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THE ROLE OF PHENOLICS IN THE INTERACTION BETWEEN OIL PALM AND *GANODERMA BONINENSE* THE CAUSAL AGENT OF BASAL STEM ROT

KHIM PHIN CHONG

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ABSTRACT

Basal Stem Rot (BSR) of oil palm (*Elaeis guineensis*) is caused by *Ganoderma boninense*, and is one of the most commercially devastating diseases in South East Asia. It can kill more than 80 percent of stands by the time they are halfway through their normal economic life. High incidence of BSR results in economic losses, due to zero yield from dead palms and significantly reduced weight and number of fruit bunches in infected but living palms. Many methods have been attempted to control BSR. These include clean clearing, fallow period before re-planting, burning of waste or dead material, windrowing, use of fungicides as soil drenching or by tree injection, surgical removal of infected material, biological control and development of varieties with enhanced resistance. To date no method gives good control of *Ganoderma* infection in established plantations and some have technical limitations in application. The aim of this study was to investigate a possible source of disease resistance associated with the accumulation of phenolics, after inoculation with *G. boninense* and elicitation with the biopolymer chitosan.

The identity of isolates of *Ganoderma* from Langkon Oil Palm Estate in Sabah, Malaysia, which were used in this project, was confirmed using *Ganoderma* Selective Medium and DNA sequence analysis after PCR amplification. That latter method showed that the Sabah isolates were very similar to virulent *G. boninense* strains FA5017 or FA5035 from West Malaysia, with a maximum similarity of 98%.

A method to inoculate oil palm seedlings with *Ganoderma*, based on root treatment with macerated mycelium, was developed which facilitated experiments with consistent and rapid disease development. This proved an

improvement compared to the traditional technique of inoculation with colonised rubber wood blocks.

Ekona and Calabar varieties of oil palm were found to be more susceptible to *G. boninense* infection compared to the variety AVROS, based on a higher accumulation of ergosterol (a fungal sterol) and infection scores based on the Modified Disease Severity Score. Results from both parameters showed a strong correlation between each other and provided a quick assessment on the progress of pathogen infection for use in this project. It should be noted, however, that none of the varieties tested exhibited a high level of disease resistance.

Following preliminary studies to quantify the accumulation of total phenolics, using Folin-Ciocalteu reagent, High Performance Liquid Chromatography was used to monitor changes in the concentration of three specific phenolics, which were considered to be of primary importance. These were syringic acid, caffeic acid and 4-hydroxybenzoic acid (4-HBA). Their identification was confirmed using co-injection with pure standards and by LCMS/Q-tof. The variety AVROS showed a slightly higher content of all these three phenolics compared to Ekona and Calabar. Stimulation of their accumulation was promoted by the addition of chitosan to the plant-growing medium. By the end of the time course, the concentration of these phenolics decreased in the oil palm tissues inoculated with *G. boninense* suggesting possible metabolism by this pathogen. This loss was, however, not detected in tissues elicited with chitosan alone and was greatly reduced when *G. boninense* was combined with chitosan.

In vitro studies on antifungal activity with phenolics incorporated either in 10% Potato Dextose Agar, Oil Palm Root Agar or Oil Palm Root Broth were done, using concentrations (50-110 μg mL^{-1}) of phenolic acids typically

detected in oil palm root tissues. Syringic acid was found to be the most antifungal of the phenolics tested. Caffeic acid had lower activity and 4-HBA was virtually inactive. Concentrations of syringic acid detected in root tissues, especially in the presence of chitosan, that might be expected to inhibit growth of *G. boninense*.

The pathogen was shown to be able to degrade all of the phenolic acids tested. However, at the highest concentration tested, metabolism was greatly delayed, only occurring after a lag phase in the growth of pathogen. TLC bioassay showed the degradation products of all these phenolics were no longer antifungal. Thus, accumulation of phenolic acids, especially syringic acid, may provide a useful marker in future breeding of resistant varieties.

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LIST OF ABBREVIATIONS

AVR	AVROS
bp	base pair
BSR	basal stem rot
^{o}C	degree Celcius
Caff	caffeic acid
Cal	Calabar
cDNA	complimentary DNA
chito	chitosan
cm	centimeter
CPO	crude palm oil
d	day
Da	Dalton
dai	day after inoculation
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide 5′ triphosphate
DSI	disease severity index
ED_{50}	Effective Dose 50
Eko	Ekona
ELISA	enzyme-linked-immunosorbent assay
ESI	electrospray ionisation
et al.	et alia (and others)
FC	Follin Ciocaltaeu
FELDA	Federal Land Development Authority
g	gram
x g	x gravity (relative centrifugal force)
GAE	gallic acid equivalent

GB	*Ganoderma boninense*
Gj	gigajoule
GMAC	Genetic Modification Advisory Committee
GMO	genetically modified organism
h	hour
ha	hectare
HBA	hydroxybenzoic acid
H_2O	water
HPLC	high performance liquid chromatography
kb	kilobase
kg	kilogram
kV	kilowatt
£	Sterling pound
L	litre
LC	liquid chromatography
LCMS	liquid chromatography mass spectrophotometry
M	molar
MeOH	methanol
mg	milligram
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
MPOB	Malaysia Palm Oil Board
N	nitrogen
nm	nanometre
OPRA	oil palm root agar

OPRB	oil palm root broth
OP	oil palm
PAL	phenylalanine
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
pg	picogram
pH	negative logarithm of hydrogen ion concentration (-log[H+])
PVPP	polyvinylpyrrolidone
rDNA	ribosomal DNA
RM	Ringgit Malaysia
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
SAR	systemic acquired resistance
SDW	sterile distilled water
SPE	solid phase extraction
Sy	syringic acid
TLC	thin layer chromatography
µg	microgram
UK	United Kingdom
µL	microlitre
µM	micromolar
µm	micrometre
UPLC	ultra pure liquid chromatography
UV	ultraviolet

CHAPTER 1: INTRODUCTION

Oil palm (*Elaies guineensis* Jacq.) is "a golden crop of Malaysia," since it generates profitable export earnings for the country and is truly nature's gift for alleviating poverty (Yusof, 2007). The Malaysian palm oil industry is economically important and diversified. In 2009, the Malaysia palm oil industry recorded a satisfactory performance with export earnings from oil palm products of RM 49.6 billion (approximate £ 11 billion), (MPOB, 2010). The area of oil palm cultivation has increased from 54,000 hectares in 1960 to 4.69 million hectares in 2009, reflecting a compound annual growth of 10.06%. This expansion in planted area has occurred mainly in Sabah and Sarawak, with a combined growth of 14.9%, compared to 3.3% in Peninsular Malaysia. Sabah remains the largest oil palm planted state, accounting for 1.36 million hectares or 29% of the total planted area in the country (MPOB, 2010). Production of crude palm oil (CPO) increased from 94,000 tonnes in 1960 to 17.56 million tonnes in 2009 (MPOB, 2010), or almost 160 fold within 45 years. This represents a compound annual growth of 11.93% per year (Yusof, 2007). The total exports of oil palm products, constituting palm oil, palm kernel oil, palm kernel cake, oleochemicals, biodiesel and finished products increased 2.9% from 21.75 million tones in 2008 to 22.40 million tonnes in 2009 (MPOB, 2010)

1.1. CLASSIFICATION OF OIL PALMS

Oil palm (*Elaeis*) comprises two species of the Arecaceae, or palm family. They are widely used in commercial agriculture for the production of palm oil. The Arecaceae has always formed a distinct group of plants among the monocotyledons, which placed them in the order Arecales. The oil palm, *Elaeis guineensis* Jacq., is grouped with *Cocos* (the coconut) and other genera in the subfamily of Cocosoidae. *Elaies* is derived from the

Greek word *elaion*, meaning oil. There are currently three accepted species of *elaies* namely *E. guineensis* (African oil palm), *E. oleifera* (American oil palm) and *E. odora* (Corley and Tinker, 2003). The African oil palm *Elaeis guineensis* is native to West Africa, occurring between Angola and Gambia, while the American oil palm *Elaeis oleifera* is native to tropical Central America and South America. *E. guineensis* is the most planted species in Malaysia.

1.2. THE AFRICAN OIL PALM, *ELAIES GUINEENSIS* JACQ.

E. guineensis is a large, pinnate-leaved palm having a solitary columnar stem with short internodes (Corley and Tinker, 2003). Mature trees are single-stemmed, and grow up to 20 m tall. The leaves can reach between 3-5 m long. A young tree produces about 30 leaves a year. Established trees over 10 years old produce about 20 leaves a year. The flowers are produced in dense clusters; each individual flower is small, with three sepals and three petals. The separate upper and lower ranks of leaflets on the rachis give the palm a characteristic untidy appearance (Corley and Tinker, 2003). The female bunch bears about 2500-3000 fruits borne on 100-120 spikelets attached to a peduncle from the axil of a frond (Yusof and Chan, 2004).

The fruit takes five to six months to mature from pollination to maturity; it comprises an oily, fleshy outer layer (the pericarp), with a single seed (kernel), also rich in oil. Unlike its relative, the coconut palm, the oil palm does not produce off shoots; propagation is by sowing the seeds. The oil palm is an erect monoecious plant that produces separate male and female inflorescences. In the past, oil palm was wind and insect pollinated. Nowadays, hand-assisted pollination is a standard management practice in plantations (Corley and Tinker, 2003).

The plant physiology of oil palm has many inherent advantages. These include the high productivity and efficient carbon assimilation. Oil palm is credited for its high oil yield per unit area. The world's demand for oils and fats is being met by 17 major sources. Palm oil, though second to soya bean in terms of total production, is the number one player in the oils and fats trade. It controls 36% of the market share. The most efficient among the oil crops, oil palm commands a yield almost 10 times higher than that of soya bean, 6.9 times over sunflower seed and 6.3 times over rapeseed. These four sources accounted for 73% of the vegetable oil produced in 1996. The world's demand for oils and fats for the year 2000 was around 105 million tonnes. This can be satisfied by a mere 30 million hectares of oil palm, or otherwise individually by 189 million hectares of rapeseed, or 209 million hectares of soya bean. Without question, hectare for hectare, oil palm is the most efficient oil crop. Moreover, being a perennial, the supply of palm oil is reliable as the crop is not easily affected by the vagaries of weather and other calamities. This is in contrast to what annuals and other sources can offer. Oil palm leads many other crops in terms of energy balance. From a total input energy of 19.2 GJ/ha/year oil palm gives products with a total energy of 182.1 GJ/ha/year. This high output: input ratio of 9.5 is achievable by only a few other agricultural systems. For comparison, similar ratios for soybean and rapeseed are only 2.5 and 3.0 respectively. This energy balance reflects crop efficiency and reduces reliance on fossil fuels, a big step towards environment conservation (MPOB, 2010).

de Vries *et al.* (2010) reported oil palm from South-East Asia, sugarcane from Brazil and sweet sorghum in China as the most sustainable energy crops at present. Maize from the US and wheat in Europe have a much more negative environmental impact. This conclusion was drawn by the Plant Production Systems chair group, after testing nine energy crops

against nine sustainability criteria. Oil palms, sugar cane and sorghum make the most efficient use of land, water, nitrogen and pesticides in proportion to the amount of energy they produce. If no forest is cut down to make way for their production, they produce far smaller quantities of greenhouse gasses than fossil fuels. Major energy crops such as maize in the US and wheat in Europe score much lower on nearly all the sustainability criteria. Sugar beet and oil seed rape (Europe), cassava (Thailand) and soya (Brazil) got an intermediate score (de Vries *et al.*, 2010).

Oil palm produces two types of oil from the same fruit, palm oil from the flesh or mesocarp and palm kernel oil from the seed or kernel inside the hard-shelled mesocarp. The kernel also yields residual product known as palm kernel meal, which is mostly used for animal feed. (Yusof, 2007)

The distinguishing of varieties of the oil palm has been attempted by many workers. These attempts have in most cases been unsatisfactory since, in the wild state, each palm is a hybrid with respect to certain of its characters (Hartley, 1979). Of interest, however, the first description came from Preuss (1902) of the *lisombe* palm, a name used in Congo, Cameroon and Nigeria for the thin-shelled *tenera* fruit form and still employed in quite recent times (Corley and Tinker, 2003). This was followed by the first sample provided by Janssens in 1927 and Smith in 1935 (Hartley, 1979) which provide classifications which, in their essentials, have stood the test of time. Janssens recognized that the fruit forms *dura* and *tenera*, distinguished by thickness of shell, could be found among fruit types of different external appearance. Both the common fruit type *nigrescens* and the green-fruited *virescens* were divided by Janssens into three forms, *dura*, *tenera* and *pisifera* (Corley and Tinker, 2003). Later the most complete and logical empirical description was provided by

Beinaert and Vanderweyen in 1941 and established the use of the fruit type and form classification (Corley and Tinker, 2003).

1.3. DEVELOPMENT OF OIL PALM PLANTATIONS IN MALAYSIA

The oil palm industry in Malaysia is over a century old. Introduced as an ornamental in 1871, oil palm was commercially exploited as an oil crop only from 1911 when the first oil palm estate was established. Much has been written about the crop, its products and commercial trade (Yusof, Jalani and Chan, 2000).

In the early 1960s, the returns from oil palm were found to be better than rubber and most of the plantation companies soon had a mix of both crops as their core business. It was Tun Abdul Razak Hussein, the Deputy Prime Minister of Malaysia at that time, who called for greater reliance on oil palm. With diminishing returns from the then two major commodities of the country, tin and rubber, oil palm growth was encouraged as a change in policy to eradicate rural poverty (Yusof and Chan, 2004).

Oil palm contributes to uplifting the quality of life of people. Growing oil palm has helped alleviate poverty among landless farmers in Malaysia, through their participation in the FELDA schemes. The income derived from oil palm, based on the 2004 data is equal to RM 6323 or £ 1405 per hectare per year compared to an average of RM 5432 or £ 1207 for agricultural crops in the UK, of which about RM 891 or £ 198 was contributed by the subsidy paid to farmers in the European Union (Yusof, 2006).

Malaysia has the most mature oil palm industry in the world and overall development of the country is moving very rapidly. The industry has undoubted assets in climate, soil, plant materials and skilled labour

and management. This has resulted in great difficulty in finding more suitable sites and suitable labour in Malaysia, and has led to the Malaysian industry looking for expansion opportunities in other countries. Several Malaysian companies have joint ventures in Indonesia settling up large scale plantations (12,000 ha) in central Kalimantan, which has demanded co-ordinated action by many players (Guertz, 2000). There is also interest in investment in Papua New Guinea, Brazil, Philippines, Colombia, Guyana, Honduras, Burkina Faso, Congo, Nigeria and Ghana (Kuruvilla and Mohanda, 1997).

1.4. BASAL STEM ROT (BSR)

The devastating Basal Stem Rot (BSR), caused by *Ganoderma boninense*, is considered the most serious disease faced by the oil palm industry in Malaysia (Figure 1.1). Oil palm has an economic life span of 25-30 years. BSR can kill more than 80 percent of stands by the time they are half-way through normal economic life. For many years, BSR was considered to infect older palms, over 25 to 30 years old, due to senescence factors, although incidence of the disease in young palms had also been periodically recorded. According to Hartley (1979), this disease was discovered in 1928 and was not considered as a serious problem as it was infecting old plants. When palms were replanted in plantations of coconut and oil palm during the mid-1950s, the disease began to affect a large number of palms of 10 to 15 years old. Symptoms were also observed in much younger palms far more frequently than previously described (Turner, 1981). Recently, this disease has been found to be present in oil palm crops as young as one to three years old. The incidence increased rapidly and disease levels can reach up to 50% of palms (Singh, 1990). High incidence of BSR results in economic losses, due to no yield

from dead palms and significantly reduced weight and number of fruit bunches from living infected ones (Turner, 1981).

Figure 1.1: Basal Stem Rot (BSR) incidence in Langkon Estate Sabah Malaysia. The figure shows some oil palm trees with early symptom of BSR. A palm that is seriously affected is arrowed

Although BSR has been known for a number of years in South East Asia (Turner, 1981), efforts to control the disease have been hampered due to lack of knowledge of the dynamics of the pathogen (Pilotti, Sanderson, and Aitken, 2003).

Generally, the earliest foliage symptom is the presence of excessive spear leaves, which are normally produced at a rate of two to three per month. The foliage is also much paler green in colour than that of a healthy palm (Turner, 1981). By the time foliar symptoms appear, usually at least one-half of the cross-section area at the stem base has been killed by this pathogen. This severely restricts the supply of water and nutrients to the aerial part, leading to wilt and symptoms of malnutrition. The disease produces a dry rot of internal tissues. Some of the internal symptoms are

light brown rot of both the stem and the bole of irregular shape, with dark bands which contains swollen chlamydospore-like hyphal cells. At the edge of the infected area is an irregular yellow zone which possibly appears as a result of a defence mechanism of the palm. Roots of infected palms are very friable and their internal tissues become dry and powdery. Cortical tissue turns brown and the stele becomes black (Hartley, 1979).

G. boninense fructifications develop either at the stem base or occasionally on the infected roots close to the palm (Turner, 1981). Sporophores usually develop as the decay advances (Singh, 1990). The time of sporophore appearance depends on the internal rotting extending to the stem periphery, so there is a wide variation in palm age when *G. boninense* is seen. The sporophores first appear as small white buttons of tissues on the leaf bases or on the stem. These develop rapidly into a familiar bracket shape. The mature sporophores vary in shape, size and colour. The upper surface of the sporophores varies from light to dark brown with a white margin. The under surface is white and perforated by numerous pores (Turner, 1981).

Several factors have been identified to encourage the infection. These include the replanting technique which is commonly practiced in oil palm plantations, the type of soil, crop age and the availability of inoculum (Khairudin, 1993). Fields that ranged from 40 to 60% infection before replanting had the same level of disease nine years after replanting. This suggests a build up could occur but there is little evidence for a further increase after the second replant (Singh, 1991).

The type of soil also plays an important role in infection. Disease outbreaks were reported to occur with a high frequency in peat and laterite soil. Infection was also correlated with plant age. Infection which started at year six increased rapidly after 11 years (Khairuddin, 1991; Navaratnam, 1964; Turner, 1965 a). Nitrogen and magnesium may have some role in

combating the disease, but more recent trials have given equivocal results (Akbar, Kusnadi and Ollagnier, 1971). Potassium chloride and urea application have both increased disease incidence in some trials, and decreased it in others. High soil salinity and low soil pH appeared to discourage the disease (Singh, 1991). Similar results were found with phosphorous and potassium in three trials (two on peat and one on coastal alluvial soil) (Tayeb Dolmat and Hamdan, 1999). Conductivity can also affect BSR, incidence has been observed to be lower on acid sulphate soils and on recent marine soils where the salt content is high (Singh, 1990).

Traditionally, primary infections of palms by *G. boninense* have been considered to occur by contact of living palm roots with colonised debris within the soil (Turner, 1965 b). Secondary spread of disease has been assumed to be caused by contact of living palm roots with each other. The current assumptions of secondary disease spread from palm to palm are supported by limited observations in Malaysia that centres of infection enlarge over time (Singh, 1990). Thus, the evidence for root to root spread of the pathogen has been largely circumstantial.

Earlier research suggested *G. boninense* was a weak parasite, and it needed to develop saprophytically in large masses of dead palm tissue before it had sufficient inoculum potential to infect live plants (Turner, 1981). More recently, though, it has been shown that even isolated roots from diseased trees could provide a sufficient inoculum source (Hasan and Turner, 1998). Work with molecular markers has confirmed that this assumption is sometimes correct, as the same pathogen genotype, which was present in debris inoculum, was detected in new diseased stumps (Flood *et al.*, 2000). Some studies on spatial patterns of the disease have shown that diseased palms in some fields tend to be in clumps, which also indicates spread through root contact (Flood *et al.*, 2000).

Other investigations with the application of molecular markers and somatic (vegetative) incompatibility demonstrated that the majority of isolates found on oil palm were genetically distinct and indicated that basidiospores may have been responsible for the disease patterns observed in the field (Ariffin, Idris and Azahari, 1996; Miller *et al.*, 1995; Miller, Holderness and Bridge, 2000; Pilloti and Sanderson, 2001). However, this assumption may need further investigation. A mature basidiocarp of *G. boninense* in a plantation is able to disperse thousands of basidiospores in a day. Whereas a single basidiospore may not easily be able to initiate infection, they may provide inoculum for long-distance dispersal and saprophytic colonisation of oil palm debris, and may thus be involved in indirect transmission.

1.5. ECONOMIC IMPORTANCE

BSR incidence was first considered an important disease after it started infecting much younger palms (Turner, 1981). In 1990, it was demonstrated that *G. boninense* has the ability to infect oil palms as young as one to two years after planting, but more usually when oil palms are four to five years old, particularly in replanted areas (Singh, 1990) or areas under-planted with coconut (Ariffin, Idris and Azahari, 1996). In the latest incidences, *G. boninense* was found to attack seedlings or immature palms less than one year old in the nursery (Susanto, 2009). High incidence of BSR disease was recorded on oil palm planted in coastal soil in Peninsular Malaysia (Khairudin and Chong, 2008) especially inland, on lateritic and peat soils, which were at one time thought to be non-conducive to BSR disease (Ariffin, Singh and Lim, 1989; Benjamin and Chee, 1995; Rao, 1990; Turner, 1981). In Sabah and Sarawak, the incidence of BSR was also reported, but at low incidence (Ariffin and Idris, 2002). This may,

however, be an under-estimation. There are two kinds of losses generated by *G. boninense*; direct and indirect loss. The direct loss refers to lowered production due to dead plants, while the indirect loss refers to reduced Fresh Fruit Bunch (FFB) weight (Susanto, 2009). The disease can kill up to 80% of the stand by the time the palms are halfway through their normal economic life span (Abdul Razak *et al.*, 2004). In Sumatra Indonesia, in plantings from the late 1960s and early 1970s, there was little decline in yield until the surviving stand had fallen to about 115 palm ha^{-1}, but in more recent plantings, any loss of palm was associated with the loss of yield (Corley and Tinker, 2003). Yield of infected palms was also reduced by 20-40% compared to the year before infection was detected (Khairudin, 1993). Palms with *G. boninense* detected yielded between 13 to 21% less than healthy palms at the same age (Nazeeb, Barakabah and Loong, 2000). Heavily infected fields yielded 26% less at 11 years after planting, and 46% less at 15 years, by which time disease incidence was 67% (Singh, 1991).

1.6. *GANODERMA BONINENSE*

1.6.1. Biology and epidemiology

The genus of *Ganoderma* is categorized as a higher fungus, a polyporoid genus, within the family: Ganodermataceae, Order: Aphyllophorales, Subclass: Hymenomycetes and Class: Basidiomycetes (Idris, 2009). Basidiomycetes are the primary cause of white rot diseases of woody plants by decomposing substrates rich in lignin. Components forming cell walls of plants comprise mainly lignin, pectins, cellulose and hemicelluloses. Thus, to attack plants, fungi need to degrade these components using an array of polymer-cleaving enzymes (Susanto, 2009). Some species of *Ganoderma* produce amylase, extracellular oxidase,

invertase, coagulase, protease, renetase, pectinase and cellulase enzymes. *G. boninense* also produces manganese peroxidases (MnP) and laccases (Corley and Tinker, 2003). White rot fungi develop unspecific ligninolytic systems consisting of peroxidases and laccases (phenol oxidases: LAC), which employ an oxidative process (Paterson, 2007). Biodegradation of the cellulose component is only achieved by cleaving β-1,4-glucosidic linkages, a simple binding among glucose molecules. Based on its infection mechanism, *Ganoderma* is classified as a white rot fungus, such rot fungi have been classified by speed and production of lignolytic enzymes (Ward, Hadar and Dosoretz, 2004). While, Miller, Holderness and Bridge (2000) suggest that *Ganoderma* is mostly 'saprobic' and exploits weak host plants, so it is categorized as a parasite or secondary pathogen.

In Malaysia, the pathogen attacking oil palm was originally identified as *G. lucidum* (Thompson, 1931). In a later report, fifteen species of *Ganoderma* were listed as pathogens likely to be associated with BSR disease and a single species was considered unlikely to be the sole cause of the disease in any particular area (Turner, 1981). Among them, seven species of *Ganoderma*, viz., *G. applanatum* (Pers.) Pat., *G. boninense*, *G. chalceum* (Cooke) Steyaert, *G. lucidum* (W. curt. et. fr.) Karst, *G. miniatocinctum* Steyaert, *G. pseudoferreu* (wakef.) Overh. and Steinmann and *G. tornatum* (Pers) Bres were reported from Peninsular Malaysia (Turner, 1981). Ho and Nawawi (1985) concluded that all *Ganoderma* isolates from diseased oil palm from various locations in Peninsular Malaysia were all the same species, *G. boninense*. Ariffin, Singh and Lim (1989) suggested that other species may be involved and Khairudin (1990) concluded that two species were present, namely *G. boninense* and *G. tornatum*. These were based on morphology of basidiomata and basidiospores collected from oil palm fields or *in vitro*.

Other early work referred to the pathogen, *G. lucidum* Karts in West Africa and Malaysia as the causal of the BSR. The general consensus now appears to be that *G. boninense* is the main species pathogenic to the oil palm, especially in South East Asia (Moncalvo, 2000). Later, the real pathogenic isolates of *Ganoderma* were isolated and identified as *G. boninense*, *G. zonatum* and *G. miniatocinctum*, but *G. boninense* was found to be significantly more aggressive than the other two. While *G. tornatum*, *G. applanatum*, *G. lucidum*, *G. oregonense*, *G. pfeifferi* and *G. philippii* were not pathogenic (Idris *et al.*, 2000 b).

In vitro studies on the morphological characteristics of *G. boninense* by Idris *et al.*, (2000 a) found the colonies of *G. boninense* were white in colour on the surface and the reverse was darkened (pigmented). Cultures of *G. boninense* had an undulating surface in the darkened regions. The first indication of basidiomata formation was the appearance of a white mycelium after one to three weeks of incubation on rubber wood blocks, which then developed into a small, white, button-like structure. The apical end began expanding rapidly giving rise to bracket-like structures which were generally white when first formed, but as their length and width increased rapidly, the upper surface developed various yellowish-brown colours with concentric zonations (Idris, 2009).

G. boninense is a facultative parasite and is capable of living as a saprophyte on rotting stumps and roots. The sporing structure is illustrated in Figure 1.2. When a suitable host like oil palm becomes available it will colonise and establish a parasitic relationship. Oil palms are replanted on previously-planted oil palm and coconut land and there is a build-up of *G. boninense* inoculum on stumps and trunk tissues, so that replanted oil palm will be infected by root contact with such materials (Turner, 1981). The attached palm roots will ultimately become colonised by *G. boninense*, which can spread within a field, mainly through root contact between

healthy and diseased palms (Miller *et al.,* 1995). A study conducted by Marzita (2002) to determine the spreading pattern of BSR in oil palm plantations with different treatments, such as sterile soils with partition, non-sterile soils with partition, sterile soils without partition and non-sterile soils without partition, also confirmed *G. boninense* can infect other healthy oil palm seedlings via root contact.

Figure 1.2: *G. boninense* fruiting stage is arrowed.

A detailed demonstration of the reproducible infection of intact roots of oil palm with *G. boninense*, was reported by Rees *et al.* (2009). Infection showed penetration, followed by rapid longitudinal progression of hyphae and colonisation of the lower stem (bole) of oil palm. Light and transmission electron microscopy also showed invasion of the root cortex, with no evidence of selective progression through the vascular system or lacunae. In newly colonised tissue, *G. boninense* behaved as a hemibiotroph with numerous, wide, intracellular hyphae occupying entire

host cells that possessed intact cell walls and contained discernible cytoplasm and organelles. In the bole this phase coincided with a complete depletion of previously abundant starch grains in advance of invasion. Subsequently, in the roots and colonised stem bases, widespread necrotrophic, enzymatic attack of all layers of the host cell walls occurred. Hyphae were intra, intercellular and intramural and associated with host cell wall degradation, which was often at a distance from hyphae, resulting in cavities within cell walls. A third developmental stage was the formation of an extensive, melanized, tough mycelium, or pseudo-sclerotium, which surrounded roots and comprised many very thick-walled cells encasing more typical thin-walled hyphae. Macroscopic observation of and isolation from the bole of randomly felled, commercial palms provided confirmatory evidence that multiple infections originated in the roots before spreading into the base of long-established palms (Rees *et al.,* 2009)

Heavily injured roots could influence the speed of *G. boninense* infection in oil palm. The rate of movement of *G. boninense* within infected roots was 1.62 to 2.12 cm month^{-1}, and the average was 1.83 cm month^{-1}. (Idris, 2009). This rate is slower than the speed of *G. lucidum* at 2.3 cm month^{-1} in roots of grape (Adaskaveg and Gilberstson, 1987). At the rate of approximately 1.8 cm month^{-1}, *G. boninense* would take about four years to reach the bole if, hypothetically, a root of one meter was infected through contact with disease debris. The age when this palm showed symptoms would have been much older when one includes the time taken for roots to grow from the seedlings and to eventually come in contact with the inoculum. This slow growth speed might explain why foliar symptoms of BSR are mostly detected on older palms (Idris, 2009). The mechanism of root to root infection is also supported by group-patterned distribution of disease. Heavily diseased plants are commonly surrounded by unhealthy plants with lighter symptoms.

Numerous oil palm plants die because of BSR when an under-planting system is implemented (Susanto, 2009). This route of infection was further confirmed by Rees *et al.* (2007), who demonstrated infection using isolates of differing aggressiveness. The group also reported that severe disease occurred after eight months on rubber wood block inoculated seedlings under shade, but not on the seedlings exposed to sun. *G. boninense* is probably inhibited in exposed soil since optimal growth *in vitro* was 25 to 30°C, and there was no recovery from 45°C. Soil temperature may explain why symptoms often first appear in mature plantations when canopy formation creates shade (Rees *et al.,* 2007).

On the other hand, basidiospores are also stated to have played significant roles in spreading the disease (Pilotti, Sanderson, and Aitken, 2003; Sanderson, 2005). The role of *Ganoderma* basidiospores in disease initiation and infection spread is unclear. Although huge numbers of basidiospores are released from basidiomata in oil palm plantations (Ho and Nawawi, 1986), the majority of oil palms remain uninfected, indicating that basidiospores are either not able to initiate *Ganoderma* disease or require very specific conditions to establish infection. Studies based on artificial inoculation with basidiospores and inoculum size suggest that the spores have inadequate inoculum potential for direct infection (Turner, 1981). Their function in disease development seems to be colonisation of suitable substrates, particularly cut stumps of trunks of trees or palms left to rot in the field, which may become infection foci. Inoculation of cut young leaf bases (Turner, 1965 b) and oil palm seedlings with spores failed to produce any infection (Ramasamy, 1972). Sharples (1936) believed that spores did not play an important role in the spread of the disease. However, Thompson (1931) was of the opinion that spores were important in initiating the disease in first generation oil palms on cleared virgin jungle areas. Basidiospores, which may either be wind-borne or insect-

transmitted, would first have to colonise suitable substrates such as dead coconut or oil palm stumps, after which they could provide an infection locus in plantations.

It was suggested that spores might enter through beetle holes caused by the *Oryctes* beetle (Turner, 1981). Caterpillar larvae of *Sufetula* spp. may also be important in spreading spores of *Ganoderma* (Genty, De Chenon and Mariau, 1976). Afidah (2006) also further investigated the potential of ants involve in dissemination of *G. boninense* in the plantation and reported ants are not involved in spreading the fungus. However, no conclusive evidence has been presented linking insects and BSR incidence and development. Chung, Cheah and Nur Azarina (1998) reported that *Eumorphus politus*, *Eumorphus quadriguttatus*, *Megalodachne elongatula*, *Bolitoxenus bifurcus*, *Morophaga sistrata*, *Odontomachus simillimus* and *Microcerotermes* have been identified as being associated with basidiomata of *Ganoderma*.

1.6.2. Detection of *Ganoderma*

BSR is not new to Malaysia; it has been known to attack oil palm since the early years when the crop was introduced into the country. A survey indicated that the disease was present in more than 50% of the oil palm fields in Malaysia (Idris *et al.*, 2001 a) and 90% in Peninsular Malaysia in 2009 (Khairuddin, H. personal communication). Unfortunately, many farmers did not realize that their fields were infected with BSR. More importantly, even in situations where the disease was certainly present, the plantation managers failed to recognize either the extent of disease incidence or the specific areas of fields when BSR was a yield-limiting problem (Idris, Mazliham and Madihah, 2009). The effect of infection by *Ganoderma* is the progressive destruction of the basal tissues of the oil

palm trunk, but the external symptoms observed in the leaves are wilting and malnutrition. The disease can be identified from the dry rot at the base of the palm trunk and the presence of typical bracket-shaped basidiomata of the fungal pathogen (Idris, Mazliham and Madihah, 2009). In immature palms, the external symptoms of BSR normally comprise a one sided yellowing or mottling of the lower fronds, followed by necrosis (Singh, 1990). The newly unfolded leaves are shorter than normal and chlorotic and, additionally, the tips may be necrotic. As the disease progresses, palms may take on an overall pale appearance, with retarded growth with the spear leaves remaining unopened. Similar symptoms are observed in mature palms, with multiple unopened spear leaves and a generally pale leaf canopy. Affected leaves die, necrosis beginning in the oldest leaves and extending progressively upwards through the crown. Dead desiccated fronds droop at the point of attachment to the trunk or fracture at some point along the rachis and hang down to form a skirt of dead leaves. Often, when foliar symptoms are observed, it is usually found that the fungus has killed at least one-half of basal stem tissue (Idris, 2009)

Basidiomata may or may not develop before foliar symptoms appear. The time of basidiomata appearance depends on the internal rotting extending to the stem periphery. The basidiomata initially appear as small white buttons of fungal tissue which rapidly develop into the familiar bracket-shaped structures. The upper surface of the mature basidiomata varies in colour and has numerous minute pores. Frequently, many basidiomata are formed close together, with overlapping and fusion to form large compound structures. The location of the basidiomata provides a rough guide to the position of the diseased area inside the palm. When the palm dies, rapid colonisation of the whole trunk can be seen through the appearance of basidiomata along the entire length. Symptoms typical of the *Ganoderma* infection will only appear on foliage after a substantial

portion of the bole is infected. Oil palm extensively decayed by *Ganoderma* may fracture at the base resulting in collapse, leaving diseased bole tissues in the ground. Subsequently, numerous *Ganoderma* basidiomata are produced, especially during the rainy season (Idris, 2009). If the palm remains standing, the trunk may become hollow. The incidence of fallen palms or vacancies increases with age and can reach 26% by the time palms are 20 year old (Singh, 1990).

Currently, there are some available rapid and accurate diagnostic techniques, which are specific and readily adapted to large scale testing for detecting *Ganoderma* in oil palm at an early stage of infection. This includes the use of PCR and has been reported by numerous researchers (Hayakawa *et al.,* 2000; Idris *et al.,* 2003; Yamoaka *et al.,* 2000). Work on the use of polyclonal antibodies (PAb) against *Ganoderma* has also been described (Darmono, Suharyanto and Darussamin, 1993, Darmono and Suharyanto 1995; Darmono, 2000; Utomo and Niepold, 2000), but they were found to cross-react with other saprophytic fungi, such as *Penicillium*, *Aspergillus* and *Trichoderma*. Idris and Rafidah (2008) also reported the development of ELISA-PAb, which was able to detect the pathogenic species *Ganoderma* and also *Ganoderma* infection in both tissues of healthy-looking palms and diseased standing palms. There was, however, cross reaction with *Penicillium,* but not with *Trichoderma* and *Aspergillus*. The development of ELISA-MAb for early detection of *Ganoderma* in oil palm, using two types of antigens, mycelium and basidiospores for immunisation of mice, was also described by Shamala and Idris (2009). Mice were killed in order to harvest the spleen containing the MAbs. Cells harvested were fused with myeloma cells. Antibody MAbs F240 and F243 showed positive signal for detection of *G. boninense*. However, these MAbs were found to react with isolates of *G. zonatum*, *G. miniatocinctum* and *G. tornatum*, saprophytic fungi (*Trichoderma* and *Aspergillus*) and also

bacteria such as *Erwinia*. Utomo and Niepold (2000) reported that PCR-based assays appear to be more specific than ELISA assays in detection of *Ganoderma*, because in the PCR assays cross-reaction with saprophytic fungi was not observed. However, for detection using a large number of samples, ELISA offers advantages in term of speed, ease of use and cost effectiveness.

On the other hand, Tay *et al.* (2009) reported the biochemical response of production of pathogenesis related (PR) proteins, played a role for early detection of infection. It was reported that responses of the PR proteins from the interaction between oil palm seedlings and *Ganoderma* infection were short-lasting and activated systematically. The study also showed that the accumulation of PR-proteins in oil palm seedlings by *Ganoderma* infection is related to systemic acquired resistance (SAR). This is because there are changes in the PR proteins that can be detected in leaves, although the infection is initiated in roots. This indicated that increase in the PR proteins was a plant response to infection, and not simply a contribution of hyphal enzymes *per se*. Although the levels of PR proteins increase in the infected seedlings during infection, it appears that they are not sufficient, or have reached effective levels too late, to stop fungal growth. Phenylalanine ammonia-lyase (PAL) activity showed significant increase in inoculated seedlings by 14 d after inoculation (dai). Peroxidase activity also showed a significant increase in *G. boninense* inoculated seedlings by 14 dai. Glucanase is the first line of defence against fungal invasion by hydrolyzing glucans, which are one of the main elements of fungal hyphae. By 28 dai, glucanase activity of the *G. boninense*-treated seedlings was 1.8 fold significantly higher than in untreated seedlings. However, chitinase activity did not show any significant changes in the treatments tested. The results from this study showed that the effect of most of the biochemical changes associated with

Ganoderma infection were expressed sooner than that of the visible infection symptoms, which appeared by the third month after inoculation. This finding implied that the biochemical approach may serve as a novel early detection method for *G. boninense* infection (Tay *et al.*, 2009). This finding was also in accordance with a report by Chee *et al.* (2009) who claimed chitin is a potential fungal component which elicits glucanase and chitinase activity after 12 h exposure of oil palm seedling roots to chitin. However, PAL and peroxidase activities were not induced within their study period, suggesting that they may take longer to be induced (Chee *et al.*, 2009). As far as molecular biology is concerned, Tee *et al.* (2009) profiled the transcripts in oil palm roots infected by *G. boninense* at three to nine weeks post infection, using a cDNA microarray. They revealed that 173 genes were differentially regulated in oil palm roots infected by *G. boninense* compared to uninfected oil palm roots. Among these genes were those related to defence and stress, which formed the largest functional category (13%). Other major functional categories of differentially expressed genes in *Ganoderma*-infected tissues were genes related to gene regulation, hormones and development, transportation, structural proteins, metabolism and signal transduction. The up-regulated genes in infected oil palms included isoflavone reductase, Cu/Zn super-oxide dismutase and hydroxyproline-rich glycoprotein family protein; whereas those that down-regulated in infected oil palms included defensins, mannose-binding lectin and thioredoxin peroxidase (Tee *et al.*, 2009).

Other early detection techniques include the PODITOO™ tomography, which locates and identifies infection of *Ganoderma* in oil palm (Idris, Mazliham and Madihah, 2009). The Geographical Information System (GIS) was used, to understand the disease establishment, development and spread in oil palm plantations and was reported by Mior *et al.* (2009). The tomography technology is a non-invasive tool designed

for assessing tree decay and degradation. The equipments consist of a sound sensor and a tomography software called PODITOO™. Sound sensors are installed around a circumference of an understudy oil palm stem. A sound wave is emitted on one sensor and time of flight of the sound propagation from the emitter to the other sensor is calculated. These sound lines are then used to construct the tomography image of the stem. The detection together with the location and the size of the decay and degradation will enable planters to conduct trunk injection with appropriate fungicides. The GIS technology can help to access the distribution of BSR disease in oil palm plantation (Idris, 2009).

GIS is also being used by New Britain Palm Oil Limited in Papua New Guinea to map and monitor the spread of pests and diseases in their plantation. The *G. boninense* infected fields are surveyed and monitored on a quarterly basis using GeoDami, an in-house developed mapping and database programme. GeoDami allows mapping by individual palms points where all palms are allocated unique identification numbers automatically, based on the field, row and palm number. GPS points of field boundaries, starting points of the rows and palms, and the stand per hectare are already known, and GeoDami automatically draws in the palm points representatively across the field. Every quarter, each palm is inspected and data entered into GeoDami on a standardized form that has a list of possible pests and diseases. Each pest and disease type is demoted by a numeric value. The unique identification number given to each palm provides the link between the GIS layers of the palm points and the recorded details in the access database files. This link allows thematic maps to be generated for every survey which assists in monitoring of the spread of *Ganoderma*. Using these maps, proper sanitation and control measures can be employed by plantation management to minimize the spread (Betitis *et al.*, 2009).

1.6.3. Control of *Ganoderma boninense*

As with most soil-borne diseases, it is extremely difficult to control BSR once the disease has become established. The best approach may be by avoidance. All potential sources of infection from old stems and roots of oil palm and coconut should be destroyed (Abdul Razak *et al.*, 2004). The inoculum of *Ganoderma* can be reduced significantly in *Ganoderma* areas by destroying the diseased oil palms. This involves excavating diseased roots and stumps of infected oil palm. Removal of diseased palms by excavating the soil, stump and root masses with a size of 2 meter length x 2 meter width x 1.5 meter depth, and refilling with soil from an uninfected area is recommended (Idris, Ismail and Ariffin, 2005). By adopting this technology, productivity can be improved by reducing risk of *Ganoderma* infection in supply palms in areas with BSR incidence. This system is adopted by Sime Darby plantations in Malaysia for diseased palms less than five years old to ensure the remains of diseased bole tissues and the major part of the root system are removed (Khairudin and Chong, 2008), who also recommended diseased but productive (healthy-looking) palms older than five years old that remained should be removed as soon as the palms no longer provide economic returns.

Attempts to control this disease in the field with fungicides have been made by various workers, but the results are inconclusive, though some systemic fungicides seem to be promising (Idris *et al.*, 2002). The first field trial using Pressure Injection and Field Applications for the application of fungicides to control *Ganoderma* infected palms was carried out at Teluk Merbau Estate, Sepang, Malaysia. Seven treatments were evaluated; which were benomyl & thiram (60 g $palm^{-1}$), bromoconazole (50 mL $palm^{-1}$), hexaconazole (90 mL $palm^{-1}$), triadimefon (40 g $palm^{-1}$), triadimenol (40 mL $palm^{-1}$) and tridemorph (20 mL $palm^{-1}$). The results showed that bromoconazole and hexaconazole, both triazole groups, were

effective in delaying the death of infected palms in comparison with four other fungicides and untreated control (Idris *et al.,* 2002).

Two years later, the bioefficacy of hexaconazole with different concentrations and application methods for prolonging the productive life of *Ganoderma* infected mature palms was reported by the same group of researchers. It was shown that 90 mL of hexaconazole dissolved in 10 L of water applied with pressure-injection gave a higher percentage of palm survival and palms producing fruit bunches, when compared to other methods of application (Idris *et al.,* 2004 a). After five years, 70% of palms treated with hexaconazole were still living and producing fruit bunches. The number of surviving palms and palms producing fruit bunches were reduced to 43.3% when hexaconazole was dissolved in 5 L of water and only 3.3% when hexaconazole was applied using a syringe or through soil drenching. Untreated palms did not survive after this period (Idris *et al.,* 2004 a). The methods of fungicide application including soil drenching, trunk injection or combinations of these two methods were described by Abdul Razak *et al. (*2004). The applications were, however, very costly and unfortunately, the yield reduction and mortality rate was still high.

Conducting surgery by excision of large, discrete lesions has been practiced (Turner, 1968) which was sometimes successful but is expensive and treated palms may collapse at the end. Surgery is more applicable to younger palms rather than the older ones as a cure, as younger palms may have a greater benefit of a long productive life ahead. Some enhanced survival and better yields were reported from palms after surgery (Hasan and Turner, 1994). However, mounding of the base of diseased palms was also found to reduce the death rate from 34% to 2% over two years after surgery. Yields were then increased more than 30% (Ho and Khairudin, 1997). However, addition of dazomet to mounding gave only a small additional benefit, which was insufficient to cover the extra cost (Corley

and Tinker, 2003). On the other hand, some claimed disease could be controlled by ensuring that fruiting bodies were not allowed to develop to the point where spores were released (Sanderson and Pilotti, 1997; Sanderson, Pilotti and Bridge, 2000). However, the claim needs further investigation (Corley and Tinker, 2003).

A method of reducing the *Ganoderma* incidence at oil palm replanting time, by ridding the fields of as much oil palm tissue as possible has been tried, but the results remain unclear (Corley and Tinker, 2003). Mechanical methods including clean clearing, burning, cutting up, splitting the boles and windrowing the old oil palms are used, so the tissues decompose rapidly thus removing potential inoculum sources. The operations are costly, but have been regarded as essential in coastal areas. Clean clearing only reduces disease incidence by a small amount, if at all, although what is meant by clean clearing may differ from one organization to another (Corley and Tinker, 2003). In the meantime, a suggestion has been made for a two-year fallow period, which should precede replanting, but this would be only accepted if another high value commercial crop could replace oil palm within the period (Hasan and Turner, 1994). Underplanting, even with 80% *Ganoderma* losses by 20 years, was reported to be more profitable than a system based on a clean clearing, with a fallow period (Wood, 1999).

Some work to evaluate the potential for biological control of BSR has also been reported. *Trichoderma*, *Penicillium*, and *Aspergillus* have been found to have some activity as biocontrol agents against *G. boninense* (Khairudin, 1993). The use of conidia and chlamydospores of *Trichoderma koningii* as a biofungicide has also been described, but no data were presented on its effects (Soepena *et al.*, 2000). Studies on the effects of *T. hazianum*, alone and in factorial combinations with a mycorrhizal preparation, dried palm oil mill effluent and calcium nitrate on

Ganoderma development in inoculated seedlings have been conducted. The results suggested that $Ca(NO_3)_2$, dried effluent and mycorrhizal inoculation all had some effect, but *Trichoderma* did not (Sariah and Zakaria, 2000). Some work claimed that young palms, inoculated with mycorrhiza in the nursery and planted next to diseased stumps, remained free of infection by *Ganoderma* for at least three years, whereas uninoculated plants were mostly infected (Corley and Tinker, 2003). On the other hand, Aida (2005) reported, mycorrhiza are not antagonistic to *G. boninense*, but potentially reduce the growth rate of this pathogen and enhance the infected oil palm root growth.

Jayanthi *et al.* (2009) further investigated the effect of application of *T. harzianum* (FA1132), *T. longibrachiatum* (FA1128) and *T. virens* (FA11128) as single and mixed treatments against *G. boninense*. Conidial soil drench of those combinations was applied fortnightly for the first six weeks and reduced to once every four weeks for the final two applications. Based on the Disease Severity Index (DSI), a single treatment of *T. harzianum* (FA1132) gave a significantly lower DSI value compared to other treatments. In addition, the assessment of plant biomass was also significantly higher in this treatment compared to other treatments, corresponding well with the percentage of healthy seedlings (Jayanthi *et al.*, 2009). This result is supported by Anis (2006) who carried out an *in vitro* study on the effect of *Trichoderma* against *G. boninense*. The *Trichoderma* isolate AM5 was reported to inhibit strongly the growth of the pathogen with percentage inhibition of radial growth (PIRG) up to 39.74%.

Other biological control research includes the reports on endophytes, *Agrobacterium radiobacter*, *Burkholderia cepacia* and *Pseudomonas syringae* for control of *G. boninense*. Maizatul and Idris (2009) claimed disease incidence and severity of foliar symptoms developed much slower in the oil palm seedlings pre-treated with

endophytes, in comparison to untreated seedlings. At six months after inoculation, BSR incidence was reduced by 62-75% in seedlings treated with *A. radiobacter* followed by *B. cepacia* (31-59%) and *P. syringae* (30-31%). *A. radiobacter* was claimed to be the most effective in controlling BSR disease, with seedling death of 13.3%-26.7%, followed by *B.cepacia* (33.3%) and *P. syringae* (33.3-40%), in comparison to seedlings untreated with endophytes (60%). A field study was later suggested to verify their effectiveness to control *G. boninense* (Maizatul and Idris, 2009). The compatibility interactions of the endophytic bacteria (EB) with arbuscular mycorrhizal fungi (AMF) and their antagonistic effects on *G. boninense* were also reported by Shamala *et al.* (2009). The study claimed, the EB (*P. aeruginosa* UPMP3 and *B. cepacia* UPMB3) were compatible with AMF (*Glomus intraradices* M1 and *G. clarum* M2). Good spore germination with germ tubes producing secondary branching were observed for M1 and M2 co-incubated with the bacterial cultures in dual culture plates. Furthermore, bacterial cells colonised the surfaces of the AMF spores. In addition, EB were found to be antagonistic to *G. boninense* and the established role of the AMF in nutrient acquisition, adds to the potential of using these endophytic microorganisms in the suppression of *G. boninense* infection and enhancement of vegetative growth in oil palm (Shamala *et al.*, 2009).

However, optimization of the delivery system can be a major constraint for the establishment of bacteria in the field, reducing their efficacy as bio-protectants against BSR. Development of high quality bacterial formulations may require fine carriers, which play a role as a substrate to protect and sustain the viability and efficacy of the inoculants during storage and in the field before they act on the pathogen. Ili, Sariah and Zainal (2009) suggested Luria broth (LB) and vermiculite may be used as carriers that significantly sustained high number of viable cells and permited the lowest reduction after five months storage of the bacterial

formulations at 10°C and 20°C. But, LB was not considered practical for large scale use in the field, considering the cost (Ili, Sariah and Zainal, 2009). However, the use of some of these endophytes, such as *A. radiobacter* and *B. cepacia*, is unsual. *A. radiobacter*, which is commercially available as strain K84, produces a very species-specific bacteriocin (Agrocin 84) and is normally used to protect crops against the pathogen *A. tumefaciens*, the causal agent of crown gall in crops including rose, raspberry, peach and nectarines. The potential use of *B. cepacia* in the open environment may be impossible, as specific isolates of this bacterium cause serious skin diseases in humans.

Host resistance is considered as an important and successful component in preventing and controlling plant diseases, as this method is relatively inexpensive, biologically safe and convenient for the farmer (Idris *et al.,* 2004 b). The development of oil palm genotypes resistant to *Ganoderma* may provide the ideal long-term solution to BSR (Idris *et al.,* 2004 b). In separate trials conducted in Sumatera, it was observed that African progenies of oil palm developed BSR at a much slower rate than the local Deli progenies (Akbar, Kusnadi and Ollagnier, 1971; Hastjarjo and Soebiarpadia, 1975). The existence of resistant genotypes has also been indicated in trials of 20 *dura* x *pisifera* (D x P) crosses in Indonesia (Purba, Purba and Sipayung, 1994) and in *E. oleifera* x *E. guineensis* hybrids in Malaysia (Chung *et al.,* 1994; Sharma and Tan, 1990). In a separate trial, significant differences in susceptibility among 20 commercial D x P materials was also detected (Ariffin *et al.,* 1999). A study conducted by Idris *et al.* (2004 b) showed that the most susceptible progeny was D x D, [Deli (Elmina) x Deli (Elmina)] whilst the partially resistant progeny was [D x P, (Congo x Cameroon)]. Basically, after six months, foliar symptoms were observed in all progenies but with different incidences, ranging from susceptible D x D (40%) to tolerant D x P (20%). However, all progenies

exhibited internal disease symptoms and *Ganoderma* was re-isolated from stems or roots at a level of 60% to 70% (Idris *et al.*, 2006).

Paterson (2007; 2008) and Paterson and Lima (2009) suggested the possibility of altering the content of lignin to resist the white rot fungus, *G. boninense*, while Mohamad Arif *et al.* (2007) suggested phenolic compounds are involved in oil palm resistance against *Ganoderma*.

For more than 40 years, *Ganoderma* has remained a serious problem in plantation areas of Malaysia and Indonesia. Unfortunately, to date, there is no single method proven to be reliable in suppressing or controlling disease development.

1.7. ANTIMICROBIAL COMPOUNDS

Collectively, plants produce a remarkably diverse array of over 100,000 low molecular mass secondary metabolites. Secondary metabolites are distinct from the components of intermediary (primary) metabolism in that they are generally non-essential for the basic metabolic processes of the plant. Most are derived from the isoprenoid, phenylpropanoid, alkaloid or fatty acid/polyketide pathways. This rich diversity results in part from an evolutionary process driven by selection for acquisition of improved defence against microbial attack or insect or animal predation (Dixon, 2001).

Related plant families generally make use of related chemical structures for defence (for example, isoflavonoids in the Leguminosae, sesquiterpenes in the Solanaceae), although some chemical classes are used for defensive functions across taxa (for example, phenylpropanoid derivatives). Some species produce a broad range of antimicrobial compounds (Dixon, 2001). For example, cocoa, when infected by the vascular wilt fungus *Verticillium dahliae*, accumulates the pentacyclic

triterpene arjunolic acid, two hydroxylated acetophenones and, most unusually, elemental sulphur, the only known inorganic antimicrobial agent produced by plants (Cooper *et al.*, 1996). Most antimicrobial plant natural products have relatively broad spectrum activity, and specificity is often determined by whether or not a pathogen has the enzymatic machinery to detoxify a particular host product (vanEtten, Matthew and Matthews, 1989).

Some of these compounds are constitutive, existing in healthy plants in their biologically active forms. Others, such as cyanogenic glycosides and glucosinolates, occur as inactive precursors and are activated in response to tissue damage or pathogen attack. This activation often involves plant enzymes, which are released as a result of breakdown in cell integrity. Compounds belonging to the latter category are still regarded as constitutive because they are immediately derived from pre-existing constituents (Mansfield, 1983).

VanEtten *et al.* (1994) have proposed the term "phytoanticipin" to distinguish these preformed antimicrobial compounds from phytoalexins, which are synthesized from remote precursors in response to pathogen attack, probably as a result of *de novo* synthesis of enzymes. For many years, studies of plant disease resistance mechanisms have tended to focus on phytoalexin biosynthesis and other active responses triggered after pathogen attack (Hammond-Kosackand and Jones, 1996). In contrast, preformed inhibitory compounds have received relatively little attention, despite the fact that these plant antibiotics are likely to represent one of the first chemical barriers to potential pathogens (Osbourn, 1996).

However, the distinction between phytoalexin and phytoanticipin is not always obvious. Some compounds may be phytoalexins in one species and phytoanticipins in others. A good example is the methylated flavanone sakuranetin, which accumulates constitutively in leaf glands of

blackcurrant, but which is a major inducible antimicrobial metabolite in rice leaves (Kodama *et al.*, 1988). In cases where a constitutive metabolite is produced in larger amounts after infection, its status as a phytoalexin would depend on whether or not the constitutive concentrations were sufficient to be antimicrobial. *In vivo* antimicrobial activity is inherent in the definition of a phytoalexin or phytoanticipin, but it is this feature that has proven most problematic to determine directly in the absence of methods to genetically modify the host plant's natural product profiles. In most cases, concentrations of phytoalexins have not been measured specifically in the cells that are in direct contact with the invading microorganism (Dixon, 2001). One exception is a careful study of the cellular concentrations of sesquiterpenoid phytoalexins in leaves of cotton varieties responding to the bacterial pathogen *Xanthomonas campestris* pv. *malvacearum*, in which it was shown that phytoalexin levels in and around the challenged cells were significantly higher than would be required to effectively inhibit the growth of the pathogen *in vitro* (Pierce *et al.*, 1996).

The distribution of preformed inhibitors within plants is often tissue specific (e.g., Bennett and Wallsgrove, 1994; Davis, 1991; Fenwick *et al.*, 1992), and there is a tendency for these compounds to be concentrated in the outer cell layers of plant organs, suggesting that they may indeed act as deterrents to pathogens and pests. Some diffusible preformed inhibitors, such as catechol and protocatechuic acid (which are found in onion scales), may influence fungal growth at the plant surface. In general, however, preformed antifungal compounds are commonly sequestered in vacuoles or organelles in healthy plants. Therefore, the concentrations that are encountered by an invading fungus will depend on the extent to which that fungus causes tissue damage (Osbourn, 1996). Biotrophs may avoid the release of preformed inhibitors by minimizing damage to the host, whereas necrotrophs are likely to cause substantial release of these compounds.

The nature and level of preformed inhibitors to which a potential pathogen is exposed will also vary, depending on factors such as host genotype, age, and environmental conditions (Davis, 1991; Price, Johnson and Fenwick, 1987).

There have been numerous attempts to associate natural variation in levels of preformed inhibitors in plants with resistance to particular pathogens, but often these attempts have failed to reveal any positive correlation (Osbourn, 1996). However, whereas preformed inhibitors may be effective against a broad spectrum of potential pathogens, successful pathogens are likely to be able to circumvent the effects of these antibiotics either by avoiding them altogether or by tolerating or detoxifying them (Bennett and Wallsgrove, 1994; Osbourn, 1996; VanEtten *et al.*, 1995).

An approach involving the study of fungal mechanisms of resistance to preformed inhibitors, and of the contribution of this resistance to fungal pathogenicity to the relevant host plants, offers route toward investigating the importance of these inhibitors in plant defence. A large number of constitutive plant compounds have been reported to have antifungal activity. Well-known examples include phenols and phenolic glycosides, unsaturated lactones, sulphur compounds, saponins, cyanogenic glycosides, and glucosinolates (Bennett and Wallsgrove, 1994; Fry and Myers, 1981; Grayer and Harborne, 1994; Mansfield, 1983; Osbourn, 1996). Moreover, 5-alkylated resorcinols and dienes have been associated with disease resistance, in this case, resistance of subtropical fruits to infection by *Colletotrichum gloeosporioides* (Prusky and Keen, 1993). However, only a few classes of preformed inhibitor have been studied in detail to determine their possible roles in plant defence against fungal pathogens (Osbourn, 1996).

1.8. PHENOLICS

Phenolics are one of the most ubiquitous groups of secondary metabolites found throughout the plant kingdom (Boudet, 2007; Harborne, 1980). They encompass a very large and diverse group of aromatic compounds characterized by a benzene ring (C6) and one or more hydroxyl groups. Generally, the classification of phenolics is based on the number of carbon atoms present in the molecule (Harborne and Simmonds, 1964). Phenolics are formed by three different biosynthetic pathways:

(i) the shikimate/chorizmate or succinylbenzoate pathway, which produces the phenyl propanoid derivatives (C6–C3); (ii) the acetate/malonate or polyketide pathway, which produces the side-chain-elongated phenyl propanoids, including the large group of flavonoids (C6–C3–C6) and some quinones; and (iii) the acetate/mevalonate pathway, which produces the aromatic terpenoids, mostly monoterpenes, by dehydrogenation reactions (Bhattacharya, Sood and Citovsky, 2010).

Phenolics are often produced and accumulate in the subepidermal layers of plant tissues exposed to stress and pathogen attack (Clé *et al.*, 2008; Schmitz-Hoerner and Weissenbock, 2003). The concentration of a particular phenolic compound within a plant tissue is dependent on season and may also vary at different stages of growth and development (Lynn and Chang, 1990; Ozyigit *et al.*, 2007; Thomas and Ravindra, 1999). Several internal and external factors, including trauma, wounding, drought and pathogen attack, affect the synthesis and accumulation of phenolics (Kefeli, Kalevitch and Borsari, 2003; Zapprometov, 1989). Furthermore, the biosynthesis of phenolics in chloroplasts and their accumulation in vacuoles are enhanced on exposure to light (Kefeli, Kalevitch and Borsari, 2003). Photoinhibition, as well as nutrient stresses, such as deficiencies in nitrogen, phosphate, potassium, sulphur, magnesium, boron and iron, also trigger the synthesis of phenylpropanoid compounds in some plant species

(Dixon and Paiva, 1995). These may include members of the flavonoid biosynthetic pathway (Balasundram, Sundram and Samman, 2006; Hollman and Katan, 1999).

Phenolics can serve a dual function of both repelling and attracting different organisms in the plant's surroundings. They act as protective agents, inhibitors, natural animal toxicants and pesticides against invading organisms, such as herbivores, nematodes, phytophagous insects, and fungal and bacterial pathogens (Dakora and Phillips, 1996; Lattanzio *et al.*, 2006; Ravin *et al.*, 1989). Mainly, volatile terpenoids, toxic water-soluble hydroquinones, hydroxybenzoates, hydroxycinnamates and the 5-hydroxynapthoquinones are widely effective allelochemicals. A number of simple and complex phenolics accumulate in plant tissues and act as phytoalexins, phytoanticipins and nematicides against soil-borne pathogens and phytophagous insects (Akhtar and Malik, 2000; Lattanzio *et al.*, 2006). Therefore, induction of phenolic compounds has been proposed for some time to serve as an useful alternative to chemical control of pathogens of agricultural crops (Langcake *et al.*, 1981). Plants respond to pathogen attack by accumulating phytoalexins, such as hydroxycoumarins and hydroxycinnamate conjugates in *Combretum micranthum* (Karou *et al.*, 2005). The synthesis, release and accumulation of phenolics, in particular, salicylic acid (Boller and He, 2009; Koornneef and Pieterse, 2008; Lu, 2009; Tsuda *et al.*, 2008) are central to many defence strategies employed by plants against microbial invaders.

Phenolics are synthesized when plant pattern recognition receptors recognize potential pathogens (Newman *et al.*, 2007; Ongena *et al.*, 2007; Schuhegger *et al.*, 2006; Tran *et al.*, 2007) by conserved pathogen-associated molecular patterns (PAMPs), leading to PAMP-triggered immunity (Zipfel, 2008). As a result, the progress of the infection is

restricted long before the pathogen fully invades the plant (Bittel and Robatzek, 2007; *et al.,* 2009).

The first demonstrated example of phenolics providing disease resistance was the case of onion scales accumulating sufficient quantities of catechol and protocatechuic acid to prevent onion smudge disease, caused by *C. circinans*. The coloured outer onion scales of resistant onion varieties contain enough of these two phenols to reduce spore germination of *C. circinans* to below 2%, while susceptible varieties lack these compounds and the germination rate is over 90% (Walker and Stahmann, 1955). Skadhauge, Thomsen and von Wettstein (1997) reported that a barley mutant (*ant* 18-159) showed extreme resistance to *Fusarium* attack: the hyphae were unable to penetrate the testa of this mutant. The testa layer of *ant* 18-159 accumulates, besides proanthocyanidins, small amounts of dihydroquercetin as a result of nonsense mutation in the structural gene for dihydroflavonol reductase. *In vitro* bioassays showed that dihydroquercetin is a strong inhibitor of *Fusarium* growth and macrospore formation.

In vitro studies reveal that phenolic compounds extracted from olive plants (*Olea europaea* L.), tyrosol, catechin, and oleuropein, showed antifungal activity, thus affecting plant resistance against *Phytophthora* sp. (Del Rio *et al.*, 2003). Quercetin 3-methyl ether and its 4'-O-glucoside and 7-O-glucoside, especially, completely inhibited conidial germination of the fungus *Neurospora crassa* (Parvez *et al.*, 2004). On the other hand, naringenin (flavanone), dihydroquercetin (dihydroflavonol), kaempferol, and quercetin (flavonols) have been tested for their biological activity against two fungal rice pathogens, *Pyricularia oryzae* and *Rhizoctonia solani* (Padmavati *et al.*, 1997). Naringenin and kaempferol showed a significant inhibition of spore germination of *P. oryzae* from 7 µg mL^{-1} upwards. No such inhibition was found with *R. solani*.

The fungicidal activity of a number of stilbenes and related compounds has been tested against several fungi, including some pathogens, which infect grapes during storage. A linear relationship between bioactivity and hydrophobicity of the molecule has been found. When the activity of two natural stilbenes (resveratrol and 3,5- dimethoxy-4'-hydroxystilbene, occurring in grapevine, was examined against different fungi, resveratrol was found less active than the dimethoxy stilbene (Schultz *et al*., 1991). It is also believed that the stilbene structure may be modified *in vivo* by partial methylation to further enhance its activity. A similar structure/activity relationship was observed when the fungicidal properties of some flavonoids, especially flavones and isoflavones, were tested against *Fusarium* sp., *B. cinerea*, *Aspergillus* sp. and other storage fungi (Wang *et al*., 1989).

Toxicity of tannins, hydrolysable tannins and proanthocyanidins, usually estimated by the measurement of the reduction of the *in vitro* growth of mycelium, is well documented for several filamentous fungi, for example *B. cinerea*, *Aspergillus niger*, *Colletotrichum graminicola*, *Gloeophyllum trabeum*, *Trichoderma viride*, and *Penicillium* sp. Tannins are quite potent antibiotics. In temperate trees, tannins and related phenolic compounds preserve heartwood from fungal decay and inhibit extracellular hydrolases from invading pathogens, thus preventing their rapid development in the plant (Harbone, 1980). It is possible that inhibition of extracellular fungal enzymes (cellulase, pectinase, laccase, xylanase, etc.), nutrient deprivation of substrates (metal complexation, protein insolubilization) and action on fungal membranes (inhibition of oxidative phosphorylation) are effectively involved in tannin toxicity. Lignans, a phenolic class of dimeric phenylpropanoid units linked by the central carbons of their side chains, also play a role in plant-fungus interactions. Some of the fungistatic activity of lignans is attributable to their inhibition

of the extracellular fungal enzymes, cellulase, polygalacturonase, glucosidase and laccase (MacRae and Towers, 1984).

If pre-existing antifungal phenolics are not sufficient to stop the development of the infection process, plant cells usually respond by increasing the level of pre-existing antifungal phenols at the infection site, after an elicited increased activity of the key enzymes (PAL and chalcone synthase) of the biosynthetic pathway (Dixon and Paiva, 1995; Pallas *et al.*, 1996). The increased level of phenolics provides an adequate substrate to oxidative reactions catalysed by polyphenol oxidase (PPO) and/or peroxidase that, by consuming oxygen and producing fungitoxic quinones, make the medium unfavourable to further development of pathogens. Therefore, although many phenolic compounds present in plant tissues may not have any antimicrobial activity *per se*, there are several cases recorded where the oxidation products of pre-existing phenolics might have antimicrobial activity, which is often associated with an inhibition of the cell wall degradation by extracellular enzymes produced by pathogens.

In addition, many simple low molecular phenolic compounds present in plants may be readily polymerized by oxidation to yield brown tannin-like substances (melanins) containing quinonoid groups. These can also precipitate protein and cross-link to other polymers. They are often formed in necrotic cells after the invasion by a pathogen, as shown by the browning, which takes place in and around the area (Butt, 1985; Friend, 1979). Thus, oxidized phenolics in resistant varieties of apple play an important role in the restricted lesion formation associated with the brown rot disease of fruits caused by *Sclerotinia fructigena* Aderh (Byrde, 1960) or with rotting of stored Golden Delicious apples caused by *Phlyctaena vagabunda* (Lattanzio, *et al.*, 2001). Furthermore, antifungal activity of oxidized phenolics may also be related to the necrotic reaction, e.g. the oxidative polymerisation involving phenolic compounds, amino acids and

proteins that yields brown melanins. This reaction results in the formation of an impermeable barrier to pathogenesis by plant parasites, and in a decrease of nutrients essential to the fungal development. The possibility that phenolic oxidation products could have an antifungal action by polymerizing and forming a protective seal on cell walls has been proposed by Beckman, Mueller and Mace (1974) who showed that artificial membranes of calcium oxalate-pectin, that had been infused with the oxidation products of 3-hydroxy tryptamine, were resistant to degradation by *Fusarium oxysporum* f. sp. *cubense*. Secondary responses also include the release of toxic phenols that are normally stored as less toxic glycosides in the vacuoles of the plant cells, the formation of lignin, a biopolymer resistant to the degradation by most microorganisms, the accumulation of cell-wall appositions such as papillae, and, finally, the synthesis of specific antibiotics such as phytoalexins (Nicholson and Hammerschmidt, 1992)

To date, although there are a lot of published articles on the role of phenolics in different plants effective against different pathogens, there is scarce information on phenolics related to oil palm. Some earlier research conducted on young oil palms reported dienic alcohols derived from methyl lineolate accumulate in the tissue of young oil palms after inoculation with *F. oxysporum* f. sp. *elaeidis*. These oxygenated derivatives which were not detected before infection, accumulate at different concentrations depending on the genetic origins of the progenies tested. Their accumulation in the tissues can be modulated by the application of several fungal or synthetic elicitors (Diabate, 1990).

The total phenolic content of oil palm roots (expressed as gallic acid equivalents) was reported by Mohamad Arif *et al.* (2007). Concentrations were high one week after inoculation with *G. boninense* but declined by four weeks. These preliminary finding suggest a possible role for phenolics

in this interaction. To date no specific research has been done to fully understand the function of phenolic compounds in the defence of oil palm against *G. boninense* invasion or the roles of specific phenolic compounds against the pathogen.

1.9. ROLE OF CHITOSAN

Chitin is the major component of fungal cell walls, and chitin oligosaccharides act as PAMPs in plant and mammalian cells. Microbial pathogens deliver effector proteins to suppress PAMP-triggered host immunity and to establish infection (de Jonge *et al.*, 2010). Chitosan is a cationic polymer, a deacetylated chitin with a specific structure and properties (Muzzarelli, 1977). It contains more than 5000 glucosamine units and is obtained commercially from shrimp and crab shell chitin (a *N*-acetylglucosamine polymer) (Han, Kimura and Okaauda, 1999). Chitin and chitosan are polysaccharides, chemically similar to cellulose differing only by the presence or absence of nitrogen. Chitosan is a low acetyl form of chitin mainly composed of glucosamine, 2-amino-2- deoxy-b-D-glucose (Freepons, 1991). The positive charge of chitosan confers to this polymer numerous and unique physiological and biological properties. It has wide applications in the cosmetic, food, biotechnology and pharmacology industries.

The fungicidal activity of chitosan has been well documented both in *in vitro* and *in situ* studies. Literature generally reports that the level of inhibition of fungi is highly correlated with chitosan concentration, indicating that chitosan performance is related to the application of an appropriate rate (Bautista-Bañosa, 2006). It is believed that the polycationic nature of this compound is the key to its antifungal properties and that the length of the polymer chain enhances its antifungal activity

(Hirano and Nagao, 1989). An additional explanation includes the possible effect that chitosan might have on the synthesis of certain fungal enzymes (El Ghaouth *et al.,* 1992). Recent studies have shown that chitosan is not only effective in halting the growth of pathogens, but also induces marked morphological changes, structural alterations and molecular disorganization of fungal cells (Ait Barka *et al.,* 2004; Benhamou, 1996; El Ghaouth *et al.,* 1999).

Studies conducted on seed germination and chitinase activity of soybean seeds, subjected to chitosan glutamate solutions at different concentrations (0.1%, 0.5% and 1.0%), and soaking periods (15 min and 6 h) indicated that the period of exposure to chitosan was more decisive for the increase of chitinase activity than chitosan concentration (Tejchgraber, Popper and Knorr, 1991). In tomato plants, the production of phenolics, phytoalexins or related compounds, induced by chitosan, precedes or coincides with the activities of hydrolytic enzymes of *F. oxysporum* f. sp. *radicis lycopersici* (Benhamou and Thériault, 1992). Other studies with peanut seeds confirmed that chitosan enhances the production of pre-formed free and bound phenolic acids in viable seed tissues (Fajardo *et al.,* 1995). Further studies indicated that chitosan triggers either the *de-novo* synthesis of phenolic compounds, as the first defensive mechanism which inhibited growth of this fungus, and that host β-1,3-glucans acted as a second mechanical barrier for blocking potential invasion by fungal hyphae and protecting the tissue against phytotoxic substances (Benhamou, Lafontaine and Nicole, 1994; Lafontaine and Benhamou, 1996). The same authors pointed out that contact with the pathogen is essential for signaling the plant to mobilize its defences and that plants treated with chitosan were able to express these defence reactions faster and to a greater degree than those treated with the pathogen alone. Antifungal hydrolases were reported in roots and leaves of

hydroponically grown cucumber plants treated with chitosan and artificially inoculated with *Pythium aphanidermatum* (El Ghaouth *et al.*, 1994 a). Reports have also shown that chitosan has the capacity to induce resistance to *F. oxysporum* in susceptible tomato plants, when applied as a root dressing, foliar spray and seed dressing, by restricting pathogen growth to the outer root tissues and eliciting a number of defence reactions, including the formation of structural barriers (Benhamou, Kloepper and Tuzun, 1998). This effect may be due to the massive accumulation of fungitoxic compounds at sites of attempted pathogen penetration. Because of its filmogenic property, chitosan may also act as a barrier to the outward flux of nutrients and, consequently, may reduce the availability of nutrients to a level that will not sustain growth of the pathogen. This contention is supported by the fact that fungal cells exposed to chitosan often display signs of nutrient deprevation (Ait Barka *et al.*, 2004; El Ghaouth, Smilanick and Wilson, 2000). Another example of an induced resistance response was reported in chitosan-treated groundnut, where a significant increase of endogenous salicylic acid, intercellular chitinase and glucanase activities was observed (Sathiyabama and Balasubramanian, 1998).

Chitosan was effective in reducing the production of polygalacturonases by *Botrytis cinerea* in bell pepper tissues and markedly reduced the maceration of the host cell walls by pectin degradation (El Ghaouth *et al.*, 1997). In studies on strawberries and raspberries with a chitosan coating, there was a significant increase in chitinase and β-1,3-glucanase activities of the fruits, compared with uncoated controls. It was also observed that chitosan partially inhibited the increase in peroxidase activity, associated with tissue browning (Zhang and Quantick, 1997). In tomato, chitosan impaired the production of fungal virulence factors, such as cell wall degrading enzymes (polygalacturonase, pectate lyase and

cellulase), organic acids (oxalic and fumaric acids), and host specific toxins (alternariol and alternariol monomethylether) and induced production of the phytoalexin rishitin (Bhaskara Reddy *et al.*, 1998; 2000). In grapes, chitosan enhanced phenylalanine ammonia lyase activity (the key enzyme of the phenylpropanoid pathway) (Romanazzi *et al.*, 2002). Other enzymatic activities, such as peroxidase and polyphenol oxidase, were elicited in palm roots injected with chitosan (El Hassni *et al.*, 2004). In the same study, after treatment with chitosan the presence of caffeoylshikimic acids (sinapic, *p*-coumaric and ferulic derivatives), were reported to be the major phenolic constitutive compounds in date palm roots and these are known to have antifungal activity and are precursors of lignin.

The role of the elicitation of several defence-related enzymes has also been studied (Bohland *et al.*, 1997; Vander *et al.*, 1998). These enzymes are known to participate in early defence mechanisms and to prevent pathogen infections. Chitosan and chitin oligomers have also been reported to stimulate other systems involved in resistance, such as lipoxygenase and phenylalanine ammonia lyase activities, and lignin formation in wheat leaves (Bohland *et al.*, 1997; Vander *et al.*, 1998).

The induction of structural barriers at sites of attempted fungal penetration is one of the most common processes that occurs in response to pathogen invasion. Cellular suberization and lignification are elicited during the infection process in some plant organs. Chitosan is reported to restrict, to some extent, fungal penetration and induce the formation of different structural barriers. Moderate lignification, as a result of chitosan treatment and inoculation with *B. cinerea*, of cell walls was reported in wheat leaves after 48 and 72 h (Pearce and Ride, 1982).

Transmission electron microscopy showed evidence of the formation of particular structures and new material. For example, the main reactions observed in host cells in tomato roots and leaves which were chitosan

treated and infected by *F. oxysporum* f. sp. *radicis-lycopersici* were: (1) occlusion of xylem vessels by an opaque or fibrillo-granular material or by the formation of a bubble-like structure, (2) coating of secondary thickenings and the pit membrane and (3) papillae formation (wall appositions) into the cortex and the endodermis tissues (Benhamou and Thériault, 1992; Lafontaine and Benhamou, 1996). Other host reactions on chitosan treated roots were contorted epidermal cells (Benhamou, Lafontaine and Nicole, 1994). In bell pepper fruit, structural defence responses were observed only in the first tissue layers beneath the ruptured cells, such as thickening of the host cell wall, formation of hemispherical and spherical protuberances along the cell walls, and occlusion of intercellular spaces with fibrillar material (El Ghaouth *et al.*, 1994 b, 1997). Further studies demonstrated that the combination of two methods of control, chitosan application, and biological control with *Bacillus pumilus*, increased the host defence reaction of the treated roots (Benhamou, Kloepper and Tuzun, 1998). For cucumber plants grown in the presence of nutrient solutions amended with chitosan, and inoculated by *P. aphanidermatum*, the host reactions were similar to those observed on chitosan-treated tomato roots such as plugging of intercellular spaces with electron-opaque and fibrillar material and papillae formation alongside the host cell wall (El Ghaouth *et al.*, 1994 b).

1.10. AIMS

This research project therefore aimed:

i) To isolate and confirm the identity of a pathogenic strain of *G. boninense* from Sabah

ii) To develop a reliable inoculation technique for *G. boninense* on oil palm

iii) To develop a reliable extraction and quantification method for phenolics in oil palm roots

iv) To quantify specific phenolics in different varieties of oil palm; Ekona, Calabar and AVROS using High Performance Liquid Chromatography (HPLC)

v) To evaluate the efficacy of chitosan in eliciting the accumulation of specific phenolics in oil palm

vi) To investigate the *in vitro* effect of the phenolics detected in oil palm on the growth of *G. boninense*

vii) To study the degradation of the phenolics by *G. boninense*

CHAPTER 2: ISOLATION AND IDENTIFICATION OF *GANODERMA BONINENSE*

2.1. INTRODUCTION

It was very important to make sure the fungus used in this research was the pathogenic species, *G. boninense*, the causal agent of BSR. There are several species of *Ganoderma* which are not pathogenic, that appear morphologically and microscopically similar to *G. boninense*. *Ganoderma* selective medium (GSM) was developed to isolate and confirm *Ganoderma* (Ariffin and Idris, 1992). This medium is very convenient to isolate the fungus from diseased tissue and provides a useful tool for isolating *Ganoderma*, free from other contaminants. The content of fungicides and antibiotics is optimal to control growth of bacteria and other contaminating fungi, while allowing *Ganoderma* to thrive. It does not, however, differentiate between difference species within the genus. Other techniques are required to make a species-level identification. It was reported that, using GSM, it is possible to detect *Ganoderma* in 5 to 16% of oil palms, not showing any obvious external symptoms of infection, or detected using a drilling technique (Idris, Mazliham and Madihah, 2009).

Other more rapid and reliable diagnostic techniques for detecting and confirming *Ganoderma* in oil palm, at an early stage of infection, are now available. The use of PCR for detection of *Ganoderma* in oil palm has been reported by many researchers (Bridge *et al.*, 2000; Hayakawa *et al.*, 2000; Latifah *et al.*, 2002; Utomo and Niepold, 2000). Some recent work also produced more specific PCR primers, PER44-123 which were constructed from the ITS region rDNA and used with LR1 primer to yield an approximately 580 bp product for detection of *Ganoderma* (Idris *et al.*, 2003; Yamoaka *et al.*, 2000). The sensitivity of these primers was assessed using a serial dilution of a given quantity of *G. boninense* genomic DNA as template. A dilution series of basidiospores of *G.*

boninense was also performed and numbers were confirmed by microscopic counts. The DNA of *G. boninense* was amplified at concentrations of template DNA as low as 10 pg μL^{-1}. DNA was not amplified when the template DNA was reduced to concentrations of 5 or 1 pg μL^{-1}. Results indicated that basidiospore DNA could be amplified by PCR to give products that could be detected on ethidium bromide-stained agarose gels. PCR products were consistently visible when 1000 or more basidiospores were supplied. Several other fungal genera which are occasionally found saprophytically on rotten palm tissues such as roots, stem and fruit bunches, including *Aspergillus*, *Botryodiplodia*, *Gymnopilus*, *Fusarium*, *Marasmius*, *Pythium*, *Penicilium*, *Pycnoporus*, *Rhizopus*, *Rhizoctonia*, *Schizophyllum*, *Thielaviopsis*, *Trichoderma* and *Volvariella*, have been tested using primers PER44-123 and LR1 to check for any cross reaction. A PCR fragment of 580 bp was amplified from isolates of *G. boninense*, *G. zonatum* and *G. miniatocinctum*, but no amplification product was detected from saprophytic fungi used in this study (Idris, 2009).

2.2. MATERIALS AND METHODS

2.2.1. Identification based on *Ganoderma* Selective Medium (GSM)

Ten fruiting bodies of *G. boninense* were collected from infected oil palm trees at the Borneo Samudera Langkon Estate in Sabah. Internal tissues of fruiting bodies were excised and cultured on GSM. The medium was prepared as described by Ariffin and Idris (1992) in two parts. Part A comprised Bacto Peptone (Difco) 5.0 g, agar extra pure powder (QRëC) 20.0 g, $MgSO_4.7H_2O$ (Merck) 0.25 g, K_2HPO_4 (QRëC) 0.5 g and distilled water pH 5.5 900 mL. Part B consisted of streptomycin sulphate (Sigma) 300 mg, chloramphenicol (Sigma) 100 mg, pentachloronitrobenzene (PCNB) pure (Aldrich) 285 mg, Ridomil (25% WP) 130 mg, Benlate T20

150 mg, ethanol 95% (Sigma) 20 mL, lactic acid (Sigma) 50% 2 mL, tannic acid (R & M Chemicals) 1.25 g and distilled water pH 5.5 80 mL. Part A was stirred on a hot plate at 100°C until dissolved before being autoclaved for 15 min. Part B was stirred for about 2 h at room temperature. Later part B was added to part A when the autoclaved medium has cooled down to 45°C-50°C.

2.2.2. Identification based on PCR/sequence homology

2.2.2.1. DNA extraction

DNA was isolated using the modified mini protocol for purification of total DNA from plant tissues by Qiagen. Approximately 100 mg of *G. boninense* mycelium, originally grown in potato dextrose broth (PDB), was harvested and homogenized using a bead and vortex at 1000 rpm for 4-5 min to disrupt the tissues. Buffer AP 1 (400 µL) and 4 µL of RNase A stock solution (100 mg mL^{-1}) were added to a maximum of 100 mg (wet weight). The disrupted fungal tissues were again vortexed vigorously. The mixture was incubated for 30 min at 65°C and mixed two to three times during the incubation by inverting the tube. This step lyses the cells. Buffer AP2 (130 µL) was added to the lysate, mixed and incubated for 5 min on ice. This step precipitates detergent, proteins and polysaccharides. The lysate was centrifuged for 5 min at 13,000 x g and lysate was then pipetted into the QIAshredder Mini spin column (lilac) placed in a 2 mL collection tube and centrifuged at 13,000 x g. The flow-through fraction from previous step was transferred into a new tube without disturbing the cell-debris pellet. Volumes (1.5 mL) of buffer AP3/E were added to the cleared lysate and mixed with a Gilson pipette. An aliquot of 650 µL of the mixture from the previous step, including any precipitate that had formed, was then pipetted into the DNeasy Mini spin column placed in a 2 mL collection tube. The

tube was centrifuged for 1 min at 6000 x g and the flow-through was discarded. The collection tube was used for the next step. The previous step was repeated with the remaining sample and flow through and collection tube was discarded. The DNeasy Mini spin column was placed into a new 2 mL collection tube and mixed with 500 µL Buffer AW and centrifuged for 1 min at 6000 x g. The flow through was discarded and the collection tube was reused for the next step. Buffer AW (500 µL) was added to the DNeasy Mini spin column and centrifuged for two min at 13,000 x g to dry the membrane and finally the DNeasy Mini spin column was transferred to a 1.5 mL microcentifudge tube and 50 µL of Buffer AE was pipetted onto the DNeasy membrane and incubated for 10 min at room temperature (25°C) and then centrifuged for 1 min at 6000 x g to elute.

2.2.2.2. Quantification of DNA concentration

DNA concentration was quantified using a NanoVue™ UV/Visible absorbance Spectrophotometer at 260 nm (GE Healthcare). The absorbance recorded was 0.235 with a concentration of DNA 5.8 ng μL^{-1}.

*Concentration = Abs260 * Factor*

NanoVue will default to factors 50 for double stranded DNA, 40 for RNA and 33 for single stranded DNA and Oligonucleotides.

2.2.2.3. Purity of DNA based on ratio A260/A280

Purity of the DNA was calculated based on the 260/280nm absorbance ratio. This ratio gives an indication of purity; however, it is only an indication and not a definitive assessment. Pure DNA and RNA preparations

have expected ratios of 1.7-1.9 and ≥ 2.0. The purity ratio obtained was 1.917.

2.2.2.4. PCR amplification of fungal ITS 1 and ITS 2 regions and the 5.8S gene

PCR amplification of the fungal DNA was done on ITS 1 and ITS 2 regions and the 5.8S gene using primers ITS1 and ITS4. TAE agarose 1.5% (w/v) was used for the agarose gel electrophoresis with 70 V for 40 min with a size of PCR amplicon approximately 650 bp. The concentration of PCR reagents were 1x PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.4 µM forward and reverse primers, one unit of *Taq* Polymerase (GoTaq Flexi DNA Polymerase. Promega) and 18 ng of DNA template.

2.2.2.5. Primer sequences

The primer sequences used were ITS1: 5'- TCC GTA GGT GAA CCT GCG G - 3' and ITS4: 5'- TCC GCT TAT TGA TAT GC -3' with a PCR protocol as described by Latifah *et al.*, (2002). The initial denaturation was set at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 40 s, and extension at 72°C for 50 s. The final extension was set to 72°C for 10 min.

2.2.2.6. PCR product purification (modification)

Before proceeding to cloning, the PCR product was purified using QIAquick PCR Purification Kit (Qiagen) according to the manual. PCR product (20 µL) was added to 100 µL of Buffer PB and later transferred to QIAquick spin column, centrifuged at 13,000 x g for one min. The flow through was

discarded and added to 750 µL Buffer PE and centrifuged at 13,000 x g for 1 min. The flow through was discarded again and the empty column was spun at 13,000 x g for one min. Finally, 15 µL of Buffer EB was added to the empty column and left to stand vertically for 10 min before being centrifuged at 13,000 x g for 2 min.

2.2.2.7. Cloning

The purified PCR product was ligated with pJET1.2/blunt vector as described in manual from CloneJET PCR Cloning Kit (Fermentas).

2.2.2.7.1. Blunting reaction

The blunting reaction was started by addition of 10 µL 2x reaction buffer with 5 µL of nuclease-free water and 2 µL of purified PCR product together with 1 µL of DNA blunting enzyme to make up a final volume of 18 µL. The combination of them was incubated at 70°C for five min and chilled briefly on ice.

2.2.2.7.2. Ligation

For ligation, 1 µL of pJET1.2/blunt cloning vector was added in together with 1 µL of T4 DNA ligase to make up a final volume of 20 µL and further incubated at 22°C for 30 min.

2.2.2.7.3. Competent cell preparation

E. coli TOP10 strain (Invitrogen) was picked from a single colony on the agar plate and cultured in LB broth at 37°C with continuously rotation at

200 rpm overnight. Overnight bacterial culture (1 mL) was transferred to 3 mL of LB broth and shaken at 200 rpm for 1 h. After that, 1.5 mL of culture was centrifuged at 4°C at 6,000 x g for 1 min and the supernatant was discarded. The pellet was washed with ice-cold 50 mM calcium chloride, the washings discarded. The pellet was then suspended in 1 mL of ice-cold 50 mM calcium chloride and spun at 4°C at 6,000 x g for 1 min. Finally, the supernatant was discarded and the cells were suspended in 100 µL of ice-cold 50 mM calcium chloride and stood for 20 min on ice.

2.2.2.7.4. Transformation and plating

Ligation mixture (5 mL) was added to 50 µL of chemically competent *E. coli* with gentle mixing and incubated on ice for 5 min. The cells were subjected to heat-shock at 42°C for 40 s without shaking and the tube was immediately transferred to ice for 5 min. After that, 50 µL of transformants were plated on Luria-Bertani agar containing 100 µg mL^{-1} of ampicillin. The plates were incubated at 37°C overnight.

2.2.2.7.5. Picking colonies and subculture

After culturing overnight, colonies were picked randomly using a sterile toothpick, streaked onto a fresh agar medium LBA plate containing 100 µg mL^{-1} of ampicillin and incubated at 37°C overnight.

2.2.2.8.. Plasmid isolation (Miniprep)

Plasmid isolation was done using GeneJET Plasmid Extraction Kit (Fermentas).

2.2.2.9. DNA sequencing

Due to the unavailability of the DNA sequencing service at the University of Malaysia, Sabah during the progress of this project, the DNA sequencing was done by First BASE Laboratories Sdn Bhd at Selangor, Malaysia.

2.2.2.10. Sequence analysis

The sequence obtained from First BASE Laboratories Sdn Bhd was trimmed with BioEdit software and later BLAST searched for closest matches in NCBI GenBank database. Furthermore, a phylogenetic tree was constructed to show the relationships among the homologous microorganisms. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length was equal to 0.18107685. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei and Kumar, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 253 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

2.3. RESULTS

2.3.1. Identification based on *Ganoderma* Selective Medium (GSM)

The fungi which successfully grew on the GSM after 5 d were identified as *G. boninense* according to this method (Figure 2.1) and later further examined under the Nikon YS100 microscope with magnification of 400x for their clamp connection, a common characteristic for basiodiomycetes. The presence of clamp connections were shown in Figure 2.2.

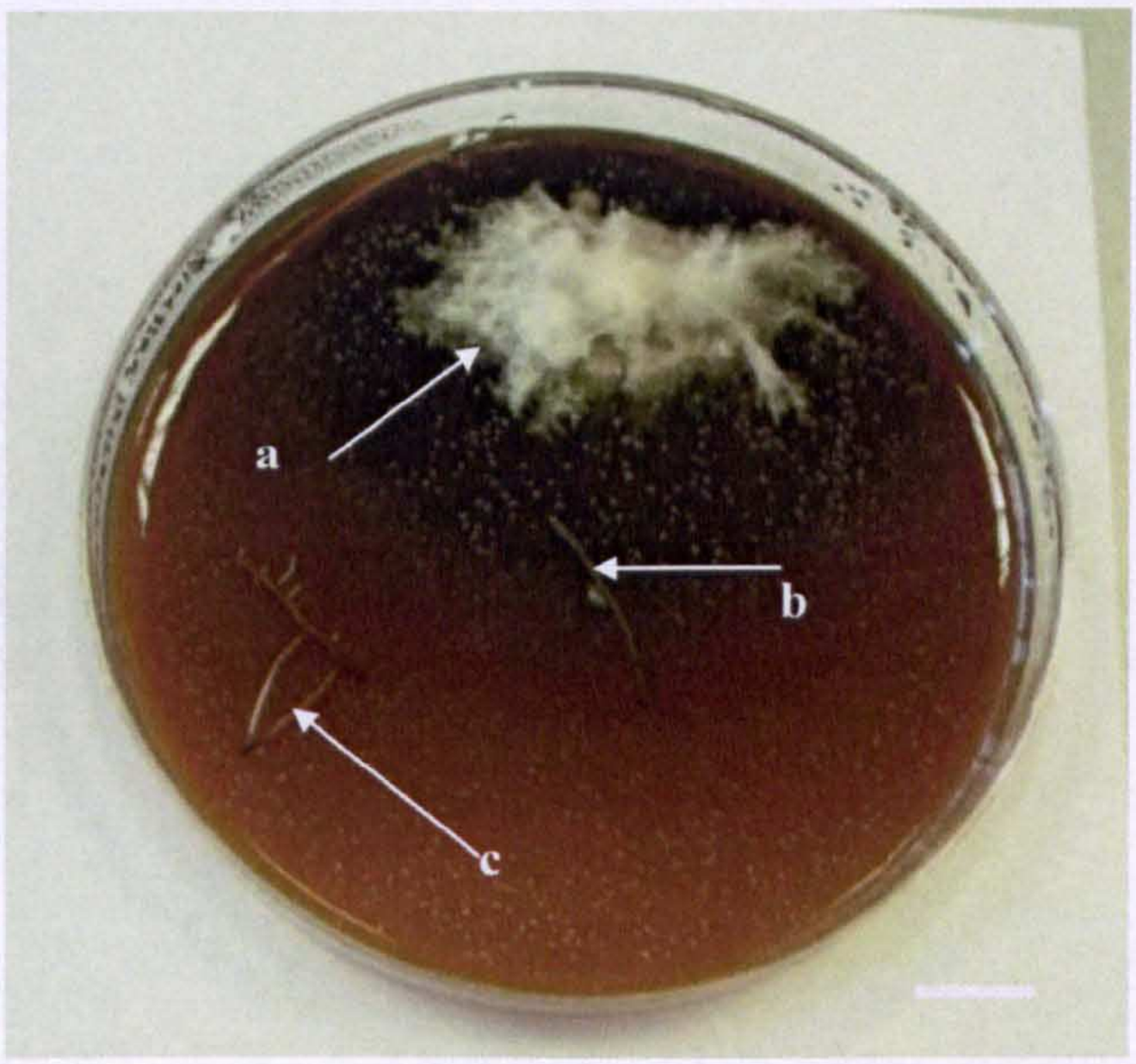


Figure 2.1: *G. boninense* vigorously growing on GSM after incubation for 5 d (Arrow a). Note the absence of other growing fungi from non *Ganoderma*-infected roots (Arrows b and c) on the same media. Bar size: 1 cm

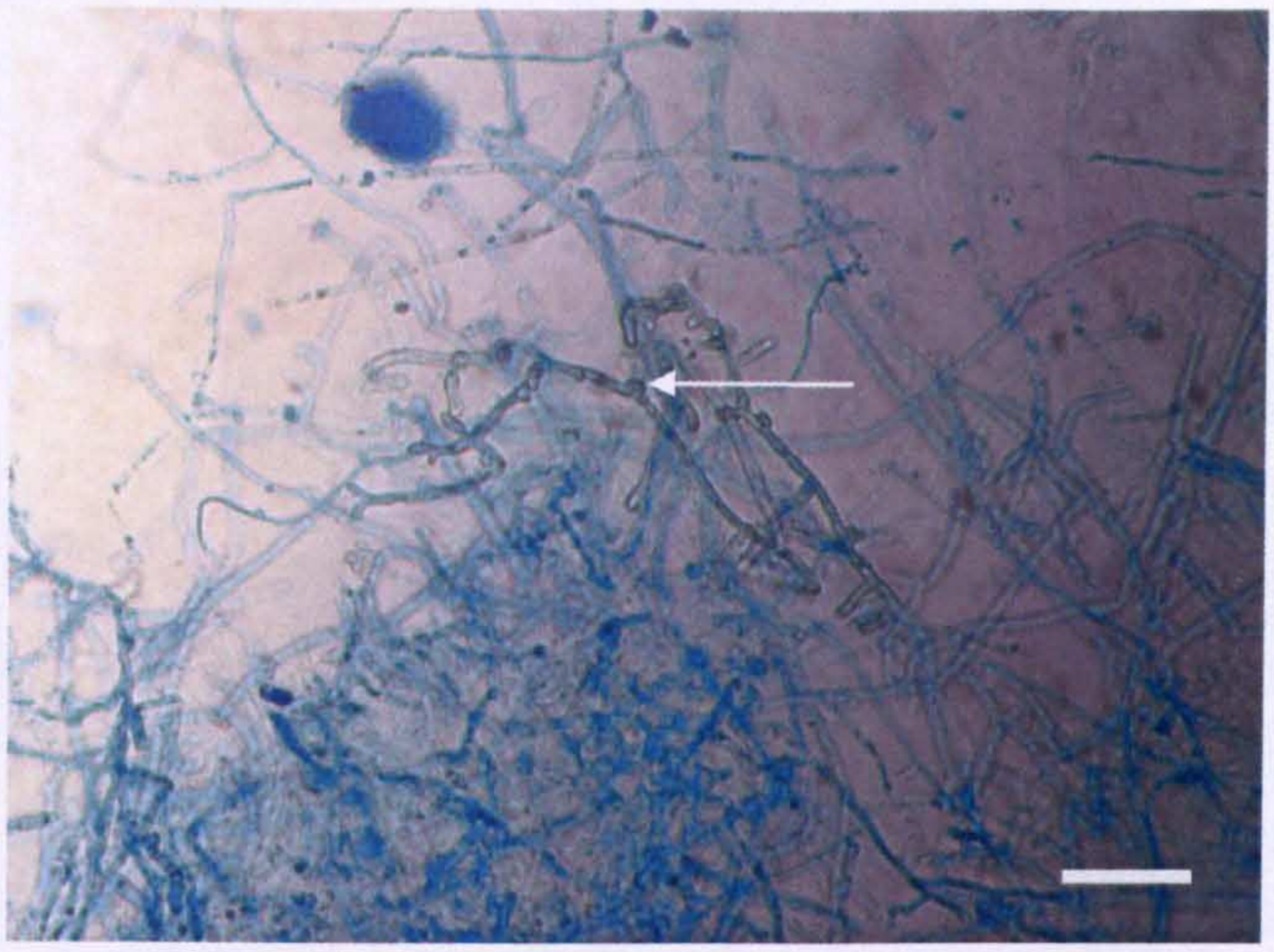


Figure 2.2: The presence of clamp connection, a common characteristic in basdiomycetes (Arrowed). Bar size= 25 µM

2.3.2. Identification based on PCR/sequence homology

The resultant electrophoresis gel from the PCR amplification of fungal ITS1 and ITS 2 regions and 5.8S gene is illustrated in Figure 2.3.

The G1_5 clone sequence from the isolate is shown in Figure 2.4.

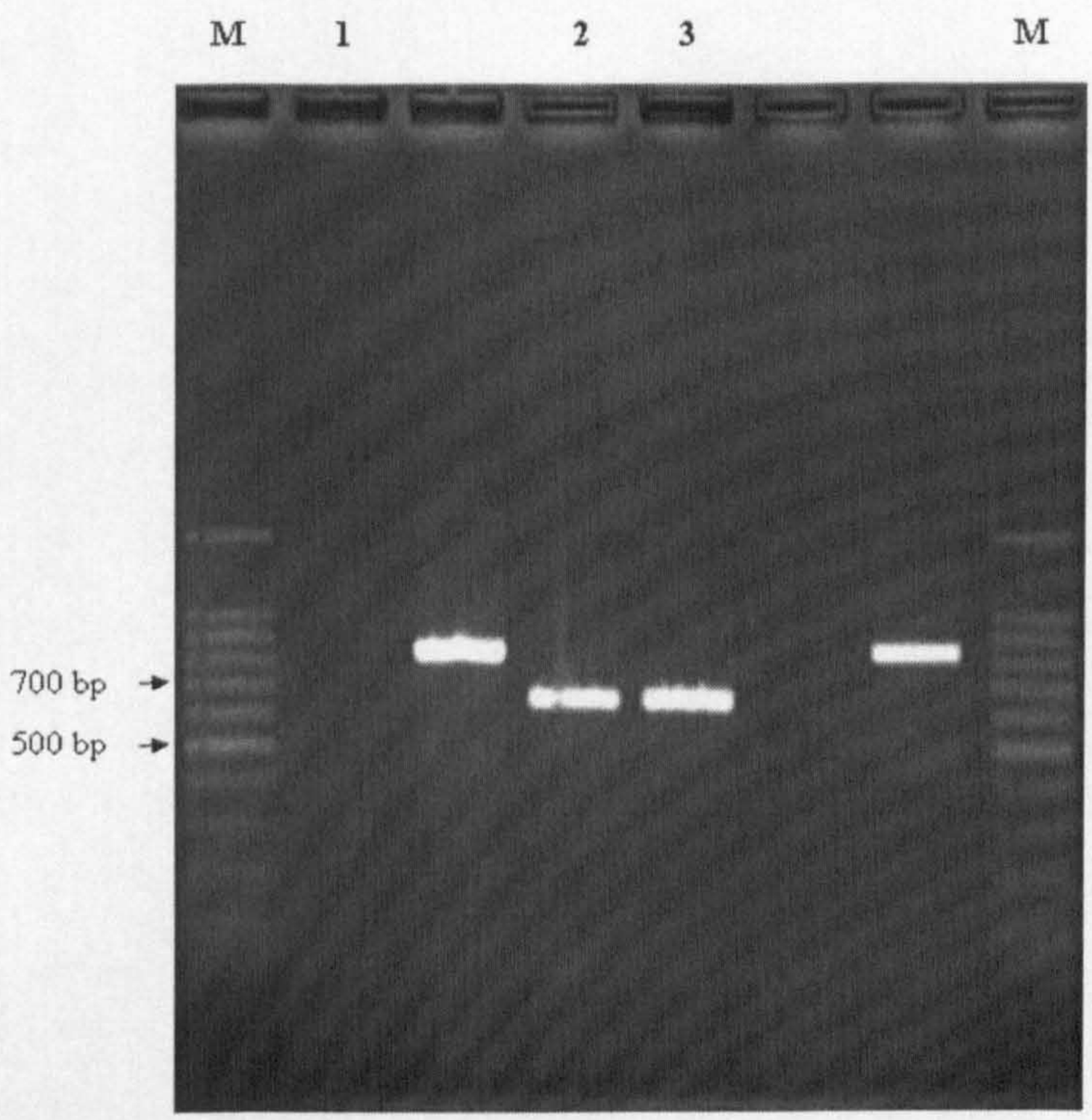


Figure 2.3: PCR amplification of the fungal ITS 1 and ITS 2 regions, and 5.8S gene. M = 100 bp DNA ladder (Promega). Lane 1: negative control (without DNA template). Lane 2 and 3: *Ganoderma* isolates.

>G1_5_CJ12F
CNNNNNNNANTCNGAGGCTCGAGTTTTCAGCAAGATTCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTTTGACTGGGTTGTAGCT
GGCCTTCCGAGGCATCGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTTACTGTGGGTTATGGATTGTGTGGAGCGAGCTCGTTC
GTTTGACGAGTTTGCGAAGCGCGTCTGTGCCTGCGTTTTTATCACAAACACTATAAAGTATTAGAATGTGTATTGCGATGTAACGCATCTA
TATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTG
AATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATCTTCAACCTACAATCTCT
TTGCGGTTCTTGTAGGCTTGGACTTGGAGGCTTGTCGGTCTTTTATTGATCGGCTCCTCTCAAATGCATTAGCTTGGTTCCTTTGCGAATC
GGCTGTCGGTGTGATAATGTCTACGCCGCGACCGTGACGCGTTTGGCGAGCTTCTAACCGTCCCGTTATTGGGACAACGCTTATGACCTC
TGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAATCTTTCTAGAAGATCTCCTACAATATTCTCAGCTGC
CATGGAAAATCGATGTTCTTCTTTTATTCTCTCAAGATTTTCAGGCTGTATATTAAAACTTATATTAAGAACTATGCTAACCACCTCATCAGG
AACCGTTGTAGGTGGCGTGGGTTTTCTTGGCAATCGACTCTCATGAAAACTACGAGCTAAATATTCAATATGTTCCTCTTGACCAACTTTAT
TCTGCATTTTTTTTGAACGAGGTTTAGAGCAAGCTTCAGGAAACTGAGACAGGAATTTTATTAAAAATTTAAATTTTGAAAAAAGTTCAGGG
TTAATAGCATCCATTTTTTGCTTTGCAAGTTCCTCAGCATTCTTAACAAAAAGACGTCTCTTTTGACATGTTTAAAGTTTAAACCTCCTGGTG
GTGAAATTNTTATCCGCTCATAATTCCNNCNNCAATTTATACGANGCCGNNAGGCANTAAAGNTGNTAAAGNCCNNNNNNNNGCNCCNN
TANNTGNG

Figure 2.4: G1_5 clone sequence from the isolate collected from Langkon Estate Sabah, Malaysia

Plasmid isolation from the selected recombinant clones contained insert of fungal ITS 1 and ITS 2 regions, and 5.8S gene is shown in Figure 2.5. The PCR product of the isolate is shown in Figure 2.6 the trimmed sequence using Bioedit software is shown in Figure 2.7.

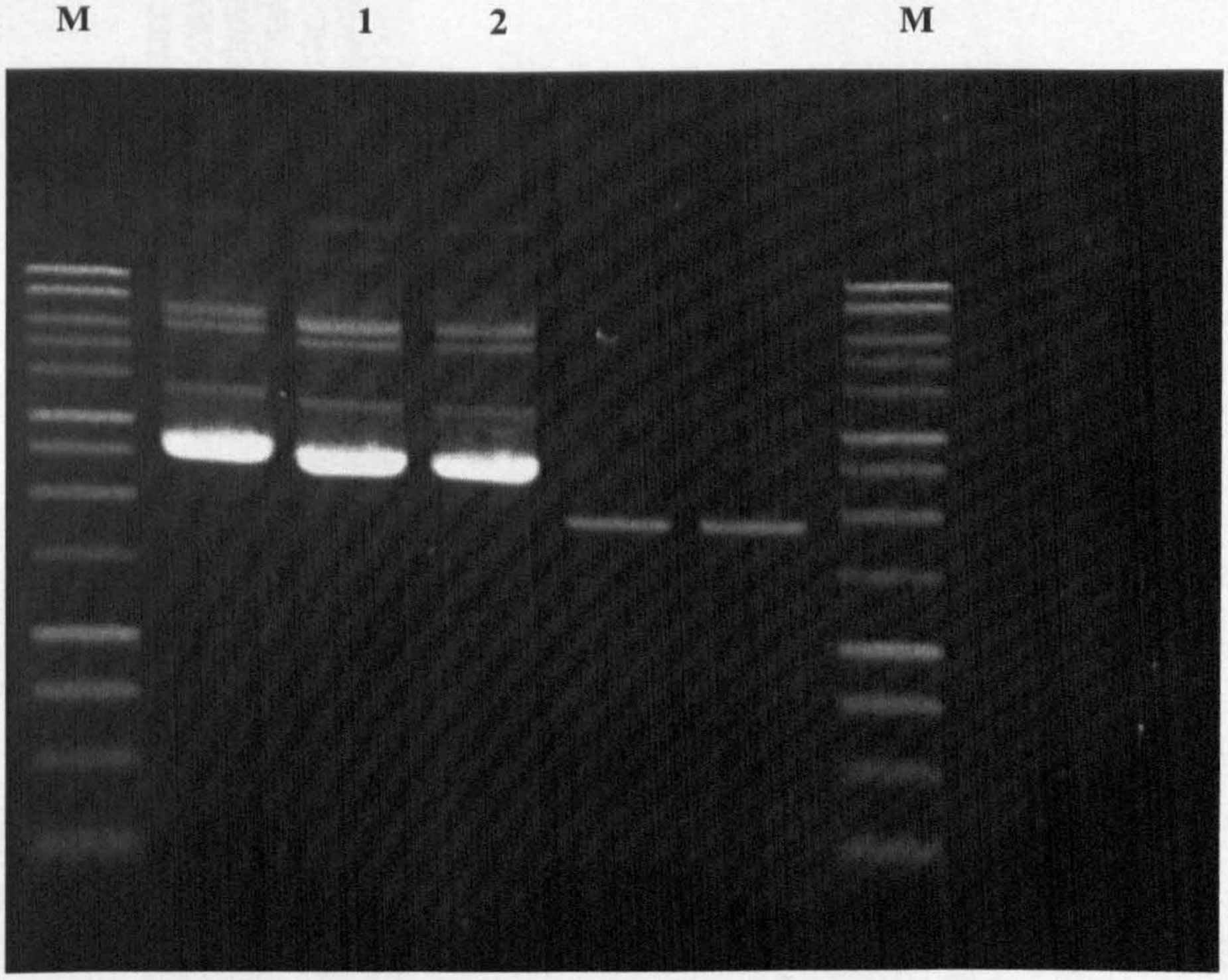


Figure 2.5: Plasmid isolation from the selected recombinant clones contained insert of fungal ITS 1 and ITS 2 regions, and 5.8S gene. M = 1 kb DNA Ladder (Promega); 1 = clone G1_5; 2 = clone G1_20.

>G1_5
TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCCTTCCGAGGCATCGTGCACGCCCTGCTCATCCA
CTCTACACCTGTGCACTTACTGTGGGTTATGGATTGTGTGGAGCGAGCTCGTTCGTTTGACGAGTTTGCGAAGCGCGTCTGTGCCTGCGT
TTTTATCACAAACACTATAAAGTATTAGAATGTGTATTGCGATGTAACGCATCTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCA
TCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTG
GTATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATCTTCAACCTACAATCTCTTTGCGGTTCTTGTAGGCTTGGACTTGGAGGCTTGT
CGGTCTTTTATTGATCGGCTCCTCTCAAATGCATTAGCTTGGTTCCTTTGCGAATCGGCTGTCGGTGTGATAATGTCTACGCCGCGACCGT
GACGCGTTTGGCGAGCTTCTAACCGTCCCGTTATTGGGACAACGCTTATGACCTCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTT
AAGCATATCAATAAGCGGA

Length: 653 bp

Figure 2.6: PCR product from the isolate collected from Langkon Estate Sabah, Malaysia

TCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCCTTCCGAGGCATCGTGCACGCCCTGCTCATCCA
CTCTACACCTGTGCACTTACTGTGGGTTATGGATTGTGTGGAGCGAGCTCGTTCGTTTGACGAGTTTGCGAAGCGCGTCTGTGCCTGCGT
TTTTATCACAAACACTATAAAGTATTAGAATGTGTATTGCGATGTAACGCATCTATATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCA
TCGATGAAGAACGCAGCGAAATG

295 bp

Figure 2.7: Sequence of isolate DNA after trimming using BioEdit software

The nine possible isolates from the NCBI GenBank database are shown in Table 2.1. Out of these nine possible microorganisms, *G. boninense* strain FA5017 and FA5035 gave a maximum identification homology of 98% with G1_5 (The alignments are shown in Appendix 1). Figure 2.8. shown a phylogenetic tree was constructed to show the relationships among the homogous microorganisms.

Table 2.1: The most homologous nine microorganism from NCBI gene bank in comparison to the Langkon estate isolate. Note the *G. boninense* strain FA5017 and 5035 with maximum identification of 98% to this isolate

Accession	Description	Max Score	Total Score	Query Coverage	E Value	Max Ident
EU841913.1	Ganoderma boninense strain FA5017 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	506	505	95%	6e-140	98%
EU701010.1	Ganoderma boninense strain FA5035 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence	505	505	95%	6e-140	98%
AJ627585.1	Ganoderma mastoporum 18S rRNA gene (partial), 5.8S rRNA gene, 26S rRNA gene (partial), ITS1 and ITS2, isolate FRIM 98	429	429	100%	4e-117	93%
AY220544.1	Ganoderma sp. SB-1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	422	422	89%	6e-115	95%
AY569450.1	Ganoderma cupreum isolate SUT H1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 25S ribosomal RNA gene, partial sequence	422	422	100%	6e-115	92%
AY593865.1	Ganoderma japonicum AS5.69 type 2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	420	420	100%	2e-114	92%
AY593864.1	Ganoderma japonicum AS5.69 type 1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	414	414	100%	1e-112	92%
DQ421109.1	Uncultured soil fungus clone 133-14 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence	411	411	100%	1e-111	92%
AF255093.1	Ganoderma sp. CBS187.31 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	411	411	100%	1e-111	92%

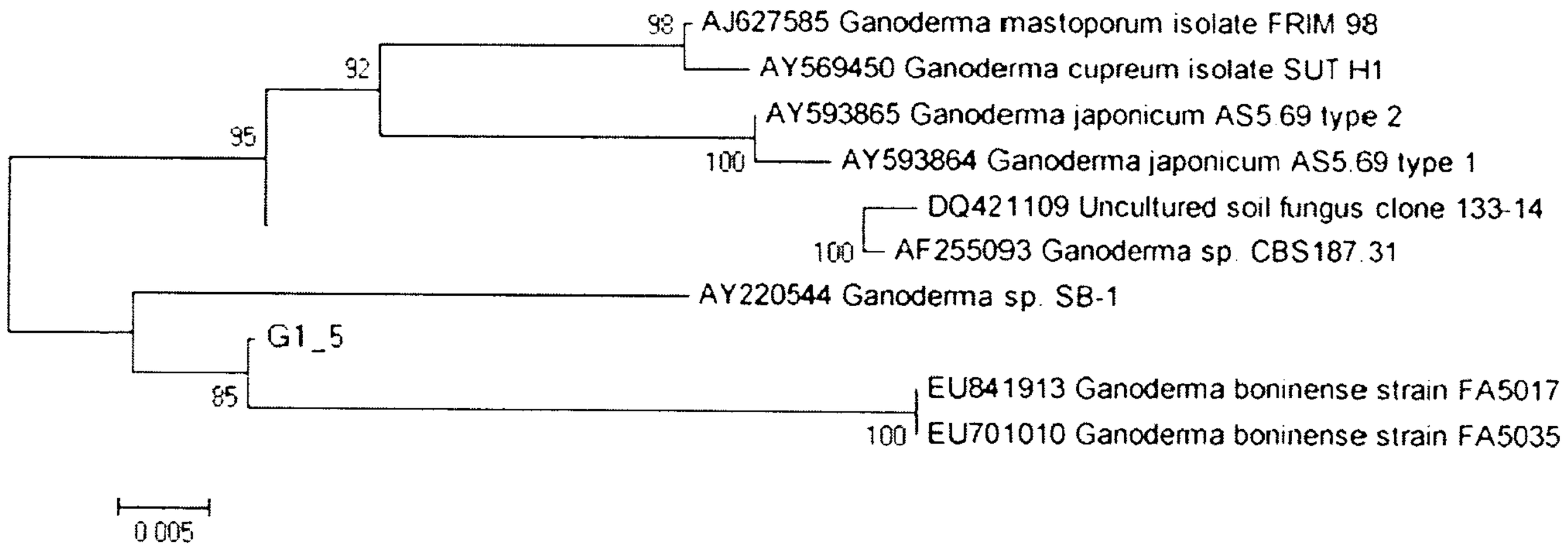


Figure 2.8: Evolutionary relationships of 10 taxa among isolate of *G. boninense* from Langon estate, Sabah, Malaysia with other nine homologous microorganisms. Note: G1_5 is the isolate from Langkon, Sabah. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.18107685 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 253 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

From Figure 2.8, there is strong evidence the G1_5 isolate from the Langkon estate is very similar to *G. boninense* strains FA5017 and FA5035. The small differences detected at the 3′ end of the sequence may reflect anomolies in the gene bank *G. boninense* sequences. Bases 427-438 show variation between the FA5017 and FA5035 isolates, compared to other *Ganoderma* sequences at these positions. This may reflect sequencing error or primer incorporation for these *G. boninense* sequences. The sequences of isolate G1_5 were divergent with other *Ganoderma* spp. Therefore, it was concluded that the isolate collected from Langkon estate was *G. boninense*, it has very high homology to known isolates from Peninsular Malaysia and it was therefore used for further experiments on oil palm.

2.4. DISCUSSION

The growth of the fungal isolate from Langkon Estate of Kota Marudu, Sabah on GSM suggested this fungus belongs to the class of basidiomycetes. Microscopic examination provided evidence of the presence of clamp connections, supporting the claim. Clamp connections are a special structure that is formed during the conjugate division of the nuclei in the tip of a growing hypha (Alexopoulos, Mims and Blackwell, 1996). Since the 1960s when *G. boninense* was first proposed as an important pathogen to oil palm industry, GSM has been widely exploited by a number of plant pathologists for identification of *Ganoderma* spp; as it provides a quick and cheap solution for the elimination of other unwanted bacteria and saprophytic fungi from oil palm infected areas. But due to the possibility of other basidiomycetes that may grow on this medium, the identification and detection using this media is inconclusive.

In recent years, progress has been made in the early detection and identification of this pathogen through more reliable methods such as enzyme-linked immunosorbent assays (ELISA) (Idris and Rafidah, 2008; Kandan *et al.,* 2009; Utomo and Niepold, 2000) as well as polymerase chain reaction (PCR) based techniques involving certain non-specific *Ganoderma* primers (Idris *et al.,* 2003; Kandan *et al.,* 2009; Latifah *et al.,* 2002; Miller, Holderness and Bridge, 2000). Molecular techniques exploiting variations in the ribosomal DNA (rDNA) have been used extensively for systematic and phylogenetic studies of fungal pathogens. Different regions of the rDNA diverged at different rates allowing the regions to be exploited at different taxonomic levels (Bruns, White and Taylor, 1991; Latifah *et al.,* 2002). Polymerase chain reaction using the ITS1 and ITS4 primers showed that isolates from Langkon Estate produced a PCR fragment of approximately 650 bp. The same fragment size does not necessarily indicate sequence similarity. The sequence was trimmed for a better BLAST search from the NCBI gene bank. The BLAST results from the NCBI gene bank confirmed the isolate collected was similar to the *G. boninense* strains FA5017 and 5035 with a homology of 98%.

Due to the importance of the oil palm industry to Malaysia's economy, the transfer of any materials that are related to *Ganoderma* is strictly prohibited from Peninsular Malaysia to Sabah and Sarawak. This basic identification which needed to be carried out for this project to confirm the identification of *G. boninense*, using PCR techniques, has never been done before in Sabah. The only report on *G. boninense* isolates in Sabah was merely based on their morphology and pathogenicity (Idris *et al.,* 2001 b). In conjunction with the morphological similarities among the different isolates, there are numerous opinions on the aggressiveness of *G. boninense* in Sabah. The Head of Research for Borneo Samudera oil palm plantation (the largest plantation group in Sabah) stated that isolates

of *G. boninense* from Sabah are less aggressive compared to those from Peninsular Malaysia (Hoong, H.W. personal communication). This may be due to lower incidence of BSR in Sabah and Sarawak compared to Peninsular Malaysia (Ariffin and Idris, 2002).

Besides speculation on the different aggressiveness of the isolates, the difference in the severity of outbreaks may due to the different re-planting factors. In Peninsular Malaysia, most of the oil palm estates are undergoing the second or the third re-planting. The increase in inoculum that remains as debris in the soil after the first and second re-planting may provide a good source for further infection of the newly planted palms through root contact. To date, it was reported that almost 90% of the estates in West Malaysia show the present of *G. boninense* (Khairuddin and Chong 2008). In Sabah and Sarawak, most of the estates are in their first planting, providing alternative hypothesis for the lower incidence of pathogen attack in these areas. The disease incidences were only reported to be 4% in Sabah, but this is distant from the real situation in estates (Idris. A.S. personal communication), who stated it is considerably higher. Big multi-billion Ringgit companies that are involved in the oil palm industries are suspected of not reporting the real statistic of the incidence in Sabah, which currently has the largest plantation areas in Malaysia, in order to avoid losses in their market share (Hoong, H.W. personal communication). Since the oil palm industry is a fast income-generating tool, data related to it maybe very secretive. Researchers, plantation managers and entrepreneurs do not share information, oil palm genetic materials or isolates of *G. boninense* with others. The quarantine and many other restrictions enforced by the government have made it impossible to obtain other, possibly more aggressive, isolates of the pathogen or other varieties of oil palm that differ in susceptibity to *G. boninense*.

CHAPTER 3: INFECTION AND DISEASE SEVERITY

3.1. INTRODUCTION

3.1.1. Infection

G. boninense is a poor competitor in non-sterile soil or in organic debris that accumulates at the frond-stem junctions of oil palm (Rees *et al.*, 2007). But once the infection is initiated, the damage caused from the establishment of the BSR can be very serious. Unlike many of the other pathogens, work on this basidiomycete is extremely difficult. The pathogen does not easily produce basidiospores under artificial conditions and efforts to trap the spores in estates always end up with high contamination from other spores. In addition to this, the role of basidiospores as inoculum to cause infection remains unclear. Basidiospores reportedly failed to initiate infection, and therefore may not serve any direct inoculation value (Ho and Nawawi, 1986; Pillotti, Sanderson and Aitken, 2003; Ramasamy, 1972; Sanderson, 2005; Sharples, 1936; Turner, 1965 b; Turner, 1981). The primary route of infection appears to be through root contact with inoculum sources in the soil. In nature, inoculum of the pathogen may remain in the soil as a free-living saprophyte after the first generation of oil palm. In the second or third generation of re-planting, the newly planted oil palm roots grow into the soil and make contact with the contaminated debris, thus initiating infection. However, the real initial point of the infection is still speculation. Rees *et al.* (2009) reported the progression of the infection using light and transmission electron microscopy. The inspections were conducted by random felling of mature palms without symptoms but suspected to be infected by *G. boninense*. This provided an insight into the progress of the infection once it was initiated. Infection of intact roots of oil palm with *G. boninense*, showed penetration followed by rapid longitudinal progression of hyphae and colonisation of the lower stem (bole). There was no evidence on selective

progression in the root during the invasion through the vascular system or lacunae.

3.1.2. Inoculation

Numerous infection trials using oil palm seedlings inoculated with large *Ganoderma*-colonised rubber-wood blocks have provided data supporting this view (Breton *et al.*, 2006; Hasan and Turner, 1998; Lim, Chung and Ko, 1992; Lim and Fong, 2005; Navaratnam and Chee 1965; Sariah *et al.*, 1994). Rees *et al.* (2007) showed that by attaching infested wood blocks to roots, much smaller inoculum sources can be used, allowing infection to occur through unwounded roots and progression and rate of invasion to be monitored. On the other hand, Idris *et al.* (2006) claimed that larger block size of inoculum provided a more successful rate of infection. A size of 6 x 6 x 6 cm gave an infection rate of 100%, based on the reisolation of *Ganoderma* on GSM, compared to 1.5 x 1.5 x 1.5 cm with only 10% of infection.

Idris *et al.* (2006) also demonstrated another technique for inoculation of oil palm germinated seeds with *Ganoderma*. The *G. boninense* inoculum was first raised on PDA in test tubes until the mycelium had completely covered the substrate in the tube. Then, a small incision was made at the side of the polypropylene bag containing the oil palm seedlings, revealing some roots. One of the primary roots thus exposed was then pulled through the opening, washed with water to remove adhering soil; 2-3 cm of the distal end of the root was then placed into the tube containing inoculum and sealed with parafilm. The whole tube was covered with brown paper to maintain darkness.

Unfortunately, most of the current methods are very labour-intensive and time-consuming. There is also a very high chance rubber blocks will become contaminated by other saprophytic fungi that remained in the blocks, although they have undergone standard or modified autoclave procedures. An

alternative method of infection that is fast, reliable and reproducible is needed for research on *Ganoderma* and oil palm.

3.1.3. Disease assessment

G. boninense is a slow growing pathogen. Early infection is usually symptomless therefore cannot be recognized visually. The progressive destruction of the basal tissues of the oil palm trunk by *G. boninense* eventually exhibits some external symptoms, such as wilting and malnutrition. Frequently, when foliar symptoms confirm the presence of the pathogen, the fungus has killed at least one-half of basal stem tissue (Idris, 2009). The most common BSR severity assessment used for analyzing the intensity of the disease is the one described by Sariah and Zakaria (2000), in which the incidence of BSR was assessed based on foliar symptoms at monthly intervals. Such that:

$$\text{Severity of foliar symptoms (\%)} = \frac{(a \times 1) + (b \times 0.5)}{c} \times 100$$

Where a: the number of desiccated leaves, b: the number of chlorotic leaves, c: the total number of leaves and where the numerical value of 1 represents the index for desiccated leaves and 0.5 for chlorotic leaves.

Alternatively, the bole of infected palm can be cut longitudinally for assessment of percentage infection of bole tissues, expressed as (d/e) x 100, where d: the lesion length (mean of two measurements) and e: the bole diameter (Teh, 1996; Teh and Sariah, 1999). Another assessment on the disease severity, as described by Abdullah, Illias and Nelson (2003) was also merely based on foliar symptoms. The researchers categorized the signs and

symptoms of the pathogen infection into four different classes in numerical values from zero to four. A healthy plant with green leaves with absolutely no mycelial development on any part of the plant is classified as zero and the highest score of four is associated with plants with the presence of well-developed basidiomata with at least 50% of the total leaf number showing severe chlorosis and the plant drying up, dying or dead.

The Disease Severity Index (DSI) is counted as:

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

Where A: disease class (0, 1, 2, 3 or 4) and B: number of plants showing that disease class per treatment

But these assessments are in generally slow and inconsistent as the infection needs a long time to develop the foliar symptoms. Because *Ganoderma*-infected roots develop symptoms more quickly, a modified scale that is based on root infection may give a better alternative to the foliar symptoms.

3.1.4. Ergosterol as an indicator to fungal biomass

Ergosterol is the main sterol in the cellular membrane of most filamentous fungi, yeasts, and some green algae and amoebae (Nielsen and Madsen, 2000; Weete, 1980). It is a constituent of mycelia as well as spores and vegetative cells. On the other hand, most higher plants, some yeasts, rust fungi and phycomycetes (with the exception of Mucorales) are not able to synthesise ergosterol (Weete, 1980). The amount of ergosterol depends on the extraction method used, fungal species, fungal isolate, substrate

composition, humidity, age of culture, developmental stage and growth phase of the fungi (Charcosset and Chauvet, 2001; Gilbert *et al.,* 2002; Newell, 1992). Despite these influencing factors the ergosterol content has been established as an indicator of fungal biomass in various samples such as soils (West, Grand and Sparling, 1987), mycorrhiza (Nylund and Wallander, 1992), aquatic systems (Padgett, Mallin and Cahoon, 2000; Suberkropp, Gessner and Chauvet, 1993), decaying plant material (Gessner and Schmitt, 1996; Newell, 1992), seeds (Richardson and Logendra, 1997), grain (Börjesson *et al.,* 1990) and tomato products (de Sio *et al.,* 2000). It seems that ergosterol describes the active, living biomass (Bjurman, 1994) as well as indicating changes in biomass (Schnürer, 1993) and total biomass in young cultures before the stationary phase is reached (Börjesson, Stöllman and Schnürer, 1990). Therefore, ergosterol analysis provides a quick assessment for the growth of *G. boninense* in this project.

3.1.5. Varietal resistance

There are several diseases and disorders in oil palm for which genetic variation in susceptibility or resistance has been demonstratedand thus breeding for resistance is feasible. Breeding for resistance has been most successful with *Fusarium* wilt (Corley and Tinker, 2003). There is still some debate as to genetics of resistance to this disease. Meunier, Renard and Quillec (1979) found parents which consistently gave crosses with a high degree of tolerance, and considered that tolerance was polygenic, but de Franqueville and de Greef (1988) suggested that it may be controlled by only two genes. The latter authors produced hypothethical genotypes at these two loci for a large number of parents in the Binga programme, but attempts to test their hypothesis with further crosses were hampered by the death, from wilt, of all the most susceptible genotypes. Renard *et al.* (1993) reported that

resistant clones could be obtained from progenies which, based on average performance, were susceptible and vice versa; this suggests segregation of a simple character, rather than additive inheritance. Rosenquist, Corley and de Greef (1990) reported that pure Dumpy Deli *dura* material appeared to be virtually immune to this disease. Two families gave 0% and 1% wilt in the nursery in Cameroon, while one family in Congo had suffered no losses after 10 years in the field, a figure recorded in only two other families out of more than 450 in the programme. For patch yellow disease in Nigeria, trials showed differences between progenies in susceptibility to the disease, which is caused by a different strain of *F. oxysporum* from that causing wilt. There is some evidence for genetic resistance to *Cercospora elaeidis*, but breeding for resistance to this disease does not appear to have been attempted (Corley and Tinker, 2003).

The phenomenon of variable susceptibility in oil palm progenies and the existence of resistant genotypes to *G. boninense* has been observed (Akbar, Kusnadi and Ollagnier, 1971; Franqueville *et al.*, 2001). These observations provide hope of generating oil palm varieties with a reduced level of susceptibility, using existing genetic materials (Mohamad Arif *et al.*, 2007). In the present market in Malaysia and Indonesia, there are a lot of varieties of oil palm that show different susceptibility to this pathogen. Some of those reported, such as the variety derived from progeny PK 2567 (D x P, Congo x Cameroon), were claimed to be partially resistant, while a variety derived from progeny PK 2724 [DxD, Deli (Elmina) x Deli (Elmina)] was declared to be very susceptible to *G. boninense* (Idris *et al.*, 2004 b). However, there are also a lot of other varieties that have been derived from different progenies for which results on susceptibility to BSR have not been reported. However, current breeding selection programmes are mostly rely on the infection developed from inoculation with colonised rubber wood blocks. A

more rapid and effective inoculation technique would be very useful to facilitate screening of resistance in breeding lines.

Selection of suitable candidates to be planted in estates may also rely on other aspects of their performance, which include fruit bunch yields, vegetative growth, oil quality and other quality aspects, besides their susceptibility to *Ganoderma* infection. The susceptibility or resistance of varieties of oil palm to this pathogen may be due to many genotypic traits. Attempts to further investigate, in this project, the factors that may lead to this different susceptibility among the varieties used by Idris *et al.* (2004 b), especially the role of phenolics, were not feasible due to the inability to obtain plant materials from the authors. Research was therefore undertaken on three other well known local Sabah varieties, which were Ekona, Calabar and the most common commercial variety of AVROS. All of them were derived from different progenies that were shown to have different susceptibility to *G. boninense* infection. Ekona was derived from PK 1231 x T 407, Calabar from BSDDS 10 x AR 301 while AVROS from BSDS 2 x AR 812; which represented their different parent materials (D x P). Calabar (D x P) and Ekona (D X P) have the same *Dura* as AVROS but with an African origin *Psifera*. Certified disease-free pre-germinated seeds, of these varieties were provided by Borneo Samudera Sdn Bhd, Sabah, Malaysia and grown to one year old in Peat Vriezenveen Substrate, Product of Holland, in the net house at the University of Malaysia, Sabah.

3.2. MATERIALS AND METHODS

3.2.1. Development of a reliable artificial inoculation method for *G. boninense* infection of oil palm

Fruiting bodies of *G. boninense* were collected from infected oil palm trees at the Borneo Samudera Langkon Estate in Sabah. Internal tissues of fruiting bodies were excised and cultured on *Ganoderma* Selective Medium (GSM) (Ariffin and Idris, 1992). Once a pure culture of *Ganoderma* was isolated, it was transferred and maintained on Potato Dextrose Agar (PDA) at 25°C. Seven to eight day old cultures were used to inoculate oil palm seedlings. The pathogen was later re-isolated from infected oil palm seedlings onto GSM. Procedures were repeated throughout the project to maintain the pathogenicity of *G. boninense.* The artificial inoculation method developed for inoculating oil palm roots with *G. boninense* was very similar to some common methods used for fungal inoculation, but it was the first time it was used for this pathogen. Briefly, the mycelium of *G. boninense* which covered the entire surface of a 9 cm Petri dish was scraped off and suspended in 20 mL PDB, blended and 50 µL of Tween 20 added. Infection, however, was failed to be intiated without the present of PDB (Data not presented). The oil palm bare roots were carefully uprooted from the peat and sprayed with a suspension of *G. boninense* fragments, with approximately 17.5 mL of the inoculum per seedling and replanted into the peat. The confirmation on the success of the inoculation method was conducted by re-isolating the pathogen from the infected roots on GSM after three weeks with surface sterilisation (10% v/v Clorox; 5000 ppm available chlorine) and observation of foliar symptom development was conducted 18 weeks later for similar plants in the group (Figure 3.1).

Figure 3.1: (A) Healthy oil palm seedlings. (B) *G. boninense* infected oil palm seedlings with artificial inoculation method. (C) Foliar symptoms developed 18 weeks after inoculation with *G. boninense.* Note: the infected and necrosed roots and chlorotic leaves are arrowed in (B) and (C). Bar size= 5 cm

3.2.2. Oil palm root elicitation with chitosan

Chitosan stimulation of the defence mechanisms was conducted on one year old seedlings. In order to minimize the impact to the environment that may be caused by the addition of chitosan, a series of preliminary studies were conducted to evaluate the lowest concentration of chitosan that effectively elicited changes in the palm roots (data not presented). Different concentrations of chitosan were incorporated into the oil palm substrates. From numerous trials, the ratio of 5 g of chitosan to 5 kg of oil palm substrate was found to be the minimum rate that gave significant differences in oil palm

disease severity. Therefore, for further work, 5 g of chitosan (R & M Chemicals, Essex, UK) was incorporated into 5 kg of Peat Vriezenveen Substrate (supplied by Lee Kian Kimia Sdn Bhd, Sabah) by mixing to homogeneity. Roots were then grown and watered daily for three, four, five and six weeks before harvesting and rinsing under running tap water. Untreated seedlings served as a control.

3.2.3. Evaluation of *Ganoderma* fungal colonisation by ergosterol analysis and quantification

The infected roots and uninfected roots (as a control) were extracted as described by Genney, Alexander and Hartley (2000). Root samples (100 mg) were extracted in methanol using bead beating to physically crush the sample. Polyvinylpyrrolidone (PVPP) (Sigma-Aldrich) was added (10% w/v) to the methanol to precipitate phenolic compounds. The extract was centrifuged at 15,000 x g for 5 min and the supernatant was made up to 1.5 mL before being filtered through a 0.45 µm acetate syringe tip filter. An Agilent Series 1200 Chromatography System comprising degasser G1313B, Quat Pump G131A, autosampler ALS G1329A with ChemStation for data manipulation software was used with an Agilent G1313B HPLC VWD detector for the analysis and quantification. A reversed phase Eclipse XDB-C_{18} 4.6 mm x 150 mm with 5 µm particle size column was used for separation. The wavelength of the UV detector was set to 282 nm, and the isolated peak elution at about 5.5-5.8 min retention time was identified as ergosterol based on its co-chromatography and identical absorption spectrum with a pure standard (20 µg mL^{-1}) (Figure 3.2) at the flow rate of 1.5 mL min^{-1}. The system was run isocratically with 100% methanol.

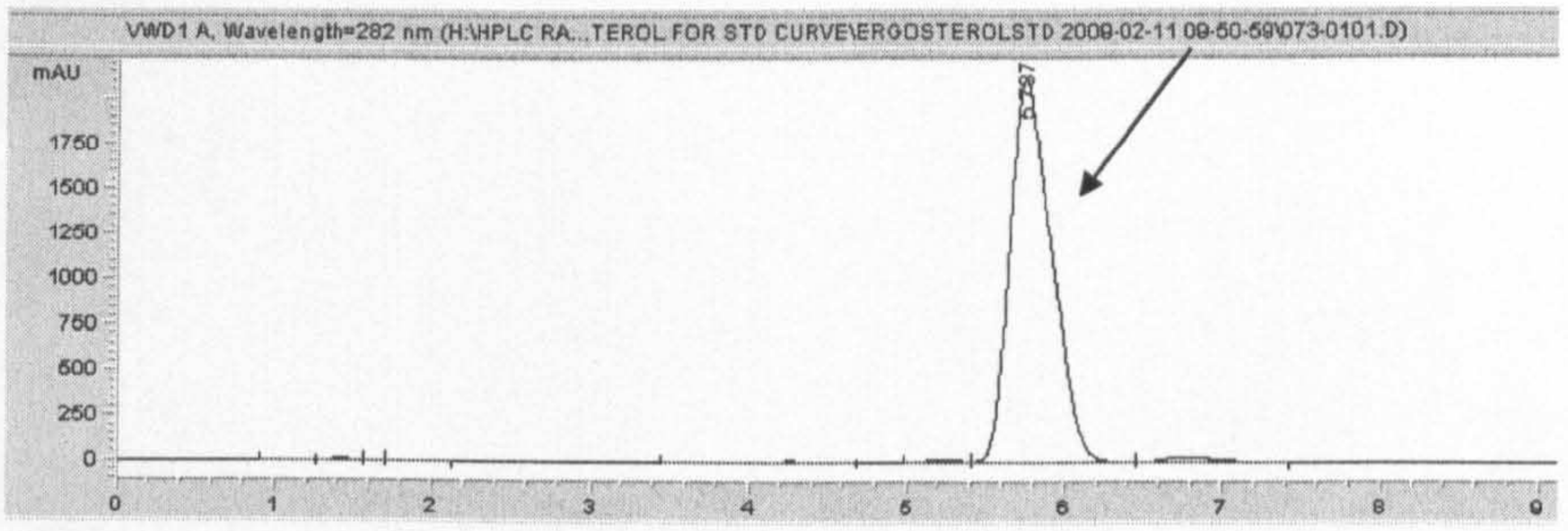


Figure 3.2: Chromatogram showing the detection of pure ergosterol by variable wavelength detector (VWD) after separation by HPLC. The peak corresponding to ergosterol is arrowed. Noted the retention time was 5.787 min

A serial dilution with a range of 5-500 µg mL^{-1} of the ergosterol standard was injected into the HPLC system to develop a standard curve as shown in Figure 3.3. The developed standard curve was used for further ergosterol quantification from oil palm roots.

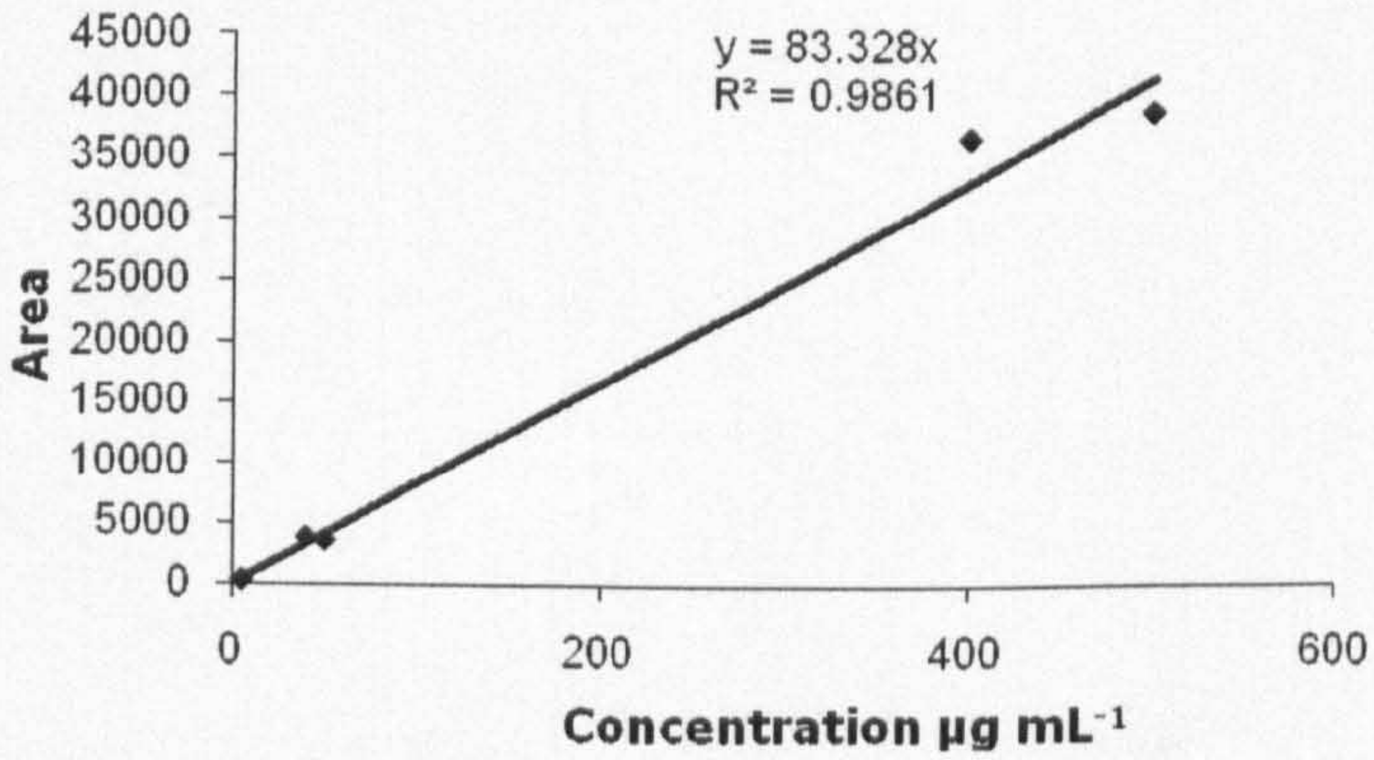


Figure 3.3: Integration of absorbance at 282 nm plotted against the concentration of standard ergosterol

3.2.4. Modified disease severity scale (MDSS)

The disease severity of the oil palm roots artificially infected with *G. boninense* was assessed based on a faster root-based modified disease severity scale (MDSS) from Horsfall and Barratt (1945) (Figure 3.4). This is the first report of use of this root symptom assessment key in the oil palm–*Ganoderma* interaction. Other workers commonly used assessment of foliar symptoms.

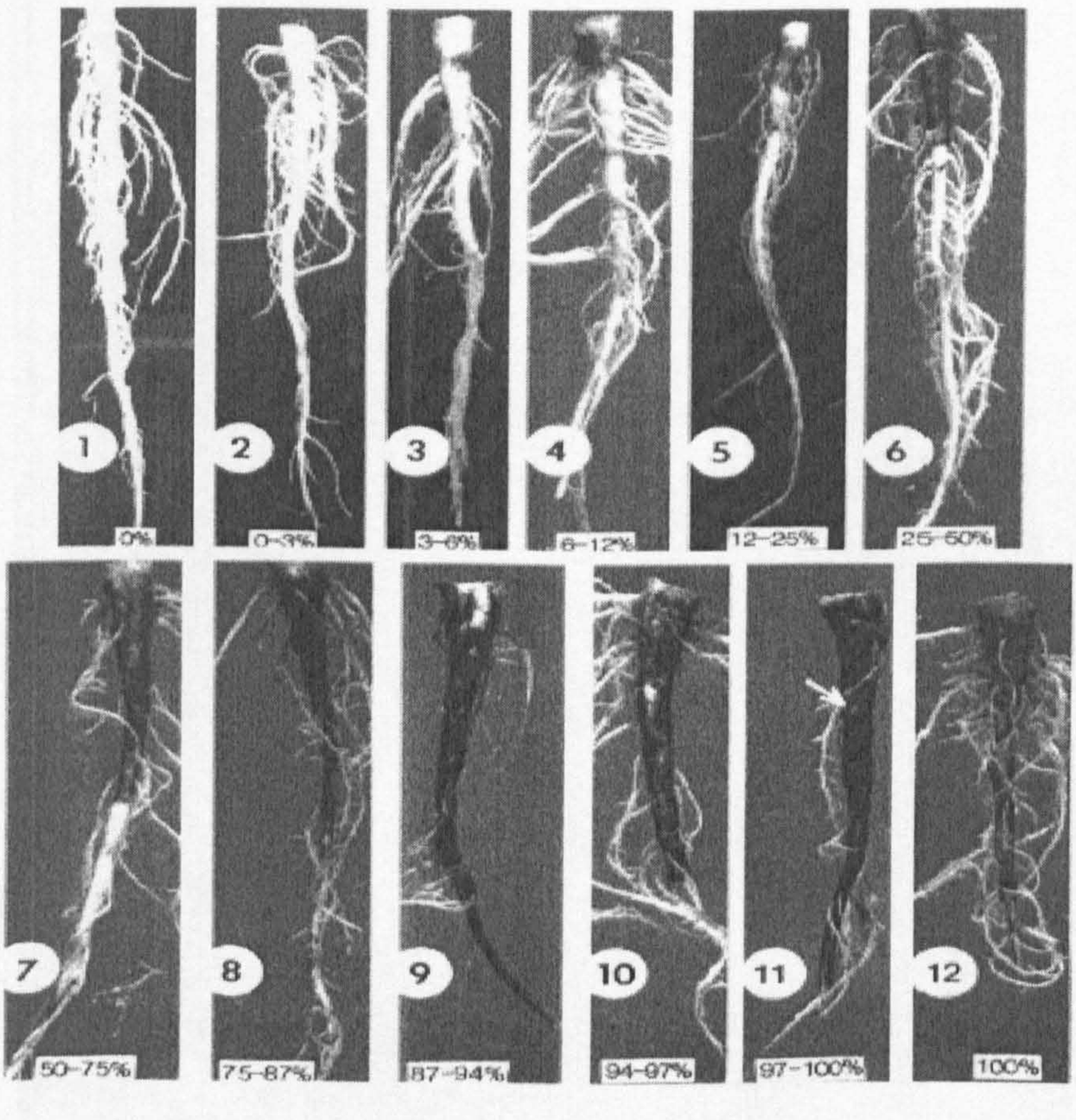


Figure 3.4: Modified disease severity scale (MDSS). Upper numbers refer to scale levels. Lower numbers are percentage ranges for each level. The scale is based on percent surface area of root showing typical brown discoloration (Horsfall and Barratt, 1945).

3.3. RESULTS

3.3.1. Ergosterol content

Statistical analyses were carried out using the SPSS 15.0 statistical package for Windows. Each treatment comprised five replicates (each replicate with one plant). Data were subjected to analysis of variance. Differences were compared using a Tukey test at a significance of $P \leq 0.05$.

Data given in Figure 3.5 show the accumulation of ergosterol increased significantly from week three to six in all varieties, except in chitosan-treated seedlings for all the three varieties in weeks four and five. There was no significant different in ergosterol content among the varieties and treatments at week three. Although intensity of the infection also increased significantly in oil palm seedlings treated with chitosan, the treatment seems to reduce the extent of the colonisation in all varieties. There were large increases in ergosterol content in all varieties from week five to six for the palms not treated with chitosan. By week six, the variety of Calabar, untreated with chitosan, accumulated almost three fold more ergosterol than the chitosan-treated group. The chitosan treated plants remained low in ergosterol content up to week six indicating substantial reduction in disease intensity. Ergosterol was not detected in any control plants throughout the study, indicating all such plants used in the experiment were disease-free (Figure 3.6). The ergosterol content in AVROS treated with chitosan was significantly lower compared to two other varieties in weeks five and six. The increase of the ergosterol content in Calabar treated with chitosan from week five to six was 1.5 fold more than the increase in AVROS. Ekona treated with chitosan was slightly less susceptible to *G. boninense* compared to Calabar with the same treatment with an increase of 8 µg of ergosterol from week five to six, but the accumulation was significantly higher than that detected in AVROS. There was no significant difference among all varieties in the concentration of egosterol accumulated if no chitosan was added.

In summary, AVROS was found to be less susceptible to *G. boninense* compared to Ekona and Calabar if chitosan was added.

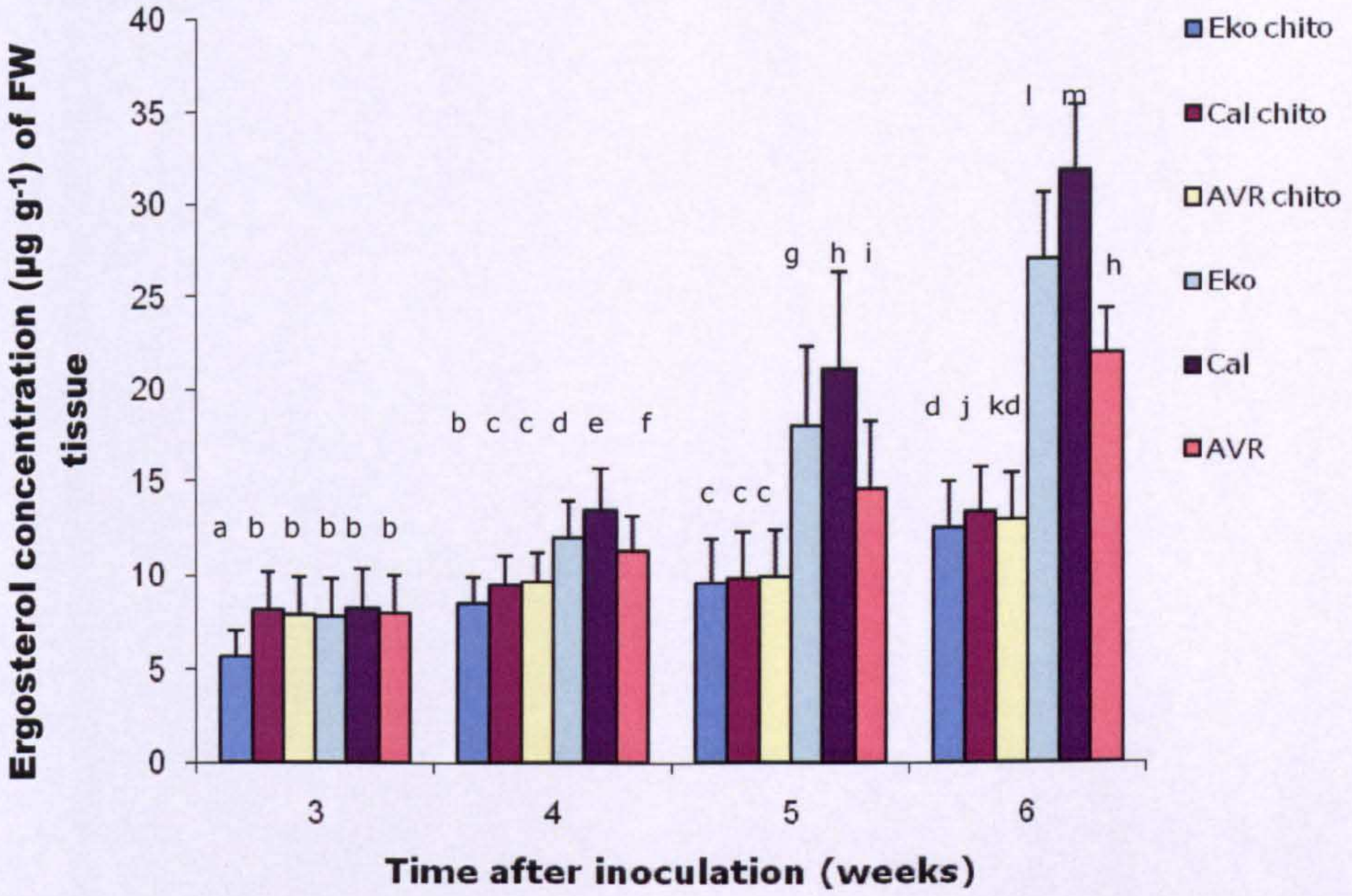


Figure 3.5: Accumulation of ergosterol in different varieties of oil palm root at different weeks infected by *G. boninense* with or without addition of chitosan. Chito denotes chitosan; Eko: Ekona; Cal: Calabar; AVR: AVROS; FW: Fresh weight Bars: standard deviations. Five replicates in each treatment.

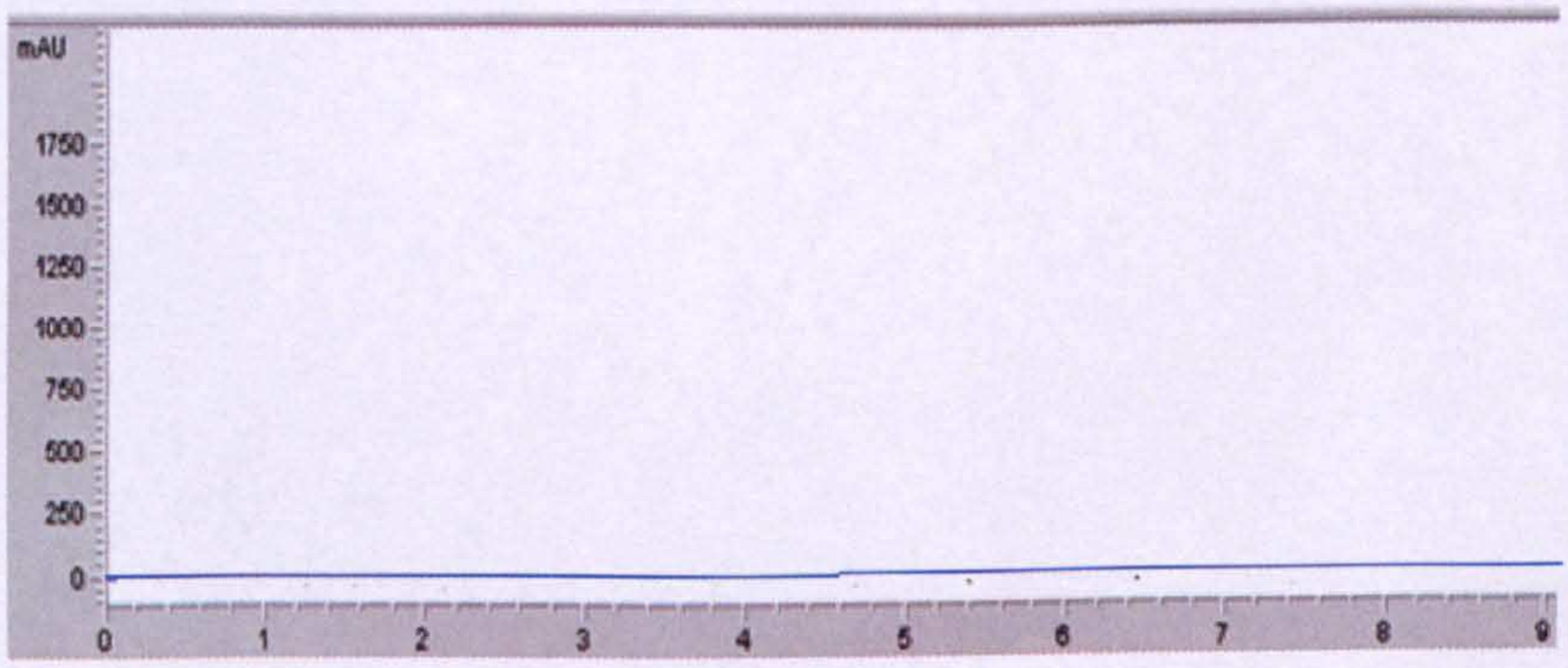


Figure 3.6: Chromatogram showing the absence of ergosterol in healthy roots. The retention time for ergosterol is 5.5-5.8 min.

3.3.2. Disease scoring

The results based on the disease severity score (Figure 3.7 and 3.8) were in accordance with the findings from the ergosterol analysis given in Figure 3.5. All control plants scored less than 2.0 in weeks three to six, indicating the plants were healthy and not infected by *G. boninense* or any other fungus at the beginning of the experiment. From Figure 3.7, the results also suggest oil palm seedlings from all varieties that were treated with chitosan showed a significantly lower disease severity, in comparison to untreated seedlings. Among the varieties, Ekona and Calabar were found to be more susceptible to infection by *G. boninense* compared to AVROS, but some of the differences are not significant. AVROS treated with chitosan scored 8.71 or a disease severity of 75-94% compared to Ekona and Calabar chitosan-treated plants with scores of 9.6 (87-97%) and 10.4 (94-100%) respectively in the last week of observation. Small, but non-significant differences in disease score were observed between varieties in the absence of chitosan amendment. None of the varieties tested showed a high level of *Ganoderma* resistance.

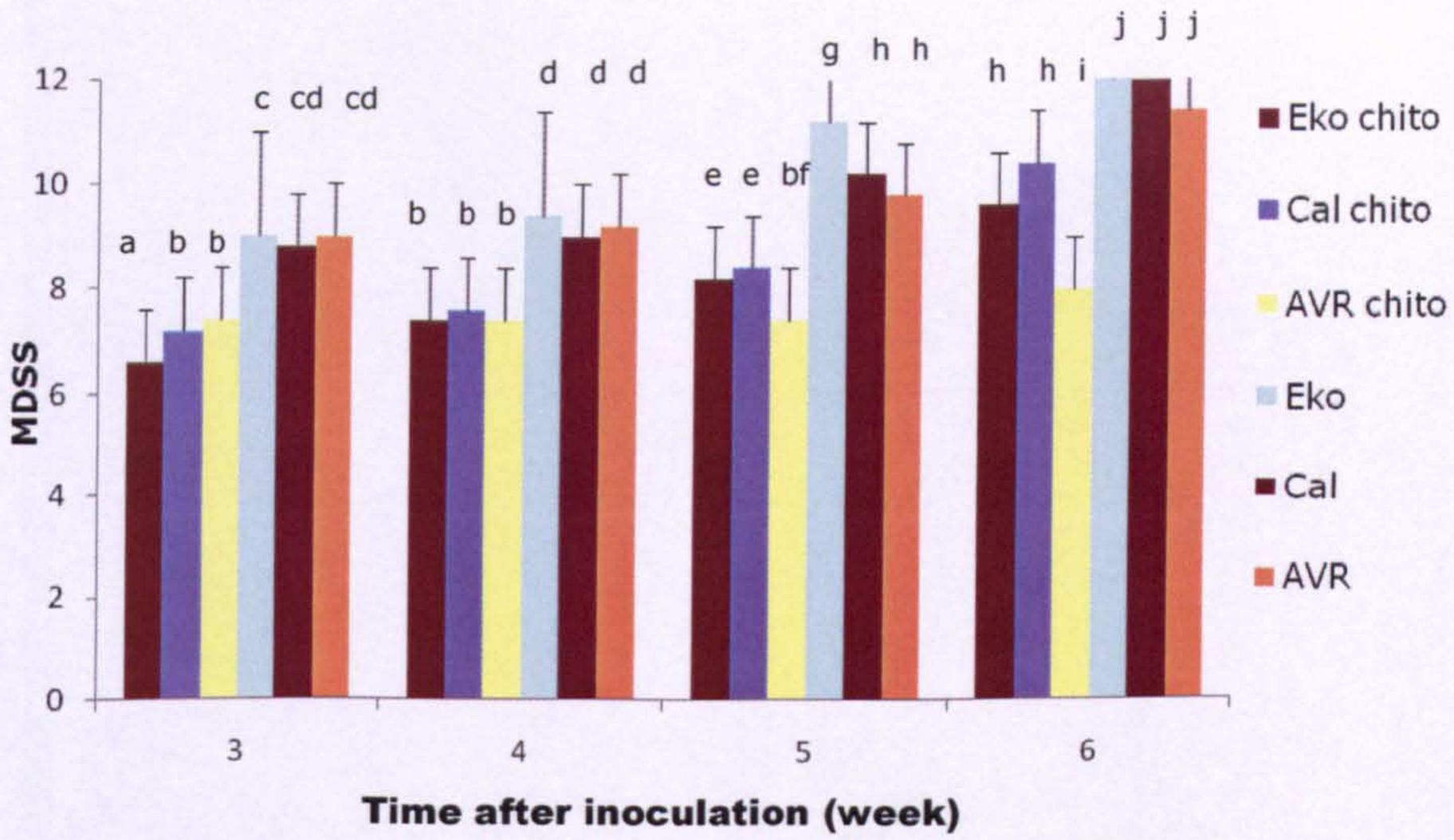


Figure 3.7: Disease severity scoring of oil palm root after infected by *G. boninense* according to the Modified Disease Severity Score (MDSS) from week three to six after inoculation. Chito denotes chitosan; Eko: Ekona; Cal: Calabar; AVR: AVROS; Bars: standard deviations. Five replicates in each treatment.

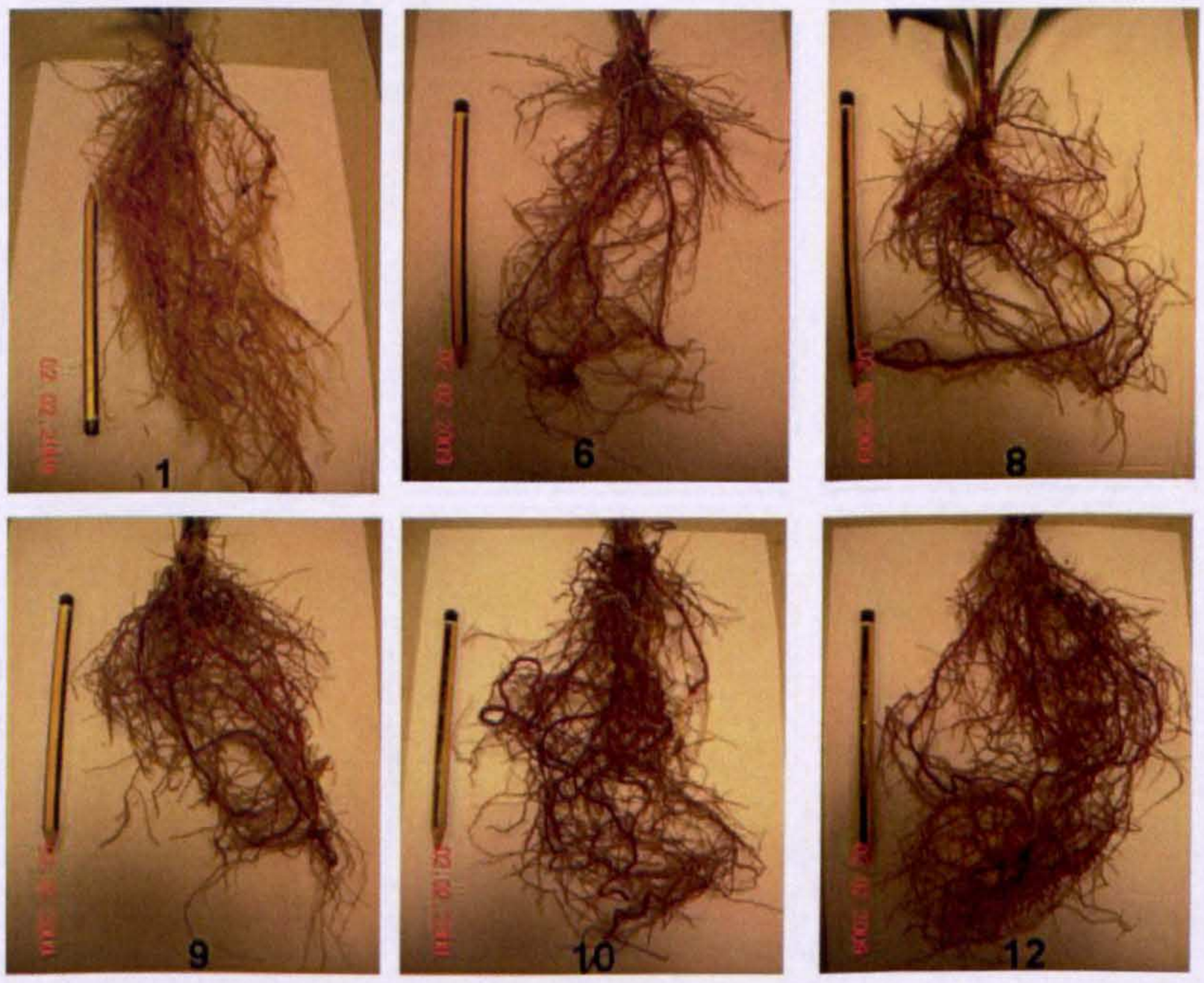


Figure 3.8: Some common scores on oil palm roots based on the Modified Disease Severity Score (MDSS). Note the score in bold.

3.3.3. Correlation between ergosterol analysis and MDSS

Both the ergosterol analysis and MDSS index provided results for infection caused by *G. boninense*, with the artificial inoculation method used. There is a lot of simililarity shown by both parameters for the role of chitosan in reducing the disease severity and the suggestion that AVROS is a less susceptible variety in comparison to Ekona and Calabar. A strong correlation was also shown between the two assessment methods (Figure 3.9) with R^2 =0.7809.

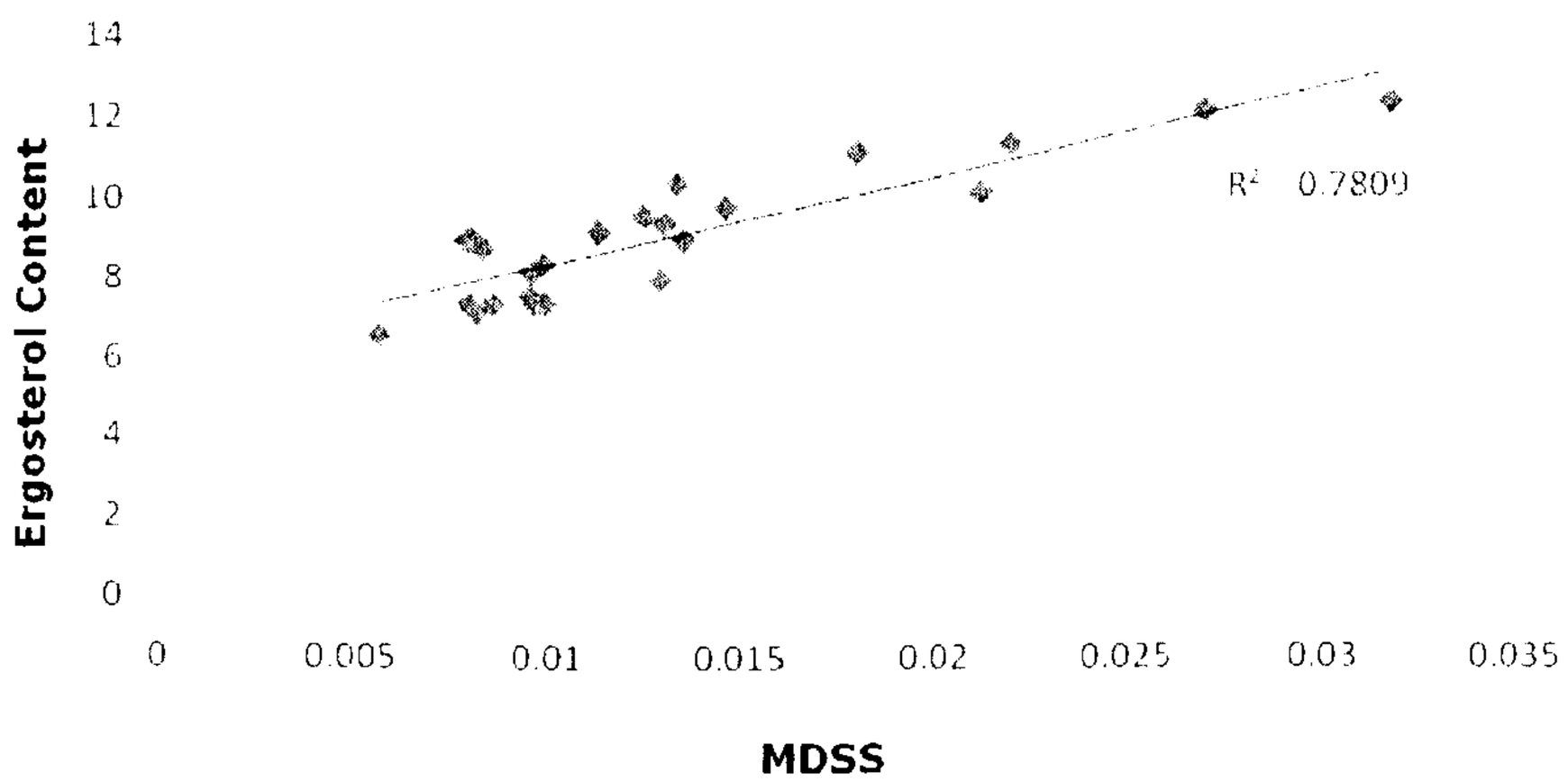


Figure 3.9: Correlation between ergosterol content and Modified Disease Severity Score (MDSS)

3.4. DISCUSSION

The development of oil palm varieties resistant to *G. boninense* may provide the ideal long term solution to the devastating BSR. Unfortunately, to date there is no proven variety resistant to *G. boninense*. The results from the ergosterol analysis and MDSS suggest none of the tested varieties were highly resistant to *Ganoderma*. Both the ergosterol analysis and MDSS clearly showed that all varieties were infected by this pathogen at week six, without the presence of chitosan. However, in the presence of chitosan, the susceptibility of the varieties was significantly different, with AVROS exhibiting both low ergosterol content and MDSS score, in comparison to Ekona and Calabar. The differences in susceptibility of these varieties were small. Chitosan, however, possibly plays an important role in preventing the disease development. Chitosan may activate early defense mechanism in AVROS. But whether chitosan acts as an elicitor of resistance mechanism in oil palm roots or is directly fungitoxic to *G. boninense* needs further investigation.

In general, induced defence reactions in plants are highly correlated with enzymatic responses. Several studies have demonstrated that chitosan is an exogenous elicitor of host defence responses, including accumulation of chitinases, β-1,3-glucanases and phenolic compounds, induction of lignification, synthesis of phytoalexins by the infected host tissue and inhibition of host tissue maceration enzymes (Arlorio *et al.*, 1992; Bhaskara Reddy, Asselin and Arul, 1997; Bhaskara Reddy *et al.*, 1999, 1999; Fajardo *et al.*, 1995; Tejchgraber, Popper and Knorr, 1991; Zhang and Quantick, 1998). It has also been reported that chitosan alone increased the amounts of genistein and 20-hydroxygenistein monoprenyls and isoflavonoids in the exudates of roots of white lupin. However, in that study no microorganisms were involved (Gagnon and Ibrahim, 1997).

The most common techniques currently employed to detect the presence of *G. boninense* use ELISA or PCR. The ELISA technique provides a cheaper detection for large number of samples but was reported to be non-specific, having cross reaction with other microorganisms (Darmono, Suharyanto and Darussamin, 1993; Darmono and Suharyanto, 1995; Darmono, 2000; Idris and Rafidah, 2008; Shamala and Idris, 2009; Utomo and Niepold, 2000). ELISA is mainly used for detection purposes and it is not easy to employ as a tool for monitoring quantitative progress of disease development recorded in this project. PCR is a more specific method for detecting *G. boninense* but it is rather expensive and needs some well-trained personel and thus may not provide a fast, reliable method for the oil palm planters. Other less common techniques, which include studies on the biochemical responses during infection (Tay *et al.*, 2009) and utilisation of sound sensors in early detection (Idris, Mazliham and Madihah, 2009) are under investigation. The use of Geographic Information System (GIS) may help in monitoring the disease intensity in a large plantation scale (Mior *et al.*, 2009).

The indication of the presence of *G. boninense* using the ergosterol assay may not be as specific as molecular markers but it is a fast and reliable method of quantifying the possible presence of the fungus. It worked as a convenience tool for disease monitoring in this project. The membrane lipid ergosterol is found almost exclusively in fungi, and is frequently used by microbiologists as an indicator of living fungal biomass, based on the assumption that ergosterol is labile, and therefore rapidly degraded after the death of fungal hyphae (Mille-Lindblom, von Wachenfeldt and Tranvik, 2004). The main advantage with ergosterol compared to other biomarkers, such as chitin and ATP, is its specific association with fungi. Low amounts of ergosterol can be found in algae and protozoa (Peeler *et. al.* 1989; Raederstorff and Rohmer, 1987), but

generally it is safe to use it as a specific biomarker for fungi (Newell, 1992). In this project, the ergosterol assay provided a more conclusive indication of *G. boninense* infection in roots, compared to MDSS. The disease index is mainly based on visualization and may be very subjective between different observers. However, the strong correlation between the ergosterol results and MDSS (Figure 3.9) suggested that this scale could be utilised in future *G.boninense*-BSR research. The index, which is mainly based on visual changes on the roots, provides a faster reference for the oil palm planters in comparison to some other complex indices that depend on slow development of foliar symptoms (Abdullah, Illias and Nelson, 2003; Sariah and Zakaria, 2000; Teh, 1996; Teh and Sariah, 1999).

CHAPTER 4: OIL PALM ROOT PHENOLIC COMPOUNDS

4.1. INTRODUCTION

4.1.1. Biosynthesis, classification and nomenclature of phenolics

Chemically, phenolics can be defined as substances possessing an aromatic ring bearing one or more hydroxyl groups, including their functional derivatives (Shahidi and Naczk, 2004). Plants contain a large variety of phenolic derivatives including simple phenols, phenlypropanoids, benzoic acid derivatives, flavanoids, stibenes, tannin, lignans and lignins. Together with long chain carboxylic acids, phenolics are also components of suberin and cutin. These rather varied susbstances are essential for the growth and reproduction of plants and also act as antifeedants and antipathogenic compounds (Butler, 1992). The contribution of phenolics to plant pigmentation is also well recognised. In addition, phenolics function as antibiotics, natural pesticides, signal substances for establishment of symbiosis with rhizobia, attractants for pollinators, protective agents against ultraviolet (UV) light, insulating materials to make cell walls impermeable to gas and water and as structural materials to give plants physical stability. Many properties of plant products are associated with the presence, type and content of their phenolic compounds (Shahidi and Naczk, 2004).

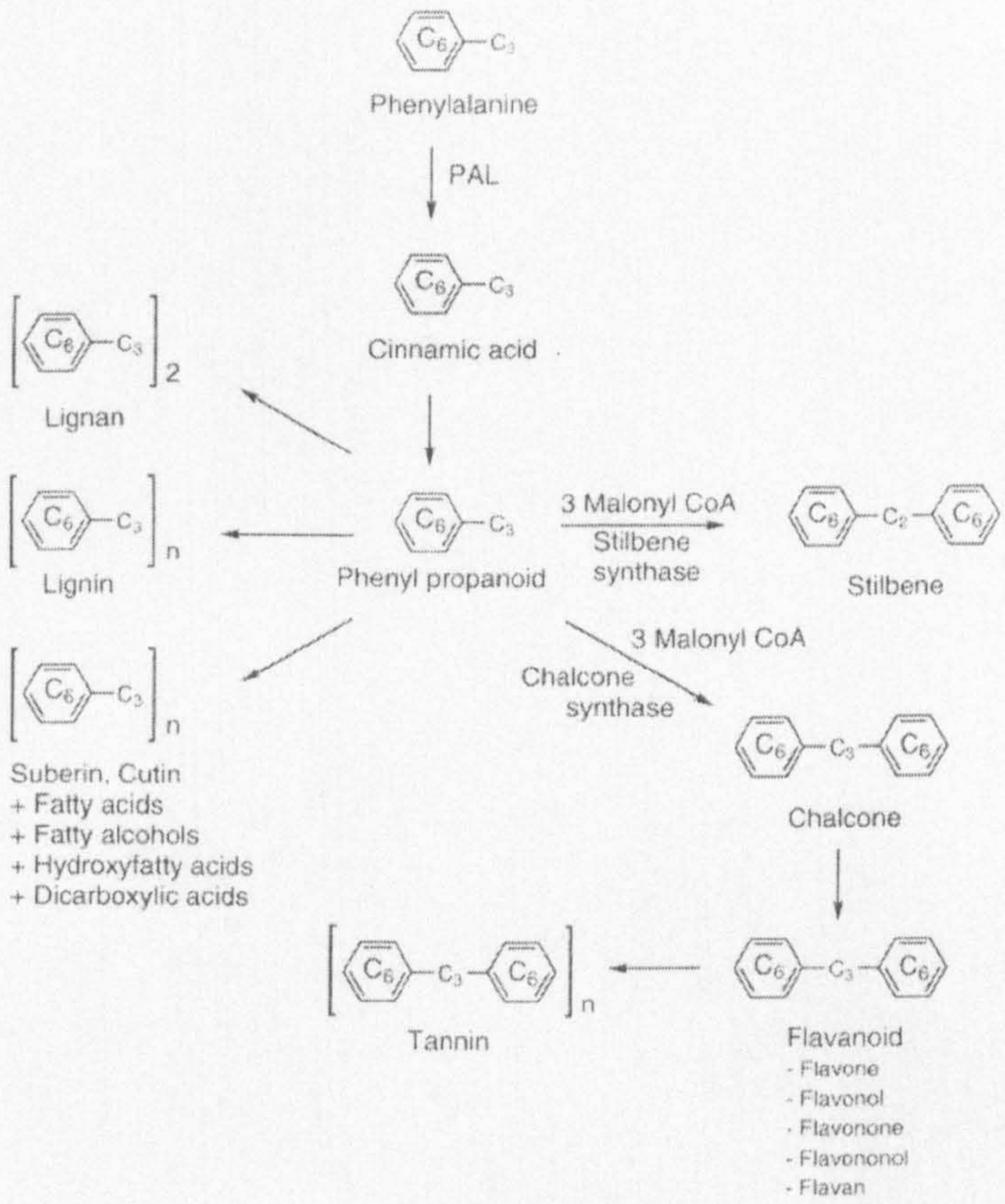


Figure 4.1: Production of phenylpropanoids, stilbenes, lignans, lignins, suberin, cutins, flavonoids and tannins from phenylalanine. PAL denotes phenylalanine ammonia lyase (Shahidi and Naczk, 2004)

Phenylalanine ammonia lyase (PAL) catalyzes the release of ammonia from phenylalanine and leads to the formation of a carbon-carbon double bond, yielding *trans*-cinnamic acid. In some dicotyledonous plants and grasses tyrosine is converted into 4-hydroxybenzoic cinnamic acid via the action of tyrosine ammonia lyase (TAL). Introduction of a hydroxyl group into the *para* position of the phenyl ring of cinnamic acid proceeds via catalysis by monooxygenase utilising cytochrome P_{450} as the oxygen binding site. The *p*-coumaric acid formed may be hydroxylated

further in positions 3 and 5 by hydroxylases and possibily methylated via O-methyl transferase with S-adenosylmethionine as methyl donor; this leads to the formation of caffeic acid, ferulic acid and sinapic acids (Figure 4.2 and 4.3).

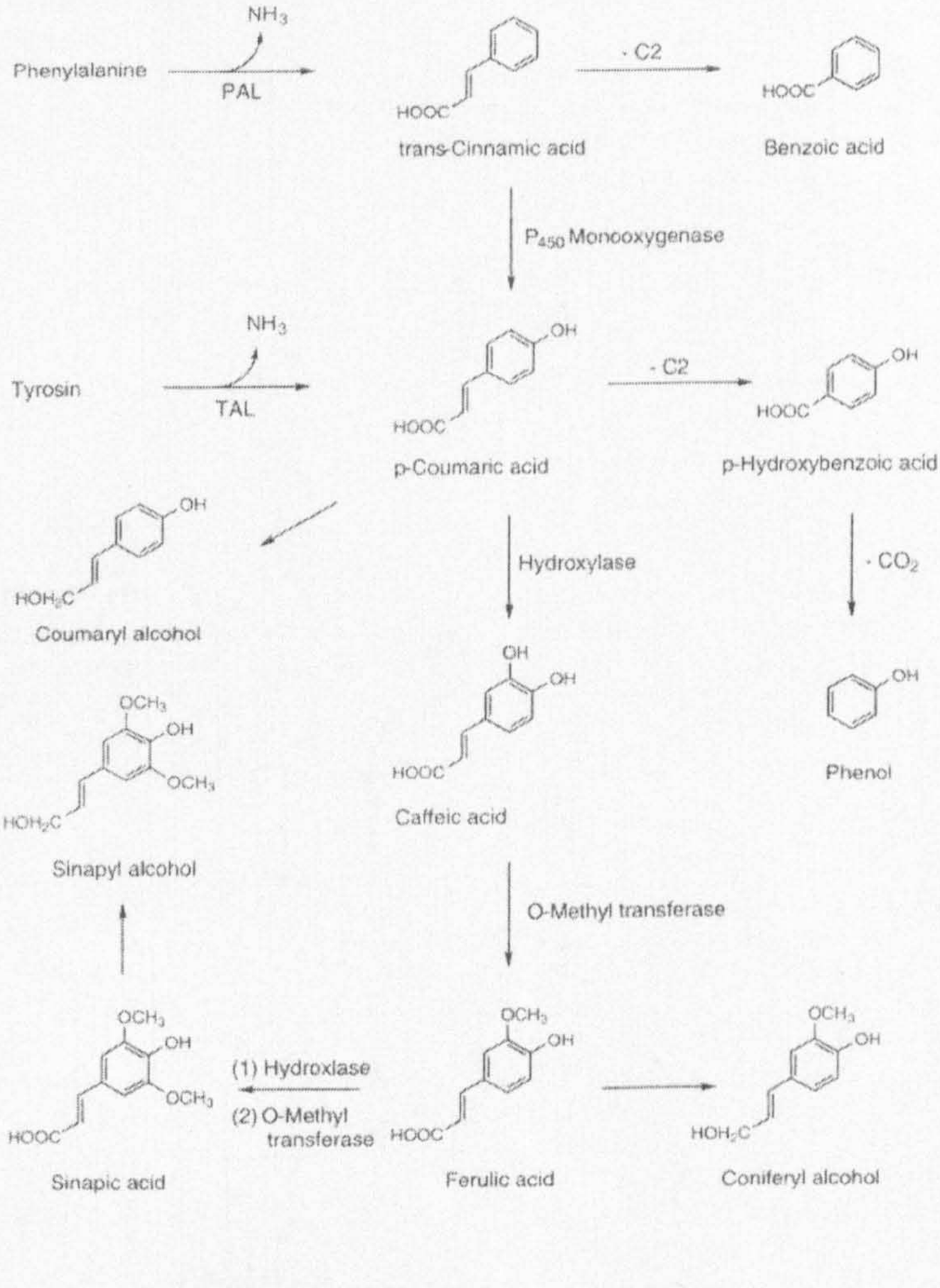
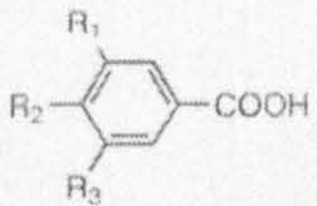


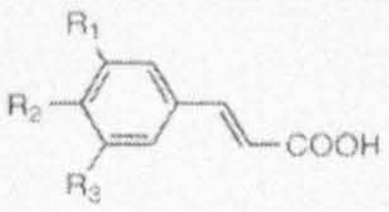
Figure 4.2: Formation of phenylpropanoids of cinnamic acid family as well as benzoic acid derivatives and corresponding alcohols from phenylalanine and tyrosine. PAL denotes phenylalanine ammonia lyase and TAL denotes tyrosine ammonia lyase (Shahidi and Naczk, 2004).

These compounds possess a phenyl ring (C6) and a C3 side chain and are thus collectively termed phenylpropanoids, which serve as precursors for the syntheis of lignins and many other compounds (Shahidi and Naczk, 2004). Benzoic acid derivatives are produced via the loss of a two-carbon moiety from phenylpropanoids.

Salicylic acid is a benzoic acid derivative that acts as a signal substance (Raskin, 1992). After infection or UV irradiation, many plants increase their salicylic acid content, which may include the biosynthesis of defence substances. Similar to phenylpropanoid series, hydroxylation and possibly methylation of hydroxybenzoic acid (HBA) leads to the formation of diHBA (protocatechuic acid), vanillic acid, syringic acid and gallic acid (Figure 4.3).



Acid		R_1	R_2	R_3
p-Hydroxybenzoic	4-Hydroxybenzoic	H	OH	H
Protocatechuic	3,4-Dihydroxybenzoic	OH	OH	H
Vanillic	4-Hydroxy-3-methoxybenzoic	OCH_3	OH	H
Syringic	3, 5-Dimethoxybenzoic	OCH_3	OH	OCH_3
Gallic	3,4,5-Trihydroxybenzoic	OH	OH	OH



Acid		R_1	R_2	R_3
p-Coumaric	4-Hydroxycinnamic	H	OH	H
Caffeic	3,4-Dihydroxycinnamic	OH	OH	H
Ferulic	4-Hydroxy-3-methoxycinnamic	OCH_3	OH	H
Sinapic	4-Hydroxy-3,5-dimethoxycinnamic	OCH_3	OH	OCH_3

Figure 4.3: Common phenolic acids of cinnamic and benzoic acid families (Shahidi and Naczk, 2004).

4.1.2. Phenolic compounds in palm

Despite knowing the importance of phenolic compounds in plant biology and the availability of sophisticated technologies, research on oil palm phenolics is not fully developed. Throughout the years, research has been focused on the palm oil components, as it is an important source of edible oil worldwide. Because most published studies focus on tocopherols and tocotrienols (tocols) in palm oil, available information on phenolics in the whole plants is still fragmentary (Wanasundara, Shahidi and Shukla, 1997). The information on phenolics from oil palm, especially roots, and their role in disease resistance is scarce and therefore needs further investigation.

Plants within the same family often share some common characteristics of metabolites. Oil palm belongs to the same family as date palm, for which the role of phenolics has been widely investigated against *Fusarium* diseases. *De novo* accumulation of phenolic compounds in date palm callus elicitated with filtrates of *Fusarium* cultures were reported greater in the resistant than the susceptible cultivars, which indicates a possible role of phenolic compounds in the resistance to Bayoud disease (Daayf *et al.*, 2003). Therefore, the role of phenolic compounds in oil palm against *G. boninense* was explored in this work.

4.1.3. Methods of analysis and quantification of phenolic compounds

The Folin-Ciocalteu assay is often used to determine the total content of phenolics. The total phenolics are assayed colorimetrically and the content of phenolics is expressed as gallic acid equivalents. HPLC techniques are now widely used for separation and quantification of

phenolic compounds. Various supports and mobile phases are available for such analyses (Senter, Robertson and Meredith, 1989). The introduction of reverse phase columns considerably enhanced the HPLC separation of different classes of phenolic compounds (Hostettmann and Hostettmann, 1982) and several reviews have been published on the application of HPLC methodology for the analysis of phenolics (Daigle and Conkerton 1983, 1988; Karchesy *et al.,* 1989; Merken and Beecher 2000; Robards and Antolovitch, 1997). Phenolics are commonly detected using UV-Vis and photodiode array (DA) detectors (Carando *et al.,* 1999; Edenharder *et al.,* 2001; Siess *et al.,* 1996; Tomas-Barberan *et al.,* 2001; Wang, Kim and Lee, 2000; Wang *et al.,* 2000; Zafrilla, Ferreres and Tomas-Barberan, 2001). Other methods used for detection of phenolics include electrochemical colorimetric array detector (EC) (Mattila, Astola and Kumpulainen, 2000; Sano *et al.,* 1999), chemical reaction detection technique (de Pascual-Teresa *et al.,* 1998), and fluorimetric detector (Arts and Hollman, 1998; Carando *et al.,* 1999). Mass spectrometry (MS) detectors coupled to high performance liquid chromatography (HPLC-MS tandem) have been employed for structural characterisation of phenolics. Electrospray ionisation mass spectrometry (ESIMS) has also been used for structural confirmation of phenolics (Shahidi and Naczk, 2004).

4.2. MATERIALS AND METHODS

4.2.1. Estimation of total phenolics content with Folin Reagent

Roots from oil palm seedlings were homogenized using the ika A11 basic grinder from Werke, before 1 g aliquots were collected for analysis. Methanol (10 mL 80%) was added to the root homogenates and vortexed for 5 min. The homogenates were then centrifuged at 3000 x g for 5 min and the supernatants were collected. Determination of total phenolic compounds was achieved by means of the Folin method. Folin Ciocalteu (FC) reagent (Frankfurter std, Darmstadt, Germany) as described by Singleton *et al.* (1999) was used. A 100 µL sample of the supernatant was mixed with 2 mL of milli-Q ultra pure water, 200 µL of FC reagent and 1 mL of 15% of Na_2CO_3 and further incubated for 2 h at 25°C. After incubation, the absorbance was read at 765 nm using a Genesys 20 Thermo Spectronic spectrophotometer. The absorbance of a blank (supernatant was replaced by 80% MeOH) was set as zero. Gallic acid (Sigma) was used to prepare a standard curve (Figure 4.4) for estimation of the amount of soluble phenolics in each sample. The concentration of phenolic compounds in the roots was subsequently expressed as gallic acid equivalent in mg g^{-1} fresh weight of sample.

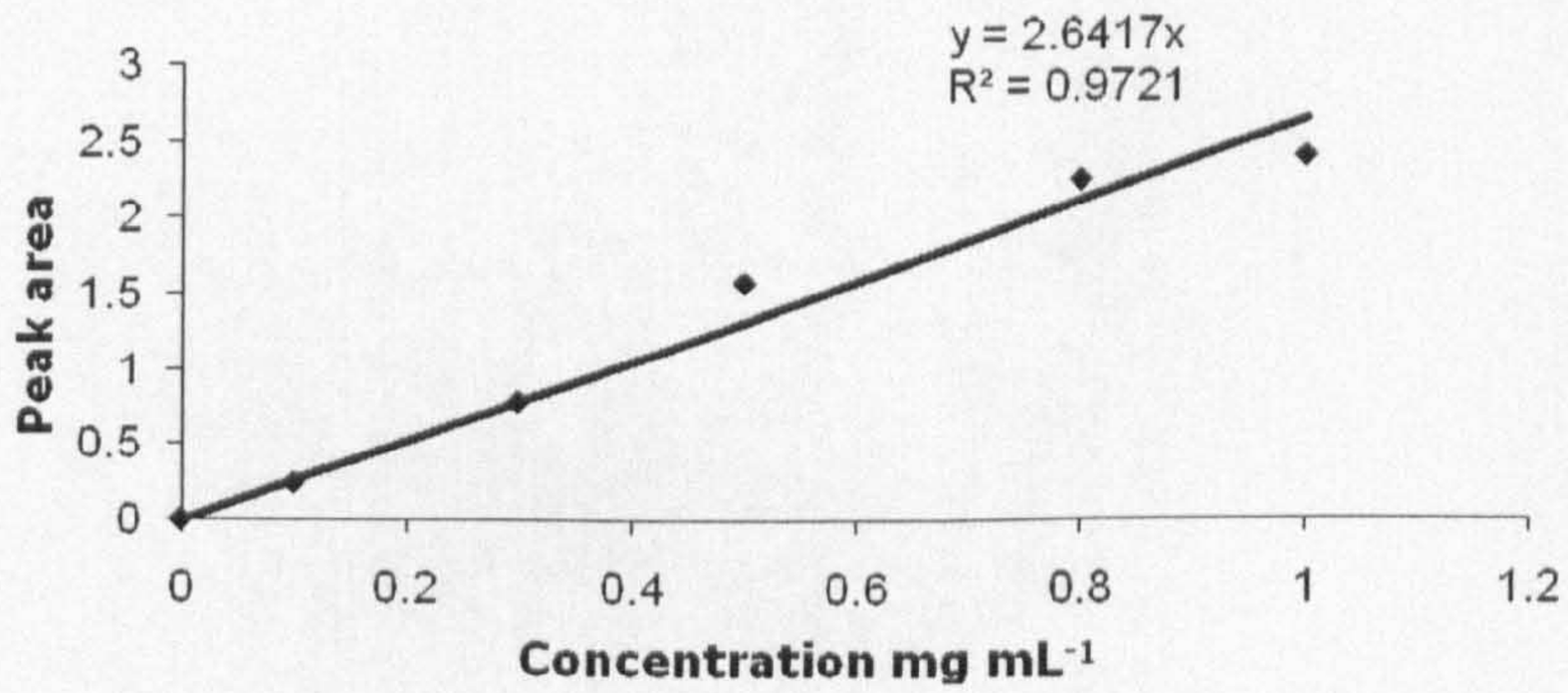


Figure 4.4: Integration of absorbance at 765 nm plotted against the concentration of gallic acid

4.2.2. Extraction of phenolic from roots for SPE and HPLC analysis

Roots (100 g) were homogenized using the same iKa A11 basic grinder and soaked in 500 mL of methanol for 2 d before filtering through Whatman No. 1 filter paper, dried with a Buchi rotary evaporator and resuspended in milli-Q ultra pure water to give a final volume of 1 mL per 5 g of tissue. Samples were centrifuged at 15,000 x g for 15 min before solid phase extraction (SPE).

4.2.3. Solid phase extraction (SPE)

Strata X 33 µM Polymeric Reversed Phase (200 mg 6 mL^{-1}) by Phenomenex cartridges were chosen for this project. Methanol (4 mL) was used for conditioning and activating the sorbent bed of the cartridge. Cartridges were later equilibrated with 1 mL of 0.1 M HCl to prepare the sorbent for optimized interaction with the analyte. Samples were then loaded in the cartridges. The cartridges were washed with 4 mL of 0.1 M of HCL to remove any impurities from samples and finally the cartridges were eluted with 4 mL of 0.1M HCL: MeOH (1:4; v/v). The aliquots were taken to dryness by rotary evaporation at 20°C. The final concentration was adjusted to 5 g of plant tissue per 1 mL of milli-Q ultra pure water.

4.2.4. Measurement of phenolic acids in oil palm roots using the HPLC and UV absorbance detection

A series of gradient systems with different ratios of acetic acid 0.3% and methanol were tested for the best elution for the phenolic acids using pure standards. The Variable Wavelength Detector (VWD) was set at 280 nm and an elution flow rate of 1 mL min^{-1} was used. Resolution was obtained with the following mobile phase and this was used for the entire project:

Acetic acid ($C_2H_4O_2$) 0.3% (A) and MeOH (B); A/B= 80:20 (0 min)→30:70 (23-24 min)

Co-injection and elution with standards was done to ensure the identity of the compounds. Reference compounds used were vanillin, transcinnamic, benzoic, 4-hydroxybenzoic, 3, 4 -dihydroxybenzoic, gallic, syringic, *p*-coumaric, caffeic, ferulic, and sinapic acids. Reference compounds were chosen in accordance to soluble phenolics found in date palm as described by Daayf *et al.* (2003), since there is no report on individual oil palm phenolics. All standards, obtained from Sigma, were prepared at a concentration of 0.02 mg mL^{-1} and 20 µL of each of the standards was injected into the HPLC system. With this system, all phenolic acids from the standards were separated into single peaks (Figure 4.5). Phenolics were identified on the basis of retention times and their characteristic spectra in comparison with standards.

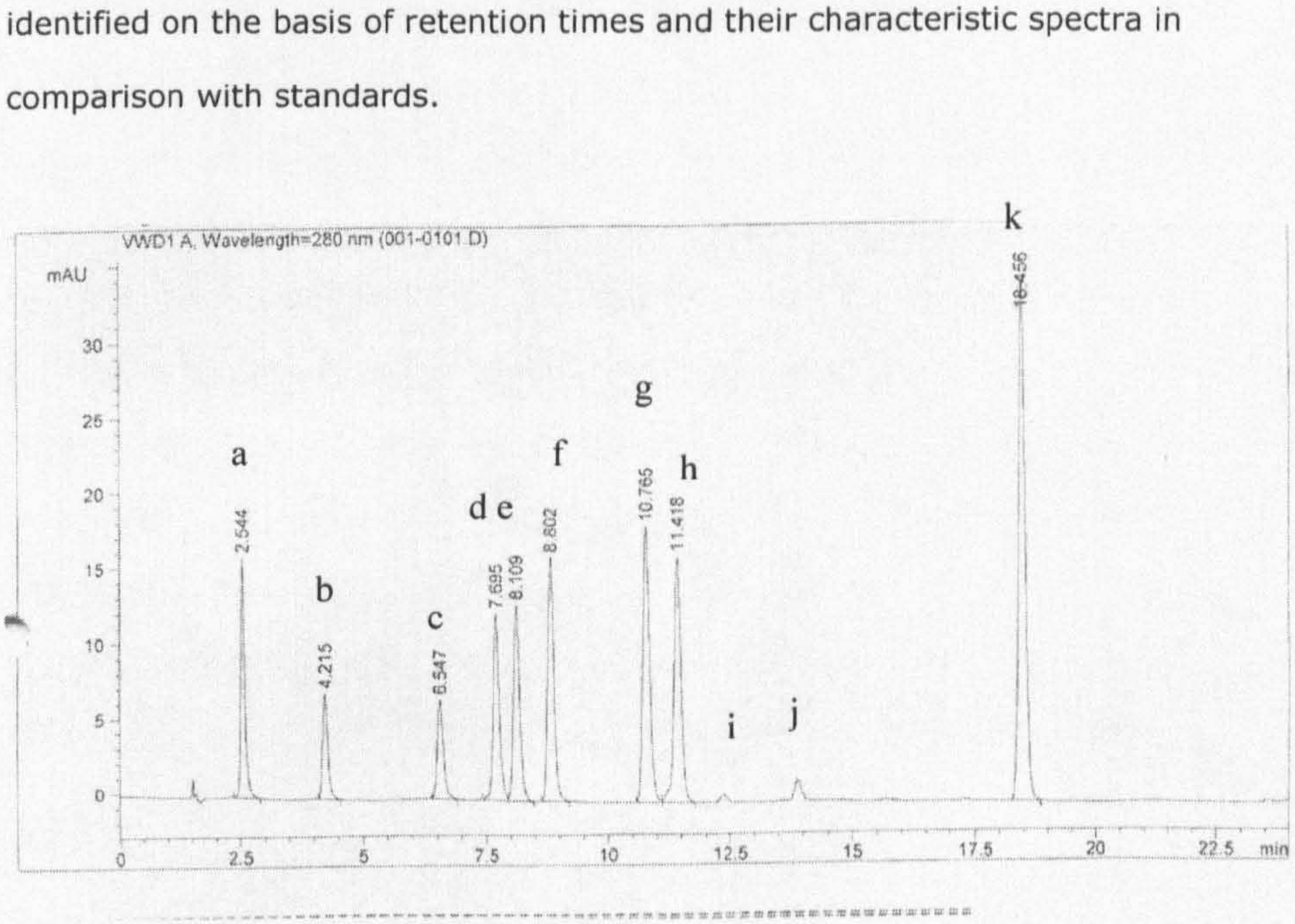


Figure 4.5: Chromatogram showing the detection of pure standards of phenolic at 280 nm after separation by HPLC. a: Gallic acid. b: 3,4- diHBA. c: 4-HBA. d: Caffeic acid. e: Syringic acid. f: Vanillin. g: *p*-coumaric acid. h: Sinapic acid. i: Ferulic acid. j: Benzoic acid. k: Transcinnamic acid

4.2.5. Confirmation of identity of phenolic acids in oil palm root using Liquid Chromatography Mass Spectrophotometry/ Q- tof (LC-MS/Q-tof)

To support further the HPLC identification of the phenolic acids present in oil palm roots, further confirmation was done using LCMS/Q-tof. The LC-MS was performed under the following condition:

LC conditions: column, a Waters Acquity UPLC BEH C18 (2.1 mm x 100 mm, particle size 1.7 µm); injection volume, 2 µL; column temperature, 40°C; PDA, 200-500 nm; flow rate, 0.5 mL min ^{1}; mobile phase, HPLC H_2O (A) and MeOH (B); A/B=100:0 (0 min)→50:50 (2.5-5 min)→ 0:100 (5-5.5 min).

MS/Q-ToF conditions: Accurate mass analysis was conducted with the same Waters LC system as described above coupled with a Waters Synapt Q-ToF system equipped with electrospray ionization positive ionization mode (ESI) probe, lock spray inlet system and mass correction was done using injection of leucine enkaphaline (556.2771 Da). Highly accurate mass acquisition of the ion of interest was performed by chromatography injection. Re-injections (2 µL) of the extracts were undertaken with a C-18 column maintained at a temperature of 15°C, using the same elution condition described before. Mass spectrometric ionization conditions were as follows: desolvation temperature, 350°C; source temperature, 90°C; capillary voltage, 2.3 kV; desolvation gas, 500 L h ^{1}, ToF detector voltage, 1700; sampling cone, 45; extraction cone, 4; trap collision energy, 6; transfer, 4.0. For samples analyzed in scan mode the scan range was 100-1500 Da.

4.2.6. HPLC analysis and quantification of phenolics

The gradient HPLC system as developed in section 4.2.4. was used for analysis and quantification for phenolics throughout the project. Serial dilutions of known concentration of standards (from Sigma) (syringic acid, caffeic acid and 4-HBA) were injected into the HPLC column and run using the same system. Areas under the peaks were integrated with the known concentrations and standard curves were produced for each of the standard Figures 4.6, 4.7 & 4.8).

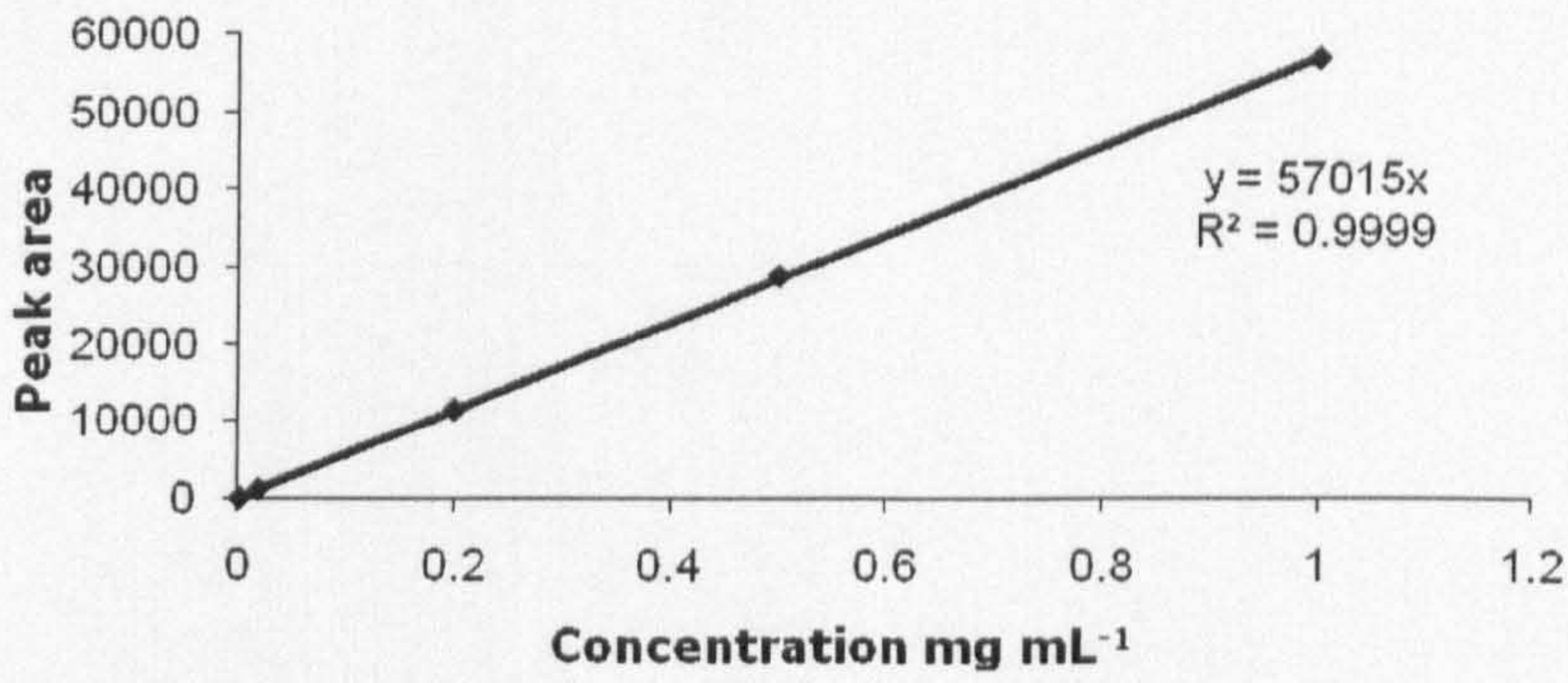


Figure 4.6: Integration output area plotted against the concentration of syringic acid

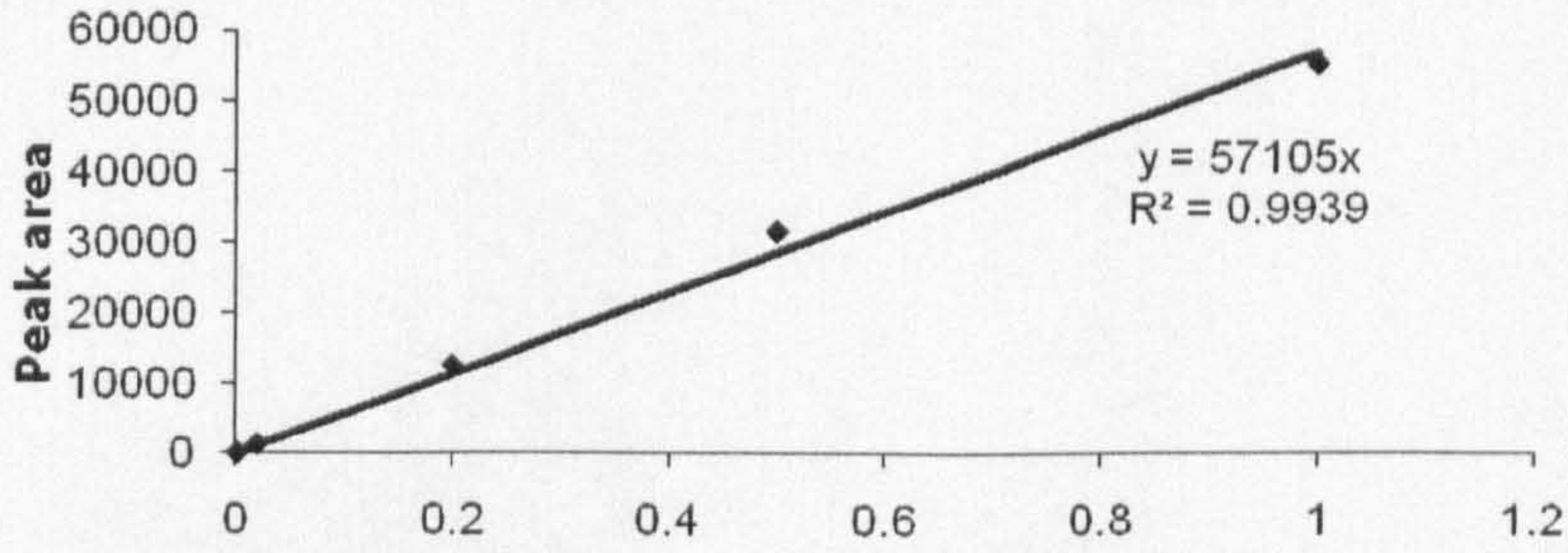


Figure 4.7: Integration output area plotted against the concentration of caffeic acid

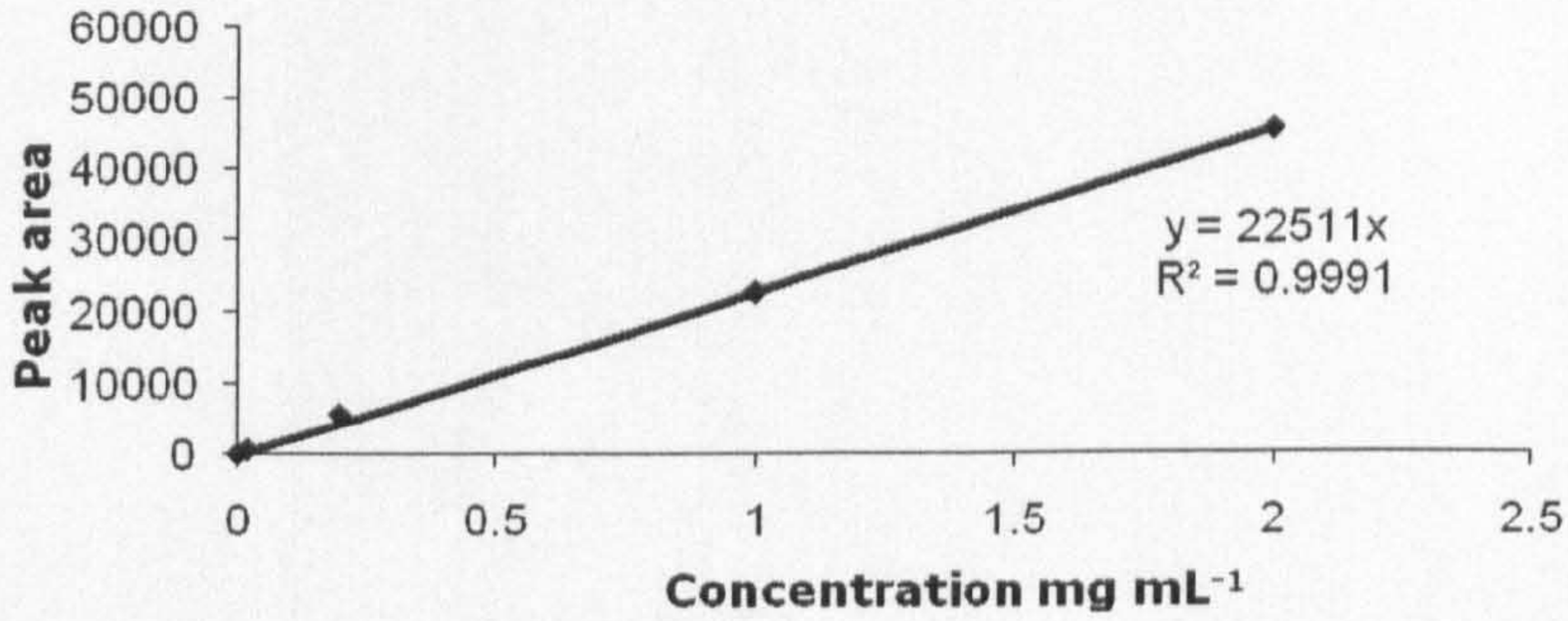


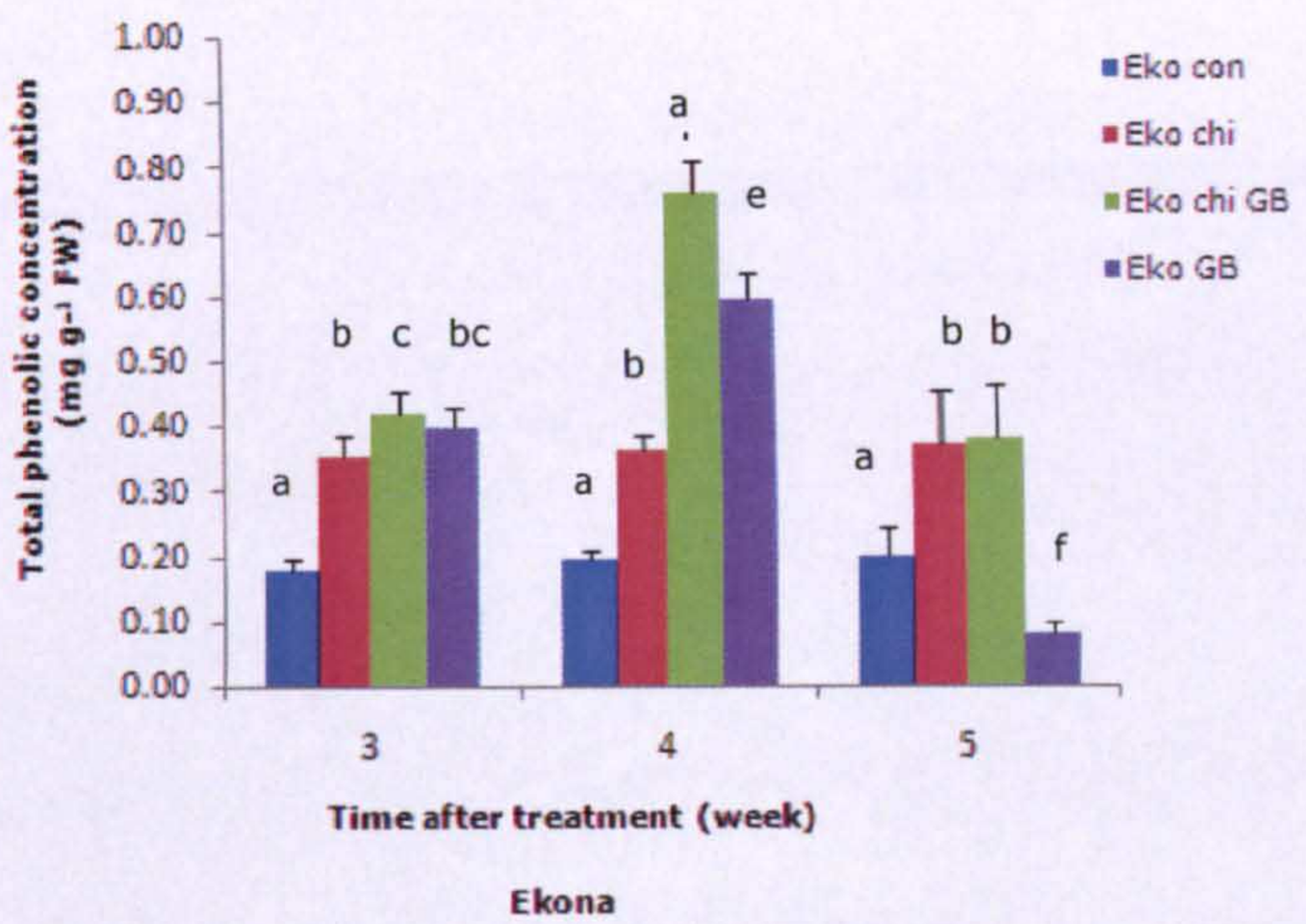
Figure 4.8: Integration output area plotted against the concentration of 4-HBA

4.3. RESULTS

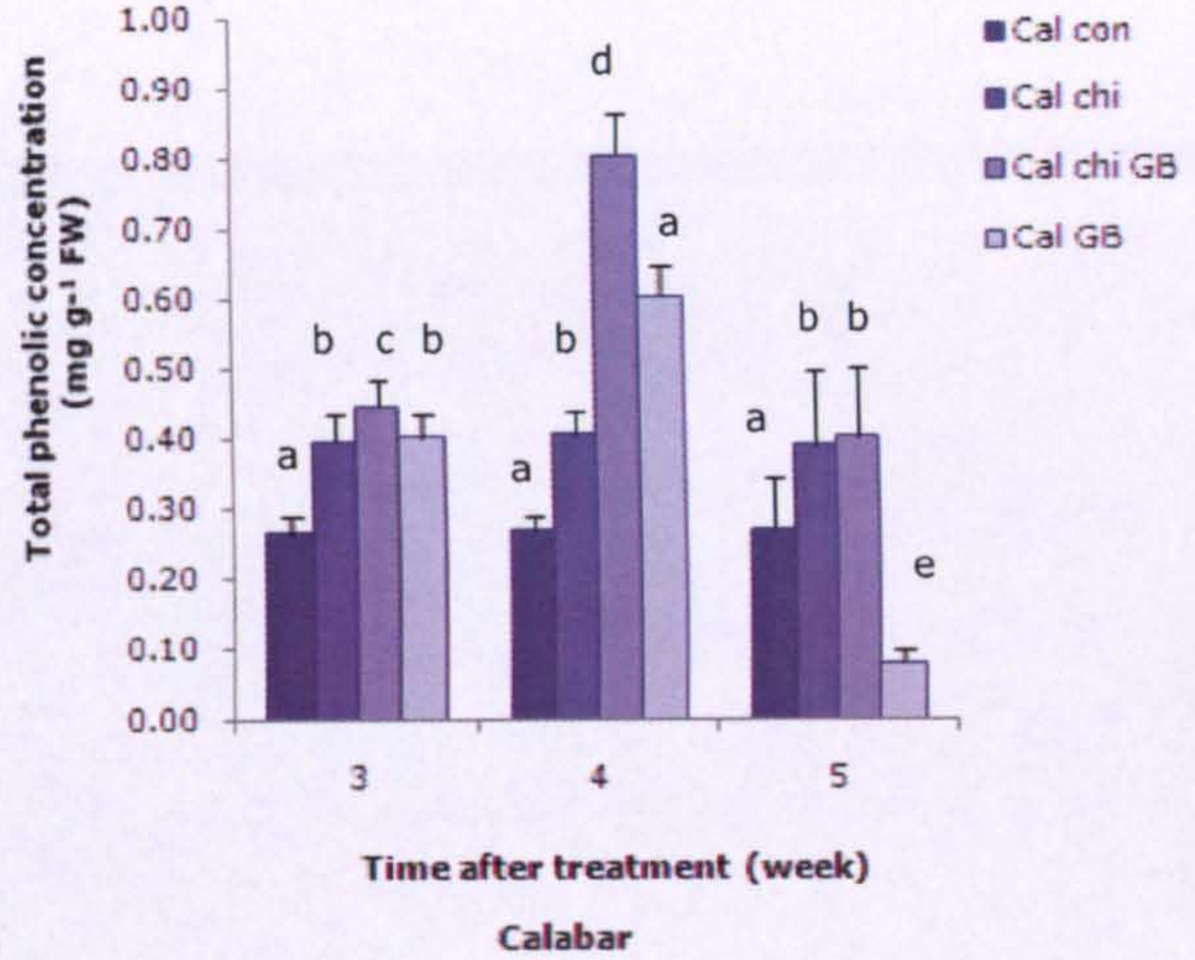
4.3.1. Total phenolic content

From the results presented in Figure 4.9, it can be seen that phenolics could be detected in all three varieties used either in control or non-challenged tissues and they were present constitutively. This background level remained constant for the duration of the experiment. Inoculation with chitosan, *Ganoderma* or both, stimulated a significant increase in total phenolic accumulation. This was most pronounced four weeks after inoculation. By week five the concentration of detected phenolics declined in comparison to those that were recorded at week four. This was most evident in treatments which contained *Ganoderma*. The presence of chitosan reduced this observed decline in phenolic concentration.

Thus, *Ganoderma* may act to elicit accumulation of phenolics early in the infection development, but also metabolises them later in the time course. There is limited evidence that the variety AVROS produces slightly higher accumulation of total phenolics in comparison to Ekona and Calabar.



Ekona



Calabar

AVROS

Figure 4.9: Total Phenolic Content in different varieties of oil palm roots at weeks after different treatments. Eko denotes Ekona; Cal: Calabar; AVR: AVROS; Chi: Chitosan; GB: *G. boninense* and FW: fresh weight. Bars represent standard deviations. Five replicates in each treatment.

In summary, the changes of the total phenolic content among the different treatments and varieties of oil palm seedlings provide a quick overview on whether these compounds may be involved in the defence mechanisms of the palm. However, total phenolic content is not specific and a more detailed study on the role of individual compound needs to be explored.

4.3.2. Monitoring changes in phenolics concentration and confirmation of identity with HPLC

From the HPLC chromatogram shown in Figure 4.10., several unknown compounds were found to be commonly present in both chitosan elicited and healthy control (non-elicited) oil palm roots. The difference observed was mainly in the amount of the compounds being produced, where the elicited roots had slightly higher accumulation in comparison to the control (data not shown).

Since there were too many compounds detected in the extract to monitor changes in all of them, the focus of the research was only on those compounds which were present in higher amounts after the elicitation or challenge with *G. boninense*, in comparison to the untreated control. The chromatogram given in Figure 4.10 indicates that three compounds accumulated more than others. Based on retention time these were tentatively identified as 4-HBA, caffeic acid and syringic acid. Confirmation of their identity was made by undertaking a matching spike HPLC run (Figure 4.11). This confirmed the identity of 4-HBA (Rt: 6.716 min), caffeic acid (Rt: 7.071 min) and syringic acid (Rt: 8.214 min). These compounds were monitored in later work.

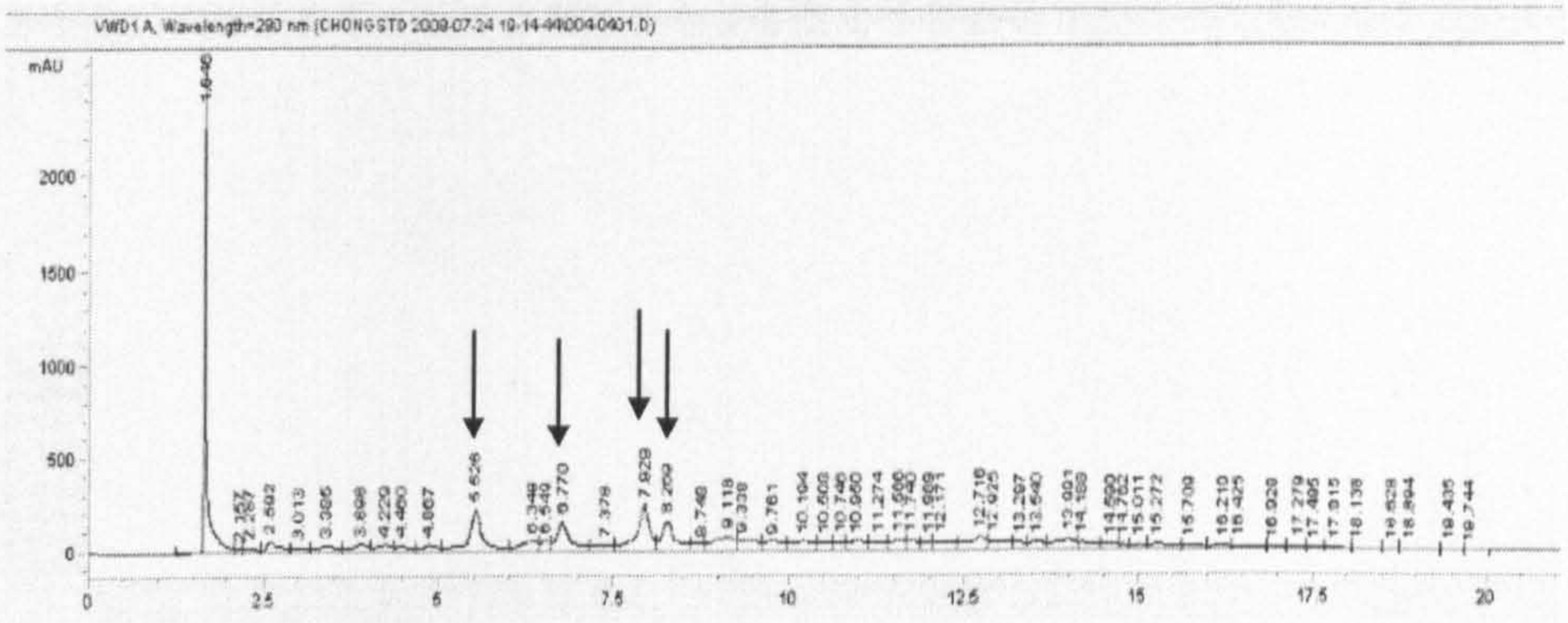


Figure 4.10: Chromatogram showing the peaks commonly present in oil palm root extract both in control and elicited roots detected by variable wavelength detector (VWD) after separation by solid phase extraction (SPE) and High Performance Liquid Chromatography (HPLC). Note; some peaks with higher accumulation are arrowed

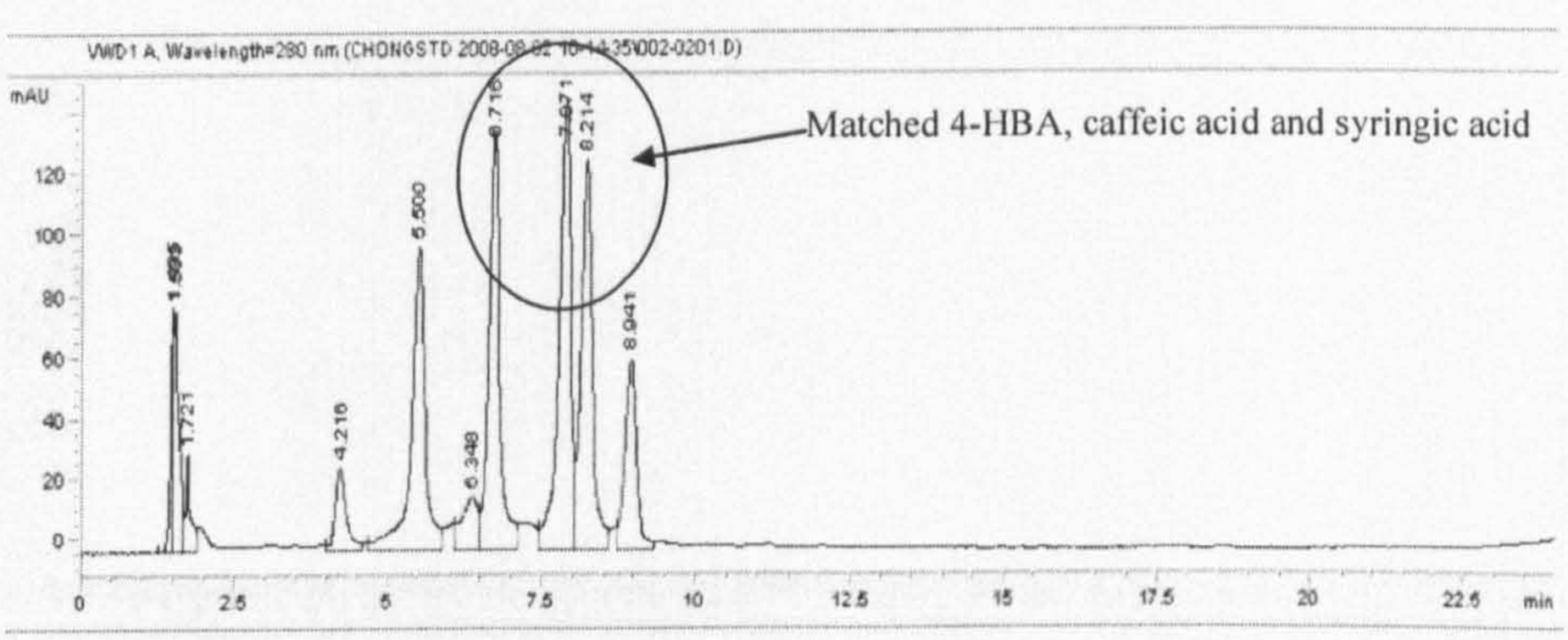


Figure 4.11: Chromatogram showing injection with root sample and standards that match the compound of interest. Arrowed: 4-HBA, caffeic acid and syringic acid

4.3.3. Confirmation of syringic acid and caffeic acid in oil palm root using LCMS/Q-tof

Using the LCMS/Q-tof two phenolic acids of possible interest were confirmed to be present in the oil palm root extracts caffeic acid and syringic acid (Figure 4.12). Surprisingly, 4-HBA was not detected during the scan mode within the range selected. This may be due to a low concentration of 4-HBA in the sample of the elicited oil palm roots during the scan mode.

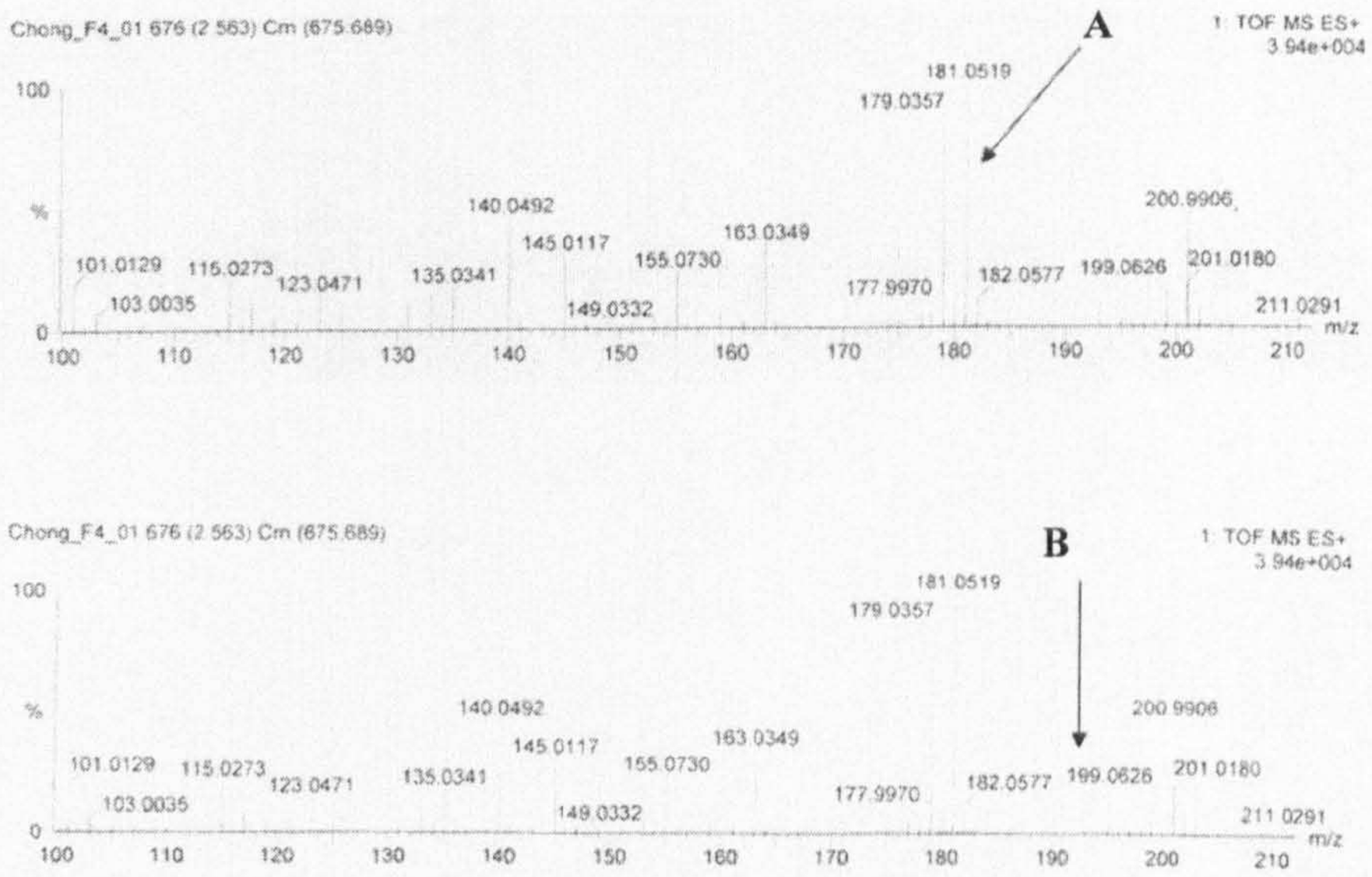


Figure 4.12: Electro spray mass spectrum of caffeic acid (181.05) and syringic acid (199.06). x-axis shows m/z ratio and y-axis shows the percentage. Arrowed: Caffeic acid (**A**) and syringic acid (**B**)

4.3.4. Quantification of syringic acid, caffeic acid and 4-HBA

Statistical analysis revealed several differences in accumulation of syringic acid among the treatments throughout the four weeks of study (Figure 4.13). Examination of this figure demonstrates that syringic acid constitutively in control, untreated seedlings and that the level remained relatively constant for the duration of the experiment. Treatment with chitosan or chitosan with *Ganoderma* increases accumulation, especially with the former. *Ganoderma* alone did not prove to be a potent elicitor of syringic acid; indeed later in the time course there is evidence of degradation by the pathogen. This apparent was greatly reduced in the presence of chitosan. There was little difference in the accumulation of syringic acid between the varieties tested, although the variety AVROS produced a small but significant increase in the level of this compound.

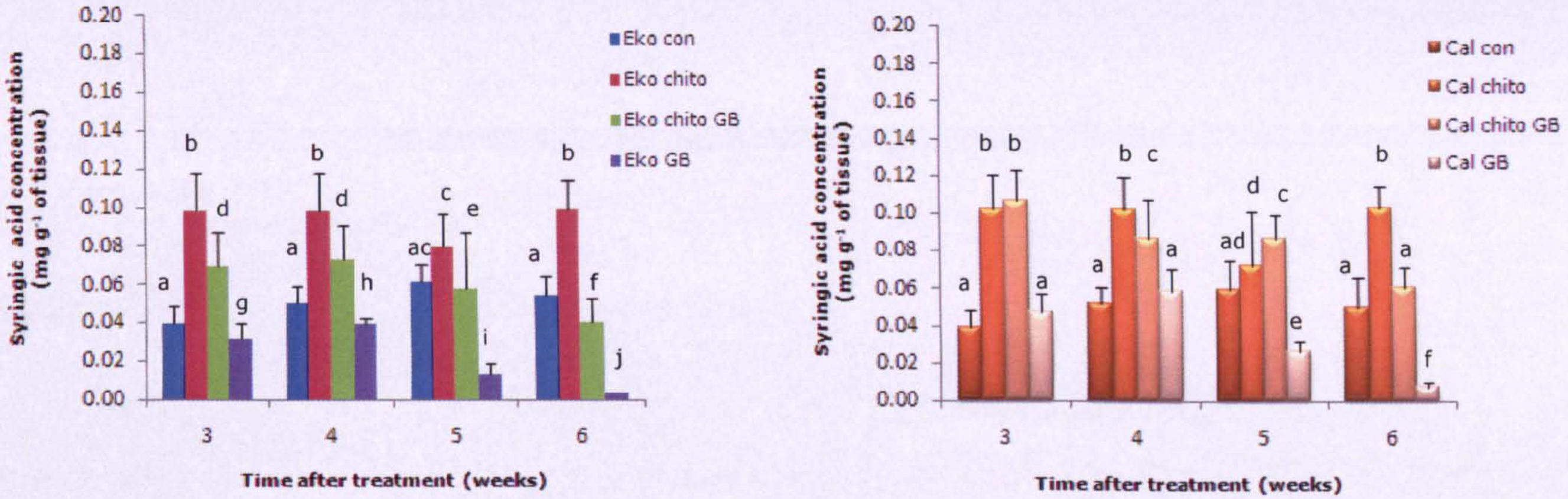


Figure 4.13: Accumulation of syringic acid in varieties of oil palm root at weeks after different treatments. Eko denotes Ekona; Cal: Calabar; AVR: AVROS; Chi: Chitosan and GB: *G. boninense*. Different alphabets indicate significant ($P<0.05$) among treatments in the same variety. Bars represent standard deviations. Five replicates in each treatment

Accumulation of caffeic acid in oil palm roots shows a similar trend to syringic acid (Figure 4.14). Significant differences in accumulation of this phenolic were found among the treatments throughout the four weeks of study. The results confirmed that chitosan stimulated phenolic accumulation but reduction in accumulated levels observed between four and six weeks after inoculation. Once again there was little evidence of varietal differences, although AVROS produced small but significantly higher levels of caffeic acid.

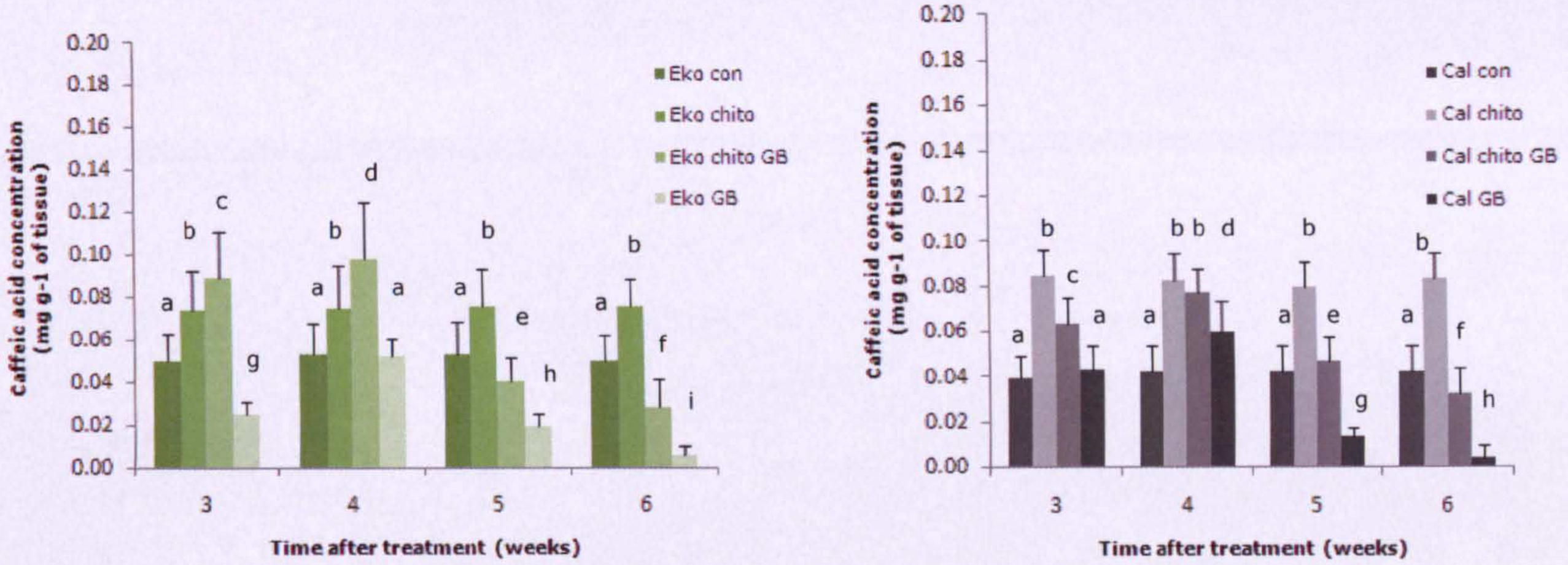
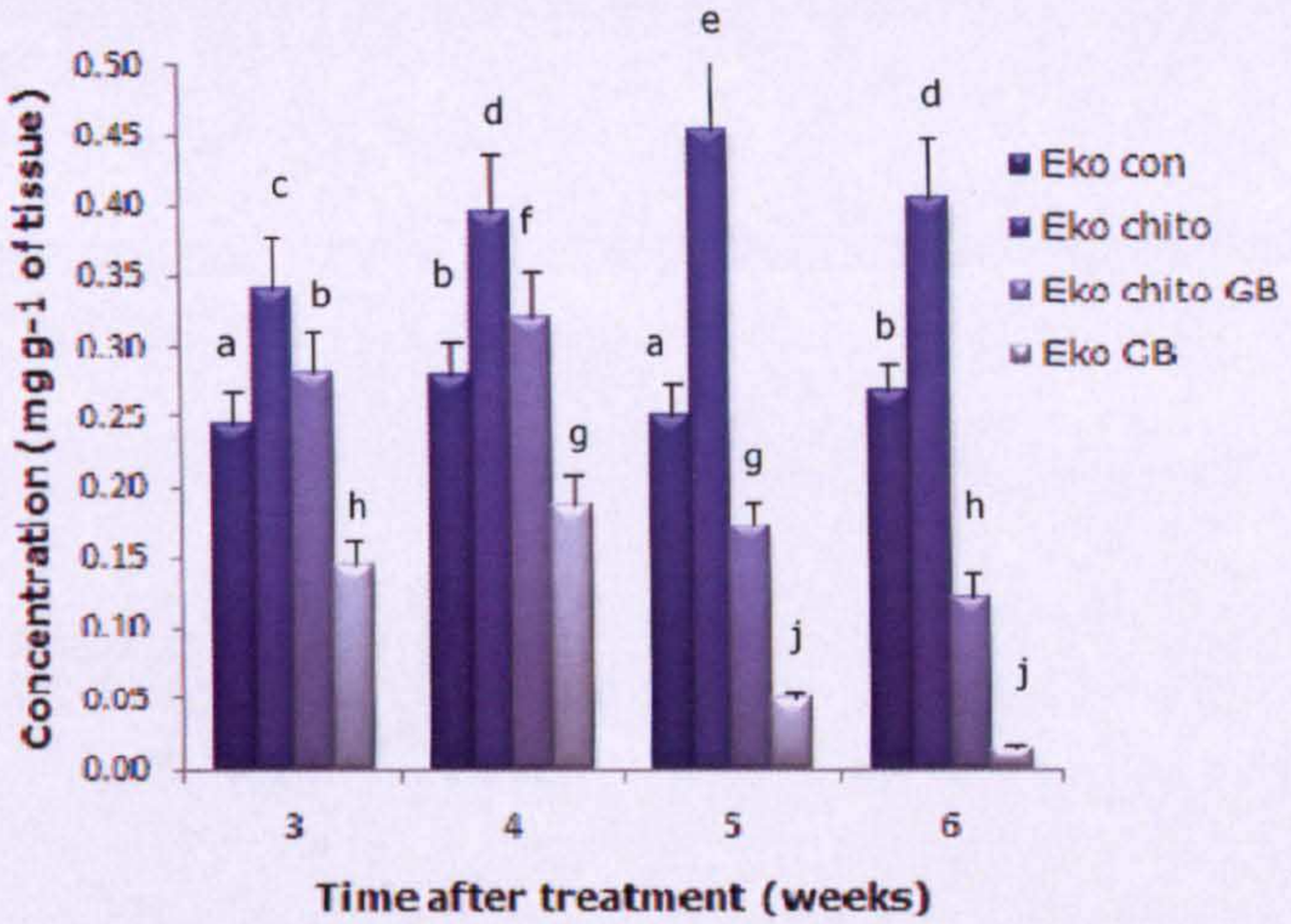
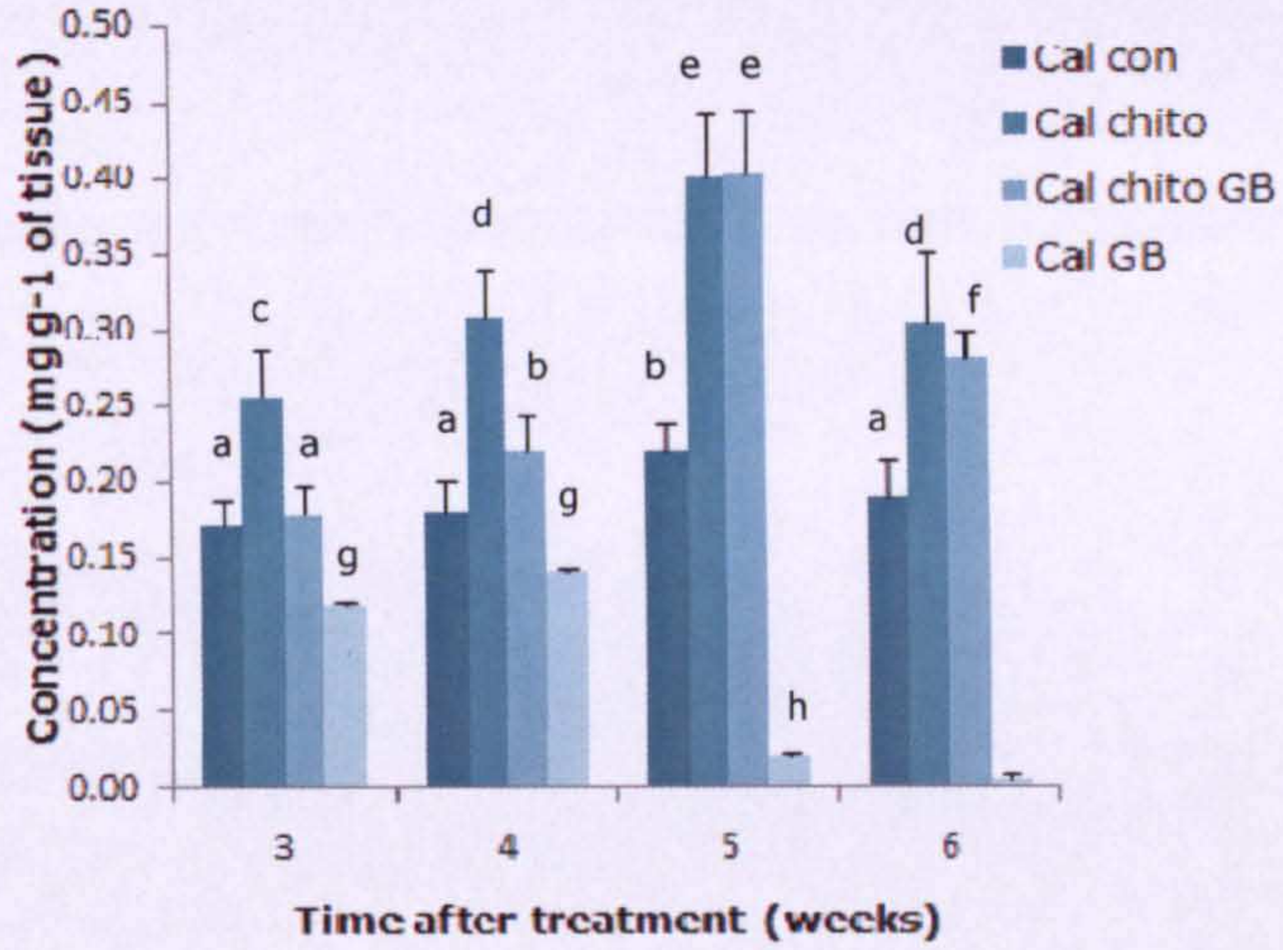


Figure 4.14: Accumulation of caffeic acid in varieties of oil palm root at weeks after different treatments.
Eko denotes Ekona; Cal: Calabar; AVR: AVROS; Chi: Chitosan and GB: *G. boninense*. Different alphabets indicate significant ($P<0.05$) among treatments in the same variety. Bars represent standard deviations. Five replicates in each treatment

4-HBA is the most abundant phenolic acid detected in oil palm roots among the three phenolics that were monitored in this study (Figure 4.15). In all three different varieties, 4-HBA accumulated almost two fold more in comparison to syringic and caffeic acids either in control or challenged seedlings. The pattern of elicitation and metabolism of this compound, however, followed similar trends to those described for syringic acid and

caffeic acid





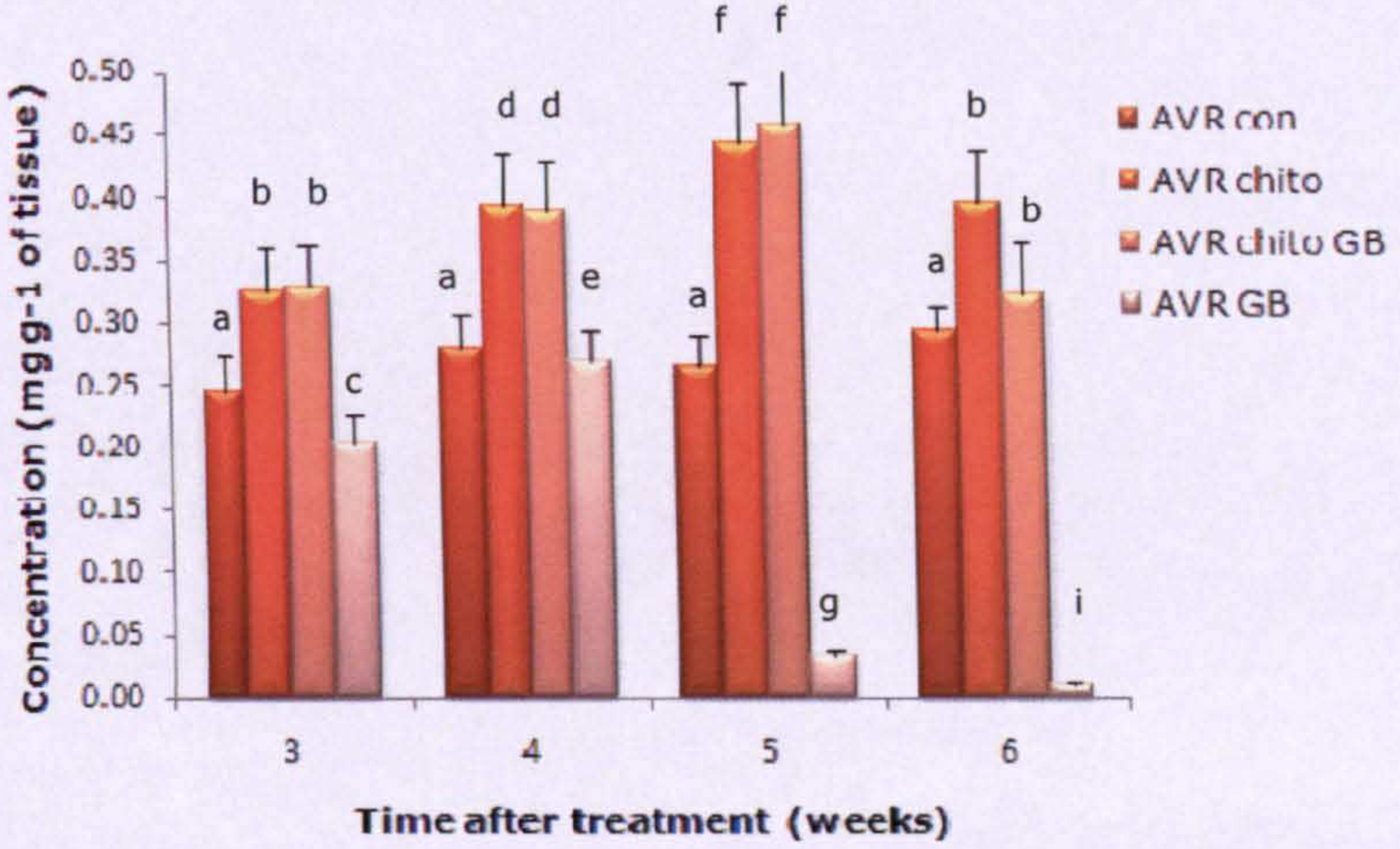


Figure 4.15: Accumulation of 4-HBA in varieties of oil palm root at weeks after different treatments.
Eko denotes Ekona; Cal: Calabar; AVR: AVROS; Chi: Chitosan and GB: *G. boninense*. Different alphabets indicate significant ($P<0.05$) among treatments in the same variety. Bars represent standard deviations. Five replicates in each treatment.

4.4. DISCUSSION

The Folin-Ciocalteu assay is one of the most commonly used spectrophotometric assays for determination of total phenolic content. Other common methods include the Folin-Denis assay and the Prussian blue assay. There are some other assays which are more specific, targeting specific compounds of interest, such as the vanillin, proanthocyanidin, collagen and hemoglobin assays. Although Folin-Ciocalteu is a common and easy assay, is not specific and will detect all phenolic groups found in extracts, including those found in the extractble proteins (Brune, Hallberg and Skanberg, 1991; Deshpande and Cheryan, 1987; Earp *et al.*, 1981; Hoff and Singleton, 1977; Maxson and Rooney, 1972). Importantly, the content of phenolics in oil palm is only expressed as gallic acid or catechin equivalents. However, in this project, the Folin-Ciocalteu assay provided a general and quick overview on total phenolic accumulation in roots of different varieties of oil palm which raised an early suggestion that the variety of AVROS may be a better candidate than Ekona and Calabar for having higher phenolic content. It remains to be determined whether phenolics have sufficient antifungal activity to play an important role in the defence mechanism against *G. boninense*. However, a better understanding of the accumulation of specific phenolics is required.

The development of more sophisticated and reliable equipment such as HPLC and LCMS, and the availability of standards from chemical suppliers, such as Sigma, helped the development of reliable HPLC quantification and analysis of oil palm roots in this project. Syringic acid, caffeic acid and 4-HBA, in low concentrations, are easily dissolved in polar solvents such as methanol which facilitated simple HPLC resolution. LCMS confirmed the presence of two of these important phenolics in oil palm roots.

From the figures shown in section 4.3.4. chitosan was found to enhance the accumulation of total phenolics, in comparison to *G. boninense*-only inoculated plants, in all three tested varieties. The findings here suggested that the total phenolics in oil palm roots may correlate with the disease intensity, as indicated by ergosterol analysis and MDSS reported in section 3.3.1. and 3.3.2. Seedlings that were pre-elicited with chitosan still became infected by *G. boninense* but the intensity of the disease was much lower and the total phenolics content was higher compared to plants that were inoculated without the presence of chitosan. This is in accordance with the finding reported by Mohamad Arif *et al.* (2007), which showed susceptible oil palm roots had low phenolic content, four weeks after inoculation with *G. boninense*, whereas one week after inoculation, high phenolic content was detected. Gallic acid concentrations decreased in the week four roots of infected susceptible palms compared to healthy seedlings. Determination of total phenolic content in infected palm seedlings roots (D x P) also showed low phenolic content (148.5 GAE ppm) compared to the non-infected palm seedlings roots (D X P) (162.8 GAE ppm). The results obtained from the preliminary study indicate that phenolic compounds are involved in oil palm resistance to *G. boninense*. Unfortunately, the work conducted by Mohamad Arif *et al.* (2007) only focused on the role of total phenolics and provided very little information. The work reported here supported this proposed involvement of phenolics in the interaction between oil palm and *Ganoderma* and also provided a more detailed description of the accumulation of specific phenolic acids. Moreover, a potentially important effect of chitosan in moderating the response was also observed.

Chitosan has been shown to control numerous pre and postharvest diseases on various horticultural commodities. It has been reported that both soil and foliar fungal plant pathogens, bacteria and viruses may be

controlled by chitosan application (Benhamou, Lafontaine and Nicole, 1994). In addition to its direct antimicrobial activity, other studies strongly suggest that chitosan induces a series of defence reactions correlated with enzymatic activities. Chitosan has been shown to increase the production of glucanohydrolases (Bautista-Baños *et al.,* 2006), phenolic compounds (Fajardo *et al.,* 1995; Tejchgraber, Popper, Knorr, 1991) and the synthesis of specific phytoalexins with antifungal activity (Benhamou and Thériault, 1992), and also reduce the activity of macerating enzymes such as polygalacturonases and pectin methyl esterase (Bautista-Baños *et al.,* 2006).

The role of phenolics in the defense mechanisms of oil palm is supported by the finding from the use of callus cultures from two cultivars of date palm (*Phoenix dactylifera*), resistant (BSTN) and susceptible (JHL) to 'Bayoud disease', caused by *F. oxysporum* f. sp. *albedinis. De novo* accumulation of phenolic compounds occurred in date palm callus in response to elicitation with filtrates from *Fusarium* cultures. Based on their chemical characteristics, most of these compounds were shown to be hydroxycinnamic derivatives. An increase was also recorded in the concentration and the degree of polymerization of flavans. All of these reactions were greater in the resistant than the susceptible cultivars of date palm, which indicates a possible role of phenolic compounds in the resistance to Bayoud disease (Daayf *et al.,* 2003).

Among the three tested varieties, AVROS contained significantly higher amounts of syringic acid, caffeic acid and 4-HBA either in healthy (control), *G. boninense*-infected or chitosan-elicited seedlings and this variety had a lower disease intensity compared to Ekona and Calabar. The role of these phenolics needs further investigation to confirm if the higher accumulation of phenolic acids in AVROS can provide a sufficiently antifungal environment to lead to lower infection in this variety.

CHAPTER 5: *IN VITRO* ANTIFUNGAL ACTIVITY OF PHENOLICS AND METABOLISM BY *G. BONINENSE*

5.1. INTRODUCTION

Results from section 4.3.4. showed an increase in the concentration of syringic acid, caffeic acid and 4-HBA, after inoculation with *G. boninense* or treatment with chitosan in different varieties. The addition of chitosan to the potting compost was shown to retain the phenolics for a longer period and at a higher concentration. The variety of AVROS, which had a higher accumulation of all these three phenolics, was less susceptible to infection compared to Ekona and Calabar, based on ergosterol analysis and MDSS as reported in sections 3.2.3. and 3.2.4. Whether the phenolics play a role in the defence of oil palm seedlings against *G. boninense* and contribute to lower susceptibility in AVROS needs further investigation.

To provide evidence to support a role for these phenolic acids, their antifungal activity against *G. boninense* needs to be examined. Bioassays are one of the most common methods for investigating such activity. There are several common techniques that can be implemeted for this purpose, including assays based on agar diffusion and growth in a liquid medium. The nutritional status and pH of the support media are important parameters when designing a bioassay system.

5.1.1. Bioassay

There are a few considerations that should be made when choosing an appropriate bioassay. The use of liquid culture systems has the advantages that (i) initial effects of antimicrobial compounds can be observed immediately after their addition, and (ii) localized depletion of antimicrobial compounds is avoided, thus simplifying quantification (Skipp & Bailey, 1977). Adrian *et al.* 1997 also reported that inhibitory effects of

resveratrol from grapevine on *B. cinerea* conidia were difficult to profound in solid medium due to crystalline suspension of the resveratrol. This makes it difficult to determine the concentrations actually evaluated, however, the study was later favored by the liquid medium for the rapid growth of the fungus. Work on bean phytoalexin with *Colletotrichum lindemuthianum* showed phaseollin was not completely soluble in agar medium therefore the concentration exposed to the fungus would be lower. A similar phenomenon was reported by Nonaka, (1967) who reported that the level of pisatin in the agar medium was less than that present before the agar was inoculated with pathogen of interest.

On another hand, the hole-plate method requires aqueous suspensions of plant extracts (Kudi *et al.*, 1999), while the filter paper disc technique may need the tested materials to be water soluble (Kim *et al.*, 2003). However, to favor most of the conditions, agar diffusion and liquid dilution methods have been chosen in this study for the *in vitro* bioassay to study the antifungal activity of syringic acid, caffeic acid and 4–HBA against *G. boninense*, with a range of concentrations of these phenolics that are commonly found in oil palm root tissues. The possibility that the pathogen may overcome these phenolics by degrading them to less toxic or non toxic compounds was also investigated.

5.1.2. Metabolism of antimicrobial compounds

Many experiments have shown that pathogens have the ability to metabolise and degrade antimicrobial compounds and phenolic acids to other less or non-toxic compounds in order to invade the host plant. Some of the examples are degradation of syringic acid by *Exophiala jeanselmei*, *F. eumartii* and *Paecilomyces variotii* (Bergbauer, 1991) and *P.putida* (Tack, Chapman and Dagley, 1972); degradation of phenolics in tomato by

bacteria (Sundin and Waechter-Kristensen, 1993; Waechter-Kristensen, Sundin and Jensén, 1994) and degradation of camalexin by *B. cinerea* (Chong, 2006). The changes in phenolic acid concentration observed during the development of disease, and the observation of growth *in vitro*, which accelerated after a period of incubation in the previous sections, suggested that phenolic degradation in oil palm might be an important factor in the interaction between the oil palm and *G. boninense*.

5.2. MATERIALS AND METHODS

5.2.1. Phenolic acids incorporated into agar

A series of concentrations of 0, 50 µg 70 µg, 90 µg, and 110 µg mL^{-1} of syringic acid, caffeic acid and 4-HBA were incorporated into 10% Potato Dextrose Agar (PDA) and Oil Palm Root Agar (OPRA), with the phenolics being first dissolved in acetone: water (50:50; v/v) before incorporation into the medium. Agar without phenolics served as a control. OPRA was prepared by boiling 300 g (freshly harvested, washed with sterile distilled water (SDW) and air dried) oil palm roots in 500 mL of SDW for approximately 1 h. The boiled extract was filtered through muslin cloth to remove solid impurities. Technical agar (20 g) was added to the filtrate to solidify the medium. 10% PDA was prepared by the addition of 3.9 g powder to 1 L of water and to ensure the medium solidified 12 g technical agar was also added. All media were adjusted to pH 4.5 using 10 mM tartaric acid (*G. boninense* infected oil palm roots, had a pH of 4.5±0.2, data not shown). A lower concentration of nutrients such as 10% PDA, was chosen to avoid an over-growth of *G. boninense* on the media during the incubation period and to provide a closer simulation of the nutritional status of oil palm roots. Plugs (8 mm) of *G. boninense* were taken from the edge of 7-8 d old cultures, using a sterile micropipette tip, and introduced

to the middle of the plates. The growth of the pathogen was expressed as radial growth (cm).

5.2.2. Phenolic acids incorporated into oil palm root broth (OPRB)

A series of concentrations of syringic acid, caffeic acid and 4-HBA were incorporated into the Oil Palm Root Broth (OPRB). The phenolics (0, 50 µg 70 µg, 90 µg, and 110 µg mL^{-1}) were first dissolved in acetone: water (50:50; v/v). Broth without phenolics and acetone served as a control. The procedure for preparation of OPRB is the same as OPRA (in section 5.2.1.) but only without the addition of technical agar. Mycelial plugs (8 mm) were taken from the edge of 7-8 d old vigorously growing *G. boninense* colonies using a sterile micropipette tip. The mycelium was excised from these plugs with a sterile scapel. To ascertain the dry weight of the inoculum, a preliminary experiment was undertaken, in which the plugs were washed in SDW, oven dried at 80°C for 8 h then weighed. Thus the final weight of mycelium, after culturing, could be corrected for the known weight of introduced inoculum. One fresh mycelia plug was introduced into 20 mL aliquots of OPRB (with phenolic acids), contained in 50 mL Falcon tubes. Cultures were grown at 25°C and aerated by shaking at 200 rpm on a Protech Model 719 orbital shaker. Five replicate culture tubes for each treatment were harvested on a daily basis for 15 d. The resultant mycelia were collected on Whatman No. 4 filter paper, washed, dried at 80°C, weighed and the final weight corrected as described above. The direct fungitoxicity of the relatively lower concentration of chitosan used to treat oil palm roots (0.1%) was also tested using this OPRB method. It was shown to be non-antifungal at this concentration (Data not shown).

5.2.3. Metabolism of phenolics by *G. boninense*

A preliminary experiment was carried out in 50 µg, 70 µg, 90 µg and 110 µg mL^{-1} of syringic acid, caffeic acid and 4-HBA to determine the possibility that *G. boninense* degrades the phenolics and to quantify the percentage loss of each molecule. Plugs (8 mm) of *G. boninense* from 7-8 d old cultures were harvested using a sterile micropipette tip from the edge of vigorously growing colonies. The mycelia on the top of the harvested discs was scraped with a sharp, sterile scalpel and immersed into 20 mL aliquots of OPRB containing different concentrations of phenolics. The samples were agitated on Protech Model 719 orbital shaker at 200 rpm at 25°C. Readings were taken at 1 d intervals for 15 d. To determine whether chitosan has any effect on metabolism, this molecule was also incorporated into media at a similar concentration to that used in peat.

5.2.4. Thin Layer Chromatography (TLC) bioassay of products extracted by n-butanol after degrdaded by *G. boninense*

To determine whether the metabolic products of the phenolic acids produced after incubation with *G. boninense* still possess antifungal activity, a futher experiment was undertaken. Broth samples (20 mL), which initially contained 110 µg mL^{-1} syringic acid, caffeic acid and 4-HBA were collected from 15 d old cultures, partitioned into 40 mL n-butanol, dried *in vacuo*, redissolved in 1 mL ethanol and separated using TLC. Ethanolic aliquots (0.1 mL) were loaded onto 1 cm origins of a TLC plate (Merck Kiesel 60 F254 Silica gel). The plate was developed in chloroform: ethanol (90:10 v/v) as suggested by Komsta and Szewczyk (2009) and run up to 16 cm from the origin. After resolution, the TLC plates were dried and bioassayed by spraying with spores of *A. niger* in Potato Dextrose Broth

(PDB) and incubated for 2 d at 25°C in 100% relative humidity. *A. niger* would not grew on the areas of the plate containing antifungal molecules. For comparative purposes similar samples obtained from the three phenolic acids at 50 µg mL^{-1} in OPRB without incubation with *G. boninense* were also applied to the TLC plates.

5.3. RESULTS

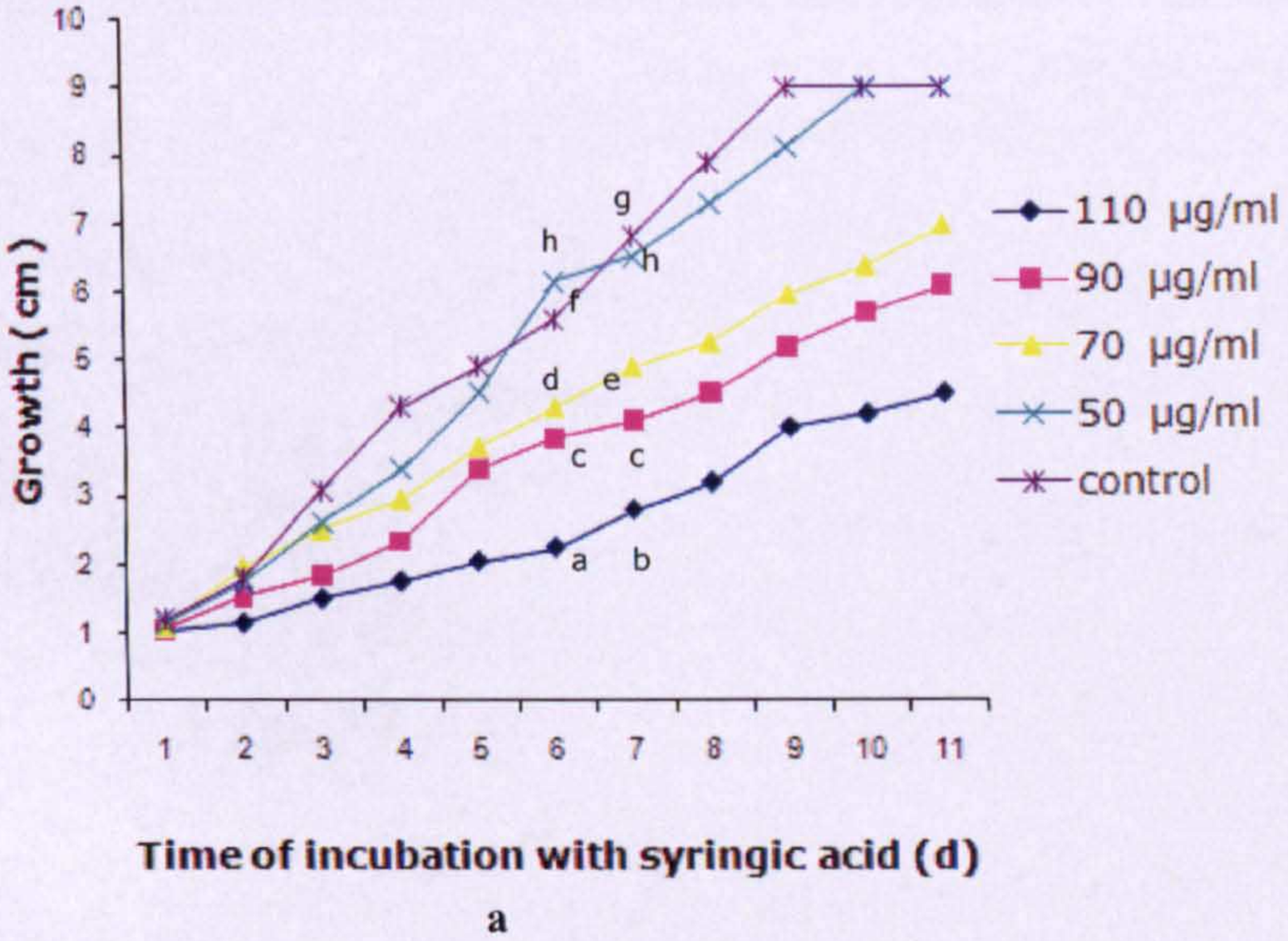
5.3.1. *G. boninense* growth on PDA and OPRA

All concentrations of syringic acid failed to stop completely the growth of this pathogen within the range of 50 to 110 µg mL^{-1} (Figure 5.1 a). However, the highest concentration tested, 110 µg mL^{-1} of this compound gave significantly slower growth of *G. boninense* in comparison to the unamended control. Inhibitory effects of the lower concentrations tested were progressively less. All concentrations showed significant ($p \leq 0.05$) lower radial growth for *G. boninense* at the last day of observation compared to the control, except 50 µg mL^{-1} syringic acid. OPRA was used to provide an alternative and possibly more representative condition to the environment of the *G. boninense*-oil palm root interaction (Figure 5.1 b). Although there was once again evidence of inhibition of *G. boninense*, the level of antifungal activity was less than that observed on 10% PDA.

A similar situation was observed with caffeic acid but with lowest antifungal activity compared to syringic acid (Figure 5.2 a and b). By day 11, in all caffeic acid tested concentrations, either in 10% PDA or OPRA, *G. boninense* grew to the maximum size of the Petri dishes (9 cm).

4-HBA was virtually inactive against *G. boninense* both in 10% PDA and OPRA (Figure 5.3 a and b). The pathogen grew steadily, except in the highest 4-HBA tested concentration. In summary, antifungal activity of

syringic acid and caffeic acid was detected even at the lowest concentration tested but it was weak for 4-HBA. Therefore, low concentration of syringic acid, caffeic acid and 4-HBA may unlikely to play any role at these low toxicities.



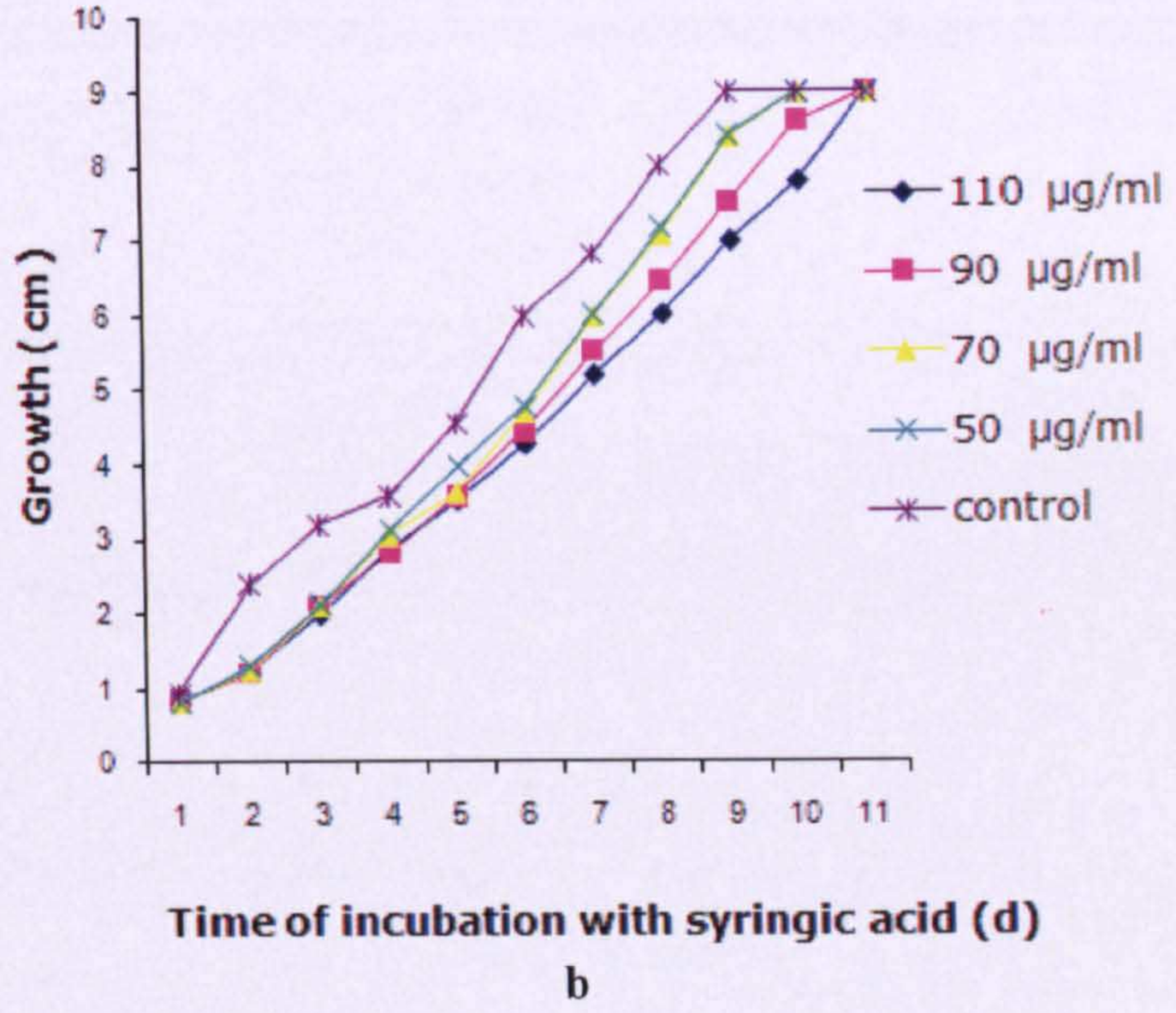
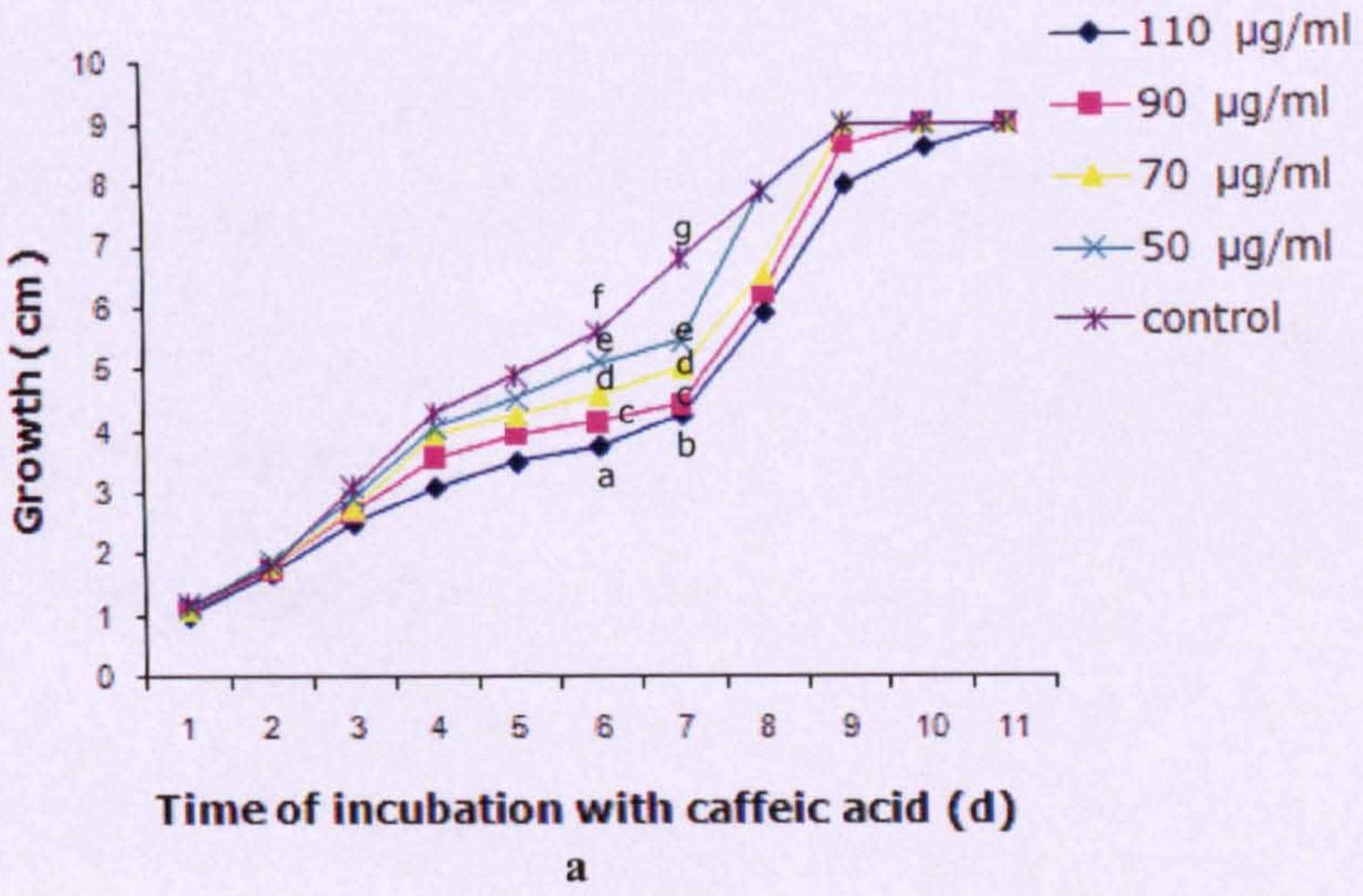


Figure 5.1: Radial growth of *G. boninense* on 10% PDA (**a**) and OPRA (**b**) with different concentrations of syringic acid incorporated into the agar. Five replicates in each treatment. Different alphabets indicate significant at $P<0.05$. Dry weight increased significantly ($P<0.05$) on OPRA in all treatments throughout the study. ED_{50}= 104 µg mL^{-1} **(a)** and 266 µg mL^{-1} **(b)**.



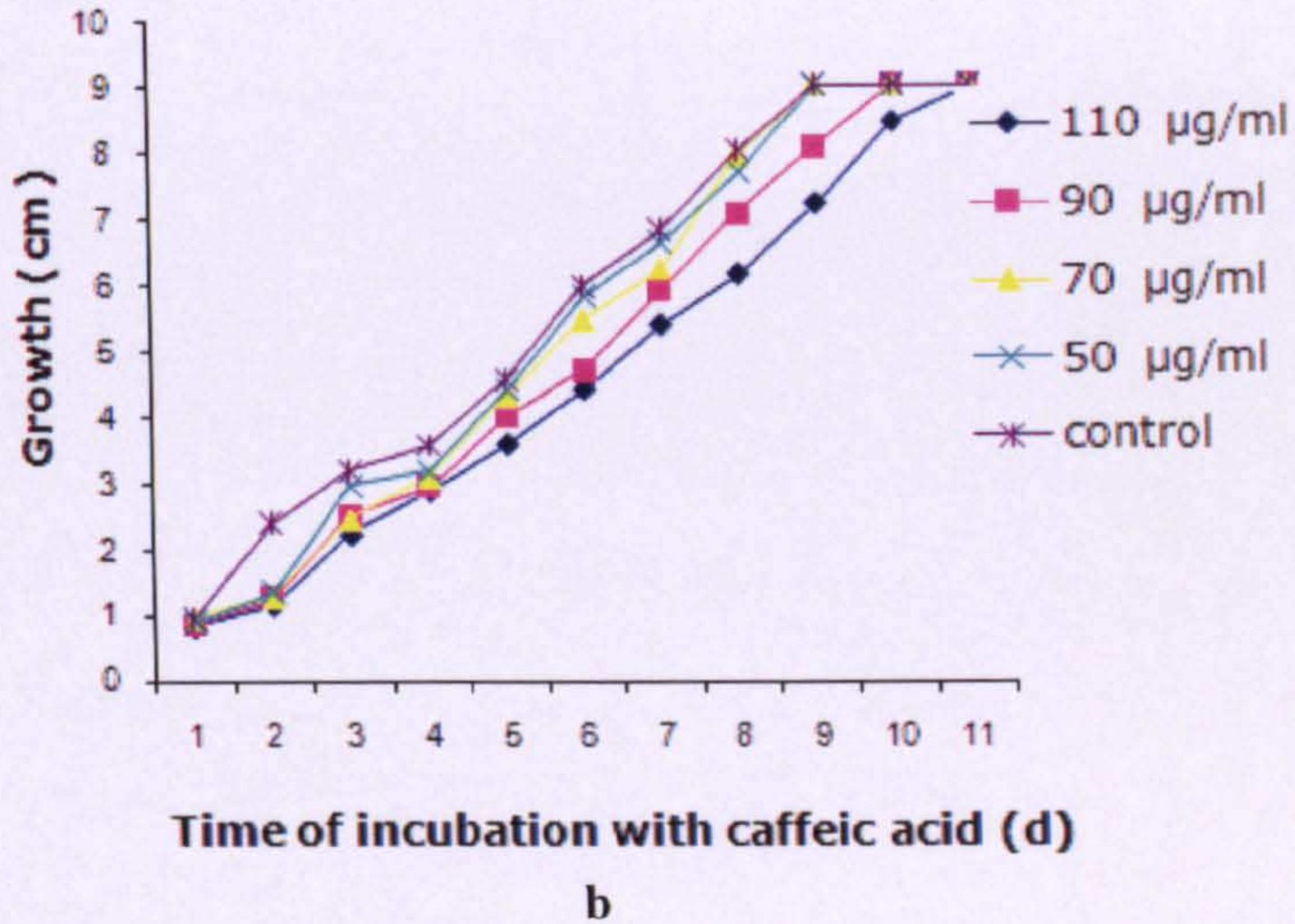
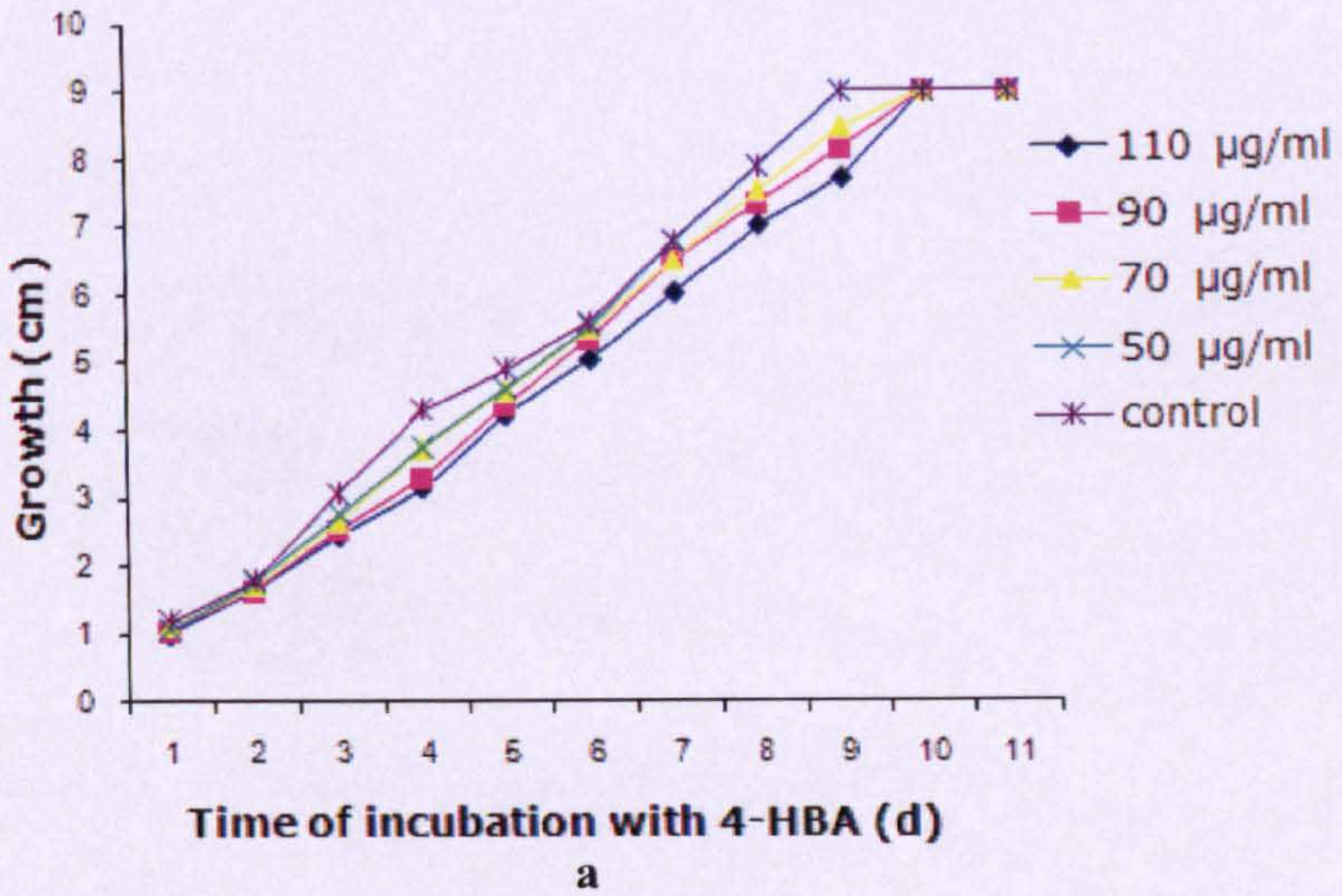
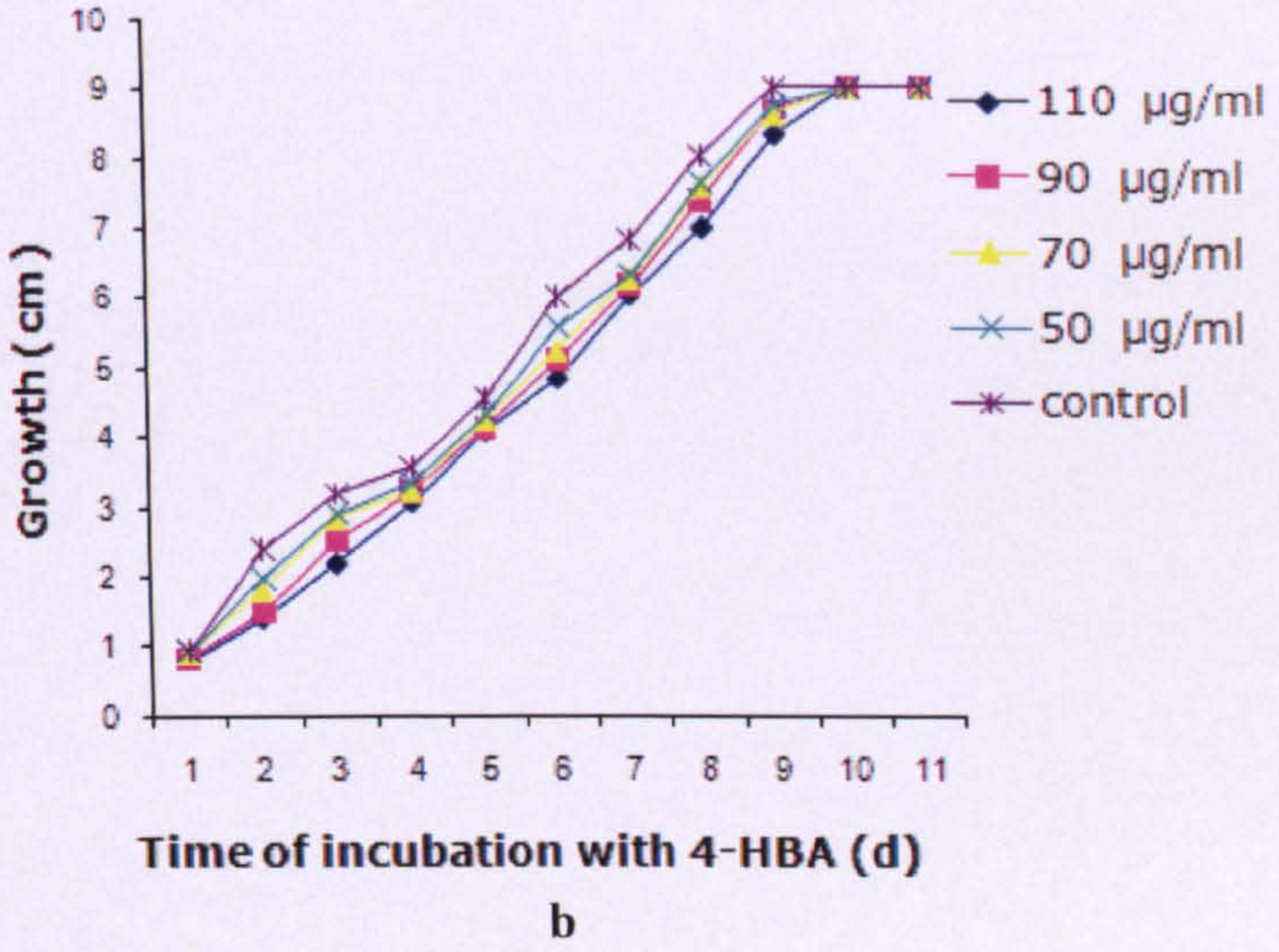


Figure 5.2: Radial growth of *G. boninense* on 10% PDA (**a**) and OPRA (**b**) with different concentrations of caffeic acid incorporated into the agar. Five replicates in each treatment. Different alphabets indicate significant at $P<0.05$. Dry weight increased significantly ($P<0.05$) on OPRA in all treatments throughout the study. ED_{50}= 656 µg mL^{-1} **(a)** and 299 µg mL^{-1} **(b)**.



a



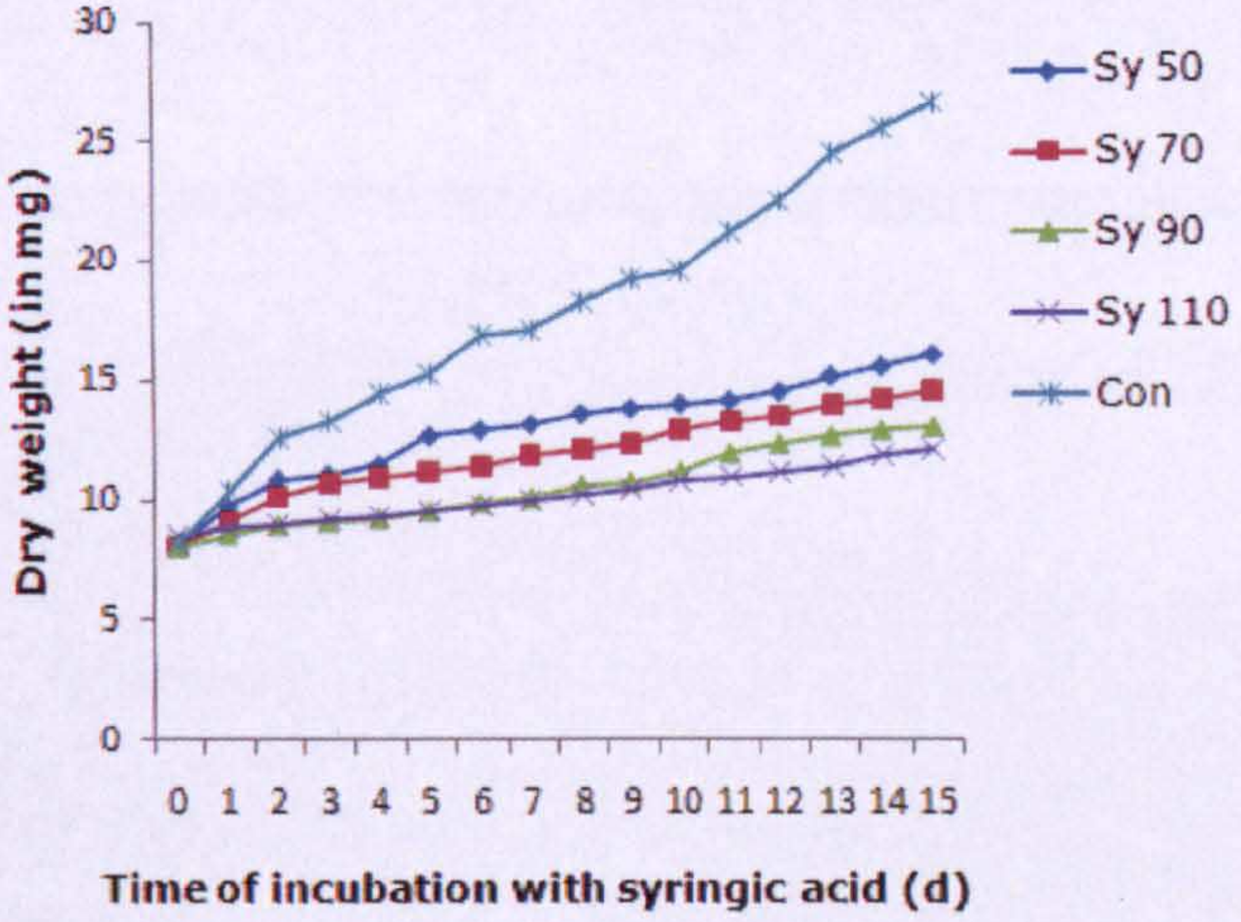
b

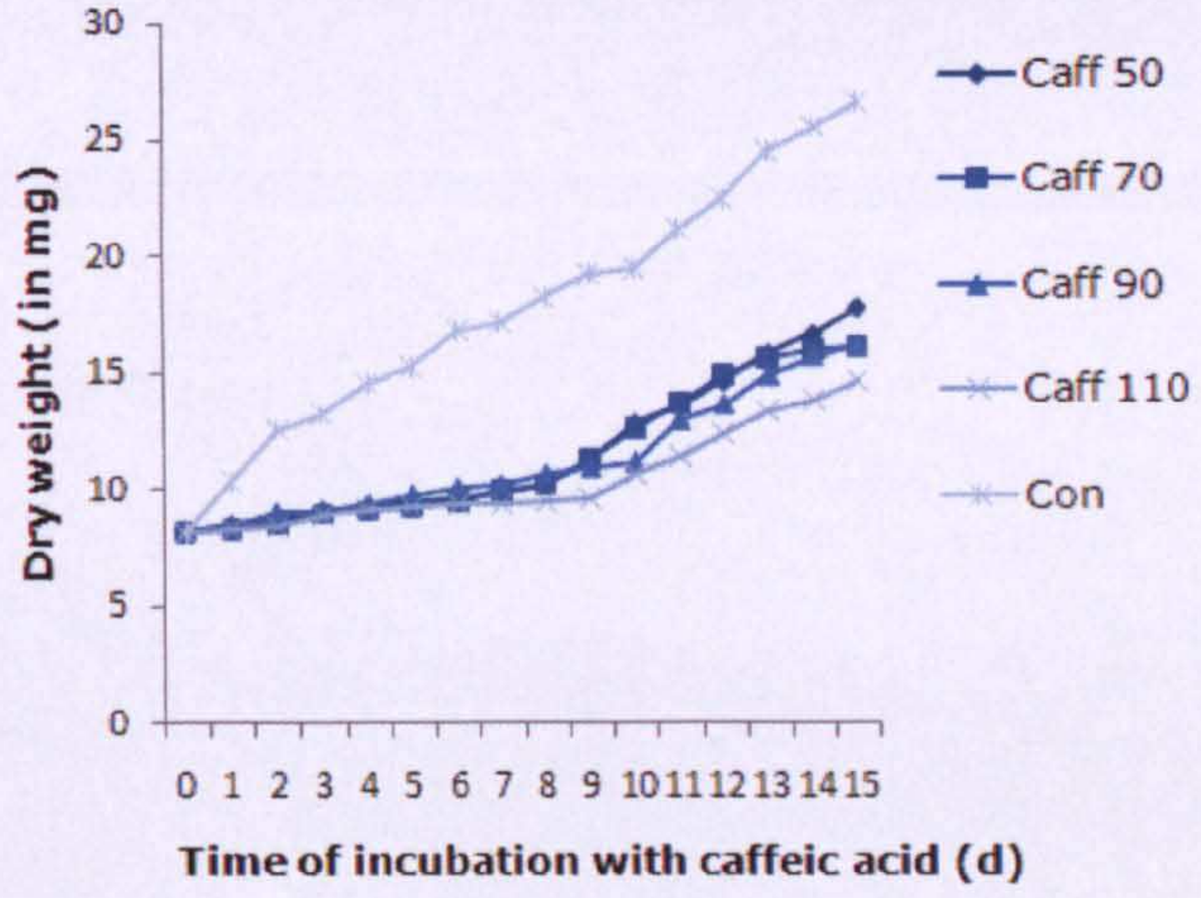
Figure 5.3: Radial growth of *G. boninense* on 10% PDA (**a**) and OPRA (**b**) with different concentrations of 4-HBA incorporated into the agar. Five replicates in each treatment. Significant increase ($P<0.05$) of dry weight in time course from day 1 to 11 in all treatments. Note: This phenolic found not fungitoxic to *G. boninense*.

5.3.2. *G. boninense* growth in OPRB

Ganoderma growth curves are given in Figure 5.4, from which it can be seen that all the phenolics tested inhibited the growth of the fungus in the liquid medium. There are some similarities observed in agar tests and the broth assay. Syringic acid was the most antifungal molecule tested. With the highest tested concentration (110 µg $mL^{-1)}$ of syringic acid, there was no significant difference in growth of the pathogen ($p \geq 0.05$) up to day 9 compared to other concentrations. Although the increase of dry weight in the same treatment after day 9 was significant ($p \leq 0.05$), it was much lower compared to that in the presence of caffeic acid and 4-HBA.

All phenolics tested found to be more fungitoxic (ED_{50}) to *G. boninense* in liquid medium in comparison to solid medium. The ED_{50} for *G. boninense* were much lower for all phenolics in liquid medium.





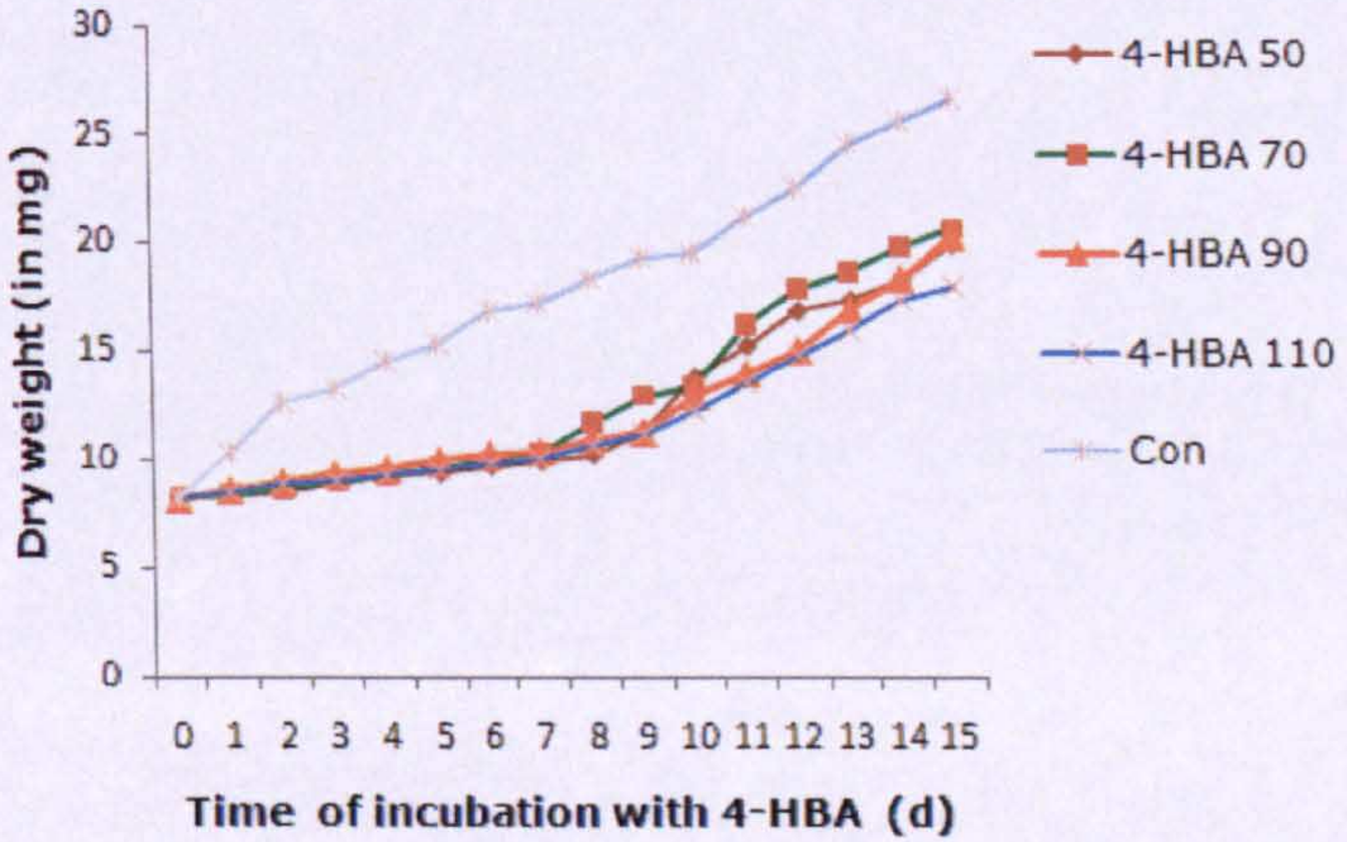


Figure 5.4: The dry weight of *G. boninense* mycelia in oil palm root broth (OPRB) with different concentrations of phenolic acids incorporated into the broth incubated for 15 d. Five replicates in each treatment. Significant increase ($P<0.05$) of dry weight in time course recorded only from day 9 onwards in all treatments. Dry weight in control increased significantly ($P<0.05$) throughout the study. ED_{50}= 82 µg mL^{-1} (syringic acid), 105 µg mL^{-1} (caffeic acid) and 163 µg mL^{-1} (4-HBA). Sy denotes syringic acid; Caff: caffeic acid; 4-HBA:4-hydroxybenzoic acid; con=control without phenolics.

5.3.3. Phenolics metabolism by *G. boninense*

In the absence of *G. boninense* inoculum, either with or without the presence of chitosan, there was no loss of any phenolic acids over the duration of the experiment (data not shown). The compounds were therefore stable. As illustrated in Figure 5.5, the losses were small and not significant ($P \geq 0.05$) in higher concentrations of syringic acid (90 and 110 μg mL^{-1}) up to day 9. This lag phase ended after day 9, with large significant ($p \leq 0.05$) losses of phenolics even at the high concentrations. All molecules were degraded at day 15. The losses were greater in lower concentrations of 50 and 70 μg mL^{-1} without a lag phase. It should also be noted that chitosan had no effect on the metabolism of syringic acid by *Ganoderma*.

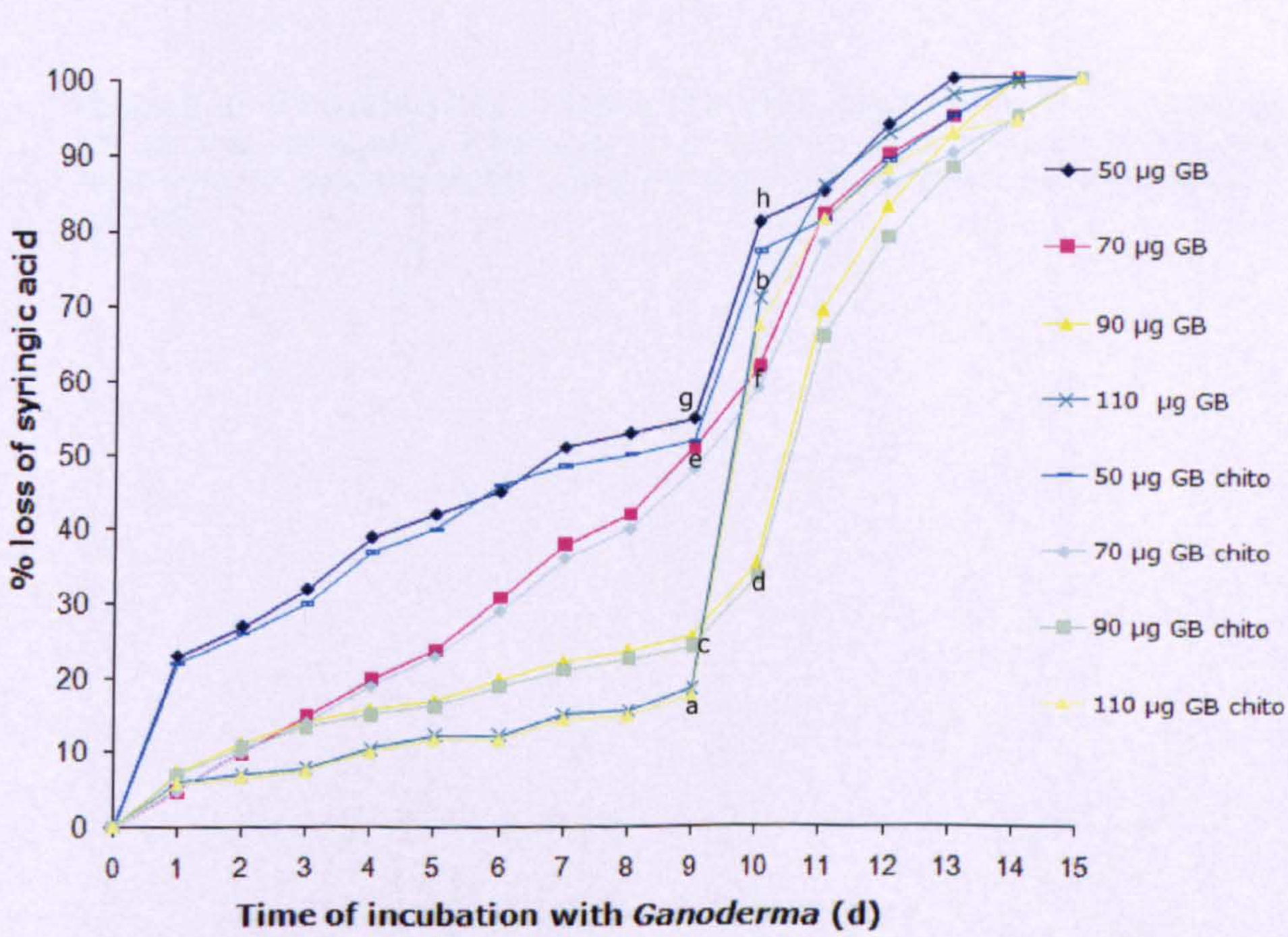


Figure 5.5: Percentage loss of syringic acid after incubation with *G. boninense*. GB denotes *Ganoderma boninense*; con: control; chito: chitosan. Five replicates for each treatment. Different alphabets indicate significant at $P<0.05$.

Figure 5.6 illustrates a similar situation with caffeic acid to syringic acid with losses which were small and not significant ($P \geq 0.05$) in the highest concentration tested (110 μg mL^{-1}) up to day 9. The lag phase ended after day 9, with large significant ($p \leq 0.05$) losses of the compound. All caffeic acid was metabolised at day 15. The losses were greater in lower concentrations of 50, 70 and 90 μg mL^{-1} with no lag phase detected.

Caffeic acid was less toxic to *G. boninese* compared to syringic acid; the pathogen metabolized the compound steadily at a concentration of 90 μg mL^{-1}. Chitosan again had no affect on this process.

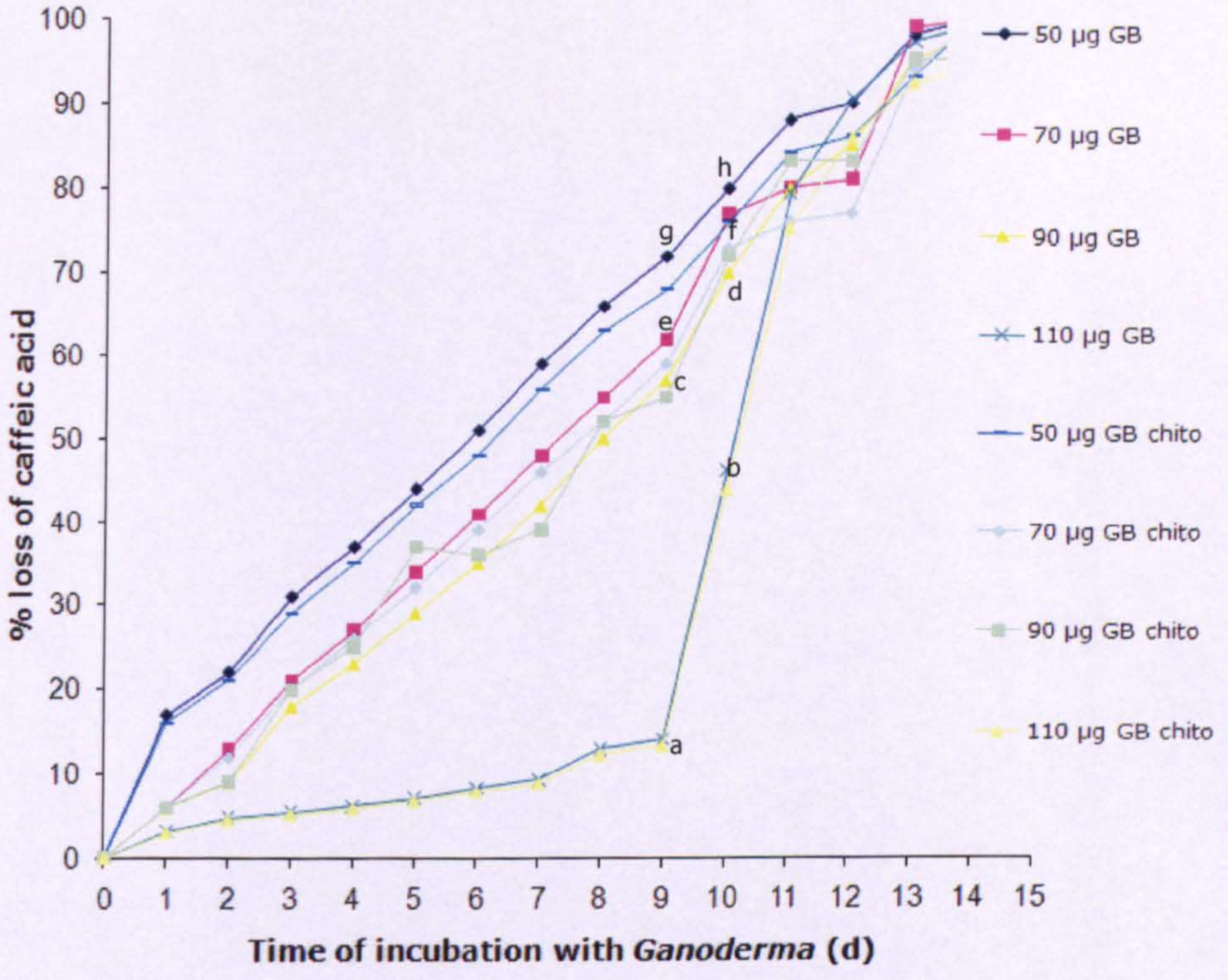


Figure 5.6: Percentage loss of caffeic acid after incubation with *G. boninense*. GB denotes *Ganoderma boninense*; con: control; chito: chitosan. Five replicates for each treatment. Different alphabets indicate significant at $P<0.05$.

4-HBA was again shown to be less antifungal to *G. boninense* compares to syringic and caffeic acid (Figure 5.7). The pathogen was able to metabolise the molecule even at the highest tested concentration. There was no obvious lag phase of losses in this compound. Degradation was significantly slower ($p \leq 0.05$) at 110 µg mL^{-1} but 4-HBA was fully degraded at the end of the experiment.

The data obtained from Figures 5.5-5.7 show some similarities between phenolic losses, with the rate of metabolism higher at low concentrations. At lower concentrations, such as 50 and 70 µg mL^{-1} the compounds underwent a steady linear loss. A lag phase occured before degradation at higher concentrations, such as 90-110 µg mL^{-1} syringic acid and 110 µg mL^{-1} caffeic acid. *G. boninense* has the ability to metabolise the molecules but this may be lost at high concentrations when it cannot grow. The data also suggest a good correlation between the antifungal activities and metabolism of those compounds. Syringic acid is the most fungitoxic to *G. boninense* of the three tested, followed by caffeic acid and 4-HBA. The pathogen was able to degrade 4-HBA even at the highest concentration, in contrast to slower metabolism in syringic acid and caffeic acid at the same concentration.

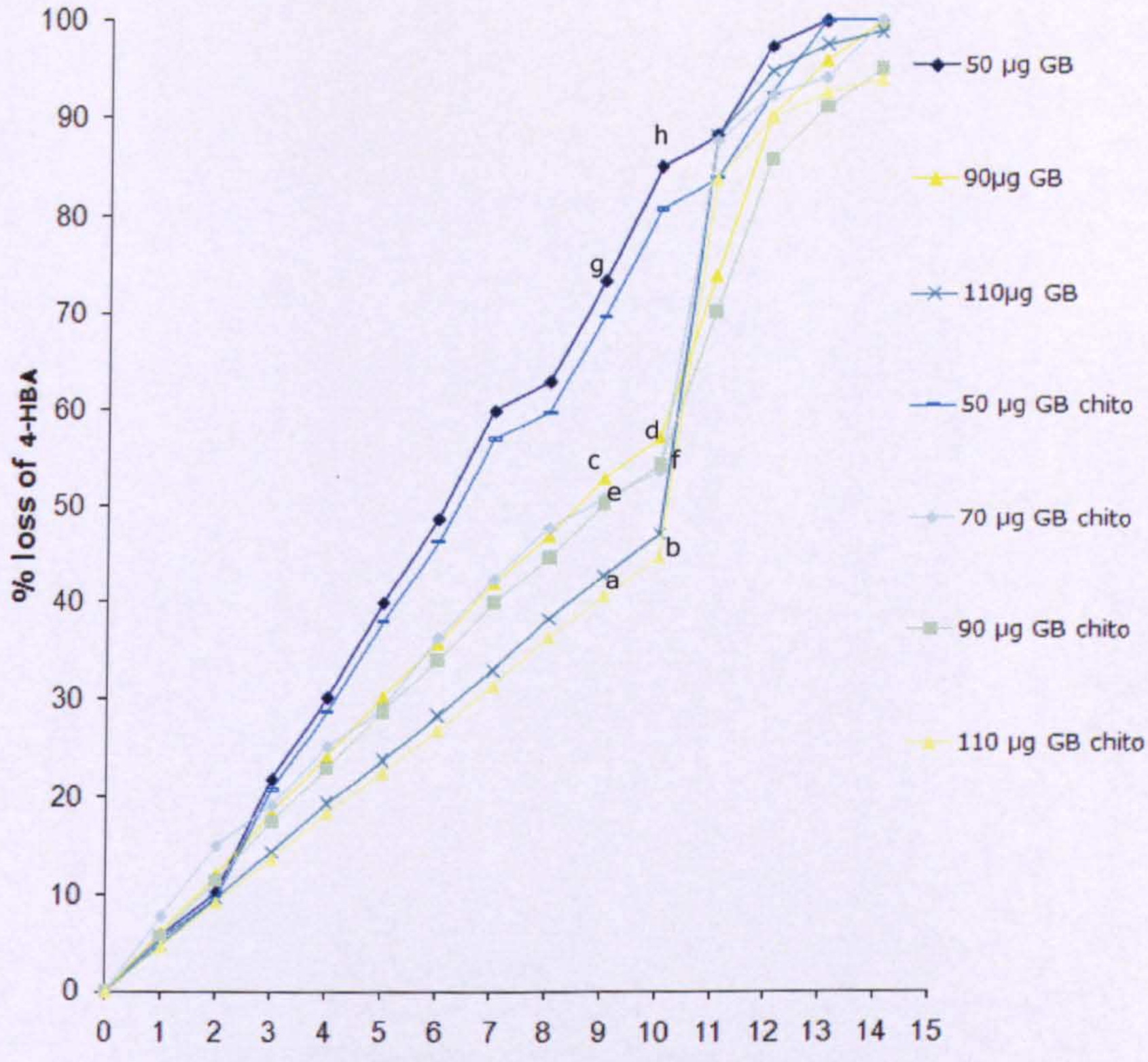


Figure 5.7: Percentage loss of 4-HBA after incubation with *G. boninense*. GB denotes *Ganoderma boninense*; chito: chitosan. Five replicates for each treatment. Different alphabets indicate significant at $P<0.05$.

5.3.4. TLC bioassay

The results obtained from the TLC bioaasay of the degraded products are shown in Figure 5.8. R_f values, as described by Komsta and Szewczyk (2009), revealed the three inhibition zones which developed were from 4-HBA (R_f: 0.4), caffeic acid (R_f: 0.2) and syringic acid (R_f: 0.58). No inhibition band was seen in samples that had been incubated with *G. boninense*. The result shows a good relationship between the loss of the phenolic acids in liquid culture and the loss of inhibition bands. Strong inhibition was found in treatments without *G. boninense*, which contained caffeic acid and syringic acid. The inhibition zone caused by the less fungitoxic 4-HBA was less pronounced.

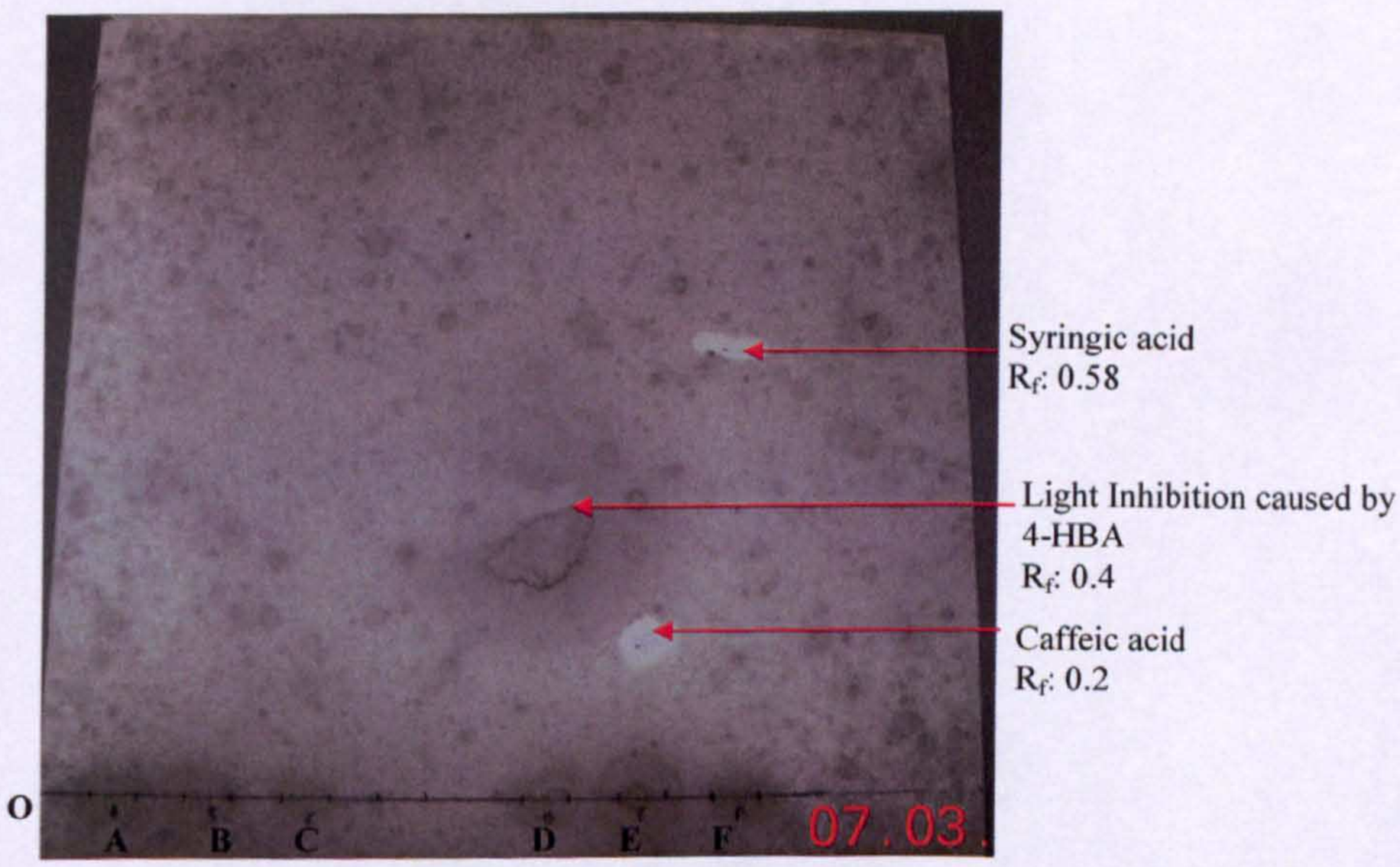


Figure 5.8: TLC biossay of products extracted with n-butanol after 4-HBA, caffeic acid and syringic acid were degraded by *G. boninense*. **A** (Degraded 110 µg mL^{-1} of 4-HBA). **B** (Degraded 110 µg mL^{-1} of caffeic acid). **C** (Degraded 110 µg mL^{-1} of syringic acid). **D** (Control 50 µg mL^{-1} of 4-HBA). **E** (Control 50 µg mL^{-1} of caffeic acid). **F** (Control 50 µg mL^{-1} of syringic acid). **O**: Origin. All inhibition bands arrowed. Developed in Chloroform: ethanol (90:10 v/v)

* All with 0.1 mL applied on 1 cm origin

In summary, *G. boninense* has the ability to degrade 4-HBA, caffeic acid and syringic acid and the degradation products were not antifungal.

5.4. DISCUSSION

Higher concentrations of syringic acid, such as 90 and 110 µg mL ^{1}, possibly play a role in limiting the growth of *G. boninese*. But the amount of this phenolic which was detected in the oil palm roots may not be sufficient to fully inhibit the pathogen. Higher concentrations may have greater effect on this pathogen. Numerous papers have been published on the effect of syringic acid in other plants but not, to date, in oil palm. Work on the resistance of raspberry to the fungus *Didymella* show syringic acid accumulated in the bordering zone of lesions forming a barrier to the fungus. The *in vitro* fungitoxicity of syringic acid was confirmed to be very high at low concentrations (Kozlowska and Krzywanski, 1994). Similarly, the sugar cane cultivar Mayarí 55-14, which is highly resistant to smut disease, showed a major accumulation pattern of syringic acid when interacting with the pathogen (de Armas *et al.,* 2007).

Although caffeic acid in oil palm root may not be as inhibitory as syringic acid, the fungitoxicity of the highest concentration, 110 µg mL ^{1}, during the *in vitro* tests should not be ignored. Caffeic acid is ubiquitously present in plants and has potent phytotoxicity affecting plant growth and physiology (Singh *et al.,* 2009). Caffeic acid was found to be inhibitory to the growth of four sweet potato pathogenic fungi. Inhibitory activity bioassays also suggested high periderm caffeic acid levels contribute to the storage root defence chemistry of some sweet potato genotypes (Harrison *et al.,* 2003).

On the other hand, the role of 4-HBA seems to be least important when compared to the other phenolic acids detected in the oil palm-*G.*

boninense interaction. From the *in vitro* test results, based on radial growth, the potential of this phenolic acid to stop the invasion of *G. boninense* is probably very small. The importance of 4-HBA was reported in rice hull against various microorganisms. An evaluation of 50% inhibition of growth (IC_{50}) revealed that most of the Gram-positive and some Gram-negative bacteria were sensitive to 4-HBA at IC_{50} concentrations of 100-170 µg mL^{-1} (Cho *et al.*, 1998). However, in the living roots, 4-HBA may contribute in providing a synergistic fungitoxic effect together with the two other phenolic acids detected. Future work should test the combination of the molecules against *G. boninense*. The availability of these phenolic acids to *G. boninense*, at a cellular level, within oil palm roots is an area which also requires investigation.

As a general observation, *G. boninense* can metabolise all phenolic acids tested, especially at low, non-antifungal concentrations. The TLC bioassay confirmed the metabolites were not antifungal. The ability of some pathogens to degrade syringic acid has been demonstrated in several studies. In work investigating the pathways for syringic acid metabolism using the white-rot fungus *Sporotrichum puluerulentum*, the detection of some identified metabolites have suggested the following reactions occur in the process of metabolism of syringic acid: reduction of the carboxyl group; hydroxylation and simultaneous decarboxylation; demethytation; and methylation of the *p*-hydroxyl group. In another study, two soft-rot fungi, *Petriellidium boydii* and *Phiuluphora mutabilis*, were found capable of rapidly metabolising syringic acid within 12 h of incubation, causing the formation of 3,4,5- trimethoxybenzoic acid, also indicating an ability within these soft-rot fungi to methylate the *p*-hydroxyl group. Some brown-rot fungi such as *Duedaleu quercina* and *Fomes pinicola* were described as poor metabolisers of syringic acid. However, demethylation was observed

with *F. pinicolu* and an unidentified product appeared in the culture solution of *D. quercina* (Eriksson *et al.,* 1984).

In a separate investigation, Bergbauer (1991) reported that six white-rot fungi were found to strongly oligomerize syringic acid, both with and without co-substrate. The main polymerization product was identified by 13 C-NMR as a 1,3-dimethylpyrogallol oligomer. Other minor metabolic products were methylated and hydroxylated derivatives. *Exophiala jeanselmei*, *F. eumartii*, and *Paecilomyces variotii* were found to have the ability to completely and rapidly degrade syringic acid (5 mg mL^{-1}) within 48 h to 100 h. Some species were able to degrade syringic acid to some extent when glucose was added. Methylated and demethylated metabolic intermediates were identified by GC/MS. Tack, Chapman and Dagley (1972) also confirmed *P. putida* has the ability to oxidize syringic acid with the presence of NADH.

The observed degradation of caffeic acid by *G. boninense* in this project is supported by some other reports in other systems. In an investigation using a closed hydroponic system, several bacterial isolates, which were obtained from the system used to grow tomato, were reduced up to 80% of 50 µM of caffeic acid within 72 h (Waechter-Kristensen, Sundin and Jensén, 1994). In work on sugarcane (*Saccharum officinarum)*, Fontaniella *et al.* (2007) examined two cultivars of sugarcane differing in susceptibility to leaf scald, a disease caused by the bacterium *Xanthomonas albilineans*, on their enzyme activities and biosynthetic metabolism. They reported *p*-coumaric, ferulic and caffeic acids, decreased in concentration, while the levels of free putrescine and ornithine decarboxylase activity increased during the interaction of the sugarcane with this pathogen. Phenolic acid metabolism also differed in the two cultivars.

4-HBA was the least antifungal among the three phenolic acids detected in oil palm. As *G. boninense* was able to degrade caffeic acid and syringic acid, the degradation of 4-HBA was not suprising. In an experiment reported by Fairley *et al.* (2002), a novel haloarchaeal strain (member of the halophile community), *Haloarcula* sp. strain D1, grew aerobically on 4-HBA as a sole carbon and energy source and was able to metabolize 4HBA via gentisic acid, rather than via protocatechuic acid, hydroquinone, or catechol. Gentisate was detected in the 4-HBA-grown cultures, and gentisate 1,2-dioxygenase activity was induced in 4-HBA-grown cells. Stoichiometric accumulation of gentisate from 4-HBA was demonstrated in 4-HBA-grown cell suspensions containing 2,2'-dipyridyl.

The pH of the media also plays an important role to the toxicity and degradation of the phenolic acids. In this experiment, caution has been taken by adjusting the pH of the media to be acidic and similar to the pH of infected roots. Diffusion of undissociated acid molecules can occur freely in an acidic medium but is limited to transport systems at neutral or basic pH values. The toxic effects associated with organic acids are the result of both anion specific effects on metabolism as well as increased internal proton concentrations. Effects on internal pH are mitigated by transport of protons out of the membrane, consumption of protons by decarboxylation reactions, and, more generally, induction of stress regulons. Anion specific tolerance mechanisms are not well characterized (Warnecke and Gill, 2005). Organic acid anions affect cell growth in a variety of ways. Increased anion concentration has been shown to lead to an increased transport of potassium ions into the cell, which increases turgor pressure (Kroll and Booth, 1983; McLaggan *et al.,* 1994). To maintain a constant turgor pressure and cell volume, glutamate is transported out of the cell (McLaggan *et al.,* 1994). This transport activity concomitantly disrupts the osmolarity of the cytoplasm, which in turn lowers the cell's growth

potential and viability. In addition to this general anion effect, there are also effects specific to each organic acid. It has been proposed that enzymes involved in protein synthesis are sensitive to a combination of two unrelated mechanisms, including the acidification of intracellular pH and the formation of an anionic pool (Roe *et al.*, 2002). Although this finding implies that the organic inhibition due to the anion pool could be acid specific, the details describing this dual inhibition mechanism remain unclear (Warnecke and Gill, 2005).

In a study reported on *E. coli* and organic acids, one of the primary factors contributing to the toxicity of the acids is their ability to diffuse across the microorganism cellular membranes, when undissociated as opposed to the restricted passage of dissociated protons and anions (Poole and Halestrap, 1993; Walter and Gutknecht, 1984). Diffusion of dissociated acids is limited to secondary transport, which is known to involve H^+ or monocarboxylic acid symporters. However, the detailed mechanism and specificities of the transporters remain unknown (Poole and Halestrap, 1993). *E. coli* maintain a cytoplasmic pH (intracellular pH = 7.5) that is most often higher than that of the external media and typically well above the pKa of organic acids (Goulbourne *et al.*, 1986; Maurer *et al.*, 2005). As a result, organic acids exist in the dissociated form within the cytoplasm. Thus, diffusing organic acids entering into the cytoplasm will dissociate and disrupt the intracellular pH and anion pool of the cytoplasm. The resulting increase in internal acidity can affect the integrity of purine bases (Choi, Baumler and Kaspar, 2000) and result in denaturing of essential enzymes inside the cell (Roe *et al.*, 2002) both of which negatively effect cell viability.

In a report on the effect of *p*-coumaric acid on four bacterial phenolic acid decarboxylases (PAD) (from *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Bacillus subtilis*, and *B. pumilus*) expressed in

E. coli, using medium supplemented with different concentrations of phenolic acids and with differing pHs, the effect of *p*-coumaric acid was highest at pH 5.2 and decreased with an increase in the initial pH of the growth medium. Rossall (1978) also reported the activity of wyerone acid, a phytoalexin found in broad bean, was higher at lower pH values. Therefore, the *in vitro* conditions provided for the interaction between *G. boninense* and the phenolic acids were chosen to be close to the *in planta* situation, to favour the antifungal effect of those phenolic acids on the pathogen.

Besides the importance of pH, the availability of nutrients maybe another important factor that contributes to infection of the host and metabolism of the phenolic acids. Based on the understanding of this factor, 10% PDA was used as one of the tested media to provide a low amount of nutrients for the pathogen, while OPRA and OPRB provided a close simulation of the *in planta* situation.

In summary, syringic acid was found to be the most active phenolic against *G. boninense*, followed by caffeic acid, with 4-HBA being the least toxic to this pathogen. Inhibition could occur *in planta* with higher levels of syringic acid and caffeic acid especially with the presence of chitosan. Chitosan is a stimulant on the production of these metabolites in living oil palm tissues and has no effect on *in vitro* metabolism by *G. boninense*. Metabolism can occur at a faster rate at concentrations with less antifungal activity. A TLC bioassay suggested that metabolites found after the degradation were no longer antifungal.

However, if syringic acid, caffeic acid and 4-HBA were oxidized, it might possess far greater antifungal activity. Gomez *et al.* 2004 reported that phenolic levels in cells of elicitor-challenged cassava (*Manihot esculenta*) suspension cells and leaves were not enhanced and were, theoretically, too low to be inhibitory. However, in combination and when

oxidized they may contribute to defence, because oxidation of esculetin and scopoletin by peroxidase and of esculetin by tyrosinase enhanced their fungitoxicity up to 20-fold.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUDING REMARKS

The molecular identification of *G. boninense* isolates from Langkon, Sabah, which was reported in chapter 2 should reduce the current debate on the aggressiveness of isolates of *G. boninense* in Sabah, compared to Peninsular Malaysia (Hoong, H.W. and Idris, A.S. personal communications). The PCR and sequence homology technique has confirmed that the isolate used in this project is very similar to *G. boninense* strains FA5017 or FA5035 from Peninsular Malaysia, with a maximum identification homology of 98%. These are the common aggressive isolates of Peninsular Malaysia, that cause BSR and major losses in plantations. The difference in disease severity between the Peninsular Malaysia and Sabah maybe due to the longer history of oil palm plantations in the former, thus providing more inoculum of the pathogen which may build up after several generations of oil palm in the older estates. The concern in the beginning of the project was choosing the right isolate to work with, as there are many strains of *G. boninense* that show different aggressiveness to oil palm. The strain isolated was shown to be pathogenic and thus provided a viable system to study the role of phenolic acids in the interaction between oil palm roots and *G. boninense*.

The development of a reliable artificial inoculation technique for this disease, reported in this project, also contributed to a faster and more reproducible system, in comparison to the most common and traditional rubber wood block method. In the conventional technique, wood blocks from rubber or oil palm trees (Idris *et al.*, 2006) were first inoculated with *G. boninense*. The block could only be used as inoculums after *G. boninense* had fully colonized the tissue; this took 30 to 60 d. As well as this long incubation time, the possibility of contamination was extremely high, when the technique was tested in this project. This may be due to saprophytes remaining in the blocks, although they were autoclaved

several times. Besides the rubber block technique, Idris *et al.* (2004 b) also proposed an alternative artificial inoculation technique, but the method was tedious and difficult to work with. The inoculation technique which was developed in this project may benefit many plant pathologists working on the *G. boninense*-oil palm interaction.

The application of ergosterol analysis for quantification of fungal biomass has been reported in other systems including; *Calluna*: *Hymenoscyphus ericae* (Genney, Alexander and Hartlet, 2000), fungal biomass in atmospheric aerosols (Lau *et al.,* 2005) and moldy bread caused by *Rhizopus nigricans* (Parsi and Gorecki, 2006). It has also been reported in oil palm but with other pathogens, such as *Hydnum* and *Polyporus* (Paterson *et al.,* 2000), but this is the first time it has been applied to the oil palm-*G. boninense* interaction. To date, no research has been published which compared BSR disease score and ergosterol content. Ergosterol analysis provided a fast and reliable representation of *G. boninense* infection in roots. Other more specific diagnostic methods have recently been developed. These include Loop-Mediated Isothermal Amplification (LAMP) (Iwamoto, Sonobe, and Hayashi, 2003; Tomlinson, Boonham and Dickinson, 2010; Yoshida *et al.,* 2005) and antibody-based lateral flow devices (Tomlinson, Boonham and Dickinson, 2010). Although these methods are available for other pathogens and may be used under field conditions, they are not available for *Ganoderma* infection of oil palm. Ergosterol analysis proved a dependable tool for understanding the relationship between disease development and phenolic accumulation. The MDSS was also a reliable method, as it offered a quick and early indication of *G. boninense* infection, instead of waiting for the establishment of foliar symptoms, which may be too late for any possible treatment. The MDSS detects early infection which takes place on roots, but assessment is somewhat subjective.

Information on the role of phenolic acids in oil palm root against *G. boninense* is scarce. However, through this project, the role of total phenolic acids and three important components, syringic, caffeic and 4-HBA, which were identified via HPLC and LCMS/Q-tof, were documented. The role of these phenolics was related to the establishment of *Ganoderma* infection of roots. There is evidence that phenolics are involved in the resistant of oil palm to *G. boninense.* Future selection of resistant plant material may utilize the selection of progenies with higher content of phenolics, especially syringic acid. Easy detection using the HPLC method developed here may be an important criterion in future breeding programmes or in the selection of palms to be planted in estates. To date, most current selections for resistance to *G. boninense* in oil palm progenies are based on the slow development of foliar symptoms (Akbar, Kusnadi and Ollagnier, 1971; Ariffin *et al.,* 1999; Chung *et al.,* 1994; De Franqueville and Durand-Gasselin, 2005; Durand-Gasselin *et al.,* 2005; Hastjarjo and Soebiarpadia, 1975; Idris *et al.,* 2004 b; 2006; Purba, Purba and Sipayung, 1994; Sharma and Tan, 1990).

The accumulation of phenolic acids may play a role in the defence of oil palm roots against *G. boninense* invasion. However, the amount which is available naturally in roots may not be sufficient to stop invasion of this pathogen. In cultivars tested in future breeding programmes, it would be interesting to evaluate phenolic accumulation in varieties with a higher level of resistance. In addition to this, the ability of *G. boninense* to metabolise low concentrations of the phenolic acids may also enhance the virulence of this pathogen. Since the concentration of the phenolic compounds reported here increased with time, there is a requirement for living host cells to undergo the synthesis. The concept of the accumulation may therefore share some similarities to the concept of phytoalexin accumulation. A key component of the proposal is that phytoalexin

synthesis can be induced in living cells by factors termed endogenous elicitors, which are released from the cells undergoing hypersensitive cell death. The phytoalexins accumulate within the dead cells to inhibit fungal growth and any excess in living tissue is metabolized by the plant when the lesion development has been restricted (Mansfield *et al.*, 2000).

However, in the case of syringic, caffeic and 4-HBA, these compounds were detected early in unchallenged or uninfected roots, but increased profoundly when induced either by the abiotic elicitor, chitosan, or with *G. boninense* alone. There may be a possibility of enhancing and maintaining the amount of these phenolics in oil palm germplasm. If the model of phytoalexins is taken into the discussion, accumulation depends on the rate and duration of phytoalexin synthesis, which will be greatly affected by the speed at which cells are killed by the advancing pathogen, and the ability of the fungus or bacterium to tolerate and detoxify the compounds to which they may be exposed. Consideration of these factors has led to the concept of a balance between the phytoalexin production by the plant and phytoalexin degradation by the pathogen occurring at microsites within the infected tissue (Mansfield *et al.*, 2000).

However, with three genotypes examined in this research, the speed of phenolic accumulation was still not sufficient to totally inhibit *G. boninense*, as the seedlings became infected by the end of the experiment. Infection was, however, reduced in the interactions with highest phenolic acid accumulation which was stimulated by addition of exogenous chitosan. It is proposed that detoxification of phenolic acids by *Ganoderma boninense* is analogous to phytoalexin degradation. The most probable scenario for disease development relates to the balance between phenolic acid production by oil palm roots and degradation by the pathogen. Roots of the cultivars tested here failed to accumulate sufficient

phenolic acids to restrict the invasion of the pathogen, before being degraded by *G. boninense*.

With the knowledge that two of the three phenolic acids detected are fungitoxic to G. *boninense*, a strategy may be feasible of applying high amounts of syringic acid and caffeic acid to oil palm roots before the infection occurs, and may help to prevent a BSR outbreak. A formulation of a biofungicide which is derived from a combination of the appropriate concentration of phenolic acids may help in reducing the incidence of BSR. Since the phenolic acids are easily and readily available in the market, an active product may be prepared by purchasing the compounds and formulating with adjuvants such as surfactants and emulsifiers, for a better absorption into the oil palm roots. A suitable delivery system maybe needed to supply the product to the target.

But a more practical, faster and reliable solution maybe the introduction of chitosan to the oil palm to stimulate phenolic acid synthesis before infection. Chitosan is easily and cheaply available from the abundant waste processing of the crustacean products in Sabah. In 2006, the export of crustaceans, molluscs and other aquatic invertebrates, either as prepared or preserved products, from Malaysia was over 9000 tonnes (Department of Fisheries Malaysia, 2010). Currently, there are more than 22 prawn processors/exporters registered with the Department of Fisheries Sabah. Should the proposal succeed, the move will not only help to boost the accumulation of phenolic acids in the oil palm root, and suppress the occurence of BSR, but the use of chitin or chitosan from crustacean shells will also reduce the environmental problem from the processing factories.

The concentration of chitosan used in this project has taken environment issues into consideration. The concentration incorporated into the oil palm substrate tested (0.1%) was the minimum that stimulated a significant effect of phenolic acid accumulation. This level of chitosan

incorporation could be used in palm estates, without risk to the environment or to the plants (Guo, Hu and Ao, 2009; United States Environmetal Protection Agency, 2010). However, higher concentrations of chitosan were not tested in this project and it may be feasible to evaluate these in future work, to determine whether increased concentrations give enhanced disease control. Ganeson and Supramaniam (2009) reported higher concentrations of chitosan gave a reduction in *G. boninense* mycelial growth *in vitro* and the highest percentage inhibition of radial growth was observed with chitosan at a concentration of 2% w/v. Such high levels may, however, be impractical for soil incorporation in oil palm estates.

Moreover, in the work reported here, chitosan was applied was made to one year-old seedlings, simultaneously with inoculation with *Ganoderma*. In future work, it would prove interesting to pre-treat plants with chitosan at intervals before challenge with the pathogen, to ascertain whether pre-incubation could elicit advanced accumulation of antifungal phenolics, such as syringic acid, and thus further limit infection.

Chitosan has also been used to ameliorate soils contaminated with copper and cadmium (Guo, Hu and Ao, 2009). According to the United States Environmental Protection Agency (2010), chitosan is used primarily as a plant growth enhancer, and as a substance to boost plants' defence against fungal infection. It is approved for use outdoors and indoors on many plants, both grown commercially and by consumers. Given its low potential for toxicity and its abundance in the natural environment, chitosan is not expected to harm people, pets, wildlife, or the environment when used according to label directions. To date, there is no official report on the use of chitosan in managing BSR in oil palm.

Some of the older generation of fungicides such as benomyl, thiram, bromoconazole, hexaconazole, triadefon, triadimenol and tridemorph have been used to attempt control of BSR. The results showed that

bromoconazole and hexaconazole, both triazoles, were effective in delaying the death of infected palms, in comparison with some other fungicides but the palms collapsed by the end of the experiment (Idris *et al.*, 2002; 2004; Teh, 1996). Alternatively, the latest generation of fungicides may provide a better remedy to the present BSR situation. The discovery of a new group, the succinate dehydrogenase inhibitors (SDHIs), may provide a solution.

SDHI fungicides play an important role in plant protection against many phytopathogenic fungi. Succinate dehydrogenase (Complex II or succinate-ubiquinone oxidoreductase) is the smallest complex in the respiratory chain and transfers the electrons derived from succinate directly to the ubiquinone pool. SDHI fungicides specifically inhibit fungal respiration by blocking the ubiquinone-binding sites in the mitochondrial complex II and play an important role in the integrated management programmes of many plant diseases. In contrast to first generation of SDHI fungicides (e.g. carboxin), which is exceptionally active only against basidiomycetes, newer active ingredients in this class (e.g. boscalid, penthiopyrad, isopyrazam) show a broad-spectrum activity against various fungal species (Avenot and Michailides, 2010). Due to their unique mode and site of action, they show no cross-resistance with other chemical classes such as strobilurins, benzimidazoles or anilinopyrimidines and therefore are excellent candidates for managing fungicide resistance development and optimizing disease control (Avenot, Morgan and Michailides, 2008; Stamler *et al.*, 2007; Zhang *et al.*, 2007). SDHI fungicides may provide a promising chemical control strategy for the current *G. boninense* problem in South East Asia, compared to other fungicides in the market.

However, BSR is a soil borne disease and the delivery of the chemical to the target by soil drenching or injection is extremely difficult, due to the tropical environment. Such factors include heavy rainfall, which may lead to more run off and cause soil or water pollution. Alternatively, a

trunk injection can be carried out, but it is impractical due to the number of palms that need to be injected in one estate. Furthermore, the consistent use of site-specific fungicides, such as SDHIs, can result in the selection of resistant fungal genotypes which may ultimately lead to a rapid decline of fungicide performance (Avenot and Michailides, 2010).

If chemical control is not a sustainable and practical approach for eradicating BSR in hot and humid tropical countries, perhaps a more feasible holistic approach is exploiting the rich biodiversity leading to the development of biological control. Unfortunately, the microbial biodiversity of Malaysia is poorly understood and information is not well documented. Numerous trials looking for potential antagonistic microorganisms against *G. boninense* ended with inconclusive results. Current research on potential biological control using the endophytes such as *A. radiobacter* and *B. cepacia* (Maizatul and Idris, 2009) are not promising, as discussed in section 1.6.3. Future research should focus on microorganisms that are found in wild ecosystems, before the monoculture of oil palm potentially reduces microbial diversity of the soil.

The development of oil palm resistant varieties may provide a long term solution for the control of *G. boninense* infection. However, the attempt to work with oil palm with differing susceptibility, from MPOB or from Peninsular Malaysia, was hampered due to restrictions and the secretive nature of the palm oil industry. This project was therefore undertaken with the best three available varieties (AVROS, Calabar and Ekona) from Sabah, which were claimed to have different BSR susceptibility. Variation in susceptibility to this pathogen has been detected within the two *Elaeis* species, *guineensis* and *oleifera*. Within *Elaeis guineensis*, material of Deli origin is highly susceptible compared to material of African origin. It is also possible to detect differences in reaction between parents and between crosses within a given origin. The variability

of resistance to BSR within the same cross is also illustrated by the diverse responses of clones derived from palms of the same origin (Durand-Gasseli *et al.,* 2005). The differences in the genetic components that may contribute to different susceptibility to the pathogen, either between the species *guineensis* and *oleifera*, or among the varieties such as AVROS, Calabar and Ekona may a very interesting topic for further investigation. Is the content of phenolic acids higher in *oleifera* compared to *guineensis*? Or is there another defence mechanism that may be involved in resistance against this pathogen, which should be the future direction of any related research?

The completion of the oil palm genome sequencing in 2009 by MPOB and the St. Louis-based Orion Genomics, on three oil palm genomes from two species, provides a comprehensive genetic blueprint of *E. oleifera* and *E. guineensis*, including the pisifera and dura palms. In commercial seed production, the pisifera is frequently used as the paternal source and the dura serves as the maternal line. Knowledge of the genomic sequence of these oil palm varieties will enable researchers to understand genetic differences between trees that are, for example, higher yielding or more resistant to disease (Schill, 2009). Ramli and Abdullah (2010) reported the MT3-B promoter in oil palm may potentially be manipulated for use in plant genetic engineering for induced synthesis of gene products. It may be possible to enhance the resistance mechanisms in transgenic plants. Research involving genetically modified organisms (GMOs) in Malaysia is currently conducted on a small scale, mainly in government-funded research institutions and universities. As a developing country, Malaysia foresees much activity involving GMOs in the future and has therefore established a Genetic Modification Advisory Committee (GMAC), which is under the Ministry of Science, Technology and Innovation, Malaysia (Abdullah *et al.,* 2006). Use of GM oil palm may ultimately be permitted as

Malaysia has approved the importation of Roundup Ready soybean, NK603, MON810 and MON863. The Malaysian Biosafety Act 2007 was approved by the House of Representatives and entered into law on 1 December 2009. Currently, the draft genetically modified organisms (GMO) labelling regulation is being prepared to regulate the placement on the market and label products derived from GMOs (Kaur *et al.*, 2010).

The breeding of disease resistance varieties in the future should not ignore other plant disease models that are related to *G. boninense* on oil palm. In Papua New Guinea, *G. boninense* was abundant as saprophyte on dead coconut (*Cocos nucifera*) trunks and stumps. Coconut appears to be resistant to rots by *Ganoderma* spp. and indeed to many other palm pathogens (Pilotti, 2005). Thus, *G. boninense* is widely believed to live as a saprophyte on the dead coconut palms, while waiting to parasitize oil palms, as their more favoured host. If *G. boninense* can become a pathogen to oil palm, why not to coconut? Coconut belongs to the family of Arecacea, the same as oil palm. Syringic, caffeic acid and 4-HBA are derived from the shikimate or succinylbenzoate pathways, which produces the phenyl propanoids derivatives (C_6-C_3) (Bhattacharya, Sood and Citovsky, 2010). Do coconuts produce a high content of these phenolics in nature? Or do coconuