilting and yellowing (Turner, 1981). Formation of fungal basidiocarps on lower stems of infected palms during the disease cycle is a prominent characteristic of BSR (Rees *et al.*, 2007). Unfortunately, these symptoms only appear when at least half of the bole tissue has decayed, ultimately leading to the fracture and toppling of whole palms (Turner, 1981; Rees *et al.*, 2007).

A combination of cultural sanitation practices, the use of fungicides and biological management has been attempted to control fungal growth and spread of BSR disease (Susanto *et al*., 2005). Traditional cultural practices (soil digging and soil moulding), replanting techniques and surgical treatments to remove infected materials have contributed to the delay of the onset of the disease and increase the life span of infected palms (Turner, 1981; Singh, 1991; Ariffin *et al*., 2000; Flood *et al*., 2000). However these methods do not provide a complete solution due to

limitations in term of cost, logistic and technical issues which do not coincide with integrated disease management systems (Singh, 1991; Breton *et al*., 2006; Chong *et al*., 2012; Naher *et al*., 2013). Similarly, application of systemic fungicides in the field to eradicate *Ganoderma* has not been very successful due to limitation of effective fungicides and appropriate delivery methods (Ariffin *et al*., 2000). Paterson (2007) and Paterson *et al*., (2008; 2009) reported the perception of *Ganoderma* as a white rot fungus which possesses the versatile lignin degrading enzyme machinery to break down the lignin component of oil palm. They suggested the prospect of controlling *Ganoderma* by selecting oil palms with high or resistant levels of lignin and manipulating the conditions for inhibition of lignin degrading enzymes such as pH, temperature, aeration, high carbon and nitrogen and also introduction of compounds such as halides, metal chelators, heavy metals and reducing agents which could contribute to the development of new methods of disease management in field. However, the development of disease resistant cultivars as the most ideal, economical, and long term solution for BSR disease is still in experimental stage (Turner, 1981; Idris *et al*., 2004; Breton *et al*., 2006, Idris, 2009). Hence, control strategies using biological agents are still considered as a prominent and complementary approach for managing BSR disease. Microbial candidates to control *Ganoderma* include antagonist fungi such as *Trichoderma* spp., (Sariah & Zakaria, 2000; Soepna *et al*., 2000; Susanto *et al*., 2005; Nur Ain Izzati *et al*., 2008; Alizadeh *et al*., 2011) *Aspergillus* sp., (Shukla & Uniyal, 1989) and *Penicillium* sp (Dharmaputra *et al*., 1989); arbuscular mycorrhiza (Hashim, 2003) and endophytic bacteria (Sapak *et*

al., 2009; Bivi *et al*., 2010) have been screened through *in vitro* and glasshouse studies. Among them, *Trichoderma* spp. gained special interest and have been intensively researched for many years to control *Ganoderma* BSR infection. However, inconsistent experimental results due to lack of information on delivery method, timing of application and viability of *Trichoderma* have been main constrains to achieve successful control over *Ganoderma*. Furthermore Abdullah *et al*., (2003) stated that *Trichoderma* is only effective for plants at the early stage of the disease and not applicable for highly infected oil palms.

Another reason for unsuccessful attempts to control *Ganoderma* using those biocontrol agents is the limited information regarding oil palm's biochemical response against the pathogen or the genes that are involved in the establishment of BSR disease resistance. Despite, many epidemiological studies on BSR disease caused by *Ganoderma* knowledge of the basic physiology of oil palm-*Ganoderma* interaction and the stimulation of plant defense mechanisms are still lacking. Therefore in this study, chitosan as a potential fungal inhibitor and an elicitor is explored as an alternative control strategy for the control of pathogenic *Ganoderma* for the long term containment of BSR disease.

Chitosan (β-(1-4)-linked N-acetyl-D-glucosamine) is a natural polysaccharide polymer derived from the shell of crustaceans (crabs and shrimps) and fungal cell walls through deacetylation of chitin that has been widely used in agriculture, pharmaceuticals and food processing applications (Shahidi *et al*., 1999). The polycationic nature of chitosan enhances physiological and biochemical changes in fungal cell walls and in host tissues (Baustista-Banos *et*

al., 2006), hence the fungicidal action of chitosan against several pathogenic fungi including *Botryis cinerea*, *Colletotrichum spp*., *Fusarium solani* and *Rhizopus stolonifer* (Hadwiger & Beckman, 1980; El Ghaouth *et al*., 1991, 1992a, 1992b; Trotel-Aziz *et al.,* 2006; Munoz *et al.,* 2009). Chitosan also has the ability to act as a potent elicitor for plant defence responses such as the deposition of lignin (Pearce & Ride, 1982), synthesis of protein inhibitors (Walker- Simmons *et al*., 1983) production of phytoalexins (Kendra & Hadwiger, 1984), formation of callose (Kauss *et al*., 1989) and secretion of defence enzymes against pathogens such as phenylalanine ammonia lyase (PAL), peroxidase (POD) and polyphenoloxidase (PPO) (El Ghauoth *et al*., 1992a, 1992b, 1994). These previous studies have supported the antifungal effect of chitosan on diverse fungal pathogens and crops; however its application on *Ganoderma* pathogen of oil palm remains unexploited. Thus this study aims to evaluate the ability of chitosan as a biofungicide approach against *Ganoderma* and BSR disease suppression.

The specific objectives of this research project are:

(1) To study the effect of chitosan on *in vitro* mycelia growth and spore germination of *Ganoderma*

(2) To investigate the effect of chitosan on *in vitro* production of lignin degrading enzymes (LDE) by *Ganoderma* under an optimized culture condition

(3) To evaluate the effect of chitosan application on the growth of oil palm and control of *Ganoderma* basal stem rot (BSR) infection under controlled condition

(4) To study the effect of chitosan on the induction of plant defense enzyme activity the interaction between host-pathogen, oil palm and *Ganoderma*

CHAPTER 2

LITERATURE REVIEW

2.1 Oil Palm

2.1.1 Origin and classification of oil palm

Oil palm is an ancient tropical plant believed has been originated from Africa based on fossil, historical and linguistic evidence (Corley & Tinker, 2003). Other reports have mentioned uncertain fact linked to the origin of oil palm such as the fat residues found in an Egyptian tomb from 5000 BP were presumed as palm oil (Raymond, 1961) and botanical characteristics of related genera were suggested as an origin in South America (Cook, 1942). However, oil palm as an African origin was strongly proved by the discovery of pollen deposits evidence from various part of Africa and clear literatures which reports on the exploration of oil palm in Africa (Corley & Tinker, 2003).

Oil palm is from a distinct group of plants among monocotyledon with 16 pairs of chromosomes (diploid number, 2n = 32). It is classified under the family of Arecaceae or Palmae and genus of *Elaeis*. Three species generally accepted belongs to this genus are known as *E. guineensis* Jacq. (African oil palm), *E. oleifera* (American oil palm) and *E. odora* (Corley & Tinker, 2003). *Elaies* is adapted from the Greek word '*elaion*' which means oil whereas Jacquin was first

who introduced botanical name of oil palm and *guineensis* refers to its origin, Guinea coast (Corley & Tinker, 2003). The African oil palm are planted and commercialized in Southeast East Asia particularly Malaysia and Indonesia.

2.1.2 Botanical and morphology characteristic of oil palm

The botanical and morphology characteristic of the African oil palm, *E. guineensis* were well documented by Corley & Tinker (2003) and Verheye (2010). Generally, the oil palm is large, unbranched, pinnate-leaved palm having solitary columnar stem with short internodes and grows up to 20-30 metre high. The arrangement of leaves which separates the upper of lower ranks of leaflets in various stages of development on the rachis resembles an untidy appearance of palm tree The roots developed from the base of the hypocotyl and formed as basal bole of stem at later stage. The primary roots were formed deep at the base of the trunk but remain short when water table is high and the secondary, tertiary and quaternary roots formed as dense mat in the immediate neighbourhood of the tree.

Oil palm is normally monoecious with male and female and sometimes mixed inflorescences found on the same tree developing in the axils of leaves. The development of the inflorescences to fruits usually takes about 42 months and the formed fruit are large and in bunches appearance wedged in the leaf axils of the palm (Verheye, 2010). The fruit has three pericarp layers whereby the exocarp (skin), mesocarp (fruit pulp which contains red palm oil) and endocarp (a hard shell surrounding the kernel which contains kernel oil) (Corley & Tinker, 2003;

Verheye 2010, Naher *et al*., 2012). Three main cultivars of oil palm are named based on the fruit thickness and yield or commercial value: dura (thick shell), pisifera (absence of shell) and tenera (thin shell). Tenera is a hybrid formed between dura and pisifera with higher oil extraction content compared the other cultivars (Harminder *et al*., 2010).

2.1.3 Economic importance and production of oil palm

Two types of oil extracted from oil palm known as crude palm oil and palm kernel oil have been used for many purposes such as for food, oleochemicals, pharmaceuticals, health supplement and as a potential source of biofuel (Ntsefong *et al*., 2012). The trans-fat free content of palm oil favours the food industry which considered the oil as an alternative for animal fat and hydrogenated vegetable oils (NetBalance Foundation, 2013). Potential use of vegetable oils for biofuel production had gained great attention recently and this directly had caused the demand for palm oil increased. Malaysia and Indonesia known as the key growing countries are in the direction to manipulate palm oil blend for biofuel use in domestic transport sector (Leow, 2010; Saxon & Roquemore, 2011).The developed countries such as Europe and United States are the main importers of palm oil to be used as biofuels in order to substitute fossil fuels for electricity generation and energy security directive respectively (Saxon & Roquemore, 2011; NetBalance Foundation, 2013).

At present, production of oil palm surpassed other commercially grown vegetable oil crops and sustain as the highest oil yielding corps in the world (Oil World, 2013). This domination phenomenon attributed to advantages of oil palm in term of producing higher yield per hectare, competitive price, diversified products, limited processing requirements and potential renewable energy resource (NetBalance Foundation, 2013). The world-wide production has increased tenfold over the decades and currently are estimated about 50 million tonnes per year and expected to rise up to 50 percent of further growth by 2050 (Rahman *et al*., 2008). Three main countries ranked as the main producers of oil palm are Asia, Africa and Latin America. Although oil palm is known as native to Africa, the productions are greater in Malaysia and Indonesia whereby it's accounted for 85 % of total world production of oil palm due to favourable climatic condition for the growth of oil palm (Oil World, 2013; NetBalance Foundation, 2013). Consumption of palm oil is increasing in developing and developed countries and the major consumers include China, India, Indonesia, Malaysia and European Union (Oil World, 2013). Taken all this together, the increasing demand for palm oil consumption can be summarized due to the expansion of human population, changing diets, efficiency of oil palm production and usage of palm oil globally (Corley & Tinker, 2003; NetBalance Foundation, 2013).

2.1.4 Development of oil palm industry in Malaysia

Oil palm was introduced to Malaysia as an ornamental plant. The first oil palm plantation in Malaysia, Tenamaram Estate was established by 1917 in Selangor state (Corley & Tinker, 2003; Sime Darby, 2014). During 1960s, the expansion of oil palm industry was rapid replacing coconut and rubber under the Malaysians government's agricultural diversification programme (Sime Darby, 2014). These were in line with increased exploration of planted area, expanded infrastructure, improved worker productivity, diversification of palm oil product and productive researches on increasing palm oil yield and environmental aspect of oil palm cultivation (Corley & Tinker, 2003). In 1990s, oil palm cultivation was focused to East Malaysia particularly Sabah to overcome the need of large planted area and now Sabah stands as the state which has largest plantation area (Corley & Tinker, 2003) compared to any single state in Malaysia. Since the importance of oil palm industry to the country's economy was recognized, agencies such as Malaysian Palm Oil Board (MPOB), Palm Oil Research Institute of Malaysia (PORIM) and Palm Oil Registration and Licensing Authority (PORLA) were established to improve and increase the production of oil palm (Sime Darby, 2014).

The overview of oil palm production and trade in Malaysia for the year 2010-2012 is shown in Table 2.1. Malaysia was the largest producer of world production of palm oil till Indonesia took over in 2006 through its new implementation projects on land grants and foreign investments for oil palm plantations (Wetlands International, 2010). According to Foreign Agricultural

Service (2011), Indonesia has emerged as largest land of area planted oil palm and immature tress in the world which could sufficiently meet the demand of palm oil trade globally in future. This dominance position is also favoured by Indonesia's lower money currency value and export tax on crude palm oil (CPO) which enables them to sell cheaper palm oil. Despite to these challenges in oil palm trade, the contribution of the industry to Malaysia's economy could not be neglected and its role was evidenced under the Economic Transformation Programme (ETP) set up by the government. Through this programme, oil palm industry was identified as one of the key drivers or called as National Key Economic Area (NKEA) to assist the nation's economy. Implementing the new and improved approaches on oil palm production under this programme, Malaysia's palm oil sector aims to increase Gross National Income (GNI) by RM 125 billion to RM 178 billion and offer 41,600 job opportunities (PEMANDU, 2012).

Table 2.1: Overview of oil palm production and trade in Malaysia

Key statistic	2010	2011	2012
Planted area (million ha)	4.8	5.0	5.1
Crude Palm Oil (CPO) Production (million tonnes)	16.99	18.91	18.79
Export of palm oil (million tonnes)	16.66	17.99	17.58
Export of palm oil and products (million tonnes)	23.04	24.27	24.59

Source: Malaysian Palm Oil Board (MPOB), 2012

2.2 Basal Stem Rot (BSR)

2.2.1 Occurrence and economic importance

Oil palm has been affected by various important plant diseases according to their cultivation regions. Vascular wilt caused by *Fusarium oxysporium* f. sp. *elaeidis* is a serious disease in Africa and often regarded as African disease (Turner, 1981; de Franqueville & Diabate, 2005). South America faces diseases problem such as bud and spear rot caused by unknown agent and red ring disease by nematode, *Rhadinaphelenchus cocophilus* and weevil vector, *Rhynchophorus palmarum* (Gomez *et al*., 2005). In Southeast Asia mainly Malaysia and Indonesia, basal stem rot (BSR) caused by fungal pathogen, *Ganoderma* spp. is the single disease to date which causes significant economic losses and emerged as one of the limiting factors for sustainable production of palm oil.

BSR was known since 1915 (Bloomfield *et al*., 1968) and has been distributed in many parts of countries in Africa, Malaysia and Indonesia as reviewed by Turner (1981). This disease was first recorded in Malaysia in 1931 infecting oil palm of over 25 years (Thompson, 1931). During this period of time, BSR was recognized as disease of old palms associated with senescence factor. Therefore the disease was considered uneconomical and was not given much importance on the occurrence since such palm could be soon replanted (Turner, 1981; Corley & Tinker 2003). In mid-1950s, *Ganoderma*-BSR infection were increasing and became apparent in much younger palms of 10-15 years old in the

areas planted replacing coconut plantings or replanted from oil palm. By 1990s, *Ganoderma* was able to infect oil palm as early as 1-2 years after planting and increases in 4-5 year old palms mainly in replanted areas or areas under planted with coconut coconut palms (Singh, 1991; Ariffin *et al*., 2000). High disease incidences were found to have close relationships with previous crop, where the incidences were higher when young palm were planted substituting coconut plantings compared to forest or rubber crop (Turner, 1981). This phenomenon is claimed due to the lack of sanitation practice during replanting of younger palms. According to Turner (1981), high disease incidences were recorded when the old coconut stumps and trunks were poisoned and left in the ground. Additionally severe infection occurs when the old coconut trunks were buried to prevent infestation by rhinoceros beetle (*Oryctes rhinoceros*). Abundant fruiting bodies of *Ganoderma* found on coconut stumps and logs served as substantial source of inoculums to initiate disease infection explained the scenario of oil palm-BSR disease on coconut planting lands (Navaratnam *et al*., 1964). However, based on the study by Singh (1991), only marginal differences were noted in the rate of disease development in field plantings after oil palm or coconut. The appearance of high disease incidences on palm grown on peat soils were not affected by the preceding crops (Ariffin *et al*., 1990). Field surveys have shown high disease incidences in coastal areas with low-lying alluvial soils particularly in the west of Peninsular Malaysia (Ariffin *et al*., 2000; Corley & Tinker, 2003; Idris, 2009). The characteristic of coastal soils which consists of high moisture content might contribute as a suitable condition for *Ganoderma* infection (Singh, 1991). Inland

areas with lateritic and peat soils were noted less susceptible of BSR infection (Turner, 1981) until recently, where high disease incidence have been recorded (Rao, 1990). BSR disease incidence in Sabah and Sarawak are still considered relatively low compared to Peninsular Malaysia (Ariffin & Idris, 2002).

Initially BSR disease incidence is low and was recorded about 1-2 % but later when the palm tree reached 25 years, it may increased up to 25 % at replanting (Singh, 1991; Ariffin *et al*., 2000). The yield losses due to BSR according to Turner (1981) occurs through dead of productive palm trees and reduced weight and number of fruit bunches produced from diseased and subclinical infected palms. The ability of the disease to kill more than 80 % of the stands by the time they are half way through normal economic life (Turner; 1981) and the yield losses reaching up to 30 % indicates the economic importance of the crop (Idris, 2009).

2.2.2 Disease symptoms

Early BSR disease symptoms in oil palm is difficult to detect due to the fact of its slow development which enables the plant to remain symptomless for a long period of time and consequently causes extensive internal rotting. The first visual symptom of BSR infection occurs in the foliar whereby it appears when at least one half of the basal stem has been decayed by the fungus (Turner, 1981; Ariffin *et al*., 2000; Soepna *et al*., 2000; Corley & Tinker; 2003). The stem decay causes restriction of water and nutrient supply to aerial parts of the plant. However foliar symptoms are not a definite detection of BSR as it also resembles other water stress factors such as drought (Turner, 1981; Corley & Tinker, 2003). In younger palms the symptoms includes presence of unopened spear leaves, molting and yellowing of fronds which followed by necrosis (Turner, 1981; Singh 1991; Corley & Tinker). Older palms similar symptoms exhibited similar symptoms and the dead fronds usually drops, hanging downwards from the attachment point of the tree to form a skirt of dead leaves (Ariffin *et al*., 2000; Corley & Tinker, 2003) (Fig 2.1 a) . Once the young palms exhibited first symptom of infection, plant mortality occurs at about 6 months -2 years, whereas the older ones survive up to 2-3 years which doesn't make any differences in the real situation (Ariffin *et al*., 2000; Paterson, 2007). The formation of basidiocarp is the most visible and definite structure of the disease symptom which grows at the stem base, leaf base or on the infected roots close to the palm (Ariffin *et al*., 2000; Rees *et al*., 2007). Initially, the basidiocarp develops as tiny, rounded and whitish sporophores which

later forms bracket shape of basiodiocarps (Corley & Tinker, 2003) (Fig 2.1 b, c). The appearance of lesions at the stem base is one of the prominent characteristic of BSR disease. This has been a beneficial tool of BSR diagnosis whereby the internal rotting of the pathogen on the stem base could be quantified with the absence of fruiting body and in younger palms.

Figure 2.1: External symptoms of basal stem rot (BSR) disease caused by *Ganoderma* (a) Foliar symptoms showing necrotic fronds and unopened spear leaves (arrow) (b) Emergence of sporophores (c) Formation of bracket-shape basidiocarp from the base of the stem indicating infected position of the stem

Turner (1981) and Ariffin *et al*., (2000) had described the internal rotting of infected oil palm caused by *Ganoderma*. In brief, cross sections of infected stem base showed light brown colour on the lesions area marked by darker bands with an outer edge of an irregular yellow zone. It was also mentioned that the darker zones are termed as reaction zones where the gum is accumulated and the formation of yellow zones were results of defense mechanism of the palm to infection. The darker zones were termed as 'dark lines' and within the lines swollen hyphal cells in the form of resting structures were noted (Ariffin *et al*., 2000). Small cavities of white mycelium were visible within the rotting tissue of the stem. Prolonging infections causes the roots of the palms become friable and the internal tissues turned very dry and powdery (Singh, 1991). Extensive decay by *Ganoderma* on oil palm with severe internal rotting leads fracture and collapse of the palm (Corley & Tinker, 2003; Rees *et al*., 2007).

2.2.3 *Ganoderma* spp.

Causal pathogens

Ganoderma spp. belongs to the family of *Ganodermataceae* which are widely recognized as white rot fungi for their unique ability to degrade lignin components of hardwood plants (Hepting, 1971; Adaskaveg *et al*., 1991) by using ligninolytic enzymes consists of laccase and peroxidases (Ward *et al*., 2004; Paterson, 2007). The status of *Ganoderma* as a serious pathogen causing BSR disease on oil palm as 'white rot' were noted in previous reports and reviews

(Miller *et al*., 2000; Mazliham *et al*., 2007; Paterson, 2007, Paterson *et al*., 2008, 2009; Rees *et al*., 2009). However, based on the intensive reviews by Paterson (2007) and Paterson *et al*., (2008, 2009) *Ganoderma* from the perspective of white rot was ignored and not empahasized in the basic disease process of BSR. In term of infection capability, Sariah & Zakaria (2000) regarded *Ganoderma* is not an aggressive pathogen and weakly pathogenic to healthy plants. Moreover, Miller *et al*., (2000) and Soepna *et al*., (2000) addressed *Ganoderma* as saprobic and only able to attack weakened hosts as parasites or secondary pathogens. According to Paterson (2007), *Ganoderma* is also known as a facultative saprophyte and Abdullah (2000) claimed this fungus lives as an endophyte in coconuts.

Initially, the fungal pathogen was classified as *Ganoderma lucidum* Karst in Malaysia (Thompson, 1931) which is a temperate species causing disease in various hosts. Turner (1981) stated that a single species were not able to be the contributor of the disease at any particular area and listed fifteen species that may responsible across different parts of the world. These includes seven species that were reported from Peninsular Malaysia namely *G. applanatum* (Pers.) Pat, *G. boninense*, *G. chalceum* (Cooke), Steyaert, *G. lucidum* (W. Curt. et fr.) Karst, *G. minitocinctum* Steyaert, *G. pseudoferreum* (wakef.) Overh. & Steinmann and *G. tornatum* (Pers) Bres. In contrast, Ho & Nawawi (1985) asserted that *Ganoderma* isolates from diseased oil palm sampled in Peninsular Malaysia were all the same species and referred as *G. boninense*. Based on morphological characterization and pathogenicity test conducted by using artificial root inoculation methods, Idris *et al*., (2000) found that *G. boninense*, *G. zonatum* and *G. miniatocinctum* are

associated with BSR of oil palm while *G. tornatum* was categorized as saprophyte on dead palms. Among the pathogenic species, *G. boninense* is the most aggressive one and was observed mainly in high disease incidence areas (Idris, 2009). These findings suggest that generally *G. boninense* is the primary and most important pathogenic species to oil palm in Malaysia.

2.2.4 Mode of infection

BSR disease manifestation under natural condition is claimed occurs through root contact as the primary route with tissues or debris colonized by *Ganoderma* from soil source or infected palm. This mode of disease spread is evidenced by observation of same strain of *Ganoderma* were identified from infected palms or in seedlings planted close to diseased stumps (Turner, 1981; Flood *et al*., 2000) and occurrence of the disease in the pattern of clumps or grouped together (Singh, 1991; Corley & Tinker, 2003). In conjunction to support this view, infection studies on young oil palm seedlings by using artificial inoculation techniques (Sariah *et al*., 1994; Lim & Fong 2005; Breton *et al*., 2006; Nur Ain Izzati, 2008; Rees *et al*., 2007; 2009) and field observations of matured palms (Hassan & Turner, 1998; Flood *et al*., 2005; Rees *et al*., 2009) shows root infection pattern leads to establishment of BSR disease. Oil palm roots from adult palms grows up four planting rows, thus these condition facilates close contact of roots (Miller *et al*., 1999). Hassan & Turner (1998) have demonstrated seedlings planted close to BSR infected oil palm stumps from previous planting are infected and died within

6-24 months. Flood *et al*., (2005) showed infections became severely diseased in seedlings planted closed to *Ganoderma* colonised oil palm trunk. Moreover a recent study by Rees *et al*., (2009) on field palms exhibited multiple infection symptoms in different roots in a single palm before spreading into the bole of palms. Turner (1981) has stated by infecting field palms by placing blocks of infected tissues about 0.3 m^2 from diseased palms under the soil surface close to palm base, multiple infection were observed. Ariffin *et al*., (2000) mentioned that infection by *Ganoderma* occur through wounded tissues or dead roots, but the latest investigations by Rees *et al*., (2007, 2009) indicate this could occur in unwounded roots and further invades through inner tissues of the plant. *Ganoderma* are known as weak parasite or pathogen with poor competitive ability and therefore a substantial inoculum were required to initiate infection in natural plantations (Turner, 1981). Nevertheless, Hassan & Turner (1998) demonstrated that isolated diseased roots are sufficient to act as a source of inoculums. In addition, Rees *et al*., (2007) found that close attachment between the roots and *Ganoderma* seems to be more important factor than inoculums size to initiate infection.

Hassan & Turner (1998) have reported young seedlings became infected within 1-2 years when planted close to *Ganoderma*-diseased stumps. Poisoned and shorter stumps showed higher infection which may due to the factor that they may rotted quicker and ease *Ganoderma* invasion in young seedlings (Turner, 1981; Hassan & Turner, 1998). Felled trunks left on the surface gave higher infection than stumps remain in the ground and buried oil palm tissues contributes

for greater infection rate than left on the surface (Turner, 1981; Hassan & Turner, 1998). These authors have also pointed out that colonization of *Ganoderma* and the infection process in healthy oil palm stumps are slower than diseased stumps. Therefore it was suggested that this could be the reason for oil palm remain infectious for long period of time once they are infected.

Despite root contact plays a key role in the mode of BSR infection, the involvement of basidiospores in initiating and spreading the disease has been strongly agreed (Thompson, 1931; Turner, 1981; Sanderson *et al*., 2000; Sanderson, 2005; Panchal & Bridge, 2005; Paterson, 2007; Rees *et al*., 2012). Sanderson (2005) and Paterson (2007) mentioned that root contact may cause infection on young seedlings and at the later stage spores dispersal would be the dominant source of disease spread. This hypothesis arises based on genetic and molecular studies which revealed high genetic diversity of *Ganoderma* isolates within and between oil palm plantations (Ariffin *et al*., 1996; Miller *et al*., 1999, 2000; Pilotti *et al*, 2000, 2003, 2004; Pilotti, 2005, Rees *et al*., 2012). Such situations were identified in Malaysia, Indonesia and Papua New Guinea. If root contact is happen to be to the mode of spread, uniform genotypes of *Ganoderma* were expected in infected palms or field (Rees *et al*., 2007). In contrast, different isolates were found even in a single palm which is postulated due to sexual reproduction of spores which forms genetically variable genotypes are dispersed through wind or transmitted by insects (Hassan & Flood, 2003; Bridge *et al*., 2004; Corley & Tinker, 2003; Cooper *et al*., 2011; Rees *et al*., 2012). In addition, tetrapolar mating system with multiple alleles owned by *Ganoderma* promotes

outcrossing (Pilotti *et al*., 2002; Rees *et al*., 2012). Disease infection in new plantation areas which are free from *Ganoderma* inoculum sources and randomly distributed disease pattern in certain areas claimed the spread were from spores (Sanderson *et al*., 2000; Lim & Fong, 2005; Cooper *et al*., 2011; Rees *et al*., 2012). Moreover, study by Bridge *et al.*, (2001) which detects *Ganoderma* in healthy young palm without any external or root symptoms suggest the possible contribution of spores.

According to Hassan & Flood (2003) and Lim & Fong (2005), spores derived monokaryotic mycelium were able to colonize substrates but infective in causing infection whereas dikaryotic mycelium (formed through fusion of compatible hypae) are capable of both. Abundant number of spores released from fruiting bodies may act as potential inoculum source plantations (Ho & Nawawi, 1986; Sanderson, 2005; Rees *et al*., 2012). Potential and accessible site for spore infection in palm was suggested earlier through wounded surface of fronds (Thompson, 1931) and Cooper *et al*., (2011) and Rees *et al*., (2012) have stated wound sites are exposed by field management practice such as extensive harvesting and pruning of frond base. Study conducted by Panchal & Bridge using PCR method have shown presence of *Ganoderma* in cut frond base and pruned frond in asymptomatic palms which were expected originated from aerial spores. A recent study by Rees *et al*., (2012) exhibited the possible infection site of basidiospores on cut surfaces in fronds, peduncle and stem. In spite of the evidence supporting basidiospore in the life cycle of BSR disease, the potential of the spores to establish inoculum source are still uncertain due to the fact that

Ganoderma are poor competitor in non sterile soils and organic debris (Rees *et al*., 2007). Along with these, infection trials by using spores were unsuccessful and were not reported in young seedling and field palms (Turner, 1981; Flood *et al*., 2002).

2.2.5 Detection of *Ganoderma*

Detection of BSR disease caused by *Ganoderma* based on typical symptoms of infected oil palm externally and internally was discussed in Section 2.2.2. A part from this, diagnostic tools for reliable detection were developed for laboratory and field applications. Calorimetric method by using ethylenediaminetetraacetic acid (EDTA) was initially adapted to detect *G. lucidum* causing Thanjavur wilt disease in coconut (Natrajan *et al*., 1986). Also, another technique used was by drilling infected oil palm stem and the obtained diseased materials were cultured in semiselective media to isolate *Ganoderma* (Ariffin *et al*., 1993). Nevertheless, these conventional methods are not accurate, time consuming and not applicable for large scale sampling in field (Utomo & Niepold, 2000). Ganoderma selective media (GSM) has shown it's advantageous in early diagnosis of the disease whereby *Ganoderma* was able to be detected in infected tissues and asymptotic oil palm which are beneficial for field studies (Ariffin & Idris, 1991; Ariffin *et al*., 1993; Paterson, 2007).

Two main approaches for determining accurate and specific detection of *Ganoderma* was directed towards enzyme-linked immunosorbent assay (ELISA)

and application of polymerase chain reaction (PCR) methods. ELISA-based studies by using antibodies demonstrated a simple and cost effective method which is applicable for bulk screening (Darmono, 2000; Utomo & Niepold, 2000; Utomo *et al*., 2005). Nevertheless, the drawback of this test are often linked with its cross reaction with other saprophytic fungi and are considered not species specific (Utomo & Niepold, 2000; Utomo *et al*., 2005). PCR based techniques used in the detection of pathogenic *Ganoderma* were employed in previous studies (Bridge *et al*., 2000; Utomo & Niepold, 2000; Utomo *et al*., 2005, Panchal & Bridge; 2005; Idris, 2009). Information on the available *Ganoderma* sequencing data which will be useful for molecular approaches for detection were described Hushiarian *et al*., (2013). PCR techniques are considered more specific and sensitive in *Ganoderma* detection compared to ELISA. However combination of both assays would provide a better option for farmers in decision making to manage oil palm plantation from *Ganoderma* infection (Utomo *et al*., 2005).

Identification of biochemical responses and genes involved during *Ganoderma* infection in oil palm are poorly studied but these could provide essential information to control BSR disease at earlier stage of plant development. A study by Tay *et al*., (2009) has shown increase accumulation of pathogenesis related (PR) proteins such as Phenylalanine ammonia-lyase (PAL), Peroxidase and glucanase in oil palm seedlings during *Ganoderma* infection. The activation of these PR-enzymes activity is reported are related to systemic acquired resistance (SAR) and expressed before appearance of infection symptoms. Tee *et al*., (2009) demonstrated profiles of 173 genes identified in the transcripts of oil

palm roots infected with *Ganoderma* by using cDNA microarray method. In line with this, Al-Obaidi *et al*., (2010) have reported a fungal resistance gene, polygalacturonase-inhibiting protein (*EgPGIP*) were identified during BSR infection by *Ganoderma*. PGIP are known as plant defense cell wall glycoprotein which functions in inhibiting the growth of fungus producing PG during infection.

Another recent approach based on the quantification of ergosterol, fungal sterol known as membrane component of fungi by using high-performance liquid chromatography (HPLC) was investigated. Although this technique is not as accurate as molecular markers but it provides a fast and reliable method under laboratory condition (Chong *et al*., 2012).

On the other hand, techniques such as E-nose, tomography imaging and spectroscopic imaging were developed to monitor BSR detection in oil palm plantations (Mazliham *et al*., 2007; Md Shakaff *et al*., 2009). The advantages of these methods are non time consuming and convenient, nevertheless further testing were required for broad application purposes (Hushiarian *et al*., 2013).

2.2.6 Control strategy

The prospects of developing oil palm resistant progenies against *Ganoderma* attack is agreed mostly as one of the future control for BSR disease (Turner, 1981; Corley & Tinker, 2003; Idris, 2009; Hushiarian *et al*., 2013). Currently, there is no any genetic resistance present in existing palms; however the study in Sumatera, Indonesia which showed slower rate of disease incidence in African progenies

compared to local Deli progenies might be a key step for this approach (Akbar *et al*., 1971). de Franqueville *et al*., (2001) demonstrated significant differences in BSR disease incidence were noted between the *Ganoderma* families and clones tested. Successful method of artificial inoculation of *Ganoderma* in oil palm has facilitated screening for resistant progenies to BSR in nursery (Corley & Tinker, 2003; Idris, 2009). By using this technique Ariffin *et al*., (1995) have shown no significant differences between 20 progenies tested against *Ganoderma* infection. In another study, Rees *et al*., (2007) have demonstrated shading and temperature effects as influential factor in causing the disease. Idris (2009) mentioned that among 43 progenies tested, a tolerant resistant progeny (DxP, Zaire x Cameroon) was detected and will used for future breeding purposes.

Meanwhile other methods such as surgery, soil moulding, and isolation trenching system which have been employed in field trials resulted less effective and insignificant practices. Surgery was conducted to excise diseased tissues or lesions according to palm age by using a chisel (Turner, 1981). Once the lesion was excised, the surface was treated with protective covering to avoid entry of microbes and insects. This method were reported more successful in older palms, over 12 years old compared to young palms due to greater disease lesion and harder stems in old palms (Turner, 1981). Implementing soil moulding technique around the base of disease palm after surgery showed increase in vigour and yield of oil palm (Lim *et al*., 1993). Both of these methods are able to prolong the economic life span of *Ganoderma* infected oil palm but less ineffective in controlling BSR disease. Isolation of infected palms by trenching system to avoid

root contact with healthy palms depends on the type of soil and therefore the maintenance cost is considerably high.

Fungicide treatment for *Ganoderma* infection is a difficult task due to inappropriate delivery method and timing of application whereby lesions are usually established before external symptoms appear (Turner, 1981). Attempts to apply systemic fungicides such as combination of carboxin and quintozene by using trunk injection method in field trial were reported with promising recovery of infected palms (George *et al.*, 1996). These fungicides may function primarily to interfere the growth development of the plant rather than to prevent *Ganoderma* infection (Corley & Tinker, 2003). Pressurised trunk injection method to deliver hexaconazole fungicide showed significantly lower mortality rate of oil palm compared to untreated plants, treatment without pressure injection and soil drenching treatment (MPOB Annual Research Review, 2002). However fungicide treatments are not practiced commercially due to inconclusive results in controlling the disease.

Up to current knowledge, sanitation practice in existing plantations prone to BSR disease and at time of replanting is considered as the most significant method to prevent *Ganoderma* BSR infection. This approach is based on the assumption that BSR incidence increases from one generation of oil palm to subsequent one and the spread of the disease occurs through root contact (Ariffin *et al*., 2000; Flood *et al*., 2000; Sanderson *et al*., 2000; Corley & Tinker, 2003). The possible treatments and clean clearing technique were described by Turner (1981). Removal of inoculums source from stumps, trunks and roots were

recommended by making dig isolation trench or sanitation holes and shred the infected materials into small pieces and left to decay. Another practice is to plant new seedlings away from former planting area, whereby Corley & Tinker (2003) has suggested that seedlings were to be planted down the centre of interlines (4.5 m from old stumps) to avoid potential infection. Based on a trial results described by Chung *et al*., (2011), proper sanitation management during replanting stage have reduced BSR incidence compared to high percentage of the disease was noted in areas where sanitation practice was neglected.

The contribution of biological control towards *Ganoderma* control strategy has gained interest by researches as an alternative solution for BSR. These were directed trough manipulation of biocontrol agents such as fungi, endophytic bacteria and mychorriza. Among them the antagonistic fungi, *Trichoderma* spp. was widely studied to control *Ganoderma* infection (Sariah & Zakaria, 2000; Soepna *et al*., 2000; Susanto *et al*., 2005; Nur Ain Izzati & Abdullah, 2008; Alizadeh *et al*., 2011; Naher *et al*., 2011). Soepna *et al*., (2000) has mentioned that *Ganoderma* infected material should be treated with *Trichoderma* biofungicide albeit the effect were not conclusive in their report. Glasshouse and field trial study by Susanto *et al*., (2005) has shown that disease incidences were lower with plants treated with *T. harzianum*. Paterson (2006a) has mentioned the application of *Trichoderma* in oil palm should treat wisely as the contamination of the final product should be avoided as this fungus are able to secrete toxic secondary metabolites. Recent studies have shown production of cell wall degrading enzyme, chitinase and expression of essential genes in oil palm treated

with *Trichoderma* which are involved in plant defense and antioxidant scavenging mechanism (Naher *et al*., 2011). Endophytes as biocontrol agent were also screened in glasshouse study to inhibit spread of *Ganoderma* (Sapak *et al*., 2008). Arbuscular mycorrhiza (AM) which promotes the plant growth and development was also approached as an alternative control measure of *Ganoderma* pathogen (Sariah & Zakaria, 2000).

The other point of view to control *Ganoderma* infection is from considering their mode of attack involving lignin biodegradation, a typical white rot process (Paterson, 2007). This subject of matter and possible approaches has been extensively addressed by Paterson (2007), Paterson et al., (2008; 2009). In an earlier report, Paterson (2006b) had discussed on the secondary metabolites derived from the lignin of host trees which may play a role as diagnostic tool for the oil palm disease. It's well known that ligninolytic enzyme system posses by white rot fungi are responsible for lignin degradation of plant cell wall. Therefore Paterson *et al*. (2008) had suggested that (i) various parameters such as pH, temperature, and oxygen transfer (ii) high carbon and nitrogen content and (iii) halides, chelators, heavy metals and other reducing agent could inhibit ligninolytic enzymes produced by *Ganoderma* and to be tested in laboratory condition before applying in field with appropriate method. In addition Paterson *et al*., (2009) had proposed method of developing oil palm modified with lignin content which is more resistant to *Ganoderma* disease by manipulating the technology used in other plants and within the various transformations in oil palm such as determination of lignin content of *Bt* oil palm.

2.3 Chitosan

2.3.1 Definition, properties and application of chitosan

Chitosan is a β-(1-4)-linked N-acetyl-D-glucosamine biopolymer which exhibits non-toxic and biodegradable properties are derived by deacetylation of chitin (Shahidi *et al.*, 1999; Hirano & Nagao, 1989). The deacetylation process includes the removal of acetyl group by heating chitin in alkaline media, whereby the acetamide group at the position 2 of carbon is transformed into primary amino group (Il'ina & Varlamov, 2004). This primary amine grouping position 2 differentiates chitosan from cellulose as shown in Fig 2.2 (Kumar *et al.*, 1999). It is a high molecular weight which has a mean molecular mass of up to 1 MD, approximately 5,000 U of chain length depending on the product (Rhoades & Roller, 2000). Chitosan has a pK_a value about 6.3; at the lower pH values the molecule is cationic and water soluble as amines are protonated (Kumar *et al.*, 1999; Rhoades & Roller, 2000). Meanwhile at higher pH value the amines are deprotonated and become insoluble (Rhoades & Roller, 2000).

The physical properties such as solubility, degree of deacetylation and molecular weight are used to determine the antifungal activity of chitosan (Hirano & Nagao, 1989; Park *et al.*, 2002). Higher degree of deacetylation increases antifungal activity due to the formation of polyelectrolyte complexes (Park *et al.*, 2002). Kendra *et al.*, (1989) had mentioned that maximal antifungal activity were

chitosan oligomers of seven or more residues. The application of chitosan on various fields is shown in Table 2.2.

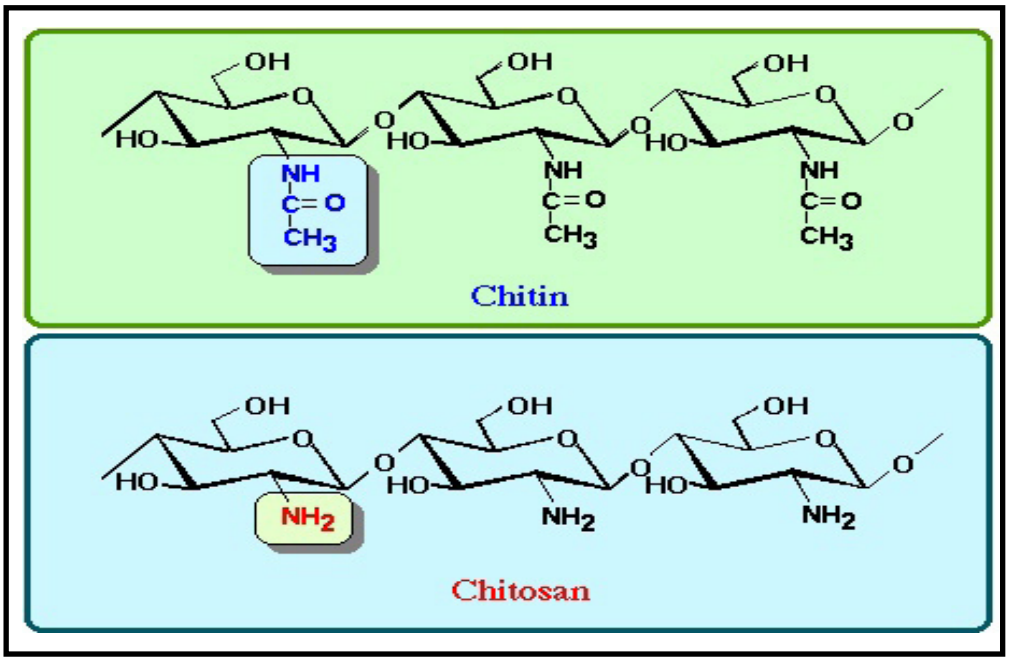

Figure 2.2: Chemical structure of Chitin and Chitosan (source from Dalwoo Corporation: http://dalwoo.tripod.com/structure.htm/, Accessed 20/1/2009)

Table 2.2: Application of chitosan in various fields

Application	Examples
Water treatment	Removal of metal iron, Flocculants/Coagulant, Filtration
Pulp and Paper	Surface treatment, Photographic paper, Carbonless copy paper
Medical	Bandages, Sponges, Artificial blood vessels, Tumor inhibition, Membranes, Dental plaque inhibition, Contact lens, Controlled release of drugs, Bone disease treatment
Cosmetic	Make up powder, Nail polish, Moisturizer, FixturesBath, lotion, Face hand and body creams, Toothpaste, Foam enhancing
Biotechnology	Enzyme immobilization, Protein separation, Chromatography, Cell immobilization, Cell recovery, Glucose electrode
Agriculture	Seed coating, Leaf Coating, Hydrophonic/ Fertilizer, Controlled agrochemical release
Food	Removal of dyes, solids, acids, Preservatives, Colour stabilization, Animal feed additives
Membranes	Reverse Osmosis, Permeability control, Solvent separation

Source: Li *et al*., 1992

2.3.2 Antimicrobial properties of chitosan

Antimicrobial effects of chitosan on wide range of microorganism have been well documented through *in vitro* and *in vivo* studies in literatures. The efficacy of chitosan as an antimicrobial agent varies depending on the type of microorganism, type and concentration of chitosan used (Benhamou, 1992; Bautista-Banos *et al*., 2006). Falcon-Rodriquez *et al*., (2012) had reviewed differences in the potency or level of inhibition by chitosan on plant pathogens, bacteria and fungi. Other important factor that contributes to the antimicrobial activity is chitosan's physical state, molecular weight (MW, degree of deacetylation (DA) and environmental

conditions (Goy *et al*., 2009; Falcon-Rodriquez *et al*., 2012). Chitosan in the form of solution has better inhibitory effect than in the dry samples due to the inability to release their energy in chemical bond to initiate reaction in the solid state (Falcon-Rodriquez *et al*., 2012). Generally, lower MW and DA of chitosan have been demonstrated and observed with greater antimicrobial activity (Jung *et al*., 2002; Andres *et al*., 2007). Reducing both of these parameters influence the solubility and charge of chitosan molecule by producing higher positive charged of free amino groups which directly correlate with higher antimicrobial effect (Goy *et al*., 2009; Falcon-Rodriquez *et al*., 2012). Chitosan is also known to act well when the pH is below 6 or pKa (protonation) between 6,3-6,5 whereby the amino groups are positively charged and enable to interact with negatively charged components of microbial cell walls (Shahidi *et al*., 1999). Environmental pH and temperature also affects the biological activity of chitosan (Sudarshan *et al*., 1992; Tsai & Su, 1999).

2.3.3 *In vitro* antifungal activity

The inhibition of mycelial growth and spore germination of pathogenic fungus have been demonstrated on *in vitro* studies using growth media incorporated with chitosan. To cite some of the studies the effectiveness of chitosan under *in vitro* condition were as shown in Table 2.3.

The effect of chitosan on fungal morphology was demonstrated by El Ghaouth *et al*. (1992a) and was observed that *Rhizopus stolonifer* treated with chitosan showed excessive branching of mycelium and suggested that these changes are related to the effect exerted by chitosan indirectly on the hyphal wall formation. Further morphological observation was carried out in *R. stolonifer* and it showed swelling, excessive branching, abnormal shape and size reduction of hyphae (El Ghaouth *et al*., 1992b). Moreover in that study ultrastructural examination of *R. stolonifer* revealed that the abnormal hyphaes were altered and surrounded by loosened cell walls. Besides that, El Ghaouth *et al*. (1994) had observed cell wall loosening, vacuolation and protoplasm disintergration in *Pythium aphanidermatum*. The observations were similar as reported by Ait Barka *et al*. (2004) and noted the large vesicle or empty cells devoid of cytoplasm in the fungal cell of *Botrytis cinerea.* However the cellular leakage is not related to abnormal cell wall when in contact with chitosan (Baustita-Banos *et al*., 2006)

Table 2.3: Summary of studies reporting on *in vitro* fungal growth in response to chitosan application

Crop	Pathogen	Chitosan concentration	References
Pea-pod	*Fusarium solani* f. sp. *phaseoli* and *F. solani* f. sp *pisi*	12-18 µg/ml^{-1} mycelium growth was inhibited	Hadwiger & Beckman, 1980; Kendra & Hadwiger, 1984
Common fruits and vegetables	*Rhizopus stolonifer, Alternaria alternata, Botrytis cinerea* and *Collectotrichum gloeosporioides*	0.75-6.0 mg ml-^{1}	El Ghaouth *et al*., 1992a
Strawberry	*Botrytis cinerea* and *Rhizopus stolonifer*	1.5-6.0 mg/ml reduce radial growth, 6.0 mg/ml spore germination and germ tube length reduced	El Ghaouth *et al*., 1992b
Cucumber	*Pythium aphanidermatum*	100-400 µg/ml, at 400 µg/ml completely inhibit mycelial growth	El Ghaouth *et al*., 1994
Tomato	*Fusarium oxysporum*	0.1-1.0 mg/ml media incorporated	Benhamou *et al*., 1994
Tomato	*Botrytis cinerea and Penicillium expansum*	0.01-1 % w/v radial growth of both fungus was reduced 0.01-1 % w/v inhibited spore germination of *Penicillium expansum* and at concentration higher than 0.01 % for *Botrytis cinerea*	Liu *et al*., 2007
Tomato and grapes	*Colletotrichum* sp.	1.5-2.5 % w/v and the most effective concentration that reduce 50 % of radial growth (EC_{50}) as 2.28 % w/v	Munoz *et al*., 2009
Groundnut	*Puccina arachidis*	100-1000 µg g^{-1} induced low percentage of germination of uredospores	Sathiyabama & Balasubramaniam, 1998
Grapevine	*Botrytis cinerea*	Radial growth was reduced on 5 % and 10 % chitogel media	Ait Barka *et al*., 2004
Grapevine	*Botrytis cinerea*	75-150 mg1^{-1}	Trotel-Aziz *et al*., 2006

2.3.4 Mode of action

Mechanism models

The direct effect of chitosan on the growth of fungi has not been fully illustrated and the mechanism is still unclear, but three possible mechanisms have been proposed. The polycationic nature of chitosan involving interaction between positively charged chitosan molecules (the amino groups) and negatively charged microbial cell membranes (phospholipids head group and protein component) has been described as a mode of action of chitosan (Baustita-Banos *et al*., 2006; Young & Kauss, 1983). This interaction results (i) changes in membrane cell wall permeability causing osmotic imbalance which leads to inhibition of microbial growth and (ii) hydrolysis of cell wall component which contributes in the leakage of proteinaceous and other intracellular constituents (Hadwiger & Kendra, 1981; Shahidi *et al*., 1999; Sudarshan *et al*., 1992; Chen *et al*., 1998). These reactions depend on the charge density on the microbial cell surface which determines the amount of adsorbed chitosan (Goy *et al*., 2009). Therefore it was suggested that this interaction mode is meditated upon the host microorganism factor (Masson *et al*., 2001). Another hypothesis by Hadwiger & Loschke (1981) stated that chitosan could interact with fungal DNA and alter its conformation by interrupting and inhibit mRNA and protein synthesis. The binding of chitosan and inhibition of mRNA synthesis occurs when the chitosan is released from the cell wall of fungal pathogen by plant hydrolytic enzymes which cause penetration into nuclei of fungi and disruptions of RNA and protein functions (Hadwiger *et al*.,

1986). The ability of chitosan which act as an effective chelating agent was also reported as a mechanism of action by chitosan. Chitosan are able to selectivity bind trace metals which consequently inhibits the production of toxin and mycelial growth (Cuero *et al.,* 1991). This phenomenon has been clearly illustrated by the inhibition of toxin production by *Aspergillus flavus* and Alternaria *alternata* f.sp. on liquid culture respectively (Reddy *et a*l., 1998; Cuero *et al*., 1991).

Elicitation of plant response

Plant signalling response

The signalling pathway which involves the starting point of the chitosan elicitor contacts the cell surface and to the final response is not well explained. Kauss (1994) described that when an oligosaccharin binds to the protein on the plant cell surface, an unknown mechanism take over which responsible to trigger defense responses by plant. Experimental evidence has shown that the depolarization of the plant membrane and changes in calcium concentrations in cell due to the presence of oligosaccharin may involved in the signalling pathway (Young & Kauss, 1983; Kohle *et al*, 1985; Amborabe *et al*., 2008).

Amborabe *et al*., (2008) have demonstrated chitosan effect membrane depolarization and increase of pH in culture media of *Mimosa pudica* motor cells. This report also stated that that plasma membrane H^+-ATPase as the primary site on chitosan's reaction initiation and exhibited its inhibitory effect on the proton pumping and the catalytic activity of the enzyme. Hydrogen peroxides (H_20_2) are

activated by oxidative enzymes due to the alkalinisation of extracellular membrane site (García-Brugger *et al*., 2006). H_2O_2 has a crucial role in plant chemical defense signal in triggering plant defense gene and served as intermediary for crosslinking proteins to reduce pathogen infection (Fauth *et al*., 1998; Ryan, 1999). Myelin basic protein kinases (MBPKs) which initiate the signaling pathway that contribute to plant defense genes had been studied on plants treated with chitosan (Stratmann & Ryan, 1997). Furthermore chitosan is able to stimulate the accumulation of jasmonic acid as the secondary signals mediate the defensive genes in plants. Doares *et al*. (1995) reported that the jasmonic acid increases in a time frame after treated with chitosan in young tomatoes.

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Physiological defense response

The stimulation of physiological changes is the mechanism where plants are able to develop structural barriers to avoid fungal penetration through leaves and roots (Baustita-Banos *et al*., 2006). Many reports have been documented regarding the potential of chitosan to induce formation of structural defense barriers. The reduction of stomatal opening and the production of hydrogen peroxide, H_2O_2 in guard cell promote decrease of stomatal aperture were identified in response to chitosan and this consequently restrict the access of fungus into inner leaf tissues through stomata (Lee *at al*., 1999).

Lignification to strengthen the cell walls of plants that are less susceptible to fungal pathogens can be induced by chitosan on various crops (Vander *et al*.,

1998 and Baustita-Banos *et al*., 2006). Benhamou *et al*. (1994) had stated that chitosan stimulate either the de-novo synthesis of phenolic substances as first mechanism to inhibit growth of fungus and the β-1, 3-glucanase as second defense response to restrict invasion of fungal cell. Chitosan and its derivatives with different physico-chemical properties were found as good inducer for lignin formation in wheat leaves (Vander *et al*., 1998). Reddy *et al*., (1999) had reported stimulation of phenolic acid and accumulation of lignin precursors in chitosan treated wheat seeds. Benhamou & Theriault (1992) had reported formation of physical barriers in tomato root tissues and leaves treated with chitosan. The visible defense structures due to the effect of chitosan treatment were observed through electron microscope and results (1) formation of papillae in cortex and endodermis tissue (2) occlusion of xylem vessels with a bubble-like structures and (3) a thick secondary coating material. The similar formation of papillae structure along host cell wall was observed in cucumber plants grown hydrophonically supplemented with chitosan and inoculated by P. *aphanidermatum* (El Ghaouth *et al*., 1994). Chitosan treated roots in this plant were observed with contorted epidermal cells (El Ghaouth *et al*., 1994). Thickening of host cell, formation of hemispherical and spherical protuberances along cell wall and occlusion of intercellular spaces with fibrillar material were the structures found in first layer of tissue in bell pepper fruit (El Ghaouth *et al*., 1994).

Pathogenesis related proteins (PRs) and phytoalexins

Chitosan plays an important role in activating plant defense related enzyme in plants. One of the extensively studied is the increase and accumulation of plant glucanohydrolases such as chitinase, chitonase and β-1, 3-glucanase in both leaves and roots (El Ghaouth *et al*., 1992a, 1994a; Trotel-Aziz *et al*., 2006; Prapagdee *et al*., 2007). Chitosan stimulated chitinase activity when directly applied to excised tissue of strawberry fruit (El Ghaouth *et al*., 1992a). Meanwhile chitosan amended with nutrient solution stimulated chitinase, chitonase and β-1, 3-glucanase enzymes in leaves and roots of cucumber plants (El Ghaouth *et al*., 1994). In groundnut treated with chitosan, increase of salicylic acid, chitinase and other glucanase were identified (Sathiyabama & Balasubramaniam, 1998). The presence of chitinase in crops treated with chitosan was also observed in soybean (Prapagdee *et al*., 2007) and grape vine leaves (Trotel-Aziz *et al*., 2006). Hadwiger & Beckman (1980) found that chitosan act as an elicitor of phytoalexin production in pea pod- *Fusarium solani* interaction, which could inhibit fungal growth and infection of the fungal. The increased enzyme activity of phenylalanine ammonia-lyase (PAL) was detected in grape vine leaves treated with chitosan (Trotel-Aziz *et al*., 2006), the polyphenoloxidase (PPO) and peroxidase (POD) (Liu *et al*., 2007) was indicated in tomato fruits which provide protection against severe fungal infection.

Inductions of phtoalexins compounds by chitosan known as toxic to various microbes were studied in several plant systems. Hadwiger & Beckman (1980) found that chitosan act as an elicitor of phytoalexin production in pea pod-

Fusarium solani interaction. Aziz *et al*., (2006) had demonstrated accumulation of phytoalexins in grapevine leaves treated with chitosan oligomers. Terpenoids and sakuranentin belongs to the same structural family of phytoalexin were induced in rice seedlings leaves treated with chitosan (Agarawal *et al*., 2002). Chitosan also found to involve in the biosynthesis of sesquiterpenes in tomato leaves (Walker-Simmons *et al*., 1984).

2.3.5 Chitosan in fungal plant disease control

Chitosan application in controlling various fungus causing plant diseases have been reported in previous literatures (Baustista-Banos *et al*., 2006; El Hadrami *et al*., 2010; Falcon-Rodriquez *et al*., 2012). However, the practical approach of chitosan on plants against pathogenic fungus are complex to be conducted (Falcon-Rodriquez *et al*., 2012), and it depends on the pathosystem, type of chitosan and derivative, concentration and formulation (El Hadrami *et al*., 2010; Falcon-Rodriquez *et al*., 2012). Chitosan are usually applied as soil amendment, seed coating and foliar spraying or in combination of this application as reviewed by El Hadrami *et al*., (2010). The ability of chitosan to reduce or control plant diseases are often associated with elicitation of plant defense responses as discussed previously in the section 2.3.4.

In term of monocots, applications of chitosan on rice disease control have been extensively studied. Lin *et al*., (2005) reported low molecular weight of chitosan showing positive control effect against inoculated *Pyricularia grisea* fungus on rice seedlings. A recent study showed application of chitosan were able

to control *Rhizoctonia solani*, causal agent of rice sheath blight disease in rice whereby inhibition of disease incidence and lesion length were reported 31-84 % and 66-91 % respectively (Liu *et al*., 2012). In addition, chitosan treatment on wheat seeds at the rate of 2-8 mg/ml were able to increase the germination rate and reduce the infection of seed borne *F. graminearum* (Reddy *et al*., 1999). Sharathchandra *et al*., (2004) have shown that seed treatment, foliar spray and combination of both applications were successful in controlling downy mildew disease in pearl millets caused by *Sclerospora graminicola* under control and field conditions. In conjunction with this, Manjunatha *et al*., 2008 reported the disease incidence was reduced up to 75.8 % and 79.08 % under glass house and field condition respectively when seeds were treated *via* seed priming at 0.5-3 g/ kg .Faoro *et al*., (2008) exhibited that foliar spraying method on barley plants was able to reduce local and systemic infection by powdery mildew pathogen *Blumeria graminis* f. sp. hordei.

A significant number of published studies are available on the effect of chitosan application on dicot plants (Baustista- Banos *et al*., 2006; El Hadrami *et al*., 2010). Under controlled bioassay, defense and protection reaction were observed in the detached grapevine leaves treated with chitosan and later inoculated with. *Botryis cinerea*. Tomato seedlings and seeds dipped in chitosan solution significantly reduced seed borne diseases caused by *Fusarium oxysporum* f. sp. radicis-lycopersici and *Phytium aphanidermatum* (Benhamou & Theriault, 1992; Benhamou *et al*., 1994; El Ghaouth *et al*., 1994). El Mougy *et al*., (2006) reported that application of chitosan by using soil amendment, root dip and seed

bed treatment at 0-6 g per 1000 units (soil or water) could reduce fungal diseases on tomato caused by *Rhizoctonia solani, Fusarium solani and Sclerotium rolfsii*. Foliar spraying of chitosan on cucumber plants reduced gray mould disease caused by *Botryis cinerea* (Ben Shalom *et al*., 2003; Ben Shalom & Falik, 2003).

CHAPTER 3

GENERAL METHODOLOGY

3.1 Source of fungus

Four isolates of *Ganoderma* (GBB 7, GBB 18, GBLS and GBU) were obtained from severely infected oil palm trees. GBB 7 and GBB 18 isolates were obtained from *Ganoderma* fruiting bodies in Balau Estate, Semenyih, Selangor Malaysia (Fig 3.1 a). GBLS isolate was collected from Lian Seng Estate, Melaka, Malaysia and GBU isolate was obtained from Plant Pathology Department, University Putra Malaysia.

The collection of spores was carried out as described by Pilotti *et al*. (2002; 2003; 2004). The spores were collected from fresh fruiting bodies in the plantations by suspending a Petri plates covered with clean Whatman filter paper 1-2 cm under the basidiocarp for 24 hours. The whole fruiting bodies and suspended plates were covered with clean plastic wrap to reduce air-borne contamination (Fig 3.1 b). The collected spore prints were processed in lab for future experiments (Fig 3.1 c). The spore discharge has been reported maximum at night (Ho & Nawawi, 1986) and it was also important to collect samples during two consecutive non- rainy days to avoid the spores being washed out from the plates by rain.

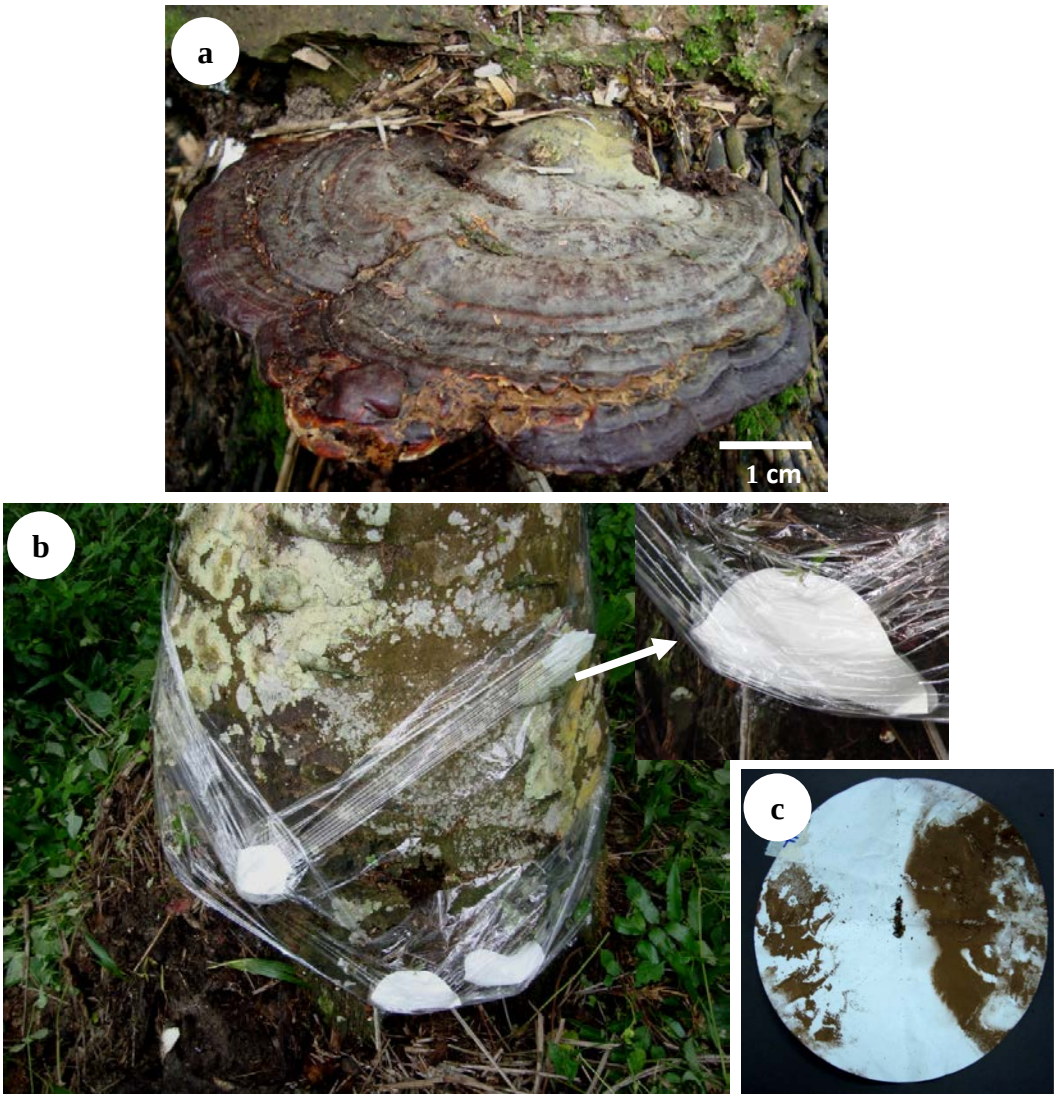


Figure 3.1: Collection of fruiting bodies and spore print of *Ganoderma* (a) Fruiting body of *Ganoderma* infected stem of oil palm (b) Spore collection by suspending plates containing filter paper and the fruiting body covered with plastic wrap (c) Collected spore print deposit on filter paper

3.2 Fungal isolation and maintenance

The pure cultures of mycelium were prepared from *Ganoderma* fruiting bodies according to Utomo *et al*., (2005) with modifications. Fruiting bodies were washed with sterile water and internal tissue was excised into 2 x 2 mm pieces. The tissue were immersed in 10 % (w/v) sodium hypochlorite for 5 minutes, rinsed twice with sterile water and blotted dry on sterile filter paper. Pieces of axenic fruiting body tissue were placed onto Potato Dextrose Agar medium (PDA; Merck, USA) amended with 250 μg/ml streptomycin sulphate and incubated for seven days in the dark, at room temperature. The pure cultures growing out of fruiting body tissue were transferred on PDA medium. Subcultures were prepared and maintained by plating a 5 mm plug of mycelium from the growing margin of the colony onto fresh PDA medium every two weeks.

3.3 Preparation of spore suspension

Spore prints on filter paper were air-dried for 2 to 3 hours and washed to remove contaminants and debris. Previous studies did not report any contamination on the collected spores from field samples so they were immediately suspended in sterile water (SW) and used for further experiments (Ho & Nawawi, 1986; Pilotti *et al*., 2002; 2003; 2004; Rees *et al*., 2012). However, in the present study a high incidence of contamination occurred using these methods, and the washing step was essential for the preparation of clean spore suspensions. The filter papers with

spore prints were cut into pieces approximately 0.5 x 0.5 cm and placed into 1.5 ml centrifuge tube. The spores from the filter paper were scrapped off using forceps and 200 µl of SW was added to each tube to make suspensions (Fig 3.2). The tubes were placed in a rotary shaker at 240 rpm for 10 minutes. Then the tubes were vortexed for few seconds and centrifuged at 12, 000 rpm for 1 min to precipitate the spores. The supernatant was discarded and SW was added again followed by the same steps. These steps were repeated three times and the final spore suspensions were made up to 200 µl with SW.

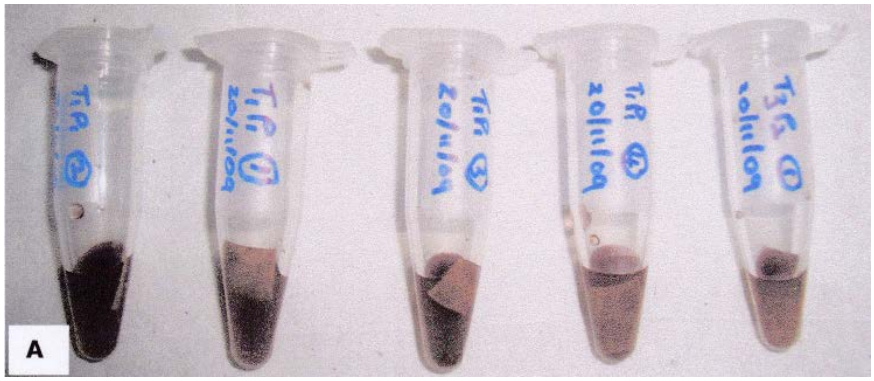


Figure 3.2: *Ganoderma* spore suspensions in 1.5 ml microcentrifuge tubes prepared from collected spore prints on Whatman filter paper

3.4 Plant source

Oil palm ramets were supplied by Applied Agriecological Research (AAR) Sdn Bhd, Sungai Buloh, Selangor, Malaysia. The rooted 90-day ramets of three to four leaf stages had been propagated through somatic embryogenesis and acclimatized in their oil palm nursery.

3.5 Artificial infection of *Ganoderma* on oil palm seedlings

3.5.1 Preparation of *Ganoderma* inoculum on rubber wood blocks (RWB)

Ganoderma inoculum on rubber wood blocks was prepared according to Sariah *et al*., (1994); Nur Ain Izzati & Abdullah (2008) and Sapak *et al.*, (2009). Rubber wood blocks (RWB) measuring 6 x 6 x 12 cm were washed, autoclaved at 121° C for 45 minutes and packed into polypropylene bags. Approximately 100 ml of molten sterilized malt extract agar (MEA) were poured on the blocks as supplementary nutrient for *Ganoderma*. The blocks were rotated to ensure proper coating of the agar on the blocks and allowed to cool for about 5-6 hours. Upon cooling and solidification, culture isolates from pre-cultured Petri plates were used to inoculate the blocks (one Petri dish per block). The inoculated blocks were then stored in the dark at 28 ºC for 8-10 weeks, to allow complete colonization of the blocks (Fig 3.3 a, b).

3.5.2 Artificial inoculation of oil palm seedlings

The oil palm seedlings were transplanted in polypropylene bags (15 x 9 cm) containing a medium comprised 2 part black soil: 1 part red soil. During planting, the primary roots of the seedlings were wounded by slicing a 1 cm^2 section from the root epidermis using a sharp scalpel (Rees *et al.* 2007) and the completely colonized blocks were placed directly in contact with the roots to avoid root

contact with external sources (Sariah *et al*., 1994; Nur Ain Izzati & Abdullah, 2008) as shown in Fig 3.3 c, d. The inoculated seedlings were maintained in shade house with regular watering (Fig 3.3 e).

3.6 Preparation of Ganoderma- selective medium (GSM)

Detection of *Ganoderma* sp. isolates from infected tissue of roots, bole and lower stem was performed on Ganoderma-selective medium (GSM) as described by Ariffin & Idris (1991). GSM medium consists of two components which were prepared separately as part A (antimicrobial) and B (non- antimicrobial) respectively. Part A contained 300 mg streptomycin sulphate, 100 mg chloramphenicol, 285 mg pentachloronitrobenzene (PCNB), 130 mg 25 % a.i. Ridomil, 150 mg Benlate, 1.25 g tannic acid, 2 ml lactic acid and 20 ml 95 % ethanol in 80 ml sterile distilled water. Part B contained 5 g bacto-peptone, 0.25 g $MgSO_4$, $7H_20$, 0.5 g K_2HPO_4 and 20 g agar in 900 ml of distilled water and was autoclaved at 121° C for 15 min. After cooling of Part B solution, Part A was added and mixed thoroughly before dispensing into Petri dishes.

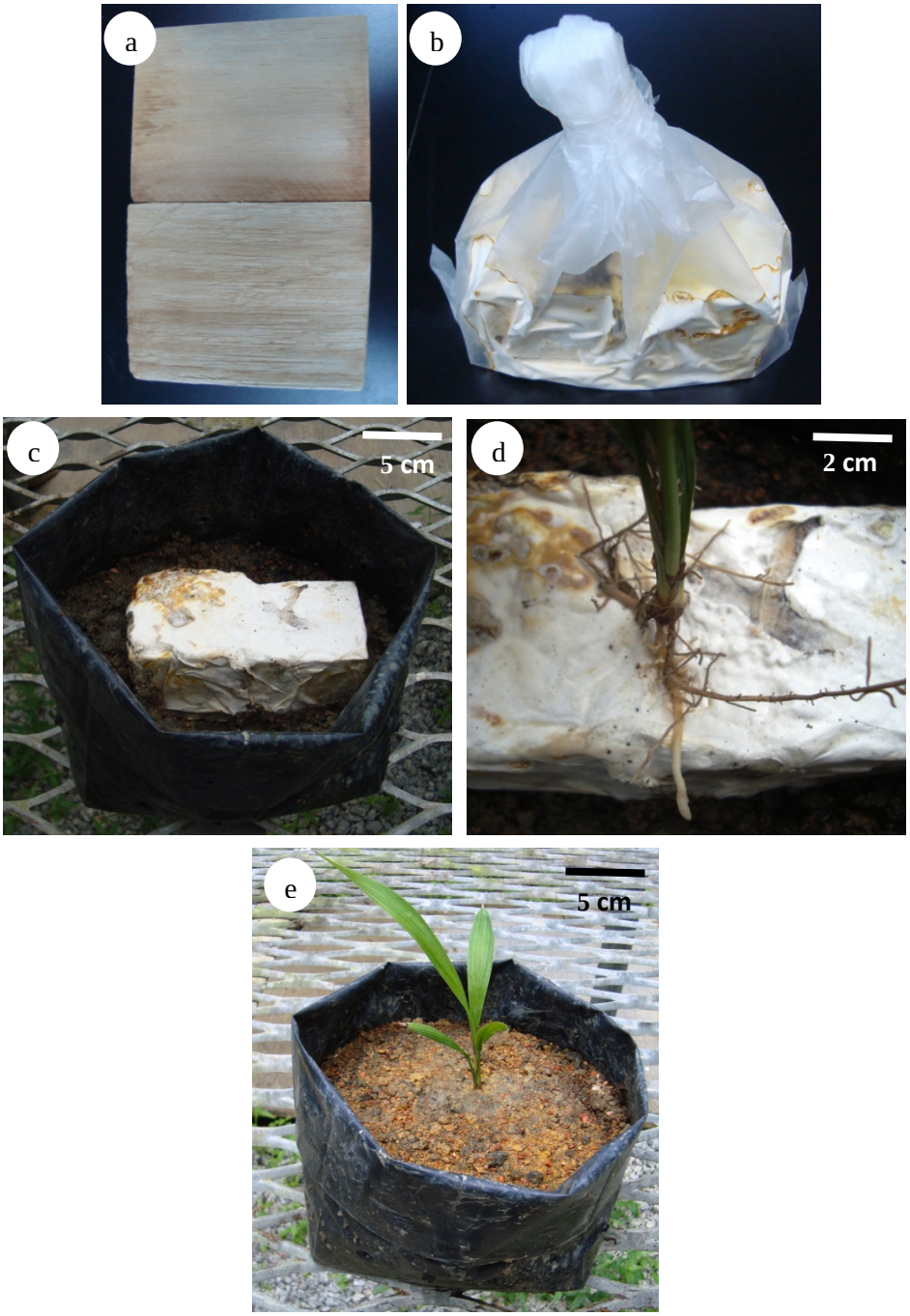


Figure 3.3: Rubber wood block (RWB) inoculation technique of oil palm seedlings with *Ganoderma* isolates. (a) RWB measuring 6 x 6 x 12 cm washed and autoclaved before inoculation (1 cm) (b) Blocks completely colonized with *Ganoderma* inoculum in MEA medium in polypropylene bags (c) RWB inoculum transferred into polypropylene bags containing unsterilized soil (d) Roots of oil palm seedlings transplanted directly in contact with blocks to avoid root contact with external sources (e) Inoculated oil palm seedling maintained under shade house conditions

3.7 Molecular diagnosis of *Ganoderma* sp.

3.7.1 DNA extraction of *Ganoderma* mycelium

Total DNA of fungal cultures was isolated using a modified CTAB method (Goes-Neto *et al.,* 2005). Seven day old mycelium cultures grown on Petri dishes were scraped using a flat spatula and ground rapidly with a pestle in mortar containing liquid nitrogen. Samples of approximately about 50 mg were weighed and placed in a preheated (65° C) 1.5 ml microcentrifuge tube containing 500 µl of extraction buffer and incubated at 65° C for overnight. The extraction buffer contained 2 % CTAB (hexadecyltrimethylammonium bromide), 100 mM Tris-HCl, 1.8 M NaCl (sodium chloride), 20 mM EDTA (ethylene-diaminetetraacetate), 1 % PVP (w/v; polyvinylpyrrolidone) and sterile distilled water. The samples were centrifuged (Eppendorf 5810R) at 10,000 g for 10 minutes and the DNA were extracted by adding an equal volume of chloroform-isoamyl alcohol (24:1) and centrifuged at 10,000 g at 4 °C for 10 min. The aqueous suspensions were transferred into new tubes and precipitated with cold isopropanol (1:1). The white pellets obtained were washed three times with 100 µl of cold ethanol 70 % and centrifuged at 10,000 g for 5 min at each time discarding supernatant and finally were diluted with 30 µl of sterile distilled water containing RNase (100 µg/ ml).

3.7.2 DNA extraction from oil palm plant infected with *Ganoderma*

Ganoderma infected oil palm samples were recovered from -80 ℃ storage and the DNA was extracted by using the method described by Moller *et al*., (1992). Samples (50 -60 mg) of infected roots and stem were ground in a sterile pestle and mortar containing liquid nitrogen. The obtained powdered tissues obtained were extracted three times with 1 ml of methanol containing 0.1 % mercaptoethanol and centrifuged between each extraction. The pellets were allowed to dry completely at room temperature and were transferred into 1.5 ml microcentrifuge tubes. 500 µl containing 100 mM Tris, pH 8.0, 10 mM EDTA, 2 % SDS (w/v; sodium dodecyl sulphate) and 50 µg of proteinase K (1 mg/ml) stock solution warmed to 60 °C was added to each pellet and incubated at 60° C for an hour. This was followed by addition of 140 µl 5 M NaCI and 65 µl of 10 % w/v CTAB and the suspension was further incubated at 65 ℃ for 10 min. An equal volume (700 µl) of chloroform-isoamyl alcohol (24:1) was added and incubated at room temperature for 30 min and centrifuged at 10,000 and 4 ℃ for 10 min. The supernatant was transferred into new tubes and 225 µl of NH4Ac (sodium acetate) was added with gentle mixing, incubated for an hour on ice and finally centrifuged at 4 ℃ 10,000 g or 10 min. Then, the supernatant was transferred into new tubes again and the DNA precipitate by adding 0.6 volume of cold isopropanol and centrifuged immediately at 10,000 g for 5 min. The visible DNA pellets were washed three times with 100 µl cold 70 % ethanol and centrifuged at 10,000 g for 5 min, each time discarding the supernatant. Finally, 30 µl of sterile distilled water containing RNase (100 µg/ ml) were added to each pellet.

3.7.3 Quantification of DNA concentration

The concentration of the extracted DNA was measured using a Nanodrop spectrophotometer (Thermo Scientific, ND-1000) at the absorbance range 260-280 nm.

3.7.4 PCR amplification

Specific primers designed as GAN 1F (5' TTG ACT GGG TTG TAG CTG 3') and GAN 2R (5' GCG TTA CAT CGC AAT ACA 3') which would produce a 167-bp PCR product, were used to identify *Ganoderma* spp. The primers were generated from the internal transcribed spacer region 1 of rDNA of *Ganoderma* (Utomo *et al*., 2005).

PCR amplification was performed by using a thermocyler (G-Storm, GS-1) programmed as 5 minutes pre-heating at 95 ℃ continued by 48 cycles of denaturation at 94 ℃ for 40 seconds, annealing at 45 ℃ for 40 seconds and extension for 45 seconds at 72 ℃ with a final 12 minutes extension at 72 ℃. The obtained PCR products were analysed by electrophoresis on 1.5 % (w/ v) agarose gel. The expected bands of DNA were visualized under UV light and photographed using a UV transillumiator (BioRad Gel Documentation XR System).

3.8 Preparation of chitosan solution

The entire chitosan product used in this study was purchased from Sigma Aldrich, USA. The chitosan solutions (Fig 3.4) were prepared by dissolving the powder in 1.0 % (w/v) glacial acetic acid and were adjusted to pH 5.6 with 1N sodium hydroxide (El Ghaouth *et al.*, 1991).

Figure 3.4: Chitosan solutions at different concentration according to the method described by El Ghaouth *et al*., (1991)

CHAPTER 4

EFFECT OF CHITOSAN ON *IN VITRO* GROWTH OF *GANODERMA*; CAUSAL PATHOGEN OF BASAL STEM ROT (BSR) DISEASE IN OIL PALM (*Elaeis guineensis*)

4.1 Introduction

Basal stem rot (BSR) is characterized as 'silent killer' due to its slow progression in expressing disease symptoms once the infection have severely established internally through oil palm root system and lower stem (Turner, 1981; Ariffin *et al*., 2000; Naher *et al*., 2013). Controlling basal stem rot (BSR) disease in oil palm remains as a challenging task due to insufficient knowledge to identify BSR pathogenic *Ganoderma* species (Rolph *et al*., 2000), determine mode of *Ganoderma* infection by spore or root to root contact (Rees *et al*., 2009; 2012) and diagnose crucial early infection symptoms (Nur Ain Izzati & Abdullah 2008; Sapak *et al*., 2008; Hushiarian *et al*., 2013; Naher *et al*., 2013). In addition the characteristic of the pathogen, *Ganoderma* sp. as a soil borne fungus associated with its persistent resting stages such melanized mycelium, basidiospores, chlamydospores and pseudosclerotia also contributes in difficulty to eradicate BSR disease (Susanto *et al*., 2005; Rees *et al*., 2009). These resting bodies of *Ganoderma* functions as food resources protector for survival under unfavourable

condition were able to form fruiting bodies and initiate infection on host plant such as oil palm (Darmono, 1998; Susanto, 2009).

At present, there is no any practical and conclusive solution in term of short or long term strategies to overcome this disease as discussed (Chapter 1; Chapter 2). Hence, the approach of this study was directed to introduce chitosan, the natural and biodegradable polymer as a new control strategy in BSR disease management context. In conjunction to this, previous studies have shown promising results by using chitosan application against wide array of pathogenic fungi associated with plant diseases under in *vitro* and in *vivo* condition as described (Chapter 1; Chapter 2).

Thus, this chapter was undertaken to evaluate *in vitro* efficacy of chitosan on the growth of *Ganoderma* BSR pathogen. The aim of the study is to (1) screen pathogenic isolate of *Ganoderma* sp. causing BSR disease under shade house condition (2) analyse various types and concentrations of chitosan for the control of mycelial growth and basdiospore germination of *Ganoderma* sp. under *vitro* condition and (3) elucidate the effect of chitosan on the process of spore germination and structural morphology of the fungal mycelium.

4.2 Materials and methods

4.2.1 Identification of *Ganoderma sp*.

Four culture isolates; GBB 7, GBB 18, GBLS and GBU used in this study was isolated and maintained as described (Section 3.2).

Macro and micro morphological identification

GBLS and GBU isolates collected from respective sample sources have been identified as *Ganoderma* sp. previously based on the macro and micro morphology characteristic upon collection (results not shown). The basic macro morphology of GBB 7 and GBB 18 isolates of *Ganoderma* sp. such as badiocarp characteristic which includes the pileus, margin of pileus, pore surface and spore characters were noted as acknowledged (Gottlieb & Wright 1999; Moncalvo & Ryvarden, 2000; Seo & Kirk, 2000).

For internal micro morphology observation, the obtained pure mycelium cultures and spores was stained with lactophenol blue and examined using an optical microscope (AZ 100, Nikon Japan).

Cultural identification

The obtained pure cultures of the isolates were grown in Potato Dextrose Agar (PDA) medium and the cultural characteristic were identified (Idris, 2009).

Molecular identification

For further confirmation of the identity of the fungal DNA of GBB 7, GBB 18 GBLS and GBU cultures was isolated using a modified CTAB method and *Ganoderma* sp. was confirmed by polymerase chain reaction (PCR) using specific primers, GAN 1F and GAN 2R as described in Section (3.7.1, 3.7.2, 3.7.3, 3.7.4).

4.2.2 Pathogenicity test

The pathogenicity of GBB 7, GBB 18, GBLS and GBU isolates was tested by direct artifical inoculation of *Ganoderma* mycelium on oil palm seedlings using rubber wood block inoculation technique as outlined (Section 3.5.1 and 3.5.2).

This experiment was designed as a randomized complete block design with four replicates. Control experiments contained seedlings that were wounded and placed directly in contact with uninoculated RWBs. The experiment was repeated twice. The effect of *Ganoderma* infection on visible symptom progression and root infection on oil palm seedlings were assessed for six months as described (Rees *et al*., 2007). The seedlings were scored as 'infection' or 'non-infection', (percentage of seedlings infected) with the total number of seedling evaluated over the 6 months of post-inoculation. Percentage of infected roots was assessed based on the necrosis of adjacent roots sections with four replicates of roots from separate seedlings. The images of developed disease symptoms were photographed and microscopy observation was done on diseased tissues (Rees *et al*., 2009). To fulfill Koch's Postulates, the roots and bole tissues from inoculated

ramets were re-isolated on *Ganoderma* semi-selective medium (Ariffin & Idris, 1991) as described (Section 3.6).

4.2.3 *In vitro* antifungal assay

Preparation of chitosan solution

Preparation of chitosan solutions were done as described (Section 3.8). The chitosan product of low molecular weight from crab shell (viscosity= 20-300 cps with 75-85 % deacetylation), medium molecular weight from crab shell (viscosity= 200-800 cps with 75-85 % deacetylation), low viscosity from shrimp shell (viscosity= < 200 cps) and high viscosity from crab shell (viscosity= > 400 cps were purchased from (Sigma Aldrich, USA). The chitosan solutions from these products were prepared as stated in Section (3.6). PDA amended with three concentrations of chitosan (1.0, 2.0 and 3.0 % w/v) was prepared as described previously by Bell *et al*., (1998). Briefly to incorporate chitosan into PDA media, the chitosan and PDA solutions were autoclaved separately and mixed thoroughly before dispensed into 90 mm diameter Petri plates to obtain the desired concentrations of 1.0, 2.0 and 3.0 % (w/v) PDA without chitosan which consists of acetic acid was prepared as the control. The volume and pH of acetic acid used for all concentrations of chitosan and control solutions was standardized to keep the salt concentration constant.

Percentage inhibition of radial growth (PIRG)

A 6 mm diameter agar plug from 14 day-old pathogenic isolate of *Ganoderma* sp. (GBLS isolate) culture was placed in the centre of PDA plates with different treatments of chitosan at 1.0, 2.0 and 3.0 % (w/v) of concentrations. The plates were incubated in the dark at 30 ºC for 20 days.

Mycelium radial extension growth technique was determined by measuring colony diameters with a vernier caliper and express as average diameter (mm) (El Ghaouth *et al*. 1992a, 1992b). The growth inhibition was calculated as the percentage inhibition of radial growth (PIRG) relative to control using the standard formula described by Siddiqui *et al*., (2008) as below:

PIRG (%) = (R1-R2 / R1) X 100 %

Where R1 is the radial growth diameter of *Ganoderma* colony in control plates and R2 is the radial growth diameter of *Ganoderma colony* in chitosan-PDA treatment plates. The experiment was conducted twice and arranged in a completely randomized design with six replicates of five plates. This experiment was repeated with a selected chitosan product which shows inhibitory effect on growth of GBLS isolate at different concentrations of 0.5, 1.0, 1.5 and 2.0 % (w/v).

Preparation of spore suspensions

The clean and contaminate free spore suspensions were prepared as described (Section 3.3). The concentration of spores in the suspension was determined with

a haemocytometer and adjusted to 1 x 10^{-6} spores / mL (El Ghaouth *et al*. 1992a, 1992b).

Basidiospore germination test

On the basis of results obtained from *in vitro* radial mycelia growth, the low viscosity chitosan was used in this experiment. Furthermore, the concentrations were selected based on a preliminary study on the sensitivity of chitosan with a range of concentrations on the basidiospore germination of *Ganoderma* (data not shown). The concentration of spores in the suspension was determined with a haemocytometer and adjusted to 1 x 10^{-6} spores / mL (El Ghaouth *et al*. 1992a, 1992b). Aliquots of 20 ml of the suspension were pipette and streaked on to each control (consists of acetic acid only) and treatment plates amended with low viscosity chitosan at 0, 0.001, 0.01, 0.1 and 1.0 % (w/v). The plates were incubated in the dark at 30 ºC for 14 days. Germination of 100 spores per plate was determined microscopically and a spore was considered germinated when the length of the germ tube equaled or exceeded the length of the spore (El Ghaouth *et al.*, 1992a, 1992b). The experiment was conducted twice and arranged in a completely randomized design with six replicates of five plates.

4.2.4 Microscopy

Light microspocy: Images of *Ganoderma* mycelium from control and treatment plates with chitosan were obtained using a Nikon 80i bright field compound

microscope (Nikon, Japan). *Scanning Electron Microscopy (SEM)*: Fungal mycelium from control and chitosan treated plates were cut at the edge of the colony, fixed onto the aluminium stub and stabilized by the carbon tape adhesive. The images were examined using a scanning electron microscope (FEI/ Quanta 400 F**)** under Environmental SEM (ESEM) vacuum mode using 10kV of electron beam with magnification ranging from 1000 x to 8000 x.

4.2.5 Statistical analysis

The data on the frequency of infection between the isolates on oil palm seedlings, differences among the treatments for percentage inhibition of mycelia growth and percentage of spore germination were pooled, arcsine-tansformed before analysis and subjected to ANOVA with a probability of 95 % confidence limits using Tukey Multiple Comparison post-tests (at $\leq P\ 0.05$). The analysis was done using GraphPad Prism Software version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com)

4.3 Results

4.3.1 Identification of *Ganoderma* sp.

Macro and micro morphological identification

The collected fruiting bodies specimens from Balau Estate Semenyih were examined for morphological characteristic and used as preliminary descriptions of *Ganoderma* before fungal isolation were done. The basidiocarp was in bracket-like shape, thick, and hard (Fig 4.1 a). The upper surface of pileus was uneven, brittle and consists of various concentric zones (Fig 4.1 a). The colour of the pileus surface varies from orange, light brown, dark reddish brown, to dark brown colour (Fig 4.1 a). The margin of the pileus was incurved, thick and white in colour (Fig 4.1 b). The pore surface was brown in colour (Fig 4.1 b) and the collected spore print was brown in colour (Fig 4.1 c).

Microscopically examined hyphae was noted with thin walled and clamp connections structures (Fig 4.2 a). The basidiospores were brown in colour, ovoid shape, smooth and slightly truncate at apex (Fig 4.2 b). The spores consist of endospore which is encased in an outer membrane (Fig 4.2 b). The observed outer membrane of the spores was found to be ruptured or intact (Fig 4.2 c).

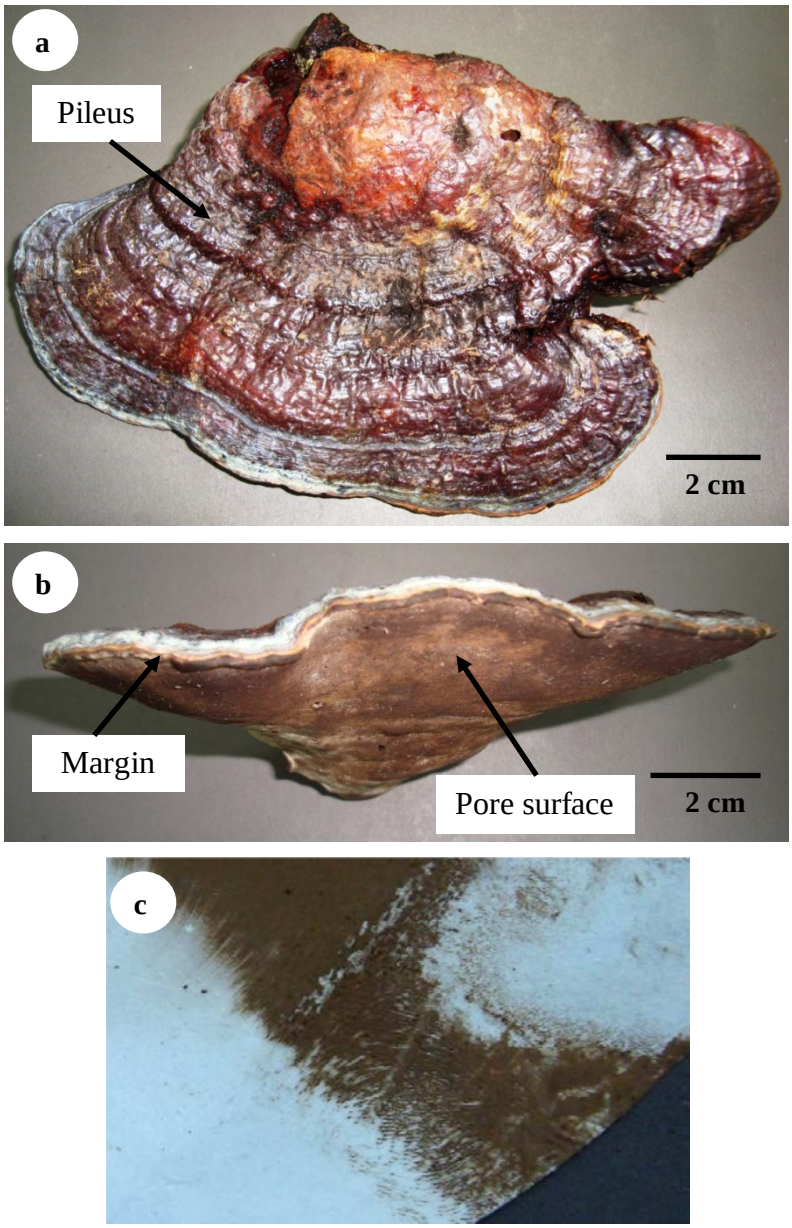


Figure 4.1: Macro-morphology characteristic of *Ganoderma* basidiocarp obtained from field sample (a) The basidiocarp showing the morphology of pileus in detail (arrow) (b) The white margin and brown pore surface of basidiocarp (arrow) (c) Brown spore print deposit on filter paper collected from fruiting body of infected oil palm tree

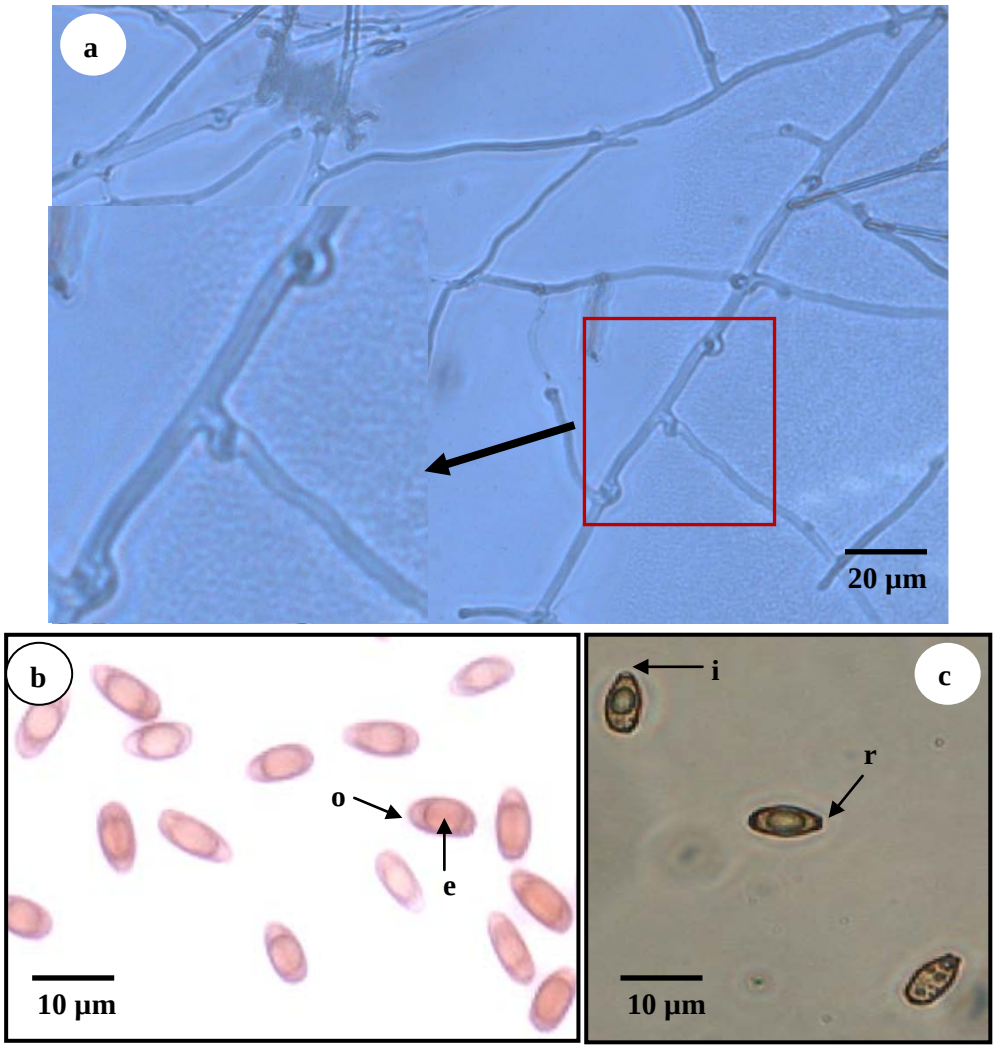


Figure 4.2: Microscopy characteristic of obtained pure mycelium culture and spores from field sample (a) Hyphae (arrow) showing presence of clamp connections, a common characteristic of basidiomycete (b) Basidiospores observed were brown in colour (c) Basidiospores observed under phase contrast; o: outer membrane, e: endospore, r: ruptured membrane, i: intact membrane

Cultural identification

The pure cultures of *Ganoderma* isolates; GBB 7, GBB 18, GBLS and GBU grown on PDA medium after two weeks of subculturing were shown in Fig 4.3. The mycelium of all the *Ganoderma* isolates was white in colour on the surface (Fig 4.3). On the reverse view, GBB 7, GBB 18 and GBLS isolates showed presence of visible brown pigmentation (Fig 4.3 a, b, c). The cultures from these isolates formed undulating surface on the pigmented region which buckled the agar medium surface (Fig 4.3 a, b, c). These observations were not noted on GBU isolate (Fig 4.3 d).

Molecular identification

Genomic DNA from pure mycelium culture isolates of GBB 7, GBB 18, GBLS and GBU was confirmed as *Ganoderma* sp. by the PCR assay in which an expected band of 167 bp was detected (Fig. 4.4).

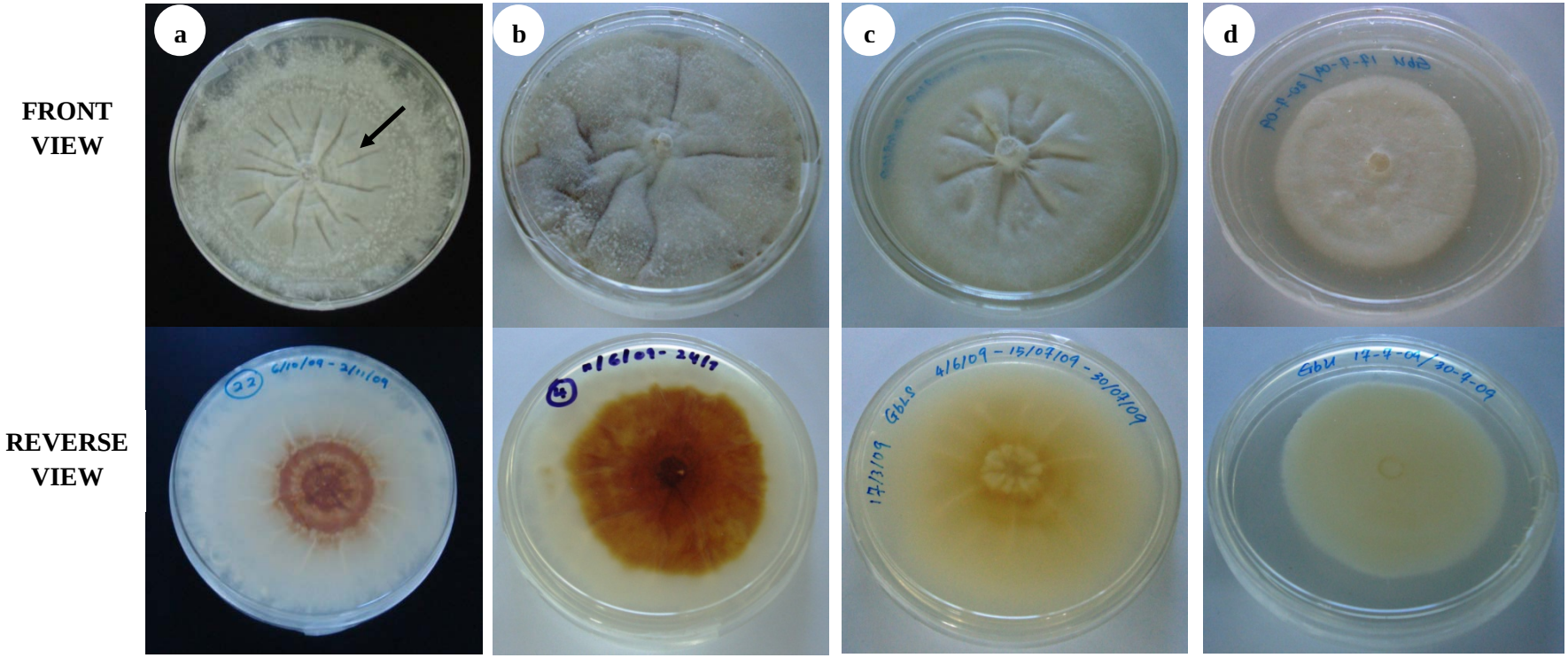


Figure 4.3: Cultural morphology of *Ganoderma* isolates grown on PDA medium after two weeks of subculturing (a) GBB 7 (b) GBB 18 (c) GBLS and (d) GBU; Front view of the plates showed white mat mycelium surface on PDA medium and reverse view of the plates showed brown pigmentation on GBB 7, GBB 18 and GBLS isolates with undulating surface (arrow) that buckled up the agar surface

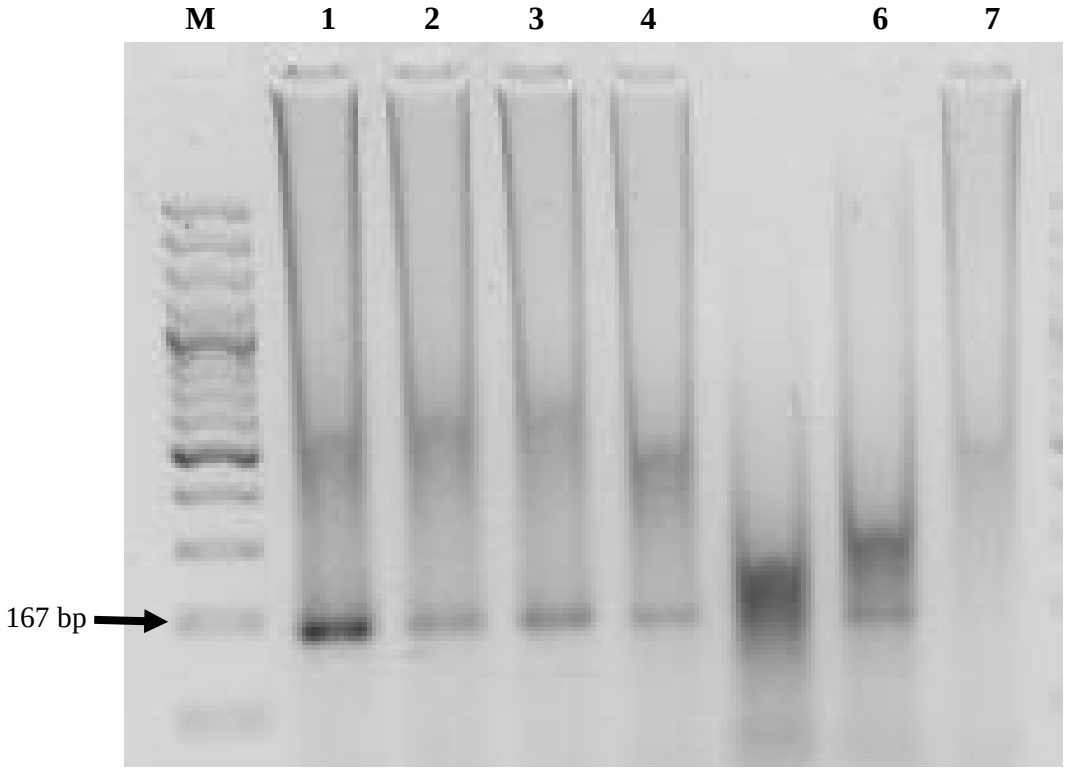


Figure 4.4: PCR amplification of 167 bp (arrow) DNA fragment of *Ganoderma sp.* region; Lane M: 100 bp marker, Lane 1-4: Mycelium sample of *Ganoderma* isolates: GBB 7, GBB 18, GBLS and GBU respectively, Lane 6: Positive control (*Ganoderma* sp. mycelium) and Lane 7: Negative control (sterile distilled water)

4.3.2 Pathogenicity test

This present study showed differences and variability in the pathogenicity and aggressiveness between the screened *Ganoderma* isolates of GBB 7, GBB 18, GBLS and GBU (Fig 4.5). GBLS appeared as the most pathogenic and aggressive isolate ($P \leq 0.05$) causing highest proportion of infected oil palm seedling and lesion roots over 80% and 50% respectively after 6 months of inoculation (Fig 4.5 a, b). There were no significant differences existed ($P \leq 0.05$) in disease infection rate on oil palm seedlings inoculated with GBB 7, GBB 18 and GBU isolates (Fig 4.5 a, b).

The oil palm seedlings artificially inoculated with GBLS isolate manifested visible and internal disease infection symptoms during 6 months period as presented in Fig 4.6. Sequential of progressive infection on seedlings coincide well with earlier observations as described by Sariah *et al.*, (1994) and Rees *et al.*, (2009). The indicative foliar symptoms infection of yellowing and desiccation from the oldest to youngest leaves began to appear after 1 month of inoculation. Within 2 to 3 months after inoculation, a white mass of mycelia observed at stem base expeditiously developed into button-like tissues initiated formation of sporophores and followed by a distinct stipe (Fig 4.6 a, b). Mature bracket shaped basidiocarp with typical white margin was prominent during 4 to 5 months after inoculation (Fig 4.6 c). Infected roots with fungal mycelia mass were marked with melanized thick pseudo-sclerotium as sheathe on the root surface emerging from infected stem base (Fig 4.6 d). Upon cross section as shown in Fig

4.6 e, infected roots emerged from stem base showed brown discolouration and the lesion on bole tissue appeared as light brown area of rotting tissues surrounded by darker brown line addressed as reaction zone (Fig 4.6 e). In between of these infected area and healthy tissue, a yellow tissue was noted as transitional zone formed as a result of defense mechanism of the plant as shown in Fig 4.6 e (Turner, 1981). Advance stage of infection revealed lesion of the bole spread into stem region as the fungus colonized rapidly (Fig 4.6 f) and the bole tissue has been decayed extensively with exposed white cellulose (Fig 4.6 g). At the end of inoculation period (after 6 months), severe foliar symptoms of GBLS inoculated seedlings were distinct compared to healthy (uninoculated) seedlings (Fig 4.6 h) and positive re-isolation of GBLS from infected roots, stem, bole tissues on GSM was accomplished on GSM medium (Fig 4.6 i).

Production of sporophores and internal infection symptoms on bole and stem region were not apparent on the seedlings inoculated with GBB 7, GBB 18 and GBU isolates and the effort to re-isolate the fungi on GSM was not achieved. Taken all these together, GBLS is assured as a pathogenic isolate causing basal stem rot (BSR) infection and was used for subsequent studies.

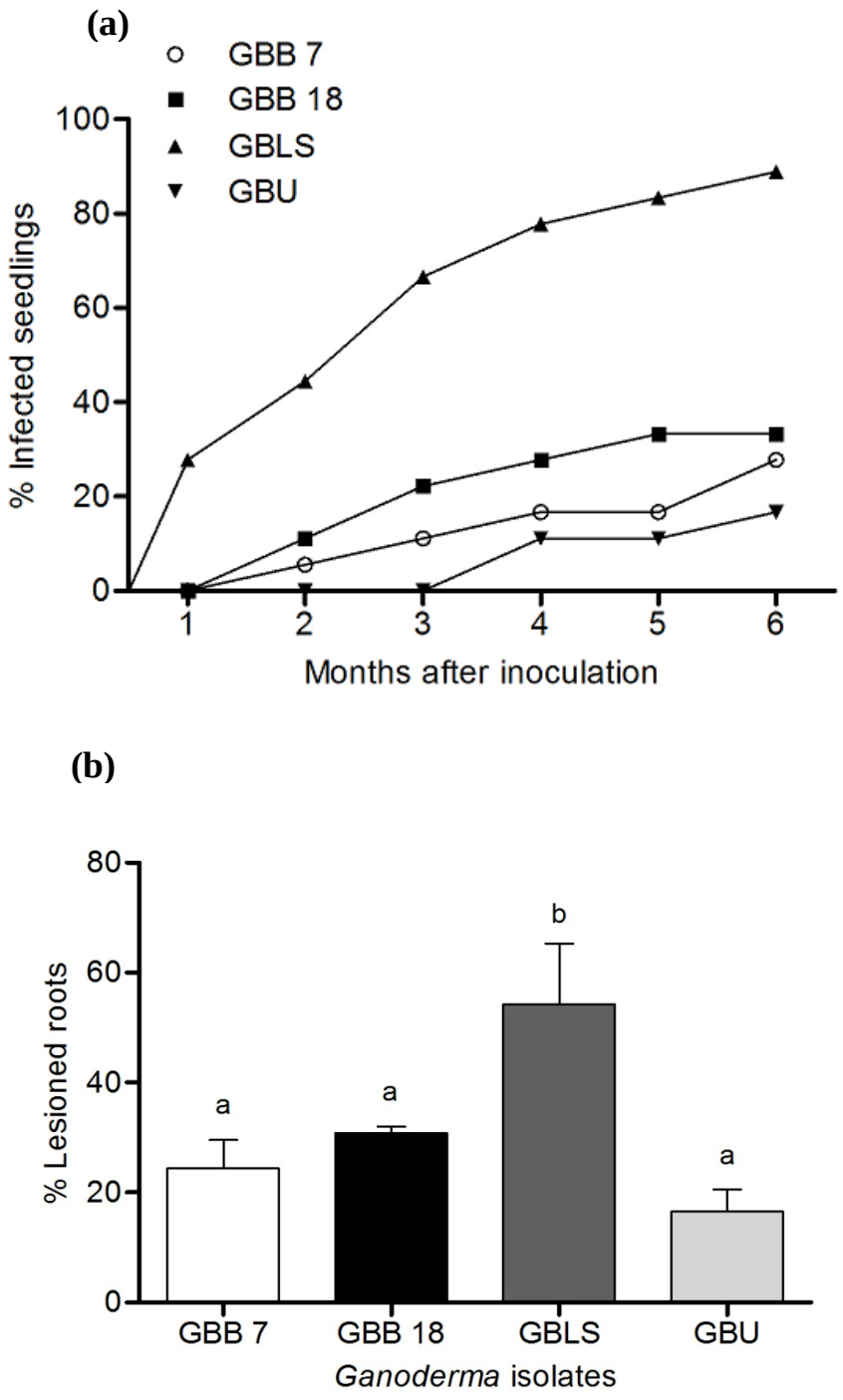


Figure 4.5: Pathogenicity and aggressiveness screening of *Ganoderma* isolates on artificially inoculated oil palm seedlings under shade house condition (a) Incidence of basal stem rot (BSR) and (b) Percentage of root infection after 6 months of post-inoculation. Bars represent mean ± standard deviation. Means with the same alphabet are not significantly different ($P \leq 0.05$) by Tukey Multiple Comparison post test

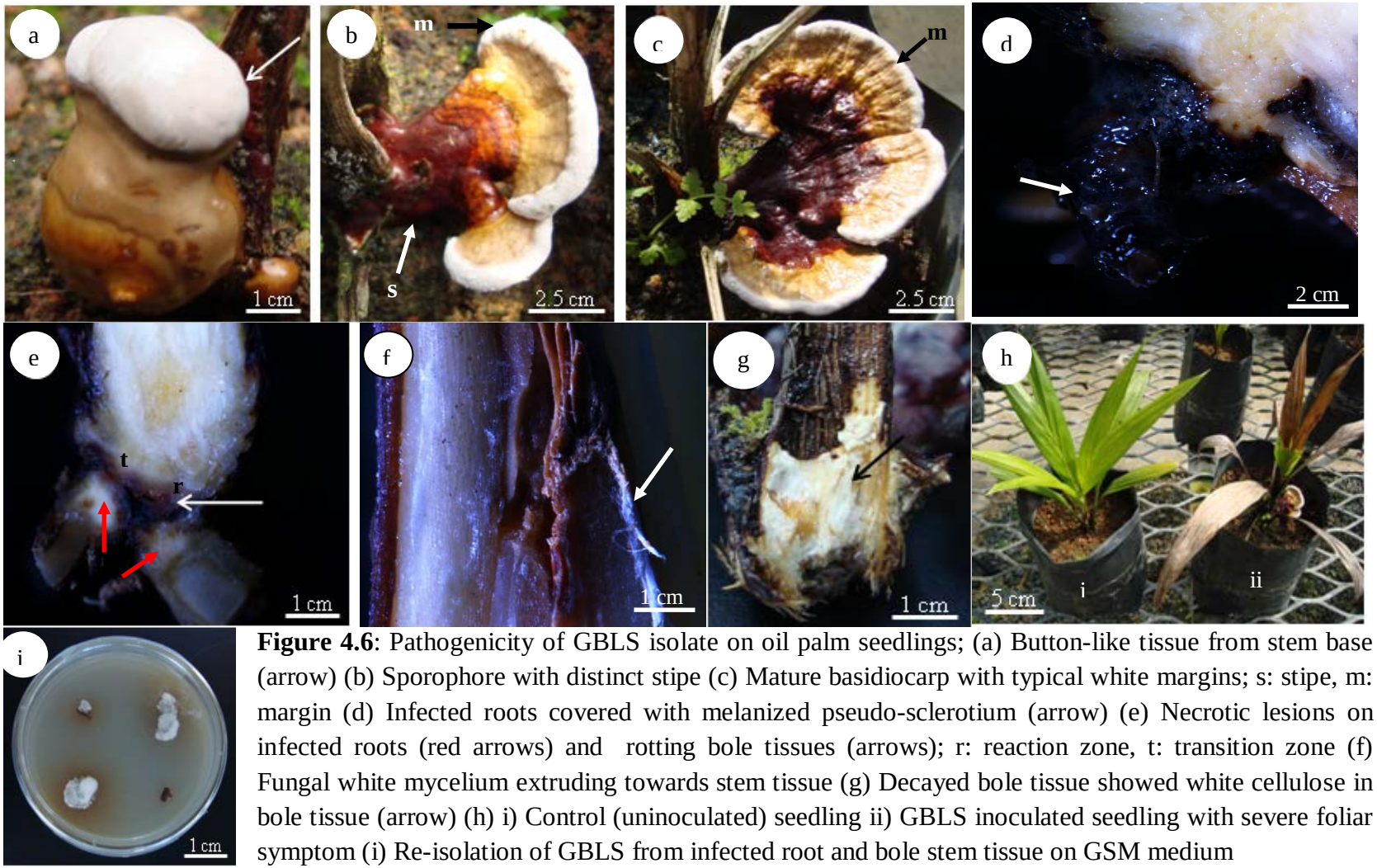


Figure 4.6: Pathogenicity of GBLS isolate on oil palm seedlings; (a) Button-like tissue from stem base (arrow) (b) Sporophore with distinct stipe (c) Mature basidiocarp with typical white margins; s: stipe, m: margin (d) Infected roots covered with melanized pseudo-sclerotium (arrow) (e) Necrotic lesions on infected roots (red arrows) and rotting bole tissues (arrows); r: reaction zone, t: transition zone (f) Fungal white mycelium extruding towards stem tissue (g) Decayed bole tissue showed white cellulose in bole tissue (arrow) (h) i) Control (uninoculated) seedling ii) GBLS inoculated seedling with severe foliar symptom (i) Re-isolation of GBLS from infected root and bole stem tissue on GSM medium

4.3.3 Effect of chitosan on mycelia growth of *Ganoderma* sp.

Mycelia growth of GBLS isolate was reduced effectively on media containing four treatments of chitosan; low viscosity (LV), high viscosity (HV), low molecular weight (LMW), high molecular weight (HMW) with all the tested concentrations after 20 days of incubation (Table 4.1). The pattern of inhibition at 2.0 % (w/v) concentration of LV chitosan displayed statistical differences ($P \leq 0.05$) compared to other types of chitosan (Table 4.1).

To determine the efficacy of LV chitosan on the mycelial growth of GBLS isolate in detail, a range of concentrations at 0.5 %, 1.0 %, 1.5 % and 2.0 % (w/v) was tested. From the results obtained, significant radial growth inhibition (at $P \leq 0.05$) was observed at 2.0 % (w/v) concentration after 20 days compared to control and other concentrations evaluated (Fig 4.7, 4.8). In this study, chitosan exhibited a fungistatic effect by markedly reducing the mycelial growth in dose-dependent manner (Fig 4.7, 4.8). The mycelial cultures of GBLS treated with chitosan at different concentrations continues to grow after being washed with sterile distilled water and transferred to new PDA medium (data not shown).

Table 4.1: Inhibition rate of chitosan treatments at different concentrations on *in vitro* mycelial growth of *Ganoderma* sp. after 20 days of incubation period

Percentage Inhibition of Radial Growth (%)				
Chitosan (%)	Low viscosity	High viscosity	Low molecular weight	Medium molecular weight
0.0	0.0 ± 0a	0.0 ± 0a	0.0 ± 0a	0.0 ± 0a
1.0	77.95 ± 0.79b	74.26 ± 0.55c	70.77 ± 0.52d	77.47± 0.17b
2.0	88.99 ± 0.32e	78.14 ± 0.26b	77.22 ± 0.44b	80.98 ± 0.99f
3.0	89.38 ±0.38e	88.05 ± 0.62e	77.55 ± 0.37b	88.41 ±0.05e

All the values represent mean ± standard deviation. Means with the same alphabet are not significantly different ($P \leq 0.05$) by Tukey's Multiple Comparison post-test

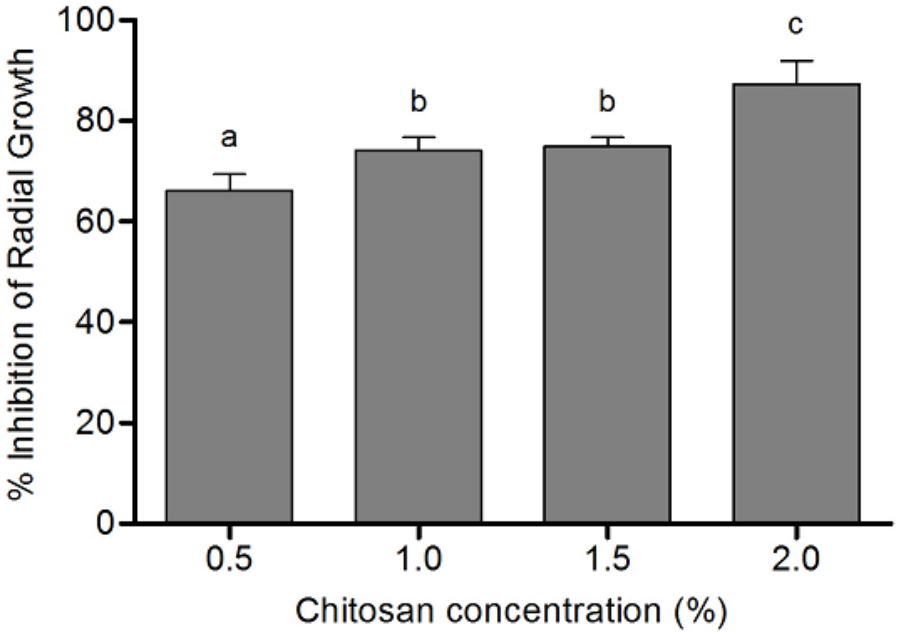


Figure 4.7: Effect of low viscosity chitosan at various concentrations on percentage inhibition of radial growth (PIRG) of GBLS isolate after 20 day of incubation. Bars represent mean ± standard deviation. Means with the same alphabet are not significantly different ($P \leq 0.05$) by Tukey Multiple Comparison post test

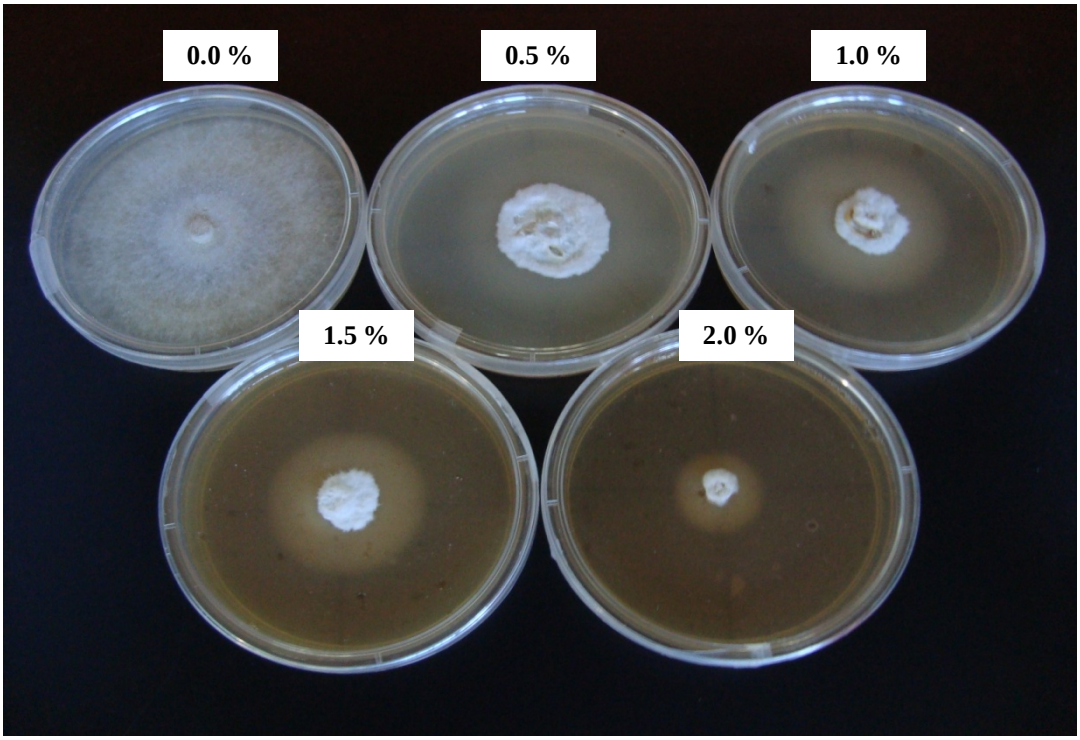


Figure 4.8: Inhibition of mycelia growth of GBLS isolate against low viscosity chitosan at different concentrations in PDA amended media after 20 days of incubation period.

4.3.4 Effects of chitosan on basidiospore germination of *Ganoderma* sp.

Chitosan concentrations tested for basidiospore germination assay appeared to be much lower compared to those needed to inhibit mycelial growth. The results as shown in Fig 4.9 demonstrated that the spores treated with chitosan medium at different concentrations (0.001, 0.01, 0.1 and 1.0 % w/v) affected the germination rate significantly ($P \leq 0.05$) compared to control treatment (29 %) after 14 days of incubation. This inhibitory effect was enhanced with increased chitosan concentration whereby the highest concentration of 1.0 % (w/v) chitosan treatment significantly ($P \leq 0.05$) reduced spore germination rate up to 5 %

followed by 0.1, 0.01and 0.001 % (w/v) (15.7, 14.5 and 10.3 % respectively). Microscopic observation of the spores exposed with different concentrations of chitosan revealed delay in sporulation and development of germ tube process after 14 days of incubation (Fig 4.10). In the control treatment, formation of germ tubes was observed within 4 to 6 days and actively expanded through elongation and branching of germ tubes during the incubation period (Fig 4.10 a, b). The spores showed increased effect of delaying germ tube emergence and elongation as the chitosan concentration marked up from 0.001, 0.01, 0.1, to 1.0 % (w/v) concentrations (Fig 4.10 c, d, e, f, g and h).

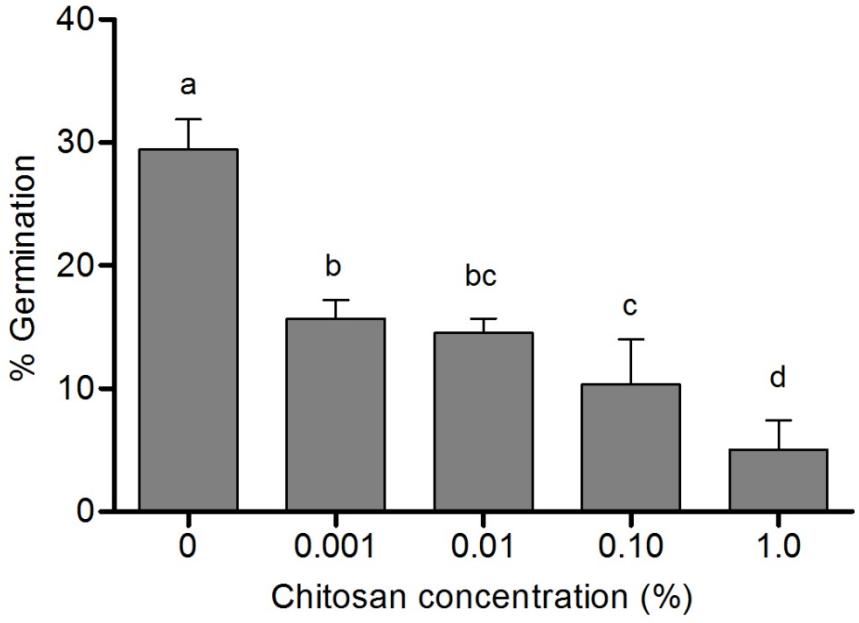


Figure 4.9: Effect of low viscosity chitosan at various concentrations on spore germination of *Ganoderma* after 14 days of incubation. Bars represent mean ± standard deviation. Means with the same alphabet are not significantly different ($P \leq 0.05$) by Tukey Multiple Comparison post test

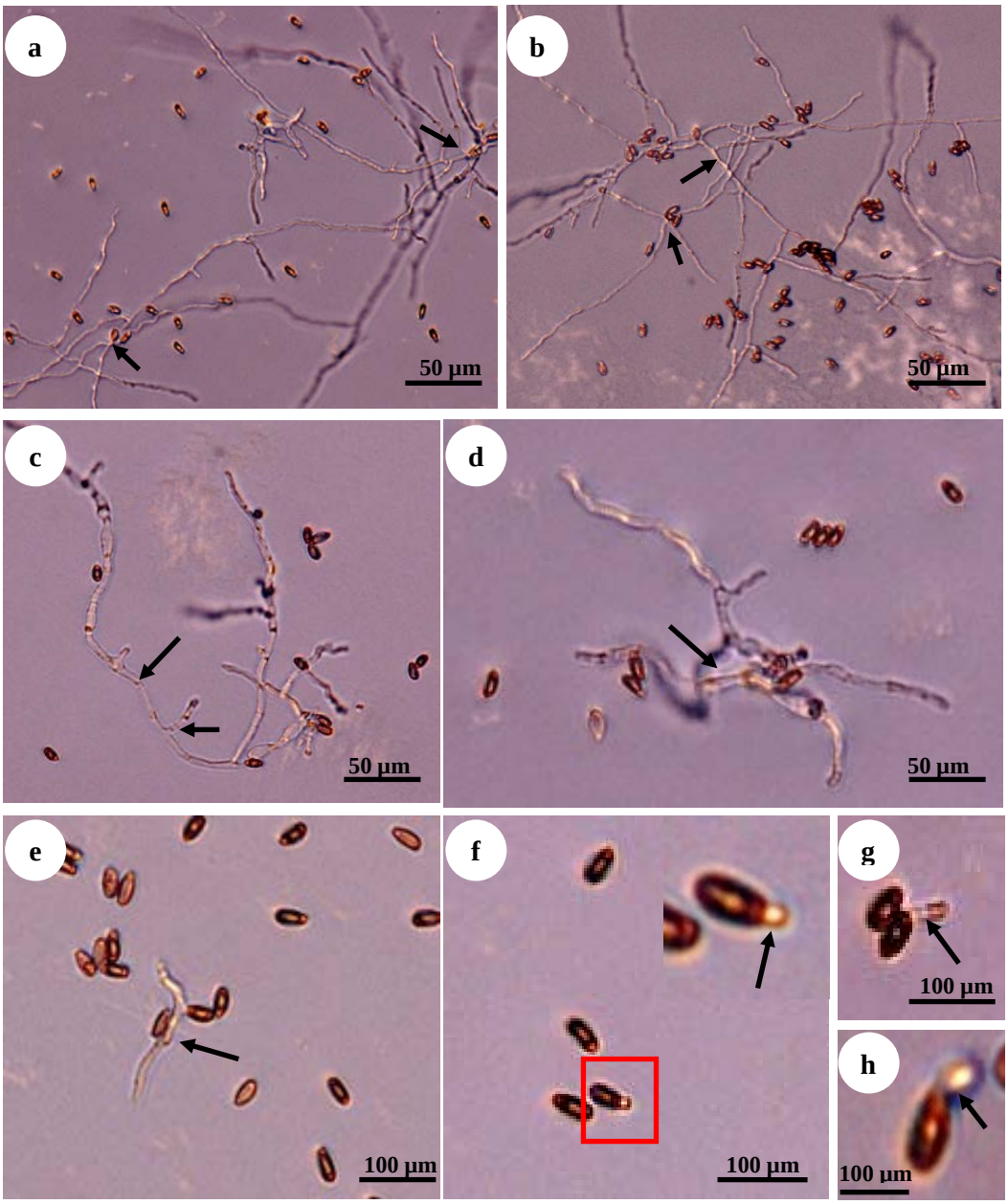


Figure 4.10: Effect of various concentrations of chitosan on basidiospore germination of *Ganoderma* after 14 days of incubation; (a, b) Control treatment (without chitosan) showing active sporulation, elongation and branching of germ tubes, Spores treated with increased chitosan concentrations showed greater effect of reduced germination rate and delay in the emergence of germ tubes (c) 0.001 % (d) 0.01 % (e) 0.1 % (f, g, h) 1.0 % (w/v). Note the germ tubes (arrows)

4.3.5 Microscopic observation on the effect of chitosan on morphological changes of *Ganoderma* sp.

Light microscopic observation showed the hyphae treated with 0.5 and 1.0 % chitosan concentrations contained small vesicles in the fungal cytoplasm (Fig 4.11 b, c). Comparison between untreated and chitosan-treated mycelium surface of *Ganoderma* using scanning electron microscopy provided a better evidence of variation in morphological alterations at all concentrations of chitosan. The control hyphae appeared to be fine with smooth surface (Fig 4.12 a, b). Corrugated and rough hyphal surfaces were observed at 1.0 % (w/v) and higher concentrations (Fig 4.12 c, d). Occurrences of excessive branching and hyphal swelling on mycelium were noted at 1.5 and 2.0 % (w/v) of chitosan concentrations (Fig 4.12 e, f). Branching protrusions or branching initials were noted on the hyphal tips, on the hyphae close to or distant from hyphal tips (Fig 4.12 e). Hyphae were found to be unevenly and abnormally swollen to differing degrees but were this not always observed (Fig 4.12 f). The surface of hyphae was also found to be severely damaged with retarded appearance (Fig 4.12 g). Furthermore at higher magnifications, extracellular layers were found surrounding the hyphae cell at 2.0 % (w/v) chitosan concentration (Fig 4.12 i). Changes in the spore morphology of *Ganoderma* treated with chitosan were not observed in any of the concentrations.

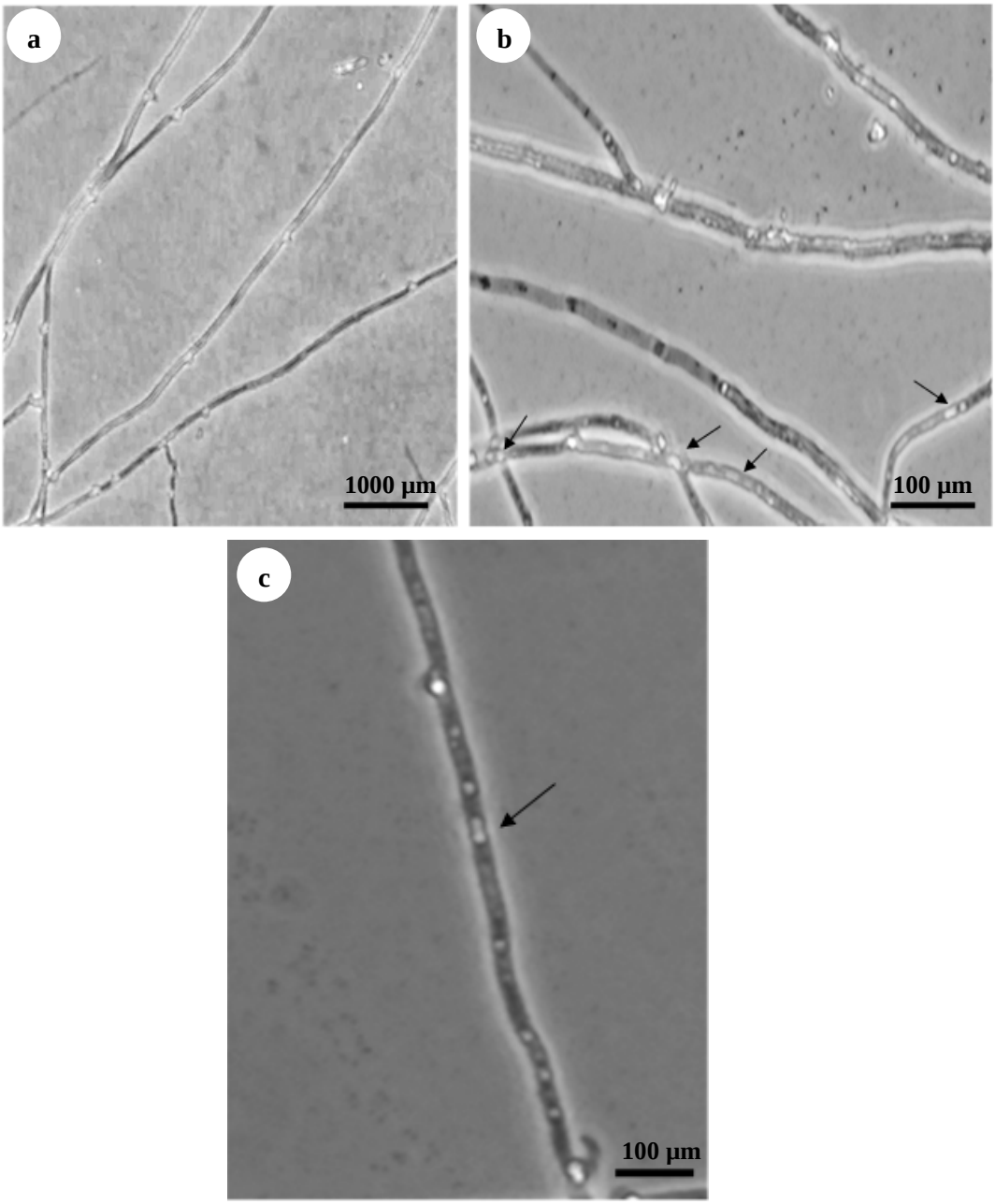


Figure 4.11: Effect of chitosan on mycelium morphological characteristic of *Ganoderma* under light microscope. (a) Control (PDA medium), (b) and (c)

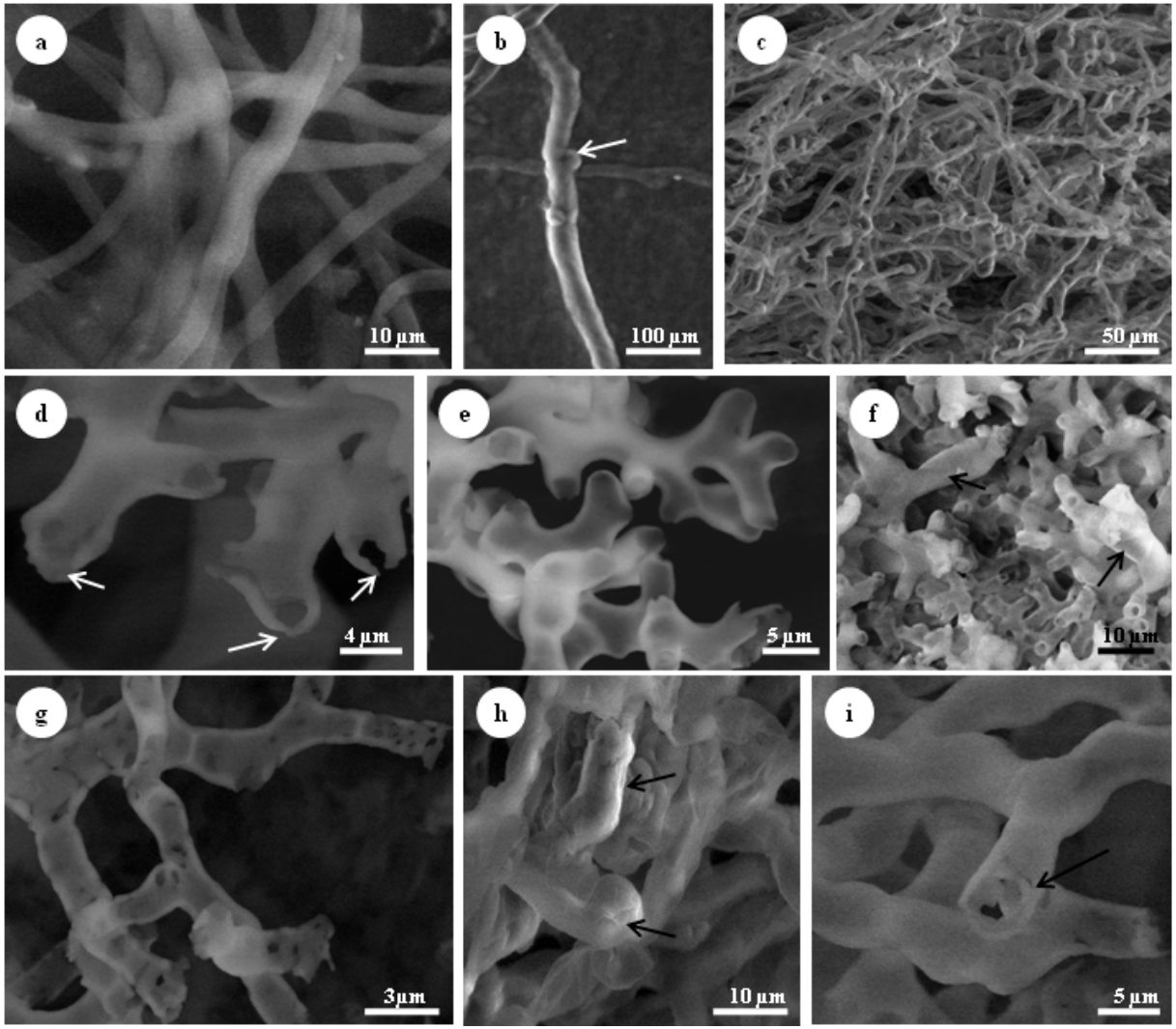


Figure 4.12: Scanning electron micrographs on the effect of chitosan on the mycelia surface morphology of *Ganoderma* (a-b) Hyphae from untreated control samples (b) Hyphae showing regular morphology with clamp connection (arrow) (c-d) Hyphae showing corrugated surface at high concentrations (e) Mycelial showing retarded growth with excessive branching (arrows). (f) Swelling of hyphals (arrows). (g) Severely damaged and collapsed mycelium (h-i) 2.0 % (w/v) chitosan treated mycelium was noted with extracellular layers surrounding the hyphal cells.

4.4 Discussion

Macroscopic descriptions of basidiocarps collected from field samples based on standard protocols (Gottlieb & Wright 1999; Moncalvo, 2000; Seo & Kirk, 2000) provided general characteristic of *Ganoderma* sp. for the fungal isolates of GBB 7, GBB 18, GBLS and GBU as described (Chang *et al*., 1992; Pilotti *et al*., 2004). In addition, microscopic examination of hyphal structure with presence of clamp connections and basidiospore morphology supported the claim of the respective fungal isolates belongs to *Ganoderma* sp. classification (Chang *et al*., 1992, Leonard, 1998; Seo & Kirk, 2000; Pilotti *et al*., 2003; Foroutan & Vaidya, 2007). Clamp connections are common among basidiomycete fungus which exists in dikaryon hyphae with two unfused compatible nuclei in each cell wall formed during conjugate division of the nuclei at the tip of growing hyphae (Miles *et al*., 1996). Moreover, morphology of basidiospore examined in this study complements well with the taxononomy feature of *Ganoderma* as reported (Adaskaveg & Gilbertson, 1986; Seo & Kirk, 2000). The growth pattern of fungal isolates colonies on PDA medium as described by Idris (2009) have also contributed in the identification of the isolates as *Ganoderma* sp. Nevertheless, a positive identification could not be confirmed based on the macro, micro and cultural morphological characteristic alone. Hence, a more reliable and accurate identification method through PCR technique was prompted in this study. Positive amplification of 167 bp fragment as confirmative results of the pure mycelium culture isolates as *Ganoderma* sp. was achieved in this study by using primers

Gan 1F and Gan 2R generated from internal transcribed spacer region of rDNA as reported in previous studies (Utomo *et al*., 2000; 2005; Kartikeyan *et al*., 2007; Kandan *et al*., 2009).

The aggressiveness and infectivity of naturally occurring *Ganoderma* isolates were quantified *via* the artificial inoculation technique using rubber wood blocks on oil palm seedlings. This method has confirmed the reproducibility and ability of isolate GBLS to induce infection on oil palm seedlings as claimed earlier by Sariah *et al*. (1994) and Nur Ain Izzati & Abdullah (2008). Based on the evaluation of disease symptoms on artificially inoculated oil palm seedlings, presence of melanized pseudo-sclerotia and validation of Koch's Postulates using GSM, confirmed isolate GBLS a pathogenic isolate of *Ganoderma* sp. causing basal stem rot disease. This study essentially requires screening of aggressive and pathogenic of different isolates in order to avoid working with discriminatory isolates in future studies as described in previous reports (Breton *et al*., 2006; Rees *et al*., 2007). Therefore, isolate GBLS was selected as a candidate for subsequent chitosan treatment experiments.

The effect of chitosan on soil-borne and post-harvest pathogens has been well documented (Stossel & Leuba, 1984; Badawy & Rabea, 2009) and the findings of this study will complement the extensive use of chitosan in plant pathology. Antifungal activities of chitosan with various properties (LV, HV, LMW, and HMW) on the pathogenic isolate GBLS were demonstrated in this study. Chitosan caused inhibition of the radial growth in proportion to the concentration used for all types of chitosan tested which is in agreement with

previous reports (Ait Barka *et al*., 2004; Munoz *et al.,* 2009). With regards to LV chitosan, the highest inhibitory effect was found at 2.0 % (w/v), however, complete inhibition of mycelial growth was not achieved even at the highest concentration and the observed growth of mycelium transferred to medium without chitosan suggests that chitosan is fungistatic rather than fungicidal. Hernandez-Lauzardo *et al.,* (2008) had noted that different molecular weights of chitosan could affect its antifungal activity against *Rhizopus stolonifer* whereby the mycelial growth was effectively reduced by low molecular weight chitosan. Guerra-Sanchez *et al*., (2009) found that chitosans with low, medium and high molecular weights showed similar radial growth inhibition on *R. stolonifer*. By contrast, high molecular weight chitosan contributed in the inhibition of mycelial growth of *Fusarium oxysporum f. sp. vasinfectum*, *Alternaria solani* and *Valsa mali* (Guo *et al*., 2006).

LV chitosan reduced germination of *Ganoderma* sp. at lower concentration ranging from 0.001-1.0 % (w/v) compared to mycelia growth ranging from 0.5-2.0 % (w/v) may due to sensitivity of spores to chitosan treatment. In addition, chitosan treated spores with increased concentrations were severely affected with prolonged emergence and decelerated elongation of germ tubes during the incubation period. These observations explained that during basidiospore germination development process, chitosan appeared to arrest the stage of germ tube formation known as isotrophic growth (swelling of spores) and its transition to polarized growth (germ tube emergence) as described (Plascencia-Jatomea *et al*., 2003; Quintana-Obregon *et al*., 2011).The presented results

exhibited similar effect as most of the studies in which the spore germination rate and germ tube growth was reduced as the concentrations increased in fungi such as *Aspergillus niger*, *Botryis cinerea*, *R. Stolonifer* and uredospore of *Puccinia arachidis* (Sathiyabama & Balasubramaniam, 1998; El Ghaouth *et al*., 1992a; Ben Shalom *et al*., 2003; Plascencia-Jatomea *et al*., 2003) It has been difficult to compare the present results with the reported literature due to the diversity of fungi (El Ghaouth *et al.*, 1992a, 1992b), different concentrations of chitosan used (Guerra-Sanchez *et al*., 2009) and differences in methods used to incorporate chitosan in growth medium (El Ghaouth *et al*., 1992a, 1992b). The antifungal activity of chitosan has been proposed to be due to the interference of positively charged chitosan with negatively charged substances in the cell wall of fungi that leads to alteration and leakage of constituents from the plasma membrane (Leuba and Stossel, 1986). However, Hadwiger & Loschke (1981) suggested that the interaction of chitosan with fungal DNA and mRNA is the basis of its antifungal effect.

Light and scanning electron microscopy revealed significant changes in the hyphal morphology of GBLS due to chitosan treatment. The alterations observed in this study, such as corrugated surface, hyphal swelling, excessive branching and increased vacuolation, were similar to those observed in other pathogenic fungi treated with chitosan. Ait Barka *et al.*, (2004) postulated that the appearance of small vesicles in hyphae was due to coagulation in the fungal cytoplasm. The excessive branching of the hyphae on PDA containing more than 1.5% chitosan has also been reported for *R. stolonifer* (El Ghaouth *et al*., 1992a,

1992b), *Sphaeropsis sapines* and *T. harzianum* (Singh *et al.*, 2008). El Ghaouth *et al.* (1992a) proposed that, due to the stimulation of chitin deacetylase activity by chitosan, the biosynthesis and turnover of chitin was interrupted and softening of fungal cell walls occurred which initiated the enhanced hyphal branching. In addition, Singh *et al.*, (2008) stated that hyperbranching of mycelium is due to unprocessed protein which enables chitosan to stimulate the function of chitin synthase by altering the plasma membrane and causing abnormal changes in the fungal cell wall. The mode of action of chitosan was found to be similar to other membrane-specific fungicides such as azoles (Pfaller *et al.*, 1992). The interesting appearance of an extracellular layer surrounding the hyphae in the 2.0 % chitosan treatment was absent at lower concentrations. This would suggest the presence of extracellular mucilaginous material (ECMM) produced by fungi when exposed to stress conditions, as described in earlier studies (Foisner *et al.*, 1985; Vasentini *et al.*, 2007).

The results presented in this study indicate that chitosan exhibited an appreciably inhibitory activity on the growth of *Ganoderma* under *in vitro* condition. These findings contribute new information on the potential of chitosan as a bio-fungicide on the growth of basidiomycete fungus. Pertaining to this framework, further studies were designed to determine the efficacy of chitosan under *in vivo* application and directs to elucidation of possible mechanism of action of chitosan during the interaction of fungal pathogen, *Ganoderma* and host (oil palm).

CHAPTER 5

EFFECT OF CHITOSAN ON *IN VITRO* PRODUCTION OF LIGNIN DEGRADING ENZYMES (LDE) FROM OIL PALM BASAL STEM ROT (BSR) PATHOGEN, *GANODERMA*.

5.1 Introduction

Lignin is an integral component of plant cell walls (formed by oxidative polymerization of p-coumaryl, coniferyl and sinapyl alcohols) which provides strength and resistance to microbial degradation (Argyropoulos & Menanchem, 1997; Breen & Singleton, 1999). It is formed as a matrix to protect the hemicelluloses and cellulose components (ten Have & Teunissen *et al*., 2001; Martinez *et al*., 2005). The main lignin degraders, the white rot fungi classified as Basiodiomycete produces laccase (p-diphenol:oxidoreductase), manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP) as the primary enzymes responsible for lignin degradation (Hakala *et al*., 2005). These essential enzymes enable recalcitrant lignin polymers of plant cell walls to be degraded during plant-pathogen interactions to allow penetration, colonization, and survival of pathogens (Esquerre-Tugaye *et al*., 2000).

Ganoderma is known as a white rot fungal pathogen causing basal stem rot (BSR) disease in oil palm (Rees *et al*., 2007; 2009). The term white rot fungus applies to *Ganoderma* due to its ability to degrade lignin in stem and root tissues using extracellular enzymes to obtain energy from exposed white cellulose

(Mazlihan *et al*., 2007; Paterson, 2007; Paterson *et al*., 2009). The perspective of *Ganoderma* as a white rot fungus on its mode of attack during its interaction with oil palm has not been given much consideration in BSR epidemiology studies. To date there have been no studies which directly link *Ganoderma* as the cause of oil palm white rot. However, a few papers have reported this possibility including Advaskaveg *et al*., (1991) who explained the decay of Canary Island date palm (*Phoenix canariensis*) by brown and white rot fungi (*G. colossum, G. zonatum*) and from Agosin *et al*., (1990) and Ferraz *et al*., (2000) who described the role of *G. australe* causing white zones on decayed woods in a southern Chilean forest through a light microscopy study of wood delignification. A part from these, the function of extracellular lignin degrading enzyme (LDE) produced by *G. lucidum* (closely related to BSR-*Ganoderma* in oil palm) in plant pathogenesis was reported in Anabe roga disease (foot rot) on arecanut by Kumari & Sirsi (1972) and in BSR disease on coconut by Rajendran *et al*., (2008).

The involvement of oil palm lignin biodegradation by *Ganoderma* in virulence and pathogenesis of BSR disease process has been reviewed by Paterson (2007); Paterson *et al*., (2008; 2009). These reports highlighted prominent information on *Ganoderma*-BSR disease establishment as a 'white rot' and possible approaches to control the infection. Schwarze (2007) and Paterson *et al*., (2008; 2009) explained that wood lignin in oil palm, which has a high ratio of syringyl compared to guaicyl units is more prone to lignin degradation. Rees *et al*., (2009) had also stated that cell wall degrading enzymes may be a crucial pathogenicity factor of *Ganoderma* in the degradation of oil palm root tissues

comprised of lignin, cellulose and suberin polymers. The extracellular LDE system in *Ganoderma* spp. has been studied and characterized by authors such as D'Souza *et al*., (1999); de Souza Silva *et al*., (2005); Elissetche *et al*., (2007); and Murugesan *et al*., (2007). The regulation of LDE by *Ganoderma* has been extensively focused for industrial applications (Orth *et al*., 1993) and in contrast, their role in plant pathogenesis has been neglected and remains unexploited.

The inhibition and sensitivity of these LDE enzymes have been tested using compounds such as metal chelating and reducing agents (Kumar & Sirsi, 1972; Cuoto & Toca, 2006). Thus, the similar approach of using chitosan, the natural polysaccharide derived from deacetylated chitin was adopted in this study as a potential LDE enzyme inhibitor or suppressor by *Ganoderma.* The inhibition mechanism of chitosan as a chelating agent has been noted to inhibit microbial growth and affects the production of pathogenic factors such as toxin by *Alternaria alternata* (Reddy *et al*., 1998; 2000), aflatoxin by *Aspergillus* sp. (Cuero *et al*., 1991; Cota-Arriola *et al*., 2010) and macerating enzymes by *Erwinia carotovora* spp. causing bacterial soft rot in inoculated potato tissue (Reddy *et al*., 1997).

Therefore, the main aim of this chapter is (1) to investigate the *in vitro* production of LDE by pathogenic *Ganoderma* sp. of oil palm (GBLS isolate) in different culture condition and (2) to demonstrate the effect of chitosan on the repression or inhibition of LDE produced by GBLS isolate in an optimized culture conditions.

5.2 Materials and methods

Ganoderma isolate GBLS, isolated as described Section 3.1, was used in this study. The fungal culture was maintained on PDA medium at 28° C in the dark. Mycelium plugs from the growing margin of the colony after 14 days were used as inoculums. Chitosan solutions were prepared as described in Section 4.2.4.

5.2.1 Determination of Remazol Brilliant Blue R (RBBR) decolourization in a plate test

GBLS isolate was screened for its ability to degrade a lignin model substrate known as Remazol Brilliant Blue R (RBBR) using a plate test. Mycelium agar plugs (6 mm) of GBLS isolate from PDA plates were placed on the centre of a Petri dishes containing malt extract agar medium (MEA) supplemented with 0.05 % w/v RBBR. The plates were incubated in the dark at 28 °C for 14 days and observed every day to determine decolourization activity of RBBR (de Souza Silva *et al*., 2005).

5.2.2 Optimization of culture condition for LDE production by GBLS isolate

Four culture conditions for solid state cultivation of GBLS isolate on rubber wood chips were prepared in a triplicate set of Erlenmeyer flasks (250 ml) as follow;

1. Rubber wood chips in malt extract broth (MEB) under stationary conditions.
2. Rubber wood chips in MEB on a rotary shaker at 120 rpm.
3. Rubber wood chips in water under stationary conditions.
4. Rubber wood chips in water on a rotary shaker at 120 rpm.

The rubber wood chips (RWC) were obtained from 10-15 years old rubber trees. Each flask contained 5 g of RWC sterilized twice at 121 ° C and 15 psi for 30 min to allow complete sterilization. Sterilized MEB (30 ml) was added aseptically to each flask and inoculated with ten mycelial plugs. This experimental procedure was based on the method reported by Murugesan *et al.*, (2007) with wheat bran substituted by rubber wood chips. The flasks were incubated under two conditions; stationary and shaken on rotary shaker (120 rpm). Uninoculated sterilized RWC chips with MEB in similar conditions were prepared as a control. A further treatment comprised replacing MEB medium with sterile water. Quantification of LDE was carried out after 3, 5, 7, 10 and 14 days of incubation.

The contents of the flasks were harvested after each culture period and the enzymes from RWC were sequentially extracted with 100 mM sodium acetate buffer, pH 5.0 by shaking for 20 minutes on a rotary shaker and were left

overnight at 4º C (Arora *et al.*, 2002; Murugesan *et al.*, 2007). The cultures were filtered on the next day by using Whatman filter paper and centrifuged twice at 10,000 g for 20 minutes. The clear supernatants obtained were used for quantification of LDE enzymes.

5.2.3 Effect of chitosan on inhibition of LDE produced by GBLS isolate and weight loss determinations

GBLS isolate was cultured in solid state medium using rubber wood chips in MEB medium under stationary conditions as described previously in Section 5.2.2 (based on the results obtained from maximum optimal production of LDE) to determine the effect of chitosan on the LDE production by GBLS isolate. Chitosan solutions of 0.02, 0.04, 0.06, 0.08 and 0.1 % (w/v) were incorporated into MEB medium after sterilization and were added to the flasks containing 5 g of sterilized rubber wood chips (RWC). This range of concentrations was chosen after completing several experiments to determine the optimal concentrations that could inhibit LDE activity and which have no toxicity or inhibitory effect on the growth of GBLS isolate. The flasks were inoculated with 10 mycelial plugs and were incubated at room temperature for 3, 5, 7, 10 and 14 days. Three types of control were prepared (i) inoculated sterilized RWC with MEB (ii) uninoculated sterilized RWC with MEB and (iii) uninoculated sterilized RWC with MEB in the presence of each concentration of chitosan. The extraction of crude enzymes was done as described in Section 5.2.2.

After extraction, the rubber wood chips were washed with water and dried to a constant weight at 80° C. Weight losses were determined based on the difference between the initial and final dry weights of the RWC (Fernandes *et al.*, 2005).

5.2.5 Analysis for lignin degrading enzyme (LDE)

Laccase (Lac)

Laccase activity was determined by using 2.2-azino-bis-ethylbenthiazoline (ABTS) substrate oxidation (molar extinction coefficient = 36, 000M^{-1} cm^{-1}) as described previously by Murugesan *et al.,* 2007. The reaction mixture consisted of 20 µl sodium acetate buffer (100 mM, pH 5.0), 160 µl ABTS solution 0.03 % (w/v) and 20 µl enzyme extract. The oxidation of ABTS was measured by monitoring the increase in absorbance at 420 nm for 5 minutes at 1 minute intervals.

Manganese peroxidase (MnP)

Manganese Peroxidase activity was measured by using guaicol substrate oxidation (molar extinction coefficient= 12, 100M^{-1} cm^{-1}) as the methodology described by Mabrouk *et al.*, (2010). The reaction mixture consisted of 20 µl sodium succinate buffer (0.5 M, pH 4.5), 20 µl guaiacol (4 mM), 20 µl manganese sulphate (1mM), 20 µl culture supernatant and 80 µl distilled water. The mixture was incubated for 2 min at 30 ºC and was initiated by addition of 20 µl of hydrogen peroxide

(1mM). The absorbance was measured at 465 nm at 1 minute intervals after addition of hydrogen peroxide.

Lignin peroxidase (LiP)

Lignin enzyme activity was evaluated based on the oxidation of the dye Azure B (molar extinction coefficient 48, $800M^{-1}$ cm^{-1} (Archibald, 1992; Arora & Gill, 2001). The reaction mixture consisted of 10 µl sodium tartrate buffer (125 mM, pH 3.0), 50 µl Azure B (0.160 mM) and 50 µl of the culture filtrate. The reaction was initiated by adding 50 µl hydrogen peroxide and measured at 651 nm at 1 minute intervals for 5 min.

All enzyme activities were measured spectrophotometrically with a microplate reader (Variouskan Skan IT, UV). The enzyme activity unit was expressed as units, where one activity unit is defined as the amount of enzyme necessary to oxidize 1 mol of substrate per min. Each value represented the mean of three replicates.

Total protein content

Quantification of total protein was determined by the method of Bradford reagent and bovine serum albumin (BSA) as standard (Appendix 1).

5.2.6 Statistical Analyses

The experiment on the optimization on culture condition and the effect of chitosan on the production of LDE enzymes by GBLS isolate were arranged in a completely randomised design (CRD). The data were subjected to One-way ANOVA and tested for significant differences among treatments by Tukey's Multiple Comparison post test at ($P \leq 0.05$) using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. All the experiments were conducted twice and the data were pooled before analysis.

5.3 Results

5.3.1 Remazol Brilliant Blue R (RBBR) decolourization

Isolate GBLS caused decolourization of RBBR after 14 days through a change from blue colour to pinkish and finally colourless (Fig. 5.1).

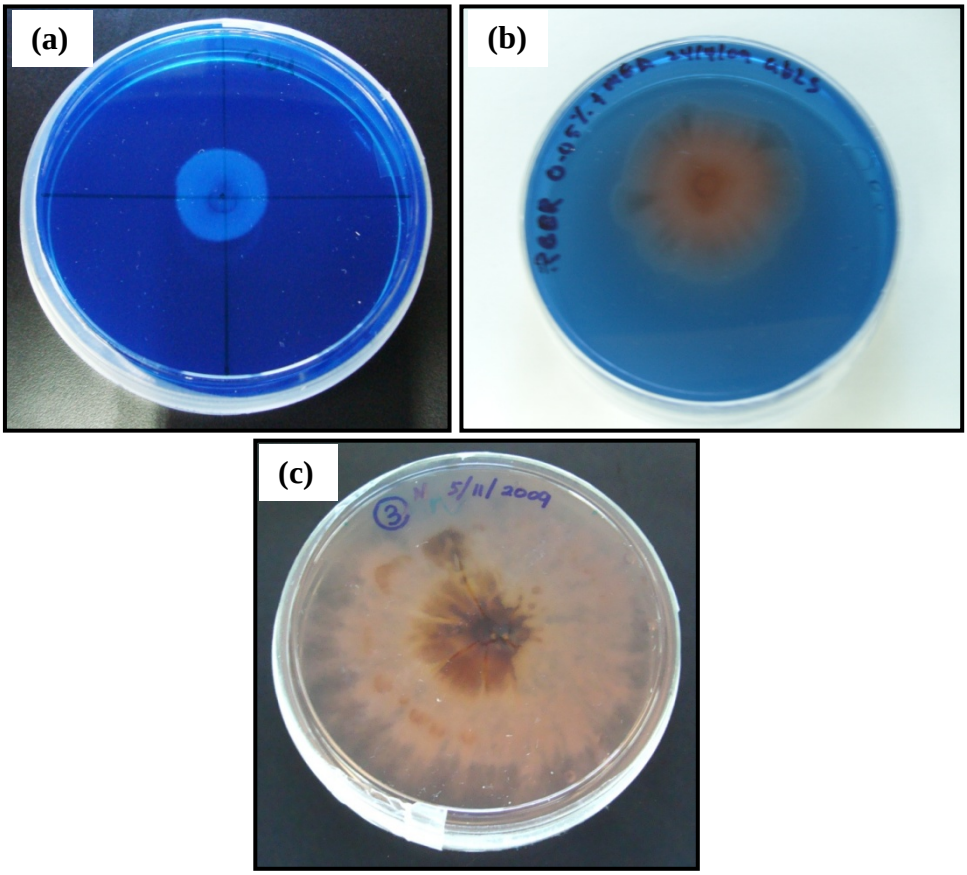


Figure 5.1: RBBR decolourization by GBLS isolate. (a) The blue dye of 0.05 % RBBR in MEA medium (b) Pink decolourization halo (c) Culture turning colourless after 14 days of incubation

5.3.2 Optimization of *in vitro* production of lignin degrading enzymes (LDE) by GBLS isolate under solid state cultivation

Activity of laccase and MnP was evident under four growth conditions during culture for 14 days (Fig 5.2, 5.3). In contrast no LiP activity was detected in GBLS. The activity of laccase compared to MnP was higher irrespective of the growth conditions (Fig 5.2, 5.3). Both of these enzymes showed higher activity in the static MEB treatment as compared to water and shaken, their activity was significantly ($P \leq 0.05$) higher in the MEB/ static cultures than in other treatments.

Laccase production peaked on day 7 and this pattern was similar for all the growth conditions. The maximum laccase activitiy on day 7 in MEB/ static cultures (40.94 U/L) was two to fourfold higher compare to the other cultures. After the 7th day, laccase activity in all the cultures began to decrease (Fig. 5.2). The maximum MnP activity of cultures in MEB medium occurred on the 10th day and for the MEB/ static cultures (14.73 U/L) was twofold higher than for the MEB/ shaken cultures (7.04 U/L) (Fig. 5.3). The peak of MnP activity peak in water medium was noted earlier on the 7th day culture and for the water/ static cultures (6.01 U/L) was also twofold higher than for the water/ shaken cultures (2.95 U/L) (Fig. 5.3).

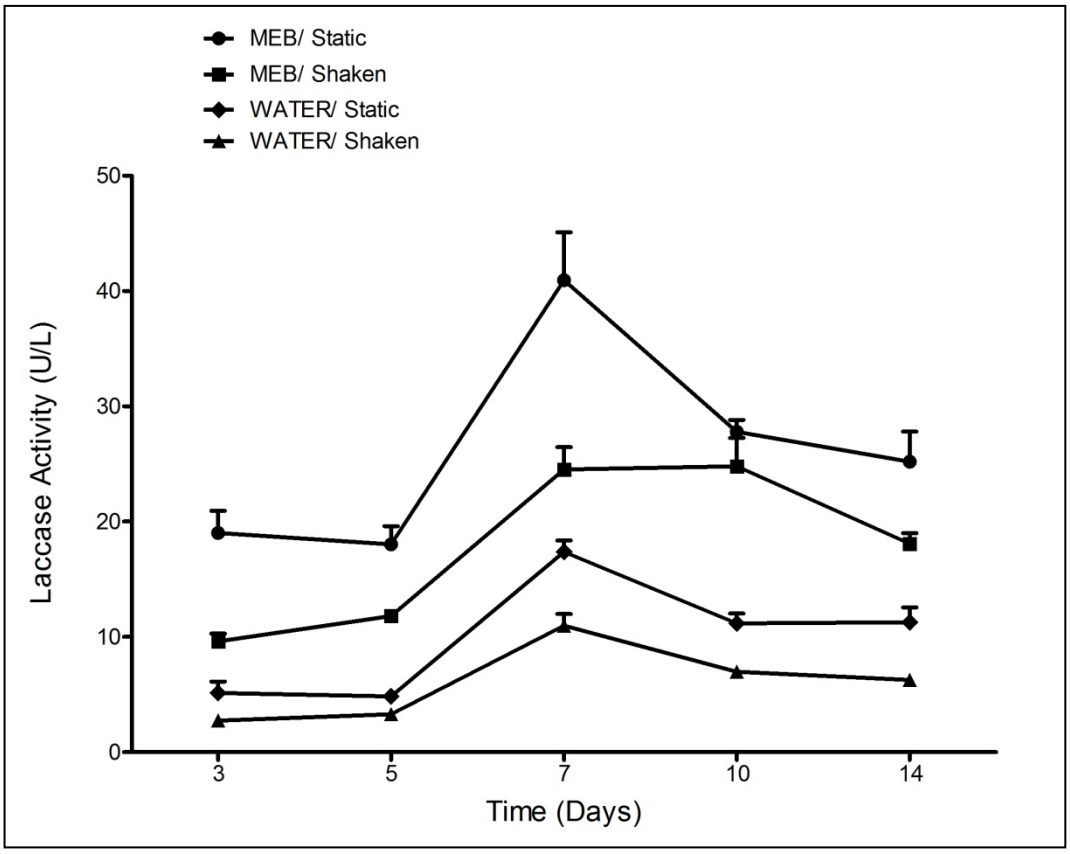


Figure 5.2: Laccase production by GBLS isolate cultured in MEB (Malt extract broth) and water medium containing rubber wood chips under static and shaken conditions. Values represent means of three replicate flasks

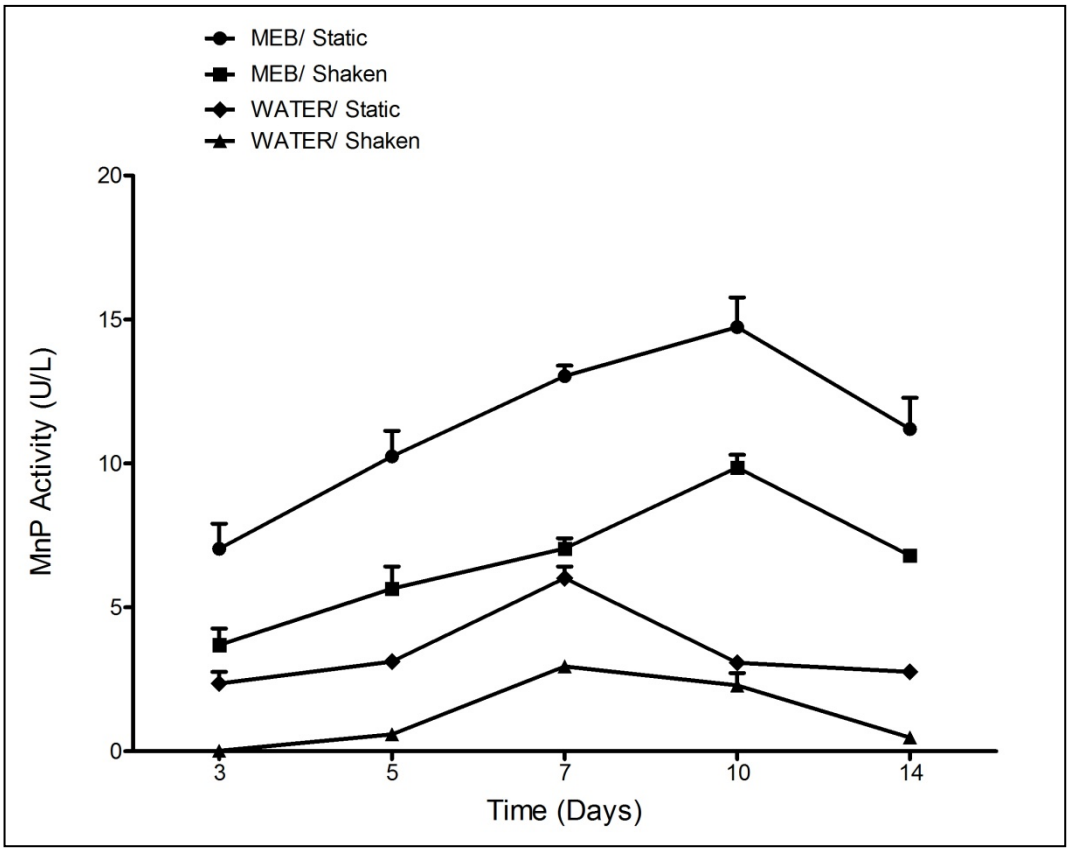


Figure 5.3: MnP production by GBLS isolate cultured in MEB (Malt extract broth) and water medium containing rubber wood chips under static and shaken conditions. Values represent means of three replicate flasks

Protein secretion in all culture conditions did not vary significantly ($P \leq 0.05$) (Fig 5.4). The concentrations of protein in MEB/ static and MEB/ shaken cultures increased from day 3 till to 10 (0.0059 and 0.0048 mg/ ml respectively) and began to decrease at the end of culture period. Protein secretion in water/ static and water/ shaken cultures reached its maximum on day 7 (0.0029 and 0.0027 mg/ ml respectively) and then decreased until the end of culture period.

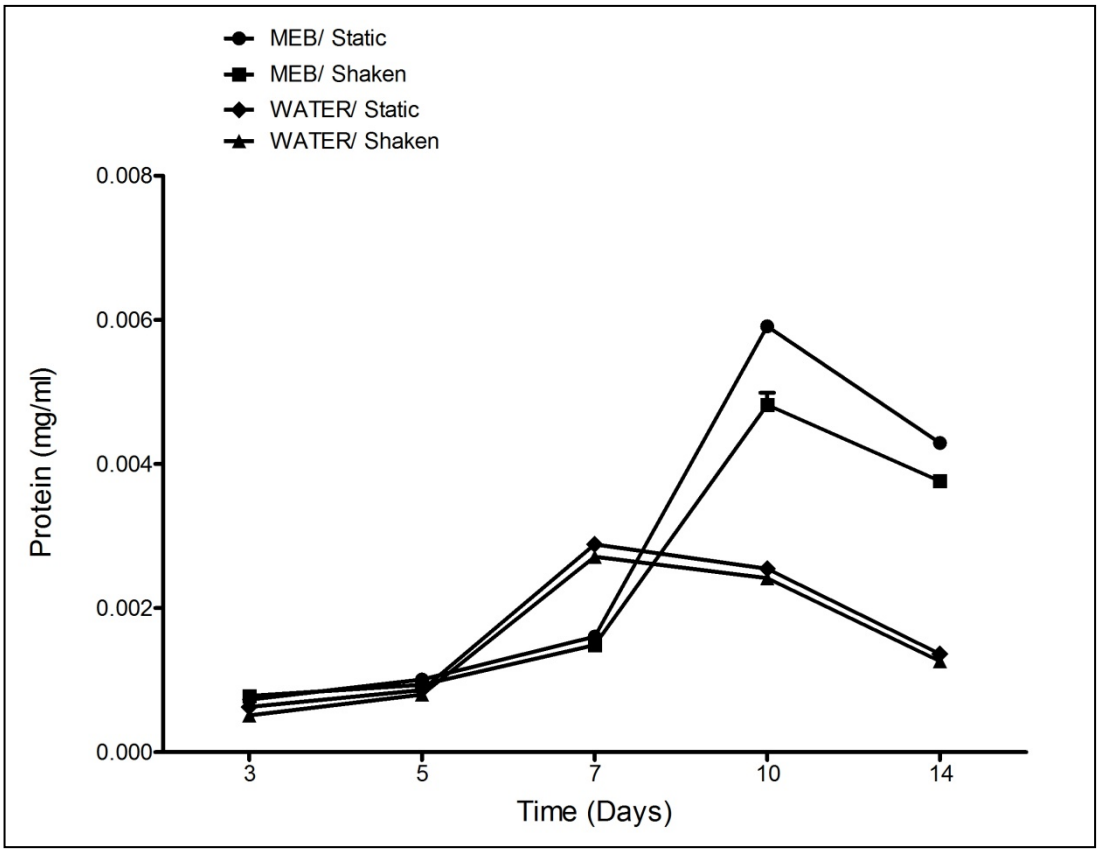


Figure 5.4: Protein concentration secretion by GBLS isolate cultured in MEB (Malt extract broth) and water medium containing rubber wood chips under static and shaken conditions. Values represent means of three replicate flasks

5.3.3 Effect of chitosan on *in vitro* production of lignin degrading enzymes (LDE) by GBLS isolate

GBLS cultures treated with chitosan at concentration higher than 0.02 % (w/v) displayed a significant ($P \leq 0.05$) inhibition of laccase activity compared with control cultures (Fig. 5.5). In contrast, the activity of MnP was significantly ($P \leq 0.05$) lower in GBLS cultures treated with all of the chitosan concentrations compared with control cultures (Fig. 5.6). Cultures treated with 0.08 and 0.1 %

(w/v) showed maximum ($P \leq 0.05$) inhibition level of laccase and MnP enzyme production (Fig 5.5, 5.6). The five concentrations (0.02, 0.04, 0.06, 0.08 and 0.1 % w/v) of chitosan evaluated in this study exhibited a consistency of dose-dependent inhibition for laccase and MnP enzyme activity during each of the incubation periods.

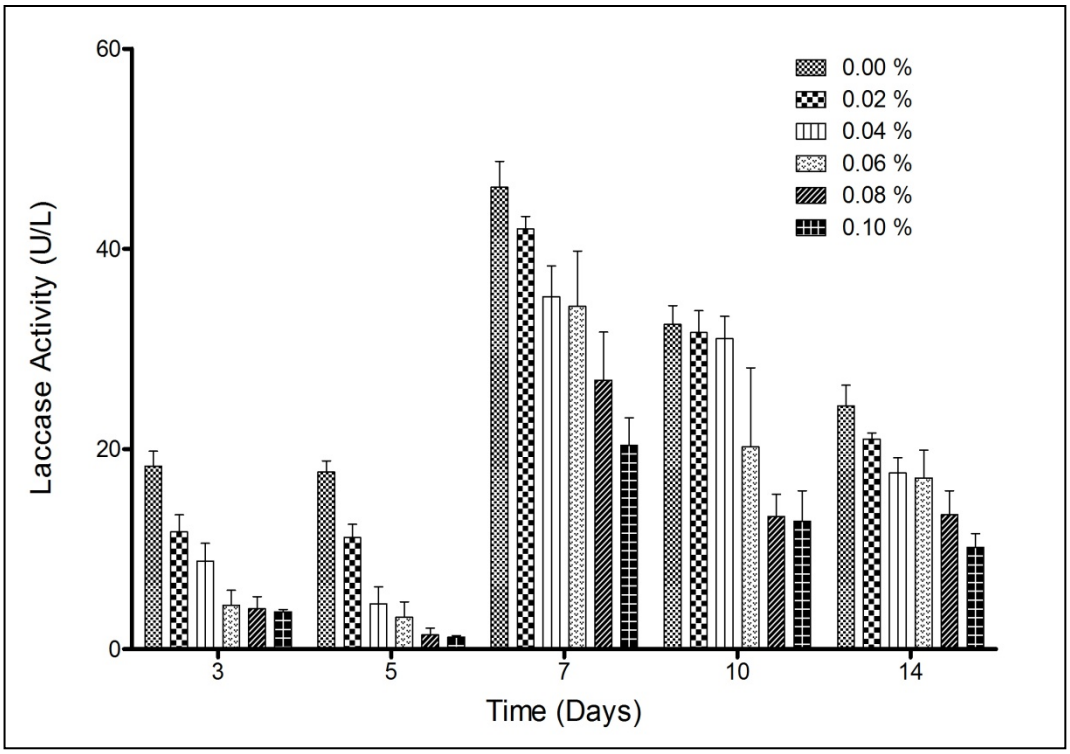


Figure 5.5: Effect of concentration of chitosan % (w/v) on laccase activity of GBLS isolate cultured in MEB (Malt extract broth) medium containing rubber wood chips under static conditions. Values represent means of three replicate flasks.

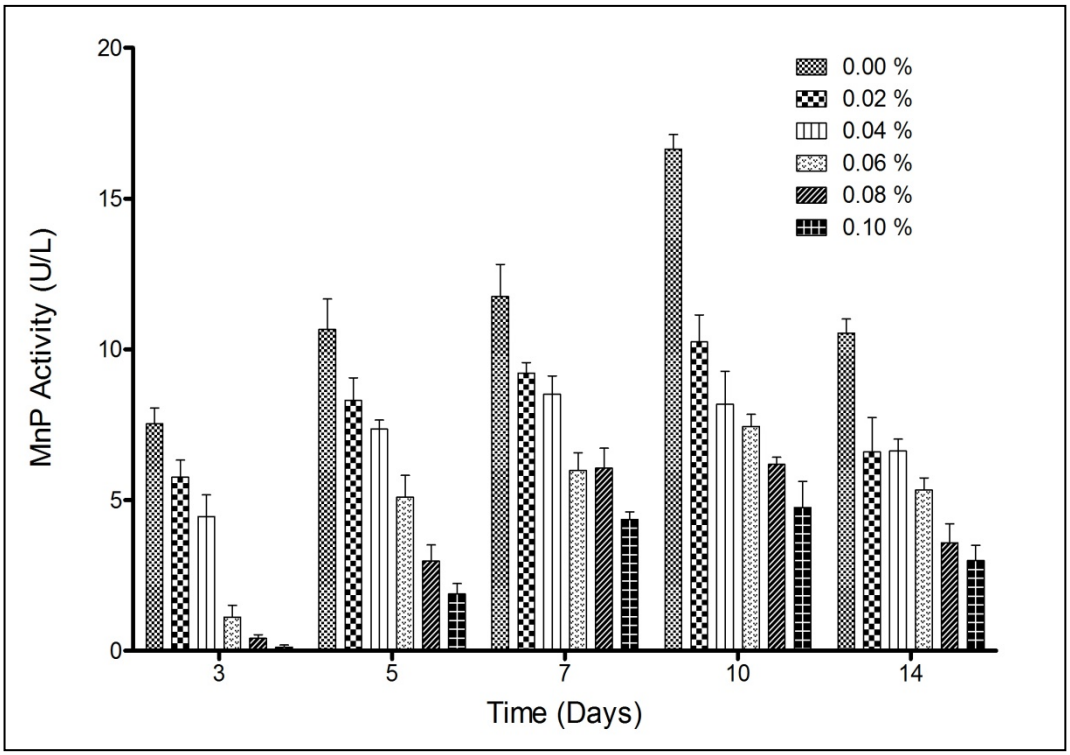


Figure 5.6: Effect of concentration of chitosan % (w/v) on MnP activity by GBLS isolate cultured in MEB (Malt extract broth) medium containing rubber wood chips under static conditions. Values represent means of three replicate flasks

The total protein content of the GBLS isolate cultured in the presence of chitosan was not significantly different ($P \leq 0.05$) compared to the control cultures throughout the incubation period (Fig 5.7). From day 7, protein content was observed to decrease with increasing of chitosan concentration.

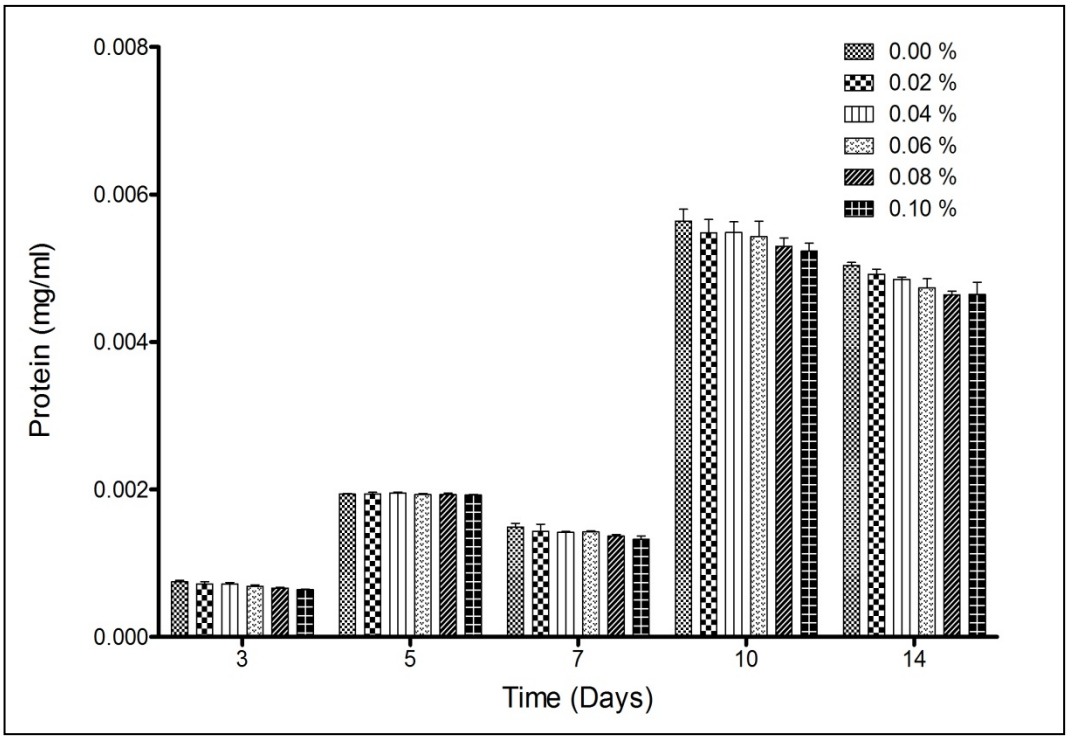


Figure 5.7: Effect of concentration of chitosan % (w/v) on total protein content by GBLS isolate cultured in MEB (Malt extract broth) medium containing rubber wood chips under static conditions. Values represent means of three replicate flasks

5.3.4 Effect of chitosan on weight loss of rubber wood chips (RWC) decayed by GBLS isolate

Chitosan at 0.06, 0.08 and 0.1 % (w/v) significantly reduced the weight loss of rubber wood chips (RWC) caused by GBLS isolate was compared to the control (Table 5.1). At higher concentrations of 0.08 and 0.1 % (w/v), there were no significant differences in the weight loss. By the end of incubation period, all the evaluated concentration of chitosan showed weight loss of RWC was reduced about two fold compared to the first day of incubation (Table 5.1).

Table 5.1: Weight loss (%) of rubber wood chips after incubation with GBLS isolate

Chitosan concentration (mg/ml)	Mean weight loss ± S. D. (%)				
Time of incubation/ Days	3	5	7	10	14
0.0	4.23±0.57	4.65±0.21	4.54±0.12	4.71±0.35	4.54±0.35
0.2	3.74±0.39	4.20±0.35	4.41±0.17	4.65±0.17	4.91±0.19
0.4	3.34±0.10	3.53±0.16	4.30±0.23	4.59±0.17	4.84±0.05
0.6	2.95±0.43	2.73±0.58	2.61±0.37	3.38±0.35	3.58±0.18
0.8	2.57±0.61	2.88±0.72	2.72±0.24	2.57±0.41	3.71±0.13
1.0	2.12±0.03	2.37±0.12	2.42±0.10	2.64±0.09	2.39±0.37

S.D. = standard deviation

5.4 Discussion

The present study demonstrates for the first time the profile of lignin degrading enzymes (LDE) produced by the oil palm BSR pathogen (GBLS isolate) of *Ganoderma* sp. and its inhibition by chitosan. The ability of GBLS to degrade lignin was illustrated in a preliminary agar plate test amended with Remazol Brilliant Blue Reactive dye which showed a series of colour changes from blue to colourless as described by Murugesan *et al*., (2007). RBBR is an anthracene derivative dye which has similar structure to lignin and its decolourization is used to determine the ligninolytic enzymes possessed by potential producers (Christian *et al*., 2005). The decolourization pattern observed with GBLS was smaller than the growth halo and may have been induced by secondary metabolic activity of older mycelium (Levin *et al*., 2004; Machado *et al*., 2005).

The laccase and MnP enzyme production by GBLS during solid state culture suggest that RWC is a good substrate to study natural secretion of LDEs. This is due to its similar lignin composition to that of oil palm which comprises syringaldehyde as the major phenolic component (Mansor *et al*., 1991; Paterson *et al*., 2008) and the established method of using RWB inoculum for pathogenicity studies of *Ganoderma* sp. in oil palm (Sariah *et al*., 1994). The combination of MEB medium and static incubation condition produced significant laccase and MnP activities compared to water medium and shaken incubation condition. The mycelial growth of GBLS was sparse in the shaken water medium. This was due to the inability of water medium to provide sufficient nutrients for the growth of

GBLS and the shaking caused shearing of the mycelia by RWC particles as described previously in the study by D' Souza *et al*., (1999).

The production of the enzymes laccase and MnP but not LiP shows that GBLS can be classified as strong white rot lignin degraders with selective lignin degradation ability (Tuor *et al*., 1995). The enzyme profile of GBLS does not coincide with others reported in the literature due to the variation in enzymes of species within *Ganoderma* genus and the highly dependence on culture conditions. Higher laccase activity detected in GBLS indicates that this enzyme may play an essential role in BSR disease on oil palm since the involvement of laccase in lignin degradation has been reported in previous literature (Hatakka, 1994; Eggert *et al*., 1997). The contribution of laccase as one of the common LDE responsible for virulence and pathogenic element in plant diseases caused by fungal pathogens has been documented. Studies by Kumar & Sirsi (1972) and Rajendran *et al*. (2008) have associated the production of laccase by *Ganoderma lucidum* as the major cause of Anabe foot disease in arecanut palm and BSR disease in coconut respectively. Further evidence for the secretion of laccase as a key factor in pathogenesis studies has been addressed by studies on *Botrytis cinerea* which infects a wide range of hosts (Bar Nun *et al*., 1988); *Cryphonectria parasitica* which cause destructive chestnut blight disease in North America and Europe (Ringling & VanAlfen, 1991); *Ophiostoma ulmi and Ophiostoma nova-ulmi* which is responsible for Dutch elm disease (Binz & Canevanscini, 1996) and *Magnaporthe grisea*, the causal agent of rice blast disease (Iyer & Chatoo, 2003).

The present study has directed a new approach for the control of oil palm BSR infection using chitosan as a potential inhibitor for LDE produced by *Ganoderma*. The findings presented have shown the inhibitory effect of chitosan at concentrations from 0.02 to 0.1 % (w/v) on the activities of laccase and MnP. The optimal concentration of chitosan was determined for inhibition of LDE activity without affecting the growth of GBLS isolate. The possible mechanism of inhibition on LDE secretion by GBLS can be attributed to the chelation of copper and manganese ions by chitosan which are required for laccase and MnP enzymes respectively (Muzzarelli *et al*., 1980; Bar Nun *et al*. 1988; Cuero *et al*., 1991). This is also supported by Paterson *et al*. (2008) who stated that laccase is a copper containing enzyme involved in oxidation of electrons to molecular oxygen, so the copper ion is susceptible to chelating or reducing agents. In relation to laccase as phytopathogenecity factor, *in vitro* experiments have demonstrated the inhibition of fungal laccase enzyme using chelating or reducing agents such as ethylenediaminetetraacetic acid (EDTA), cyanide, diethyldithiocarbamate and cysteine (Kumar & Sirsi, 1972; Bar Nun *et al*., 1988). Although the effect of chitosan on LDE has not been investigated previously, its ability to control the production of pathogenic factors such as macerating enzymes, organic acids and toxins by microbial pathogens has been reported previously. For example, Cuero *et al*., (1991) and Cota-Arriola et *al*., (2000) indicated that chitosan inhibits aflatoxin synthesis by *Aspergillus* sp. due to its chelating ability on metal compounds and Reddy *et al*., (2000, 2007) have acknowledged the tendency of chitosan as a chelating agent to control the secretion of macerating enzymes such

as polygalacturonase, pectate lyase, cellulose and host specific toxins by *Erwinia carotovora* and *Alternaria alternata*. The significant reduction in weight loss of RWC by GBLS in the presence of chitosan at 0.06, 0.08 and 0.1 % (w/v) tested indicated that lower secretion of LDE may influence the decay of RWC.

In conclusion, the present study has established new knowledge on the role of *Ganoderma* as the cause of a white rot in oil palm. The *in vitro* culture system confirmed the production of LDE by *Ganoderma* (GBLS isolate) which has been postulated in lignin degradation in oil palm BSR disease. These interesting findings could lead to manipulation of chitosan as a bio-fungicide against *Ganoderma* on oil palm under *in vivo* and in field condition.

CHAPTER 6

EFFECT OF CHITOSAN APPLICATION ON GROWTH OF OIL PALM AND CONTROL AND OF *GANODERMA* BASAL STEM ROT (BSR) INFECTION

6.1 Introduction

Basal stem rot (BSR) caused by the fungal pathogen *Ganoderma* is a major and economically important disease of oil palm in Malaysia. The increasing incidence of this disease has raised concerns about finding significant solutions to eradicate it in oil palm plantations. The current control approaches, such as conventional cultural practices, application of fungicide, manipulation of biological agents and developing disease resistant cultivars (as discussed Chapter; 1, 2) have achieved limited success. According to Chung (2011) the most appropriate control measure in commercial BSR disease management till to date especially for replanting and existing plantations is through implementing proper sanitation practices. This minimizes inoculums sources responsible for infection such as the stumps, trunks, and infected root masses of BSR palms (Flood *et al*., 2000; Chung, 2011). Nevertheless, continued research on applied aspects of oil palm BSR management is necessary to provide alternatives to existing control measures.

As an extension from *in vitro* findings which showed convincing results on the inhibitory effect of chitosan on the growth and lignin degrading enzymes

(LDE) production of *Ganoderma* (Chapter; 4, 5), chitosan is explored in this present study as a potential natural bio-fungicide which could be a possible solution for BSR disease. Chitosan has gained great attention recently due to its advantageous properties such as biodegradability, antimicrobial activity and elicitor potential (Benhamou, 1996). Furthermore, the efficacy of chitosan as an active ingredient in several chitosan based commercial products such as Chitogel (Ecobulle, France) (Ait Barka *et al*., 2004), Elexa (Glycogenesys Inc, Boston USA) (Sharathchandra *et al*., 2004) and Armour-Zen (Botry-Zen Ltd, New Zealand) (Reglinski *et al*., 2010) has been noted for its ability to protect against plant diseases caused by fungal pathogens.

This study aims to determine the appropriate concentration of chitosan, suitable mode of delivery application (*via* root dipping, soil amendment and soil drenching) and time course for multiple applications for effective *Ganoderma* disease control in artificially inoculated oil palm seedlings. In addition, the effect of chitosan treatment on vegetative growth, and ultrastructural morphology of oil palm was investigated in this study.

6.2 Materials and methods

The pathogenic GBLS isolate of *Ganoderma* sp. used in this study was maintained on PDA medium at 28° C under dark conditions as described (Section 3.2). The plant materials used throughout in the shade house experiments were described (Section 3.4). Artificial inoculation technique which includes the preparation of GBLS inoculums on rubber wood blocks and inoculation method on oil palm seedlings was optimized and carried out as described (Section 3.51, 3.5.2). The preparation of chitosan solutions was done as described (Section 3.8).

6.2.1 Shade house experiment

6.2.1.1 Effect of chitosan at different concentrations and delivery method for controlling *Ganoderma* infection

Chitosan at the range of 0.5, 1.0, 1.5 and 2.0 % (w/v) was applied *via* root dipping, soil amendment and soil drenching application with modification as described by (El Mougy *et al*., (2006); Algam *et al*., (2010). The experiment was run for four months using a randomized complete block design with three replicates with of three seedlings. Two types of control treatment were used; (1) Positive: inoculated/ untreated and (2) Negative: uninoculated/ untreated were evaluated in this study.

For chitosan application by root dipping, roots of oil palm seedlings were individually dipped in chitosan solutions for 5 min and allowed to air dry for 30 min. The treated plants were then inoculated with GBLS inoculums and transplanted in soil-based compost in polyethylene bags. For soil amendment**,** the soil compost was mixed with chitosan solutions for each of the concentrations at the rate of 100 ml/ kg of soil. The treated compost was transferred into polyethylene bags and the oil palm seedlings were transplanted into them. For soil drenching 100 ml chitosan solutions was applied to each polyethylene bag.

The plants were watered daily and weeded manually on a regular basis. Based on the results obtained from these trials, chitosan at 0.5 % (w/v) applied as soil drench application method appeared to show lower disease severity on oil palm post four months after inoculation (Table 6.2).

6.2.1.2 Effect of multiple application of chitosan *via* soil drenching for controlling *Ganoderma* infection

An experiment was set up with eight treatments (two untreated controls and six treatments with 0.5 % (w/v) applied as soil drench; Table 6.1 with three replicates of twelve seedlings arranged in a randomized complete block design. The plants were maintained for nine months in a shade house, watered daily and weeded manually on a regular basis. No additional organic fertilizer was applied. The whole experiment was repeated twice.

Table 6.1: Treatments of 0.5 % (w/v) chitosan application as a soil drench method to oil palm seedlings

Treatment		Description
Inoculated/ Untreated	T1	Positive control
Inoculated / Treated	T3	Treated once: immediately
	T4	Treated twice: immediately, then after 3 months
	T5	Treated three times: immediately, then after by 3 and 6 months
Uninoculated/ Untreated	T2	Negative control
Uninoculated/ Treated	T6	Treated once: immediately
	T7	Treated twice: immediately then after 3 months
	T8	Treated thrice: immediately, then after 3 and 6 months

6.2.2 Effect of chitosan on basal stem rot (BSR) disease severity

Severity of foliar symptoms percentage

The oil palm seedlings were recorded for disease severity at intervals of three months based on foliar symptoms as follows:

Severity of foliar symptoms (%) = (a x 1) + (b x 0.5) /c x100

where a is the number of desiccated leaves, b is the number of chlorotic leaves, c is total number of leaves and the numerical value of 1 represents the index for desiccated leaves and 0.5 the index for chlorotic leaves (Sariah & Zakaria, 2000).

Bole infection percentage

At the end of experiment by dissecting the bole of oil palm seedlings was dissected longitudinally and were visually scored based on the scale illustrated by Breton *et al*., 2006 in which:

0 = healthy: no internal rot,

1 = up to 20 % rotting of bole tissue,

2 = from 20 to 50 % internal rotting

3 = over 50 % internal rotting

4 = total rotting of bole tissue along with total desiccation of the plants.

The scores were later converted to a Disease Severity Index (DSI) based on the equation by Kobriger & Hagedorn (1983):

Disease severity index = (Disease class x Number of plants in that were scored in that class)/ (Total number of plants x 4) x 100.

Area under the Disease Progress Curve (AUDPC)

The effect of chitosan treatment on disease progression in oil palm seedlings was estimated based on the severity of foliar symptoms using the Area Under the Disease Progress Curve (AUDPC) equation of Shaner & Finney (1977) and Campbell & Madden (1990):

$$AUPDC = {}^{n-1}\sum_{i} [Y_{i} + Y_{i+2} / 2] (t_{i+1} - t_{i})$$

in which n is the number of assessment times, Y is the disease severity measurement and t is the observation time (in days). This method allows the

comparison of disease reduction between the treatments which was calculated as a measure of cumulative of disease incidence during the study.

6.2.3 Plant growth

At the end of experiment (after nine months) the growth parameters on the plant height, stem diameter, plant fresh weight and root mass weight were recorded. Plant height was measured from the soil level to the tip of growing leaves and the stem diameter was measured on the plant height above ground. Plant fresh plant weight and root mass were determined by weighing the whole plant and roots of each oil palm seedlings (Sapak *et al*., 2008).

6.2.4 Scanning electron microscopy (SEM) study

Transverse section of roots sampled from control and chitosan treated oil palm plants were fixed onto the aluminium stub and stabilized by carbon tape adhesive. The samples were examined and photographed using a scanning electron microscope (FEI/ Quanta 400 F**)** under Environmental SEM (SEM) vacuum mode using 10kV of electron beam with magnification ranging from 1000 x to 8000 x.

6.2.5 Determination of chitosan penetration in treated roots, bole tissues and stems of oil palm seedlings

The presence of chitosan tissues of oil palm seedlings was assessed calorimetrically using bromocresol purple dye (BCP, pH indicator dye; pH 4.7) as proposed by Abou-Shoer (2010) and Agrawal & Kotasthane (2012). At the start and end of the study (on the 9th month), the chitosan treated roots were cut into thin transverse sections and the bole tissues into longitudinal sections. These sections were immersed in in 20mg/ ml BCP dye solutions for 5 min. Then the excessive dye was removed using tissue papers and the sections air dried for 15 min. These samples were then observed under a microscope to determine colour changes which indicated the presence of chitosan.

6.2.6 Koch's Postulate of *Ganoderma* using molecular diagnosis technique

Total Genomic DNA from control and chitosan treated oil palm ramets (roots and leaves) were extracted according to Moller *et al.*, (1992) as explained (Section 7.2). Sufficient quantity of extracted of DNA were measured as described (Section 3.73). The specific primers designed as GAN 1F and GAN 2R which would produce a 167-bp PCR product were used for *Ganoderma* detection (Utomo *et al.*, 2005). PCR amplification were optimized and conducted as described (Section 3.74).

6.2.7 Statistical analysis

Data of disease severity percentage were transformed by arcsine and plant growth parametesr were subjected to ANOVA. Significant differences among treatments were analyzed by Tukey's Multiple Comparison post test at ($P \leq 0.05$) using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

6.3 Results

6.3.1 Effect of chitosan at different concentrations and mode of application on oil palm BSR disease severity

The results of oil palm disease severity based on severity of foliar symptoms and bole infection (Fig 6.1) were in agreement with previous study by Sariah & Zakaria (2000) and Breton *et al*., (2006) respectively. Root dipping application on oil palm seedlings was not successful for all the concentrations of chitosan tested. These plants began to show severe desiccation of leaves, formation of thick filming on the roots and died within a month after the treatment. The seedlings treated with chitosan by soil drenching application showed a lower percentage of disease severity (foliar symptoms and bole infection) compared to soil amendment application (Table 6.2). There were no significant differences in disease severity between the treatments in the seedlings treated by soil amendment application (Table 6.2). Meanwhile, the seedlings treated with 0.5 and 1.0 % (w/v) concentration of chitosan by soil drenching application recorded a significant reduction ($P \leq 0.05$) in disease severity compared to other treatments (Table 6.2). About three folds of disease severity reduction were observed in seedlings at 0.5 % (w/v) of chitosan concentration when applied as soil drenching compared to positive control seedlings. Based on the results demonstrated, soil drenching application at 0.5 % (w/v) concentration of chitosan was selected as an

appropriate delivery method with a maximum reduction in BSR disease severity for subsequent studies.

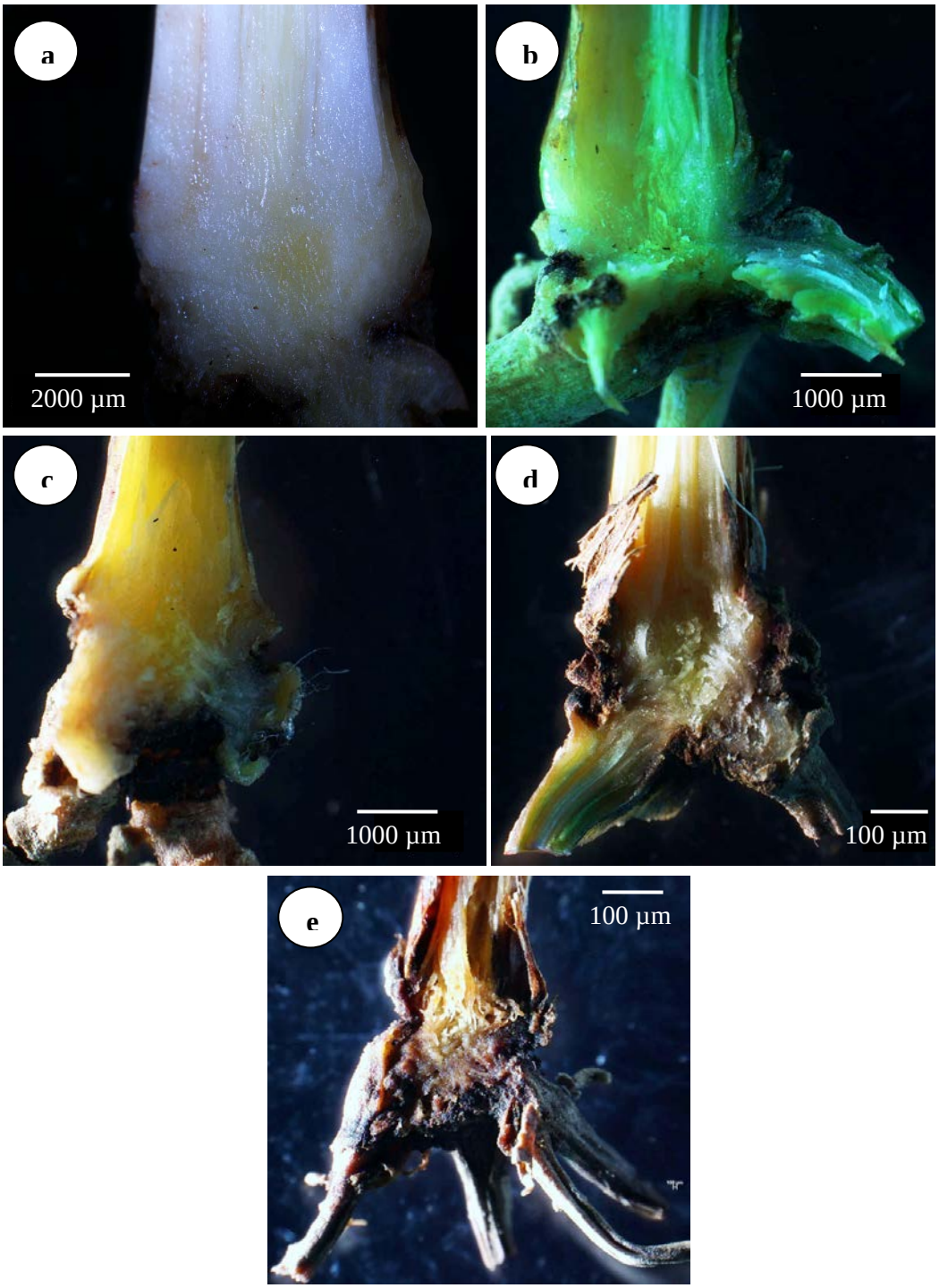


Figure 6.1: Disease severity (internal) estimation based on the proportion of bole tissues damaged by *Ganoderma* (a) 0 = healthy: no internal rot, (b) 1= up to 20 % rotting of bole tissue, (c) 2= from 20 to 50 % internal rotting (d) 3= over 50 % internal rotting (e) 4= total rotting of bole tissue along with total desiccation of the plants

Table 6.2: Disease severity of oil palm seedlings 4 months after treatment in response to mode of applications and concentrations of chitosan

	Disease Severity Assessment (%) Mode of Application			
	Soil amendment		Soil drenching	
Treatment/ Chitosan (%)	Severity of foliar symptoms	Bole infection	Severity of foliar symptoms	Bole infection
0.5	42.1a	16.7a	18.0c	7.4c
1.0	45.6a	16.7a	24.7c	10.2c
1.5	53.0a	26.0a	42.4a	21.3a
2.0	51.7a	24.0a	48.6a	23.1a
Positive control	52.5a	24.1a	52.2a	30.5a
Negative control	9.0b	0b	2.2b	0b

Means with the same alphabet are not significantly different at $P \leq 0.05$ using Tukey's Multiple Comparison post-test

6.3.2 Effect of multiple applications of chitosan as a soil drenching on oil palm BSR disease severity

All three treatments with 0.5 % (w/v) chitosan (T3, T4 and T5) reduced BSR disease severity (foliar symptoms and internal bole infection) significantly ($P \leq 0.05$) compared to positive control seedlings (Table 6.3). At nine months of inoculation, the disease severity in seedlings treated by chitosan as three applications (T5) did not differ from those treated with two applications (T4). However, in both of these treatments disease severity was significantly reduced (*P*

≤ 0.05) compared to the single application of chitosan (T3 seedlings) (Table 6.3, Fig 6.2a).

Sporophores developed only in positive control treatment after four to six months after inoculation (Fig 6.3 b & c). The areas under the disease progress curve (AUDPC) showed that highest disease reduction occurred in oil palm plants treated with T5 followed by T4 and T3.

Table 6.3: Effect of multiple addition of chitosan as soil drenching application at different intervals on basal stem rot (BSR) disease severity assessment and development on oil palm seedlings after nine months

Treatment	Severity of foliar symptoms (%)			AUDPC (units^2)	Disease Reduction (%)	Bole infection (%)
	3 months	6 months	9 months			
T1	46.1a	66.8a	82.7a	11803.2		63.8a
T2	2.4b	8.6b	11.6b			0.0b
T3	27.8c	35.8c	55.2c	6957.0	41.1	36.1c
T4	26.1c	31.7c	38.7d	5773.0	51.1	22.2d
T5	22.9c	31.3c	31.7d	5268.5	55.4	20.8d

Means with the same alphabet are not significantly different at $P \leq 0.05$ Tukey's Multiple Comparison post test.
AUDPC = Area Under Disease Progress Curve
T1= Positive control (inoculated/ untreated); T2= Negative control (uninoculated/ untreated); T3 = Inoculated/treated once (immediately); T4 = Inoculated/treated twice (immediately and after 3 months); T5 = Inoculated/treated three times (immediately and after 3 and 6 months).

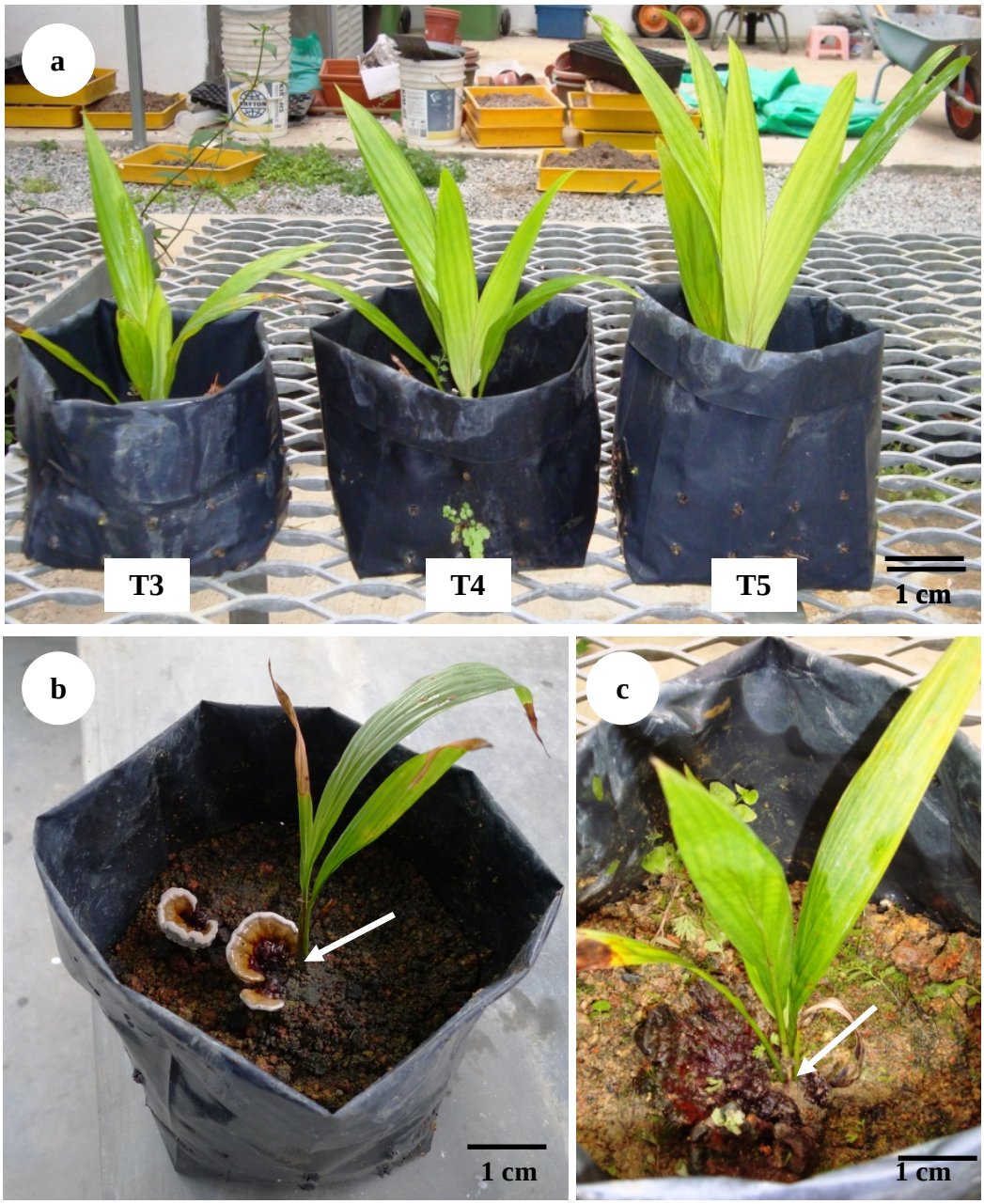


Figure 6.2: Disease development of basal stem rot (BSR) in oil palm seedlings treated with multiple applications of 0.5% (w/v) chitosan as a soil drench. (a) T3 (treated once: immediately), T4 (treated twice: immediately then after 3 months) and T5 (treated three times: immediately then after 3 and 6 months). (b) and (c) Production of sporophores in TI (positive control seedlings) after four to six months post–inoculation

6.3.3 Effect of chitosan on plant growth

By the end of the study (after nine months), all treatments (controls and chitosan at 0.5 % w/v) showed increases vegetative growth of the oil palm seedlings (Fig 6.3). Application of chitosan to seedlings not inoculated with GBLS isolate (treatments T6, T7 and T8) showed a significant increase ($P \leq 0.05$) in height, stem diameter and fresh weight compared to seedlings inoculated with GBLS (T3, T4 and T5). This trend indicates that the efficacy of chitosan application was not sufficient to maintain the growth of oil palm seedlings due to infection caused by *Ganoderma* (GBLS isolate), however the inoculated/treated seedlings (T3, T4 and T5) showed a significant improvement in vegetative growth ($P \leq 0.05$) compared to positive control seedlings (inoculated/untreated; T1) (Fig. 6.3). Increasing the applications to three times on inoculated (T5) and uninoculated (T8) seedlings resulted in a significant increase ($P \leq 0.05$) of plant height, stem diameter, fresh weight and root mass compared to respective control treatments, T1 and T2.

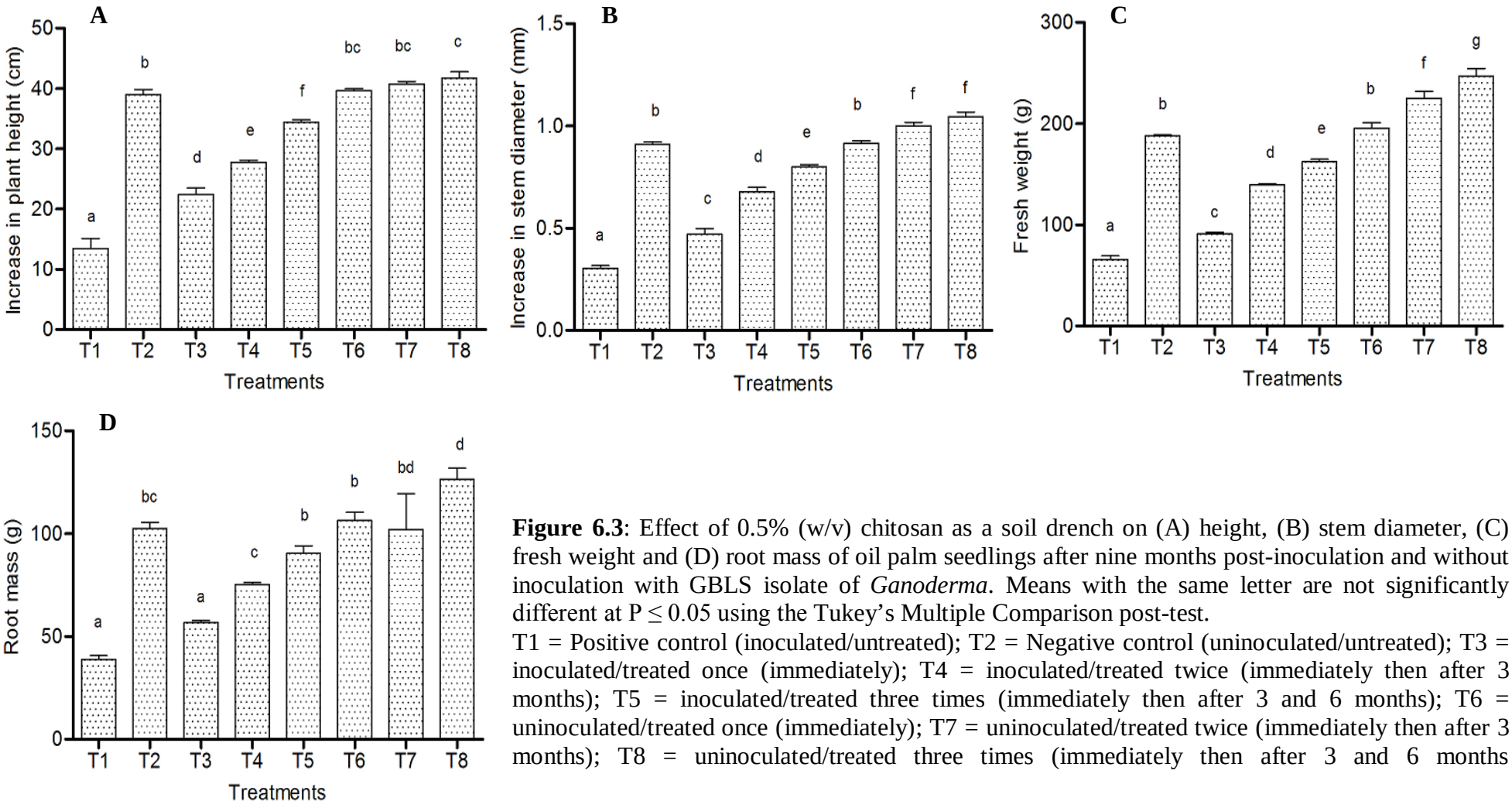


Figure 6.3: Effect of 0.5% (w/v) chitosan as a soil drench on (A) height, (B) stem diameter, (C) fresh weight and (D) root mass of oil palm seedlings after nine months post-inoculation and without inoculation with GBLS isolate of *Ganoderma*. Means with the same letter are not significantly different at $P \leq 0.05$ using the Tukey's Multiple Comparison post-test.
T1 = Positive control (inoculated/untreated); T2 = Negative control (uninoculated/untreated); T3 = inoculated/treated once (immediately); T4 = inoculated/treated twice (immediately then after 3 months); T5 = inoculated/treated three times (immediately then after 3 and 6 months); T6 = uninoculated/treated once (immediately); T7 = uninoculated/treated twice (immediately then after 3 months); T8 = uninoculated/treated three times (immediately then after 3 and 6 months

6.3.4 Effect of chitosan on oil palm surface morphology of oil palm roots

Scanning electron microscopy (SEM) study revealed alterations in oil palm roots caused by application of chitosan 0.5 % (w/v) chitosan as a soil drench (Fig 6.4 & 6.5). Uninfected roots (negative control) possessed typical arrangement of xylem vessels in oil palm primary roots (Fig 6.4 a, b). In contrast, alterations were observed in endodermis, xylem vessel and cortical cells of infected roots (positive control) (Fig 6.4 c, d).

Transverse sections of infected roots of chitosan treated seedlings (T4 and T5 treatments) after nine months revealed some changes (Fig. 6.5) which were not detected in the roots of T3, infected/ untreated (T1), uninfected/untreated (T2) and chitosan treated plants (T6, T7 and T8). Some thickening and coating of pit membranes may have been due to chitosan polymer was also evident (Fig. 6.5 a, b).

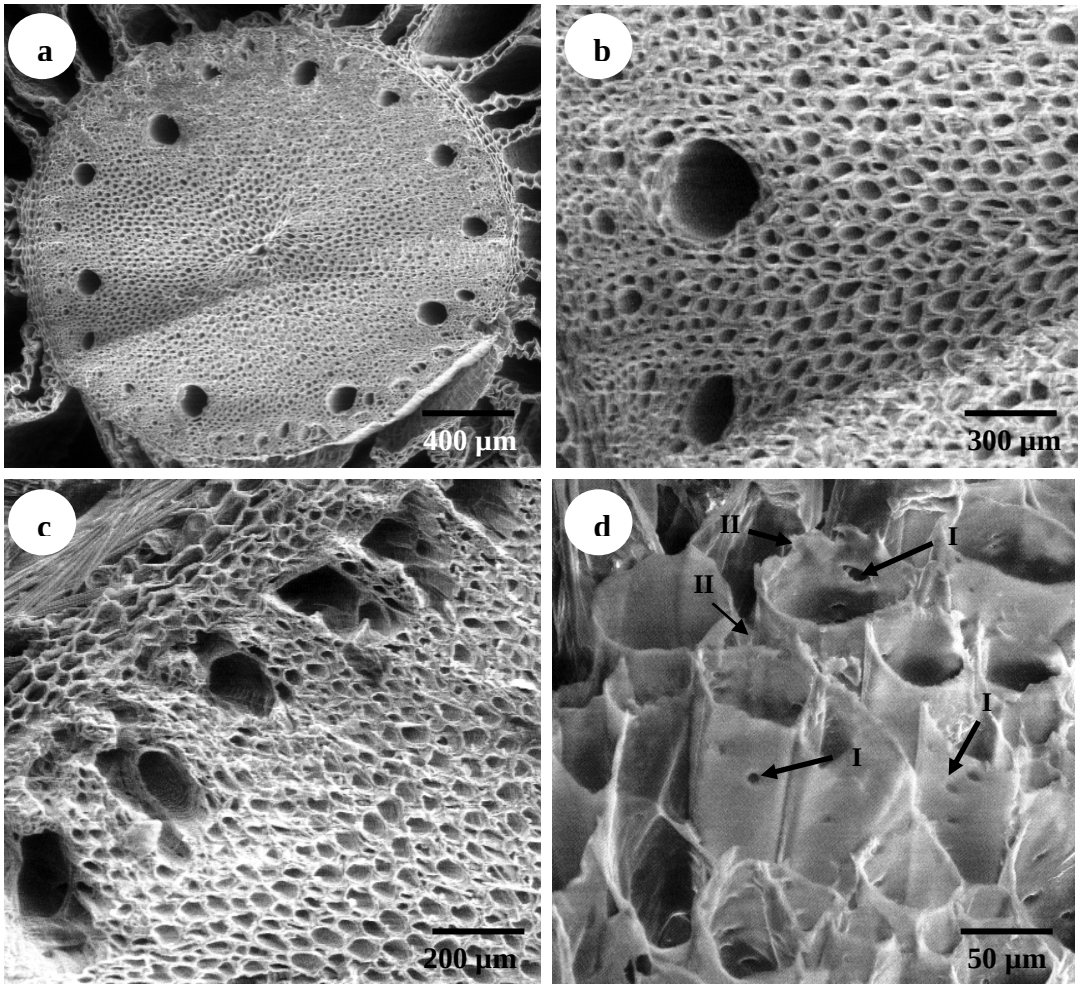


Figure 6.4: (a) Transverse section of healthy roots (T2) (b) Arrangement of xylem vessels of healthy roots (c) Transverse section of roots inoculated with *Ganoderma* (T1) (d) Lesions (I) and damage (II) in cortical cells of root inoculated with *Ganoderma*

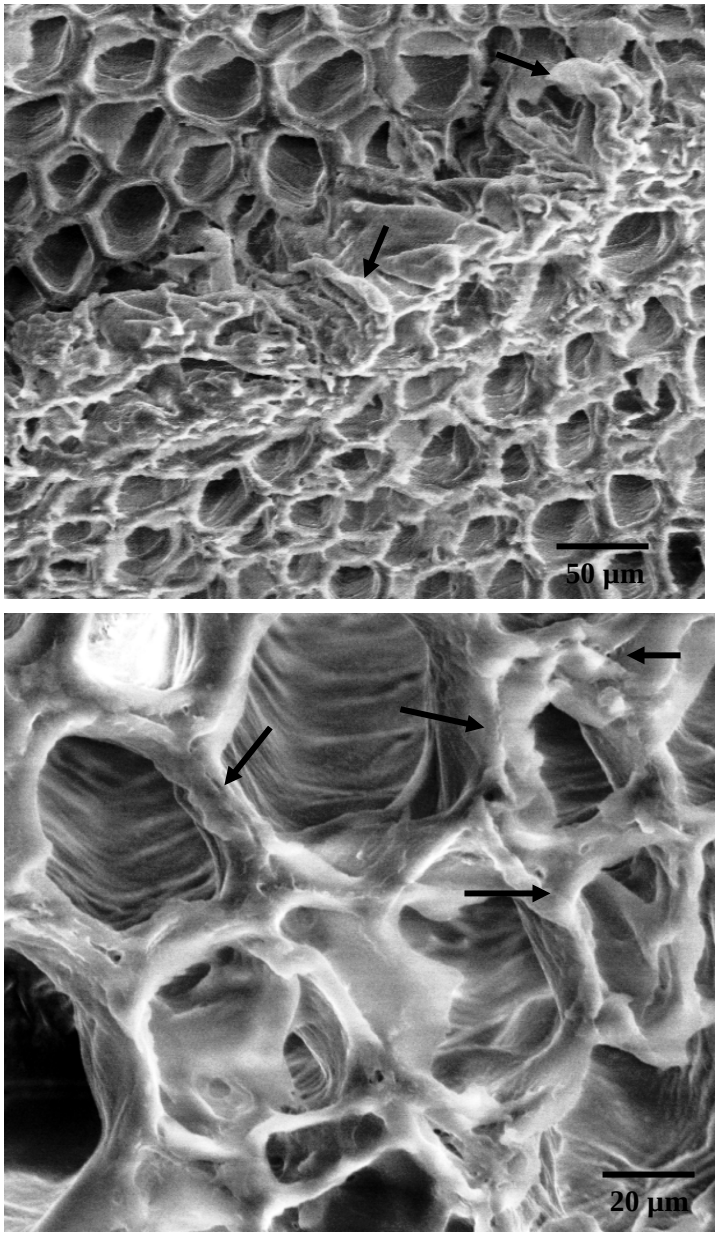


Figure 6.5: Scanning electron micrographs of oil palm roots infected by *Ganoderma* and treated with 0.5 % (w/v) chitosan as a soil drench (root samples were taken from T5 seedlings) Thickening of pit membranes (arrows) due to coating of chitosan

6.3.5 Detection of chitosan in treated bole tissues, stems and roots of oil palm

Tissue samples from treatments T4, T5, T7 and T8 stained with BCP dye showed a change in the dye colour from bright yellow to reddish purple which indicated the presence of N-acetyl glucosamine units of chitosan (Fig. 6.6).

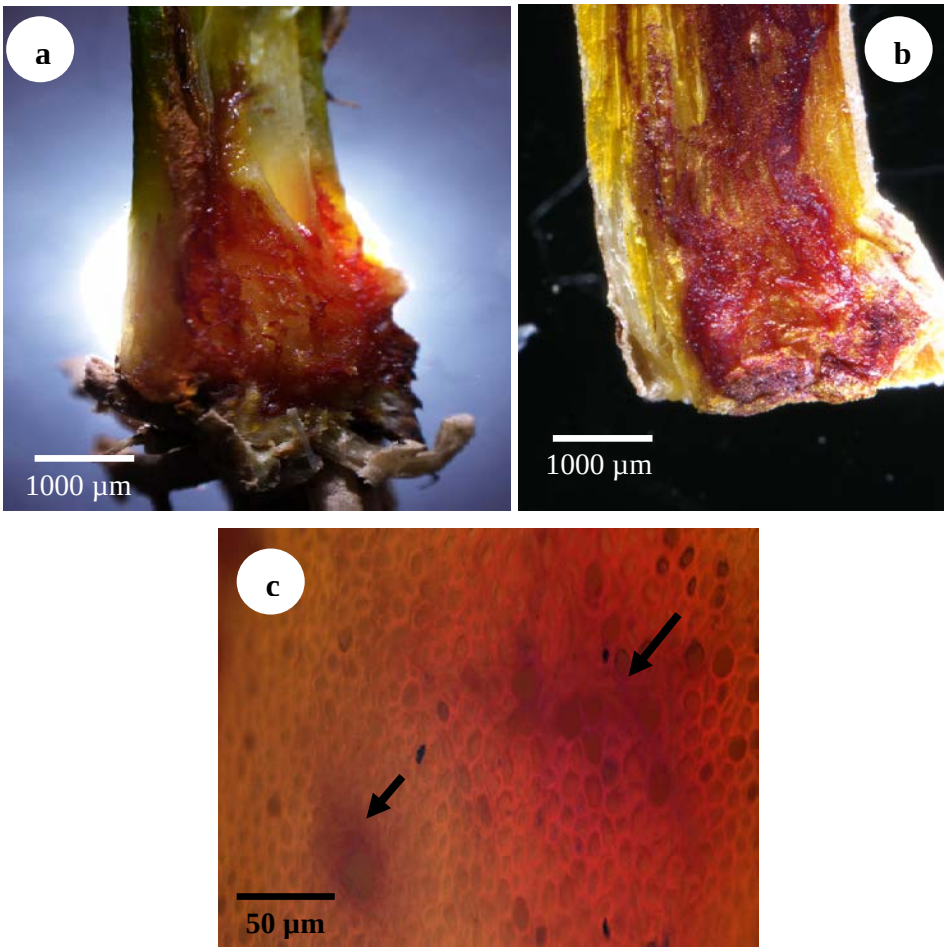


Figure 6.6: Evidence for chitosan penetration into oil palm tissues using bromoscerol purple dye. Note the colour change (arrows) from bright yellow to reddish purple in (a) bole tissue (b) stem (c) transverse section of root

6.3.6 Koch's Postulate of *Ganoderma (*GBLS isolate) by PCR amplification

The presence of *Ganoderma* in the infected roots and bole tissue from positive control plants (infected and untreated; T1), chitosan treated seedlings (T3, T4, T5) and pure *Ganoderma* mycelium was confirmed by amplification of a 167 bp PCR product (Fig. 6.7). As expected there was no amplification with the negative control plants (uninfected and untreated; T2) and this confirmed that the diseased samples would not generate false DNA bands.

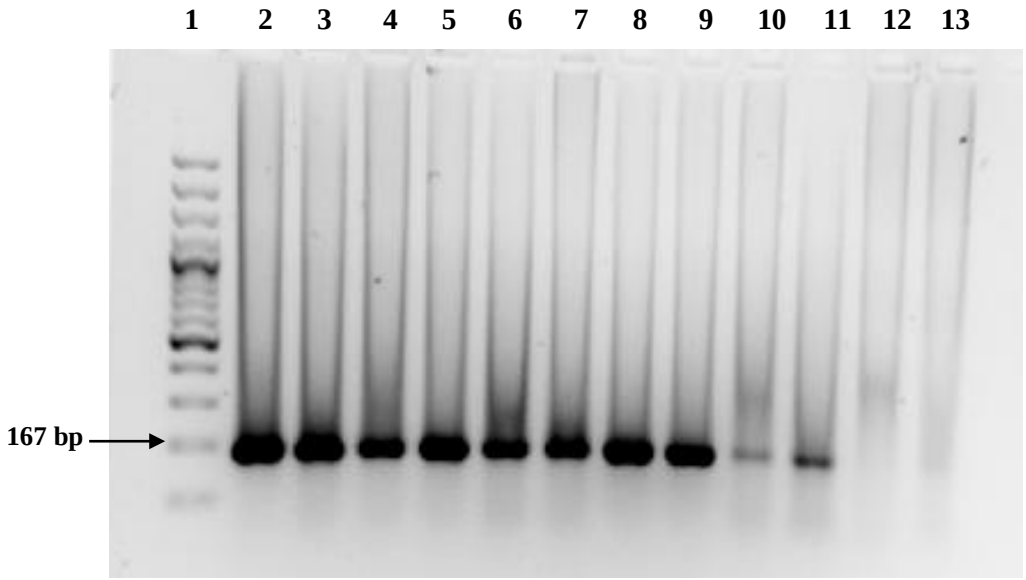


Figure 6.7: Confirmation of Koch's postulate of *Ganoderma* (GBLS isolate) through PCR amplification using GbF and GbR primers in artificially inoculated oil palm ramets. Lane **1**: 100 bp DNA ladder; Lanes **2-5**: Roots of T1, T3, T3 and T5 plants; Lanes **6-9**: Bole tissues of T1, T3, T4 and T5 plants; Lanes **10-11**: Positive control (mycelial DNA from GBLS isolate); Lanes **12-13**: Roots of T2 (negative control; uninoculated oil palm roots and bole tissue, respectively)

6.4 Discussion

This study reports the efficacy of chitosan as a biofungicide to control *Ganoderma* BSR infection on artificially inoculated oil palm seedlings grown under shade house conditions. Chitosan applied at 0.5 % (w/v) as a soil drench (100 ml/ per kg growing medium in polyethylene bags) achieved better reduction of disease severity of foliar symptoms and bole infection compared to other application methods (root dipping and soil amendment) and concentrations (1.0, 1.5 and 2.0 % w/v) evaluated. Root dipping at all the chitosan concentrations tested was not successful and with increasing the concentration of chitosan used, regardless of the application method (soil drenching and soil amendment), the severity of BSR in oil palm seedlings was found to increase as well. These phenomena may due to possible phytotoxicity of chitosan on oil palm seedlings. The best application of chitosan in this study was soil drenching and this compared favourably with other studies that have demonstrated that chemical fungicides (triazole and hexaconazole) and biocontrol agent formulations (*Trichoderma* and endopyhtic bacteria) applied on *Ganoderma*-infected oil palm in controlled environments or field conditions were effective as a soil drench or soil amendment methods (Sariah & Zakaria, 2000; Idris *et al*., 2004; Sariah *et al*., 2005; Sapak *et al*. 2008; Susanto, 2009; Nur Ain Izzati & Abdullah 2008; Alizadeh *et al*., 2011; Naher *et al*., 2012). The high disease reduction and improved vegetative growth in treatments T4 and T5 treatment suggest that a single application of chitosan immediately after inoculation, as in T3 treatment is not appropriate. To obtain

efficient disease control under shade house conditions a multiple application of chitosan treatment as in treatments T4 and T5 needs to be considered.

There have been many studies on the methods application and chitosan concentrations to control plant diseases on crops caused by microbial pathogens. The recent study by Chong *et al*., (2012) which reported chitosan as a soil amendment at a minimum rate of 5 g of chitosan/ 5 kg of growing medium showed a significant reduction in the severity of disease in oil palm as in the present study. Chitosan evaluated as a seed treatment has been shown to enhance germination and seedling vigor in wheat (Reddy *et al*., 1999), pearl millet (Sharatchandra *et al*., 2004) and chilli (Photchanacha *et al*., 2006) and reduce infection caused by *Pyricularia grisea* on rice seedlings (Rodriguez *et al*., 2007). Chitosan added to nutrient solution (400µg/ ml) was able to suppress *Pyhtium aphanidermatum* infections in hydrophonic cucumber plants (El Ghaouth *et al*., 1994). Ben-Shalom *et al*., (2003); Ben Shalom and Fallik (2003) reported that chitosan applied as a spray (0.1 % and 0.2 g/l respectively) contributed to the control of gray mould disease caused by *Botrytis cinerea*. Treatment of tomato seedlings with chitosan protected them from crown and root rot caused by *Fusarium oxysporum*. Effective protection in terms of reducing the disease incidence of root lesions was achieved by root dipping, foliar spraying at 0.5-2.0 mg/ ml (Benhamou & Theriault, 1992) and seed coating in combination with substrate amendment at 2mg/ ml (Benhamou *et al*., 1994). Abd-El-Kareem *et al*., (2006) and El-Mougy *et al*., (2006) demonstrated that chitosan applied as a soil amendment at 6kg/ soil reduced the incidence of disease in tomato roots caused

by pathogens such as *Rhizoctonia solani*, *Fusarium solani* and *Sclerotium rolfsii* in greenhouse and field trials. Algam *et al*., (2010) also proposed that chitosan improved disease control against the bacteria pathogen *Ralstonia solanacearum* in tomato when used as soil drench (10 mg/ ml and 50 ml/ per pot). Foliar spraying of groundnut leaves with 1000 ppm chitosan suppressed leaf rust caused by *Puccinia aracchidis* (Satthiyabama & Balasubramaniam, 1998). On soybean leaves sprayed at 3 mg/ ml, it effectively delayed sudden death syndrome (SDS) caused by *Fusarium solani* (Prapagdee *et al*., 2007) and, in detached leave assays (5, 10 and 20 g/L) and field experiments successfully reduced *Botryis* bunch rot in grapes (Reglinski *et al*., 2010). A plant promoting effect of chitosan has also noted on various crops such as strawberry, tomato, pearl millet, chilli and soybean (Ait Barka *et al*., 2003; Sharatchandra *et al*., 2004; Photchanacha *et al*., 2006; Prapagdee *et al*., 2007; Algam *et al*., 2010; Abdel-Mawgoud *et al*., 2010). It is difficult to compare the efficacy of chitosan in the present study with other reports in the literatures due to the variation in the uptake and translocation by different crop species, application rate/method, timing/interval of application and environmental factors (Cooke *et al*., 2001). One explanation for the potential of chitosan to suppress or minimize a wide range of plant diseases is its ability to activate and induce plant defense responses during the host-pathogen interaction. This may explain how chitosan reduce BSR severity on oil palm seedlings as discussed in the Chapter 7.

Examination of roots sections in the scanning electron microscope (SEM) provided evidence that chitosan as at 0.5 % (w/v) applied as a soil drenching had

induced structural changes in roots of oil palm seedlings (treatments T4 and T5) by nine months after inoculation. The pattern and the extent of alteration and damage on the roots of control seedlings (positive control) observed were closely similar as in the previously by Kandan *et al*., (2009) on coconut roots infected by *Ganoderma*. The appearance of coating material and thickening of pit membranes in oil palm roots treated with chitosan were in the agreement with a study on the of tomato roots infected by *F. oxysporum* (Benhamou & Theriault, 1992). This coating response may provide barriers for *Ganoderma* entry into the root system of oil palm which consequently resulted in minimum alterations of the roots. Induction of structural defense responses by a host due to application of chitosan, such as formation of structural barriers at the targeted sites of fungal penetration has been described as an important element in plant disease resistance (Benhamou, 1996; Baustista-Banos *et al*., 2006). Findings from transmission electron microscopy (TEM) have confirmed the appearance and interesting formation of structural features due to host-pathogen interaction after chitosan treatment on the roots of tomato infected with *F. oxysporum* (Benhamou & Theriault, 1992; Benhamou *et al*., 1994; Lafontaine & Benhamou, 1996; Benhamou *et al*., 1998), cucumber infected with *P. aphanidermatum* (El Ghaouth *et al*., 1994) and bell pepper fruit infected with *B. cinerea* (El Ghaouth *et al*., 1997). The general observations reported in these studies included papillae formation in the root cells, occlusion of xylem vessels by an opaque or fibrillio material and coating of cell wall thickening and pit membranes. According to Benhamou & Theriault (1992), absence of structural barriers in uninfected,

chitosan treated roots suggested that the expression of plant defense response genes may be initiated from the fungal pathogen and chitosan may only play a role in assisting the plants to respond more quickly towards fungal invasion without being involved in the stimulation of defense gene products.

The positive reaction of chitosan (amino group function polymer) with anionic dyes such as bromocresol purple (BCP) results in the production of a stable visually coloured product (Abou-Shoer, 2010). Thus, the change of BCP from yellow to purple colour confirmed the presence of N-acetyl glucosamine (chitosan) at the pH range from 5.1 to 5.6 (Agrawal & Kotasthane, 2012). Maffi *et al*., (1998) used another anionic dye for the assessment of chitosan in the fungus *Erysiphe graminis* and barey host cell walls. The nitrous acid and 2-hydrrazono-3-methylbenzothiazoline (MBTH) staining which formed a pale blue colour indicated the presence of chitosan. Staining with BCP which formed reddish purple colour in the bole tissues, stems and sections of roots from oil palm seedlings treated with chitosan (treatment T4, T5, T7, and T8) revealed that chitosan had penetrated the tissues. This was achieved using 0.5 % (w/v) chitosan applied as soil drench multiple applications increased the penetration of the tissues. No detection following a single application of chitosan (treatments T3 and T6) indicated that the chitosan polymer might have degraded during the nine months. The verification of Koch's Postulate in this study showed that the primers amplified DNA fragment of 167-bp from pure *Ganoderma* mycelial DNA and all *Ganoderma*-infected samples of roots and bole tissues after nine months post inoculation. These results are in agreement with previous studies which confirmed

the presence of *Ganoderma* in infected plant parts of oil palm and coconut (Utomo *et al*., 2005; Kartikeyan *et al*., 2007; Kandan *et al*., 2009).

In summary, this work has indicated chitosan to be potential biofungicide to control oil palm BSR disease caused by *Ganoderma*. Although this study was done in the conditions of a small shadehouse, the results on the method of application, the concentrations of chitosan and the need for multiple application of provides essential knowledge towards a BSR disease management strategy. The present findings showed that chitosan by soil drenching reduce BSR disease symptoms and infection on artificially inoculated oil palm seedlings could serve as the basis for further exploration of the efficacy of chitosan as a commercial application in field. Elucidation of how chitosan may induce a plant defense response during the host-pathogen interaction, from the scope of this study is described in next Chapter. This would further promote chitosan as viable long term solution for BSR disease control.

CHAPTER 7

EFFECT OF CHITOSAN ON THE INDUCTION OF PHENOLICS AND PLANT DEFENSE ENZYMES DURING INTERACTION BETWEEN OIL PALM AND BASAL STEM ROT PATHOGEN, *GANODERMA*

7.1 Introduction

Plants are equipped with complex inducible defense machinery responses that provide protection against pathogen attack or are activated following elicitor treatments (Montesano *et al.*, 2003; Trotel-Aziz *et al*, 2006). According to Benhamou (1996), during plant-pathogen interactions, delayed plant defense responses are most likely to favours the pathogens to invade plant cells, colonize and establish diseases rather than the absence of or inactivation of a defense reaction. Timely recognition of signals originating from pathogens or elicitor molecules and rapid activation of essential plant defense reactions are central to ensure plant protection (Benhamou, 1996; Ebel & Cosio, 1994). Once pathogen recognition has been successfully determined, plant cells stimulates early events including rapid ion depolarization in the plasma membrane, activation of kinase cascade and production of reactive oxygen species (ROS) such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Benhamou, 1996; De Gara *et al.*, 2003; Montesano *et al.*, 2003). These responses are usually followed by an immediate rapid reaction known as hypersensitivity (cell death response) which

occurs at the infection points, alteration in plant cell walls, activation of systemic acquired resistance (SAR) which occurs in non- infected parts of the plant, and stimulation of biochemical defense responses including phytoalexin synthesis, plant defense enzymes and pathogenesis-related proteins (PR) (Paz-Lago *et al*., 2000; Rabea *et al*., 2003).

The role of chitosan in integrated disease management as a controlling agent for microbial diseases and an exogenous elicitor of host defense responses have been presented in the literatures (Hadwiger *et al*., 1986; Benhamou & Theriault, 1992; Doares *et al*., 1995; El Paz-Lago *et al*., 2000; El Hassni *et al*., 2004; Trotel-Aziz *et al*., 2006). One distinct biological response in several plants is its capability to elicit plant defense enzymes against pathogens (Chen *et al*., 2000; Baustista-Banos *et al*., 2006). These enzymes are involved in stimulation of phenylpropanoid pathways, biosynthesis of antimicrobial compounds and accumulation of pathogenesis-related (PR) proteins (Chen *et al*., 2000; Karthikeyan *et al*., 2006; Trotel-Aziz *et al*., 2006).

In response to *Ganoderma* infection in oil palm, the evidence for induced defense response mechanisms, which could plays a key role in basal stem rot (BSR) establishment and control is still inadequate. According to Tay *et al*., (2009), *Ganoderma* triggered accumulation of PR proteins which were activated systemically but were insufficient to halt the fungal growth and spread. Hence, realizing an induced plant defense response by chitosan, this study would represent a novel control strategy for BSR management in oil palm. This would also improve our understanding of basic biochemical events that take place during

oil palm-*Ganoderma* (host-pathogen) interactions followed by the possible plant defense response with chitosan as a biotic elicitor.

Although studies on oil palm for the induction of defense responses are limited, notable results on the suppression of BSR obtained with chitosan treatment (Chapter 6) has prompted the investigation of the role of chitosan in defense reactions which suppress BSR. Thus, the present study aims to evaluate the ability of chitosan to elucidate the expression of phenolics and defense enzymes in oil palm seedlings inoculated with *Ganoderma*.

7.2 Materials and method

7.2.1 Shade house experiment

The sources of fungus and oil palm seedlings, techniques of inoculation using rubber wood blocks and preparation of chitosan solutions were described in Section (3.1, 3.4, 3.5.1, 3.5.2 and 3.8) respectively. The design of the experiment was similar to that described in Section (6.2.1.2). The treatments to study the effects of applying 0.5 % (w/v) chitosan as soil drenches were as follow;

T1: Positive control (inoculated/ untreated);

T2: Negative control (uninoculated/ untreated);

T3 (Inoculated/ treated immediately);

T4 (Inoculated/ treated immediately and after 3 months);

T5 (Inoculated/ treated immediately and after 3 and 6 months);

T6 (Uninoculated/ treated immediately);

T7 (Uninoculated/ treated immediately and after 3 months);

T8 (Uninoculated/ treated immediately and after 3 and 6 months)

7.2.2 Collection of samples for biochemical assay

Sampling of oil palm seedlings was done destructively at intervals of 0, 3, 6 and 9 months after treatment with two seedlings per replicate. The primary and secondary roots were pooled per seedling for the enzyme assays. The roots were washed in running tap water, dried using paper towels and stored in a freezer (-80° C).

7.2.3 Total phenolic content

Total phenolic content was determined by using the Folin-Ciocalteu method as described by Singleton *et al*., (1999). Root samples (1 g) were homogenized using pre-chilled pestles and mortars containing 10 ml of ice cold methanol (80 % v/v) and vortexed for 5 min. The homogenates were centrifuged at 13000 g for 5 min and the root extracts were collected.

The reaction mixture consisted of 100 µl of root extracts, 2 ml of distilled water, 200 µl of Folin-Ciocalteu reagent and 1 ml of sodium carbonate solution and was incubated for 2 hours at room temperature. A blank reaction was prepared by substituting root extract with 80 % methanol. Then 200 µl of the reaction mixture and blank solution were pipetted into 96 well plates and the absorbance of developed blue colour was measured at 765 nm. Total phenolic content was expressed as mg of gallic acid equivalent per gram of sample (mg g^{-1}) using gallic acid as a standard reference curve (Appendix 2).

7.2.4 Phenylalanine ammonia lyase (PAL)

Roots samples (1g fresh weight) collected at different time intervals were homogenized in 10 ml of ice-cold 0.1 M sodium borate buffer (pH 8.8) which consisted of 1.4 mM 2-mercaptoethanol and 0.3 g insoluble polyvinyl pyrrolidone (PVP). The homogenate was filtered through cheesecloth and centrifuged at 16,000 g for 15 min at 4 °C. The obtained supernatant was used as a crude enzyme extract for PAL activity assay (Kartikeyan *et al.*, 2006).

PAL was assayed as the rate of conversion of L-phenylalanine conversion to *trans*-cinnamic acid according to Dickerson *et al*., (1984). The reaction mixture consisted of 40 µl crude enzyme extract and 50 µl 12 mM L- phenylalanine in 50 µl 0.1 M sodium borate buffer (pH 8.8) and was incubated for 30 min at 30 °C. Absorbance at 290 nm was read and the concentration of *trans*-cinnamic acid was calculated using extinction coefficient value of 9630 M^{-1}. The enzyme activity was expressed as µmol of *trans*-cinnamic acid min^{-1} mg^{-1}**.**

7.2.5 Assay of peroxidases

7.2.5.1 Total peroxidases (PO)

Roots sample (1g fresh weight) were homogenized in 8 ml tris-maleate buffer 0.1 M (pH 6.5) which consisted of Triton 100 X (0.1 g/l^{-1}) as described El Hassni *et al*., (2004). The homogenate was filtered through cheesecloth and centrifuged at

16,000 g for 15 min at 4 °C. The supernatant was used as a crude enzyme extract for PO activity assay.

PO activity was assayed by adding 2 µl of the crude enzyme extract into a reaction mixture of 200 µl tris-maleate buffer (pH 6.5) and 25 mM guaicol as a substrate. The reaction mixture was initiated by adding 2 µl of H_2O_2 (10 % v/v) and the absorbance was measured after 3 min at 470 nm (El Hadrami & Baaziz, 1995). The enzyme activity was expressed as changes in absorbance min^{-1} mg^{-1} protein.

7.2.5.2 Polyphenoloxidase (PPO)

Roots sample (1g fresh weight) were homogenized in 10 ml of 0.1 M sodium phosphate buffer (pH 6.0), filtered through cheesecloth and centrifuged at 16,000 g for 15 min at 4 °C. The supernatant was used as a crude enzyme extract for the assay of PPO activity (Mayer *et al.*, 1965)

PPO activity was determined by adding 155 µl 0.1 M sodium phosphate buffer (pH 6.0), 40 µl 0.2 M catechol as a substrate and 5 µl crude enzyme extract. The absorbance at 420 nm was measured for 3 min at intervals of 30 (Chen *et al.*, 2000; El Hassni *et al.*, 2004) and enzyme activity was expressed as changes in the absorbance min^{-1} mg^{-1} protein.

7.2.6 Assay of cell wall degrading enzymes assay

7.2.6.1 β-1-3-glucanase

Roots samples (1g fresh weight) were homogenized in 10 ml of 0.05 M sodium acetate buffer (pH 5.0), filtered through cheesecloth and centrifuged at 16,000 g for 15 min at 4 °C. The supernatant was used as a crude enzyme extract for β-1-3 glucanase activity assay (Kartikeyan *et al*., 2006).

β-1-3-glucanase activity was determined by measuring the release of reducing sugar, laminarin as a substrate and using glucose as a standard reference curve (Appendix 4). The reaction was carried out at 40 °C for 2 h using 1.5 ml micro-centrifuge tubes consistaining 100 µl crude enzyme extract and 200 µl (2 mg/ml) laminarin prepared in sodium acetate 0.1 M (pH 5.0) as described by Fink *et al*., (1988) and Rodriguez *et al*., (2007). The amount of reducing sugar produced was measured by terminating the reaction with 375 µl of dinitrosalicylic acid reagent solution (DNS) followed by heating for 5 min on a boiling water bath and vortexing. 200 µl of the reaction solution were pipetted into 96 well plates and the absorbance was measured at 500 nm (Kartikeyan *et al*., 2006). The enzyme activity was expressed as the amount of enzyme which produced 1 µmol of glucose ml^{-1} min^{-1}

7.2.6.2 Chitinase

Roots samples (1g fresh weight) were homogenized in 10 ml sodium citrate buffer 0.1 M (pH 5.0), filtered through cheesecloth and centrifuged at 16,000 g for 15 min at 4 °C. The supernatant was used as a crude enzyme extract for chitinase activity assay (Kartikeyan *et al*., 2006).

Chitinase activity was determined by measuring the release of N-acetyl-glucosamine (NAG) from colloidal chitin as a substrate by using NAG as a standard reference curve (Appendix 5) as described in the protocol of Reissig *et al*., (1955). The reaction mixture was prepared as described by Nirala *et al*., (2010) with minor modification. The reaction was conducted in 1.5 ml micro-centrifuge tubes by mixing 100 µl crude enzyme extract with 300 µl chitin (0.5 % w/v dissolved in 0.05 M sodium acetate buffer pH 5.0) then incubated at 37 °C for 60 min. The reaction mixture was terminated on ice by adding 100 µl HCl 0.1 N and incubated further for 10 min to precipitate insoluble chitin followed by centrifugation at 14,000 g for 5 min.

The solution was transferred into new tubes and the obtained NAG product was measured according to the method of Miller (1959). For 100 µl reaction solution, 100 µl DNS reagent was added in the ratio of 1:1 and boiled for 10 min in a water bath. Then 40 µl potassium-sodium tartarate 40 % (w/v) was added and allowed to cool at room temperature. The total reaction solution (240 µl) was pipetted into 96 well plates and the absorbance was read at 540 nm. The enzyme activity was expressed as the amount of enzyme which produced 1 µmol of NAG ml^{-1} min^{-1}

The total protein quantification was determined by the method of Bradford reagent and bovine serum albumin (BSA) as standard reference curve (Appendix 3). All enzyme activities were measured spectrophotometrically by using a microplate reader (Variouskan Skan IT, UV). Each value represented the mean of three replicates.

7.2.7 Statistical analysis

The enzyme activity data obtained from this study were subjected to One-way ANOVA and tested for significant differences among treatments by Tukey Multiple Comparison post test at ($P \leq 0.05$) using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. The biochemical assays were carried out three times and the data were pooled before analysis.

7.3 Results

7.3.1 Total phenolic content

The pathogen inoculated control (T1) and some of the pathogen inoculated/treated seedlings (T3, T4 and T5) displayed a similar trend with the accumulation of phenolics for six months followed by a significant decline by the end of the study (Fig 7.1). These results coincide with the observation by Chong *et al*., (2012) which reports the presence of *Ganoderma* in the treatments may contribute in inducing phenolics at the early stage of disease development and metabolizes them later during the time course of study. Inoculated seedlings treated once with chitosan (T3) exhibited the same pattern of accumulation of total phenolics as inoculated control/untreated (T1) seedlings. Seedlings that received 2 or 3 chitosan applications (T4 and T5) showed significantly ($P \leq 0.05$) higher accumulation of phenolics compared to the controls and other treatments after 6 and 9 months post inoculation (Fig 7.1). The total phenolic content in these two treatments was significantly different after nine months.

The accumulation of phenolic was significantly higher in uninoculated /treated seedlings (T6, T7 and T8) compared to uninoculated/ untreated control seedlings (T2). After 9 months, uninoculated seedlings treated three times with chitosan (T8) possessed significantly higher activity of phenolics compared to uninoculated/untreated control (T2) and uninoculated /treated (T6 and T7) seedlings (Fig 7.1)

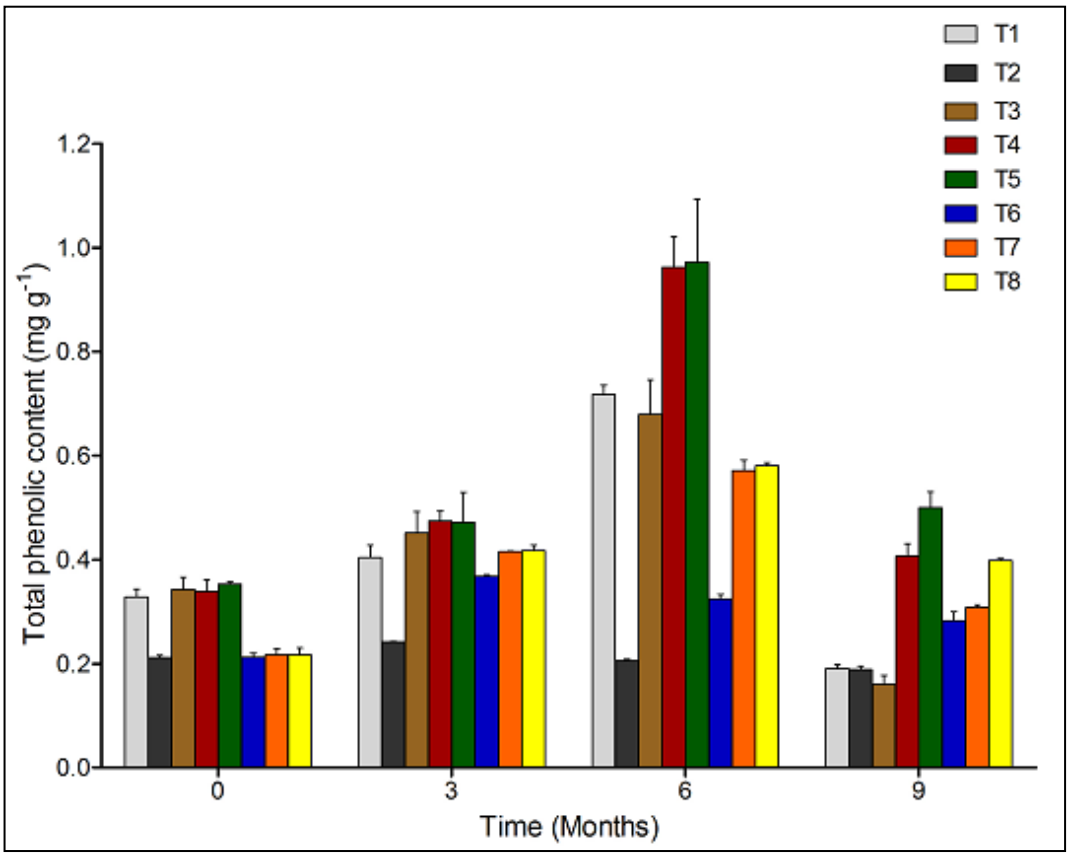


Figure 7.1: Accumulation of total phenolics in oil palm roots treated with 0.5 % chitosan (w/v) as a soil drench. Values are means of three replicates. Bars represent standard errors

T1: Positive Control (inoculated/ untreated);
T2: Negative Control (uninoculated/ untreated)
T3 (Inoculated/ treated immediately);
T4 (Inoculated/ treated immediately and after 3 months);
T5 (Inoculated/ treated immediately and after 3 and 6 months)
T6 (Uninoculated/ treated immediately);
T7 (Uninoculated/ treated immediately and after 3 months);
T8 (Uninoculated/ treated immediately and after 3 and 6 months)

7.3.2 Phenylalanine ammonia-lyase (PAL) activity

PAL activity was significantly ($P \leq 0.05$) higher in inoculated seedlings treated 2 or 3 times with chitosan (T4 and T5) compared to controls and other treatments after six and nine months (Fig 7.2). The peak of PAL activity occurred after six months in these seedlings and had declined by the end of the study. PAL activity was significantly ($P \leq 0.05$) higher in inoculated seedlings treated 3 times with chitosan (T5) compared to inoculated/untreated control (T1), inoculated/treated seedlings (T3 and T4) and other treatments, with 2.3-, 1.8- and 1.3 times higher activity compared to the control (T1), T3 and T4 respectively.

PAL activity in uninoculated/treated seedlings (T6, T7 and T8) appeared to vary very little within and between the treatmens. However there was a significant increase in the enzyme activity (1.7- and 1.8 times respectively) in the T7 and T8 seedlings compared to uninoculated/ untreated control seedlings (T2) after nine months.

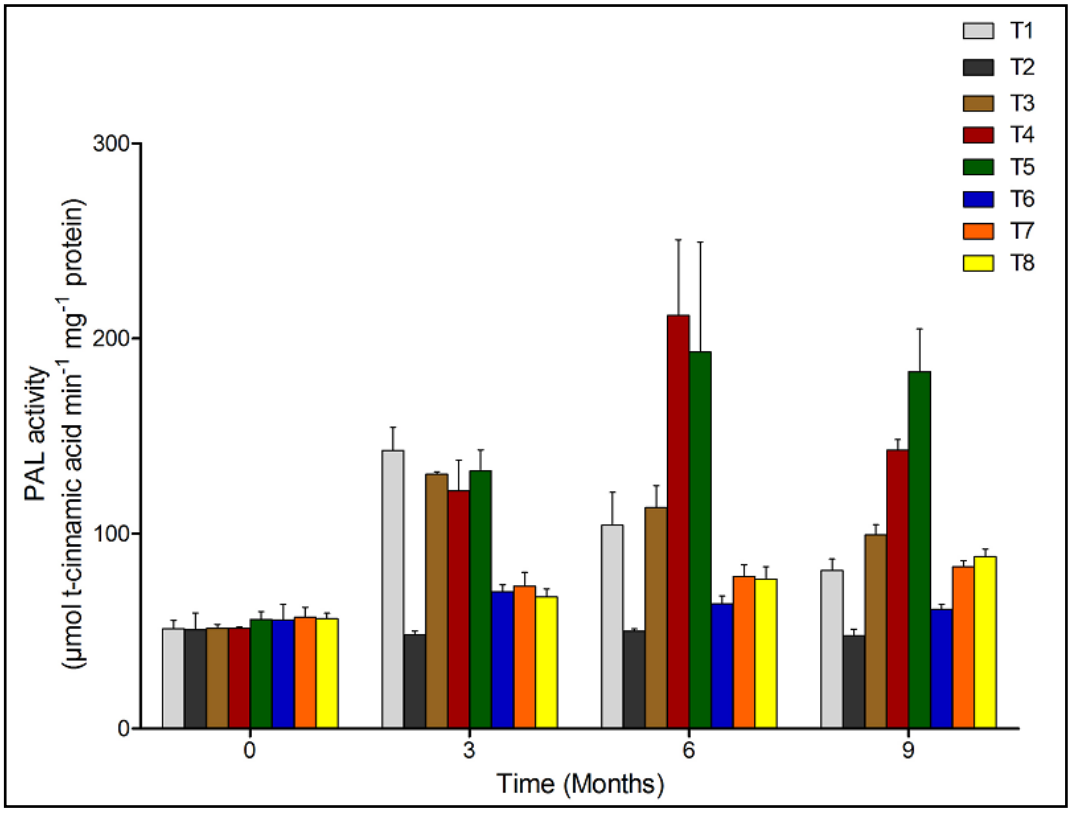


Figure 7.2: Phenylalanine ammonia-lyase (PAL) activity in oil palm roots treated with 0.5 % (w/v) chitosan as a soil drench. Values are means of three replicates. Bars represent standard errors.
T1: Positive Control (inoculated/ untreated);
T2: Negative Control (uninoculated/ untreated)
T3 (Inoculated/ treated immediately);
T4 (Inoculated/ treated immediately and after 3 months);
T5 (Inoculated/ treated immediately and after 3 and 6 months)
T6 (Uninoculated/ treated immediately);
T7 (Uninoculated/ treated immediately and after 3 months);
T8 (Uninoculated/ treated immediately and after 3 and 6 months)

7.3.3 Total peroxidase (PO) activity

PO activity of inoculated/untreated control (T1) and T3 treated seedlings showed little variation throughout the study (Fig 7.3). In contrast, in T4 and T5 seedlings the PO activity appeared to increase throughout the study. After nine months, PO activity was significantly ($P \leq 0.05$) higher in T5 seedlings compared to control

and most of the other treatments. Likewise, PO activity in the uninoculated/ treated seedlings, T7 and T8 was significantly higher compared to that in T6 and the uninoculated/untreated control (T2) after nine months.

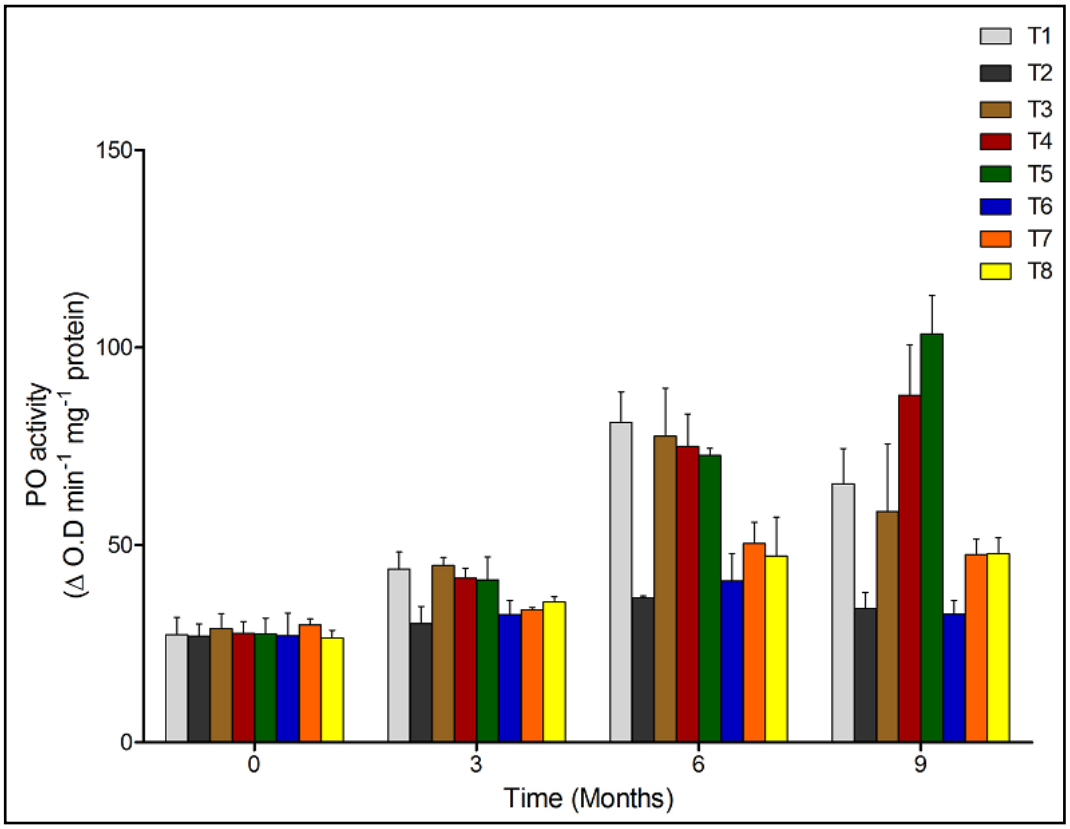


Figure 7.3: Peroxidase (PO) activity in oil palm roots treated with with 0.5 % (w/v) chitosan as a soil drench. Values are means of three replicates. Bars represent standard errors.
T1: Positive Control (inoculated/ untreated);
T2: Negative Control (uninoculated/ untreated)
T3 (Inoculated/ treated immediately);
T4 (Inoculated/ treated immediately and after 3 months);
T5 (Inoculated/ treated immediately and after 3 and 6 months)
T6 (Uninoculated/ treated immediately);
T7 (Uninoculated/ treated immediately and after 3 months);
T8 (Uninoculated/ treated immediately and after 3 and 6 months)

7.3.4 Polyphenoloxidase (PPO) activity

Differences in PPO activity in the inoculated/ treated seedlings (T3, T4 and T5) were not significant differenent between the treatments or compared with inoculated/untreated control seedlings (T1) (Fig 7.4). Similar trends were also observed between the uninoculated /treated seedlings (T6, T7 and T8) and the uninoculated/untreated control (T2).

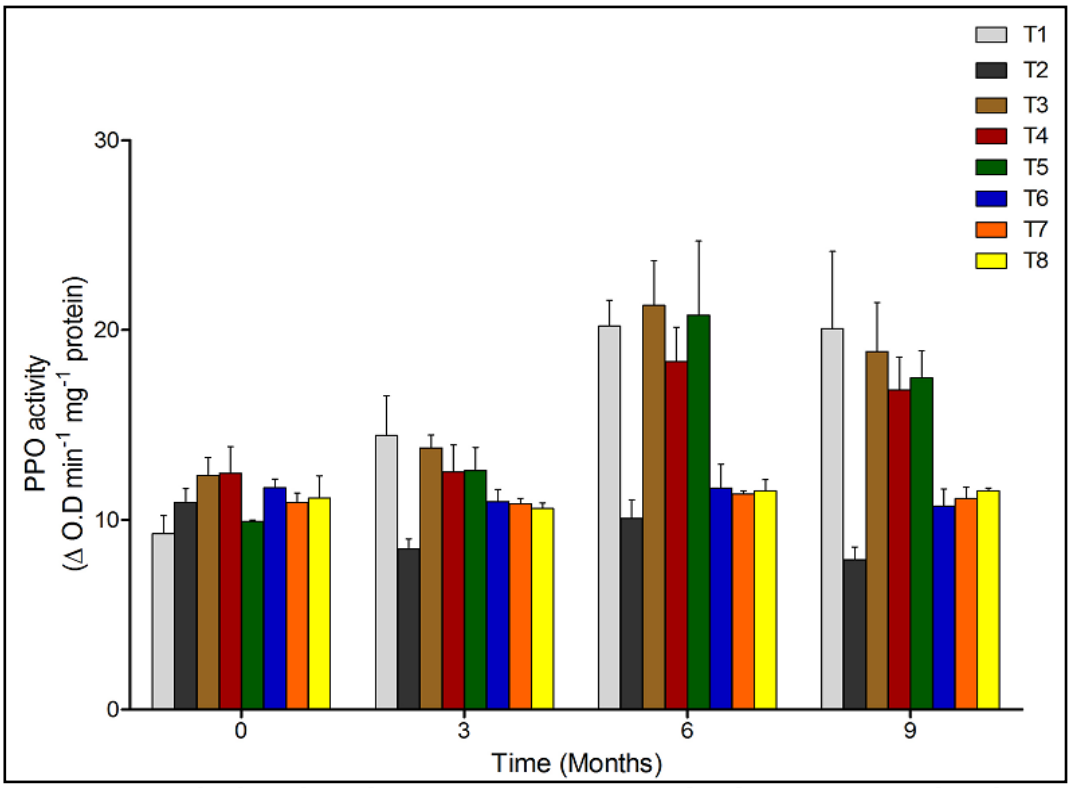


Figure 7.4: Polyphenol oxidase (PPO) activity in oil palm roots treated with 0.5 % (w/v) chitosan as a soil drench. Values are means of three replicates. Bars represent standard errors.
T1: Positive Control (inoculated/ untreated);
T2: Negative Control (uninoculated/ untreated)
T3 (Inoculated/ treated immediately);
T4 (Inoculated/ treated immediately and after 3 months);
T5 (Inoculated/ treated immediately and after 3 and 6 months)
T6 (Uninoculated/ treated immediately);
T7 (Uninoculated/ treated immediately and after 3 months);
T8 (Uninoculated/ treated immediately and after 3 and 6 months)

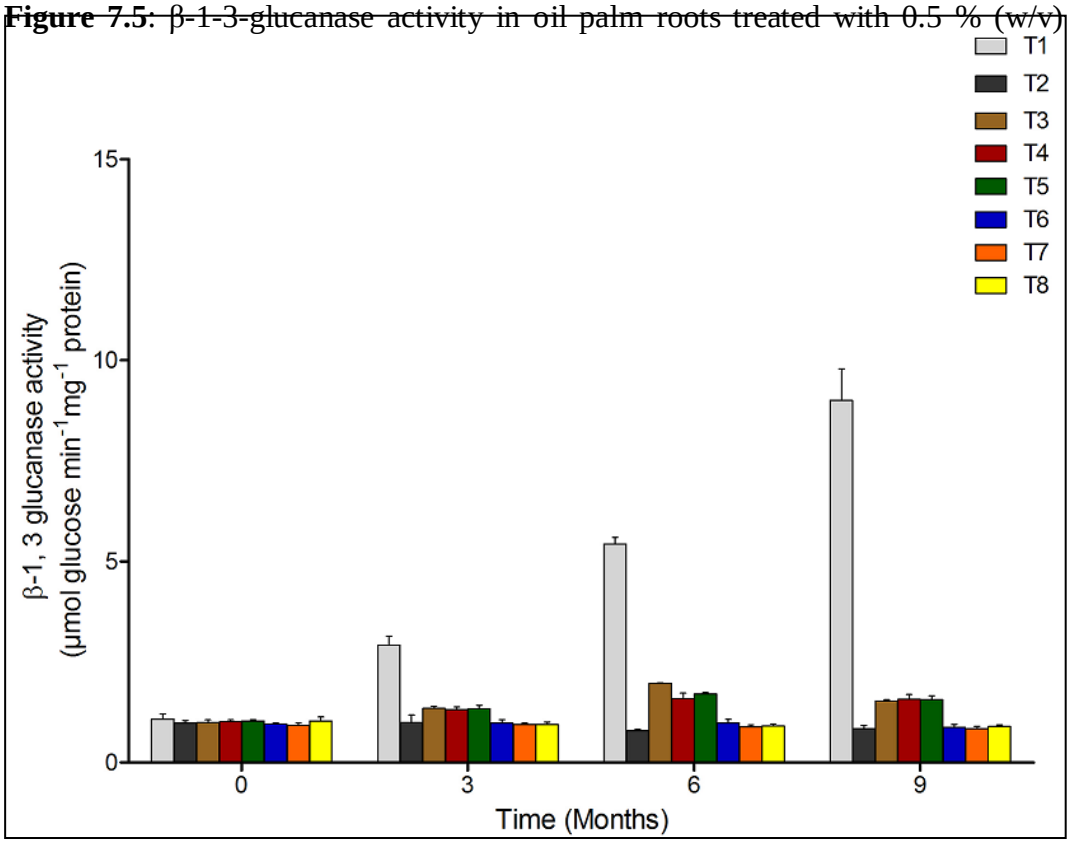
7.3.5 β-1-3-glucanase activity

The activity of β-1-3-glucanase in inoculated/ untreated control (T1) seedlings increased up to 9 months and was significantly ($P \leq 0.05$) higher than in the other treatments (Fig 7.5). In all of the chitosan treatments, either inoculated (T3, T4 and T5) or uninoculated (T6, T7 and T8), there were no differences in the enzyme activity Activity of β-1-3-glucanase in uninoculated chitosan treated seedlings (T6, T7 and T8) was low and did not differ from the control (T2).

7.3.6 Chitinase activity

Chitinase in the roots of inoculated/untreated control (T1) and inoculated/treated seedlings (T3, T4 and T5) showed a marked increase by six months and significantly ($P \leq 0.05$) higher compared to the control (T1) and other treatments (Fig 7.6). The activity in T5 seedlings was about 1.8-, 1.7 and 1.2 times higher compared to control, T3 and T4 seedlings, respectively. With the uninoculated/treated seedlings (T6, T7 and T8), differences between the treatments and compared to the uninoculated/untreated control (T2) were not significant.

Figure 7.5: β-1-3-glucanase activity in oil palm roots treated with 0.5 % (w/v)



chitosan as a soil drench. Values are means of three replicates. Bars represent standard errors.

T1: Positive Control (inoculated/ untreated);
T2: Negative Control (uninoculated/ untreated)
T3 (Inoculated/ treated immediately);
T4 (Inoculated/ treated immediately and after 3 months);
T5 (Inoculated/ treated immediately and after 3 and 6 months)
T6 (Uninoculated/ treated immediately);
T7 (Uninoculated/ treated immediately and after 3 months);
T8 (Uninoculated/ treated immediately and after 3 and 6 months)

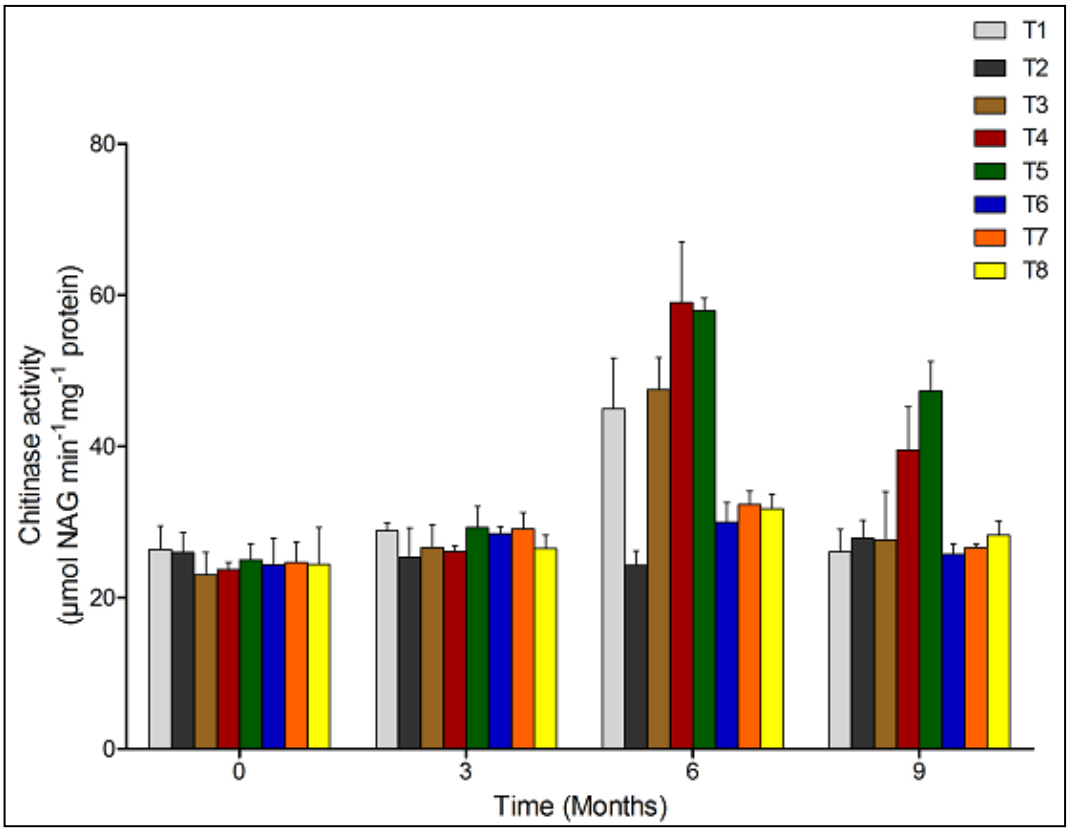


Figure 7.6: Chitinase activity in oil palm roots treated treated with 0.5 % (w/v) chitosan as a soil drench. Values are means of three replicates. Bars represent standard errors

T1: Positive Control (inoculated/ untreated);
T2: Negative Control (uninoculated/ untreated)
T3 (Inoculated/ treated immediately);
T4 (Inoculated/ treated immediately and after 3 months);
T5 (Inoculated/ treated immediately and after 3 and 6 months)
T6 (Uninoculated/ treated immediately);
T7 (Uninoculated/ treated immediately and after 3 months);
T8 (Uninoculated/ treated immediately and after 3 and 6 months)

7.4 Discussion

The accumulation of phenolics and induction of plant defense related enzymes, such as Phenylalanine ammonia-lyase (PAL), total peroxidase (PO) and chitinase by chitosan in oil palm roots occurred irrespective of whether the seedlings were pathogen-inoculated. In contrast, induction of polyphenol oxidase (PPO) and β-1-3-glucanase was not apparent in oil palm seedlings treated with chitosan. Sampling was carried out at intervals of three months due to *Ganoderma* being a slow-growing pathogen (Naher *et al*., 2012) and also based on the earlier results which showed that seedlings inoculated with GBLS isolate needed two to four months post inoculation to develop disease symptoms (Section 4.3.2.). The present study demonstrated that multiple applications of chitosan are important for effective defense response in oil palm seedlings. Higher accumulation of phenolics and higher activity of PAL, PO and chitinase were observed in the seedlings treated with multiple application of chitosan (T4, T5, T7, T8) compared to single applications of chitosan (T3 and T6) and control treatments (T1 and T2). Moreover, the highest level of chitosan application to pathogen-inoculated seedlings (T5; treated immediately and after 3 and 6 months) achieved the highest defense activity after nine months. These results correspond well with the earlier observations (Section 6.3) that the increased addition of chitosan reduced disease severity and enhanced the vegetative growth of oil palm seedlings.

Phenolic and enzyme activities elicited by chitosan are known to be associated with plant biochemical defense mechanisms which eventually lead to

enhanced protection of oil palm against *Ganoderma*. The production of phenolic compounds directly involves the PAL pathway in plants (Graham & Graham; 1991; Shahidi & Naczk, 2004). Therefore, increased accumulation of phenolics in oil palm seedlings as observed may have been due to the increased activity of PAL. PAL removes ammonia from phenylalanine and produces trans-cinnamic acid which later converts into phenylpropanoids (Tan, 1979; Shahidi & Naczk, 2004). Various secondary compounds such as phenolic derivatives, phytoalexins and salicylic acid which involves in plant defense reactions are synthesised through phenylpropanoids metabolisms (Tan, 1979; Kartikeyan *et al*., 2006). Peroxidase (PO) is an oxidative enzyme involved in the synthesis of lignin and which directly contributes to the mechanical strength of plant cells structure (Lavania *et al*., 2006). Chitinase, a hydrolytic enzyme is one of the important pathogenesis-related (PR) proteins in plant defense during infection (Prapagdee *et al*., 2007; Naher *et al*., 2012). This enzyme prevents the spread of fungal pathogens by breaking down the chitin cell wall of invading fungi (El Ghaouth *et al*., 1994). Chitinase is also commonly correlated with oil palm defense against *Ganoderma* through soil applications of *Trichoderma* spp. as a biocontrol formulation (Naher *et al*., 2012). These inducible enzymes and several other host defense responses have been extensively researched using chitosan as an exogenous elicitor in a range of crops (Fajardo *et al*., 1995; Bhaskara reddy *et al*., 1999; Bautista-Banos *et al*., 2006). Gagnon & Ibrahim (1997) stated that using chitosan alone without any microorganisms would activate and increase the activity of isoflavonoids compounds in the roots of white lupin. This result agrees

with the present study whereby there was an increase in plant defense activity in the uninoculated oil palm seedlings treated with chitosan in comparison to the negative control (uninoculated/ untreated).

Despite our good knowledge of biochemical responses during interactions between pathogens and –hosts, these responses are unclear for BSR in oil palms. Hence, this study explored the role of plant defense activity and chitosan as an elicitor during *Ganoderma* infection in oil palm seedlings. The approach of this study was supported by previous findings which demonstrated the effect of chitosan/chitin treatment on the induction of plant defense responses in date palm (*Phoenix dactylifera* L.) and coconut (*Cocos nucifera* L.) plants which are in the same family as oil palm (El Hassni *et al*., 2004; Kartikeyan *et al*., 2006). Accumulation of phenolic compounds and the activity of peroxidases in date palm roots elicited following injection of chitosan into roots play an important role in date palm resistance to Bayoud disease (El Hassni *et al*., 2004). In addition, Kartikeyan *et al*., (2006) reported that soil application of biocontrol formulations in combination with chitin stimulated high defense responses of phenolic, PAL, PO, PPO, chitinase and β-1-3-glucanase activity in coconut roots infected with *Ganoderma lucidum*. A recent report by Chong *et al*., (2012) which is more closely related to the present study had noted enhancement of phenolic acid compounds in oil palm roots elicited with chitosan *via* soil application. The findings from this study provide new information on the application of chitosan in oil palm at the whole plant level. There have been limited investigations in monocots and most of the reported studies in the literature involve dicotyledonous

plants and cell cultures. Besides the studies described above, other monocots that have been treated with chitosan or chitosan oligosaccharides such as wheat and rice were able to elicit biological defense responses (Tiuterev, 1996; Vander *et al*., 1998; Bhaskara Reddy *et al*., 1999; Agarawal *et al*., 2002; Rodriguez *et al*., 2007).

The activation of defense responses by chitosan depends on the molecular weight (MW) and degree of deacetylation (DA) of the molecule (Vander *et al*., 1989; Kauss *et al*., 1989). According to Trotel-Aziz *et al*., (2006), chitosan with MW of 1.5 kDa and DA value ≤ 20% (1.5/20) exhibited higher production of phytoalexin, chitinase and β-1,3-glucanase activity compared to oligomers of chitosan with greater MW in grapevine leaves. It has been suggested that activation of chitosan response with low DA could be due to the charges along the chitin backbone which are responsible for yielding multioligomer complexes with membrane components. In contrast, a study by Rodriguez *et al*., (2007) showed that the higher solubility of chitosan in aqueous solution contributed to greater induction of defense enzyme activity. Taken together, these studies are in the agreement with the present study which used low viscosity chitosan and may have attributed to the induction of plant defense responses in oil palm seedlings. The signalling pathway of chitosan in the defense responses initiated in plants system is not well established. The elicitation activity of chitosan is postulated to be due to its interaction as a polycationic molecule with negatively charged phospholipids rather than a specific interaction between membrane-receptor like molecules (Kauss *et al*., 1989). According to Rabea *et al*., (2003) the signalling – response process with chitosan is initiated through its binding to receptors located

in the cell membrane of plants followed by membrane depolarization step which sends a signal to nucleus that triggers activation of biochemical defense reactions.

In conclusion, this study has provided a basic understanding on the elicitation by chitosan of plant defense in oil palm during host-pathogen interaction. The earlier observation of the reduction of disease severity (Chapter 6) is likely to have resulted from these defense reactions. These findings also provide vital information for future studies of the molecular basis of defense reactions and the activity in oil palm against *Ganoderma*. They strongly support the need to explore further the application of chitosan for the control of BSR caused by *Ganoderma* under field conditions.

CHAPTER 8

CONCLUSION

This study provides a new insight on the potential of chitosan for the control of *Ganoderma* causing basal stem rot (BSR) disease in oil palm. It provides basic knowledge on the role of lignin degrading enzymes during infection by *Ganoderma* and understanding of the complex defense response during the host-pathogen interaction between oil palm-*Ganoderma*.

Main concerns when conducting this research were for the selection of a pathogenic isolate of *Ganoderma* and the appropriate type and concentration of chitosan. Pathogenicity screening was carried out and revealed that GBLS isolate which exhibited typical symptoms of BSR on artificially inoculated oil palm seedlings as a suitable candidate. This GBLS isolate were confirmed as *Ganoderma* by using Ganoderma selective medium (GSM) and molecular identification. Low viscosity (LV) chitosan from Sigma product was selected based on a preliminary screening experiment (Section 4.3.3).

In vitro, chitosan inhibited the growth and production of lignin degrading enzymes (LDE) of the GBLS isolate. Growth of mycelium was inhibited by LV chitosan in a dose-dependent manner in the concentrations range of 0.5 to 2.0 % (w/v) over an incubation period of 20 days. Alterations in fungal morphology were observed through light and scanning electron microscopy and maybe the possible mode of action of chitosan. Chitosan at concentration of 0.001 to 1.0 % (w/v) reduced the germination of basidiospores. In the subsequent work, the

efficacy of chitosan to control *Ganoderma* infection was directed towards mycelial growth rather than the basidiospores. Nevertheless the role of basidiospores as inoculum source for BSR infection in oil palm has been recognized in previous studies and future studies should focus on the effect of chitosan on *Ganoderma* basidiospores. This is the first study that has determined the secretion of LDE by GBLS isolate of *Ganoderma* under *in vitro* conditions. The lignin degrading capability of GBLS was confirmed by the production of laccase and manganese peroxidase (MnP) in cultures. Both of these enzymes were inhibited by chitosan at 0.02-0.10 % (w/v) with the maximum inhibition at 0.08 and 0.10 % (w/v). Chitosan was able to suppress the activity of these enzymes due to its chelating ability. This could provide a model system to study the inhibition of lignin degrading enzymes by chitosan as a possible control strategy for *Ganoderma*. Future studies should determine the effect of chitosan on lignin in oil palms under *in vivo* condition. Chitosan applied to oil palm seedlings at 0.5 % (w/v) as a soil drench significantly reduced the severity of BSR. Root dipping and soil amendment were not successful compared to soil drenching. The *in vitro* study demonstrated that 2.0 % (w/v) chitosan inhibited mycelial growth better than 0.5 % (w/v), hence this concentration were chosen for the trial experiments and suggested that excess of chitosan may not be favourable for oil palm seedlings *in planta* situation. Interestingly there was enhancement of vegetative growth in oil palm seedlings with this mode of chitosan treatment.

For the first time, this study has indicated that chitosan as an inducer of total phenolics and plant defense related enzymes (PAL, PO and chitinase) in oil

palm-*Ganoderma* interaction. These defense reactions were postulated to be responsible for induced resistance against *Ganoderma* in oil palm seedlings. Multiple applications of chitosan during the crucial infection period resulted in greater control of *Ganoderma* and stimulation of inducible defense reactions. Further study is required to determine the genes associated with the respective defense enzymes expressed in response to chitosan treatment.

From the results of this study, chitosan could be proposed to eradicate or minimize BSR disease on oil palm during its seedling stages. The results provide information for effective implementation of chitosan under shadehouse conditions such as type of chitosan, concentration, method of application, frequency and timing of application. Chitosan is highly biodegradable and non-toxic which confers advantage to its development as a potential bio-fungicide on the seedlings of oil palm. Although determining the efficacy of chitosan in field for BSR disease control against *Ganoderma* is important, the concern that should taken into consideration would be whether the results obtained from shadehouse will complement the study in field condition. There are complex interactions in the field such as environmental elements, variation in pathogen epidemiology and abiotic factors. Therefore before chitosan compounds are applied in the field, these interactions should be studied to optimise chitosan treatment in oil palm plantations. There is a potential for chitosan to be an alternative to existing control measures against *Ganoderma* including biocontrol agents and synthetic fungicides.

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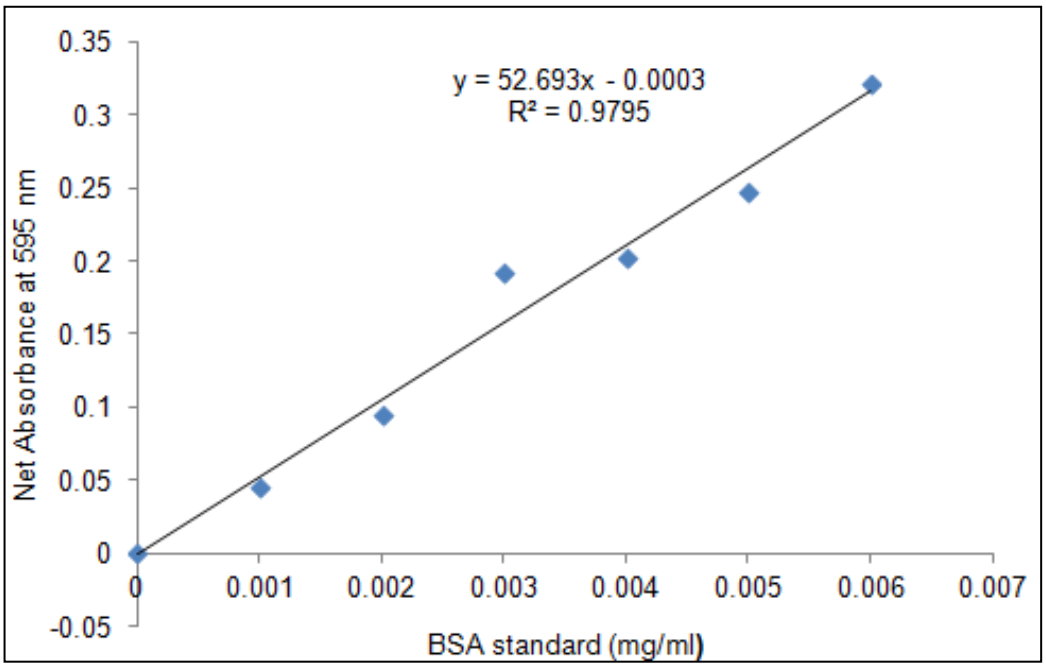
APPENDICES



Appendix 1: Standard curve for bovine serum (BSA) showing linear regression between absorbance at 595 nm and BSA concentration (mg/ml)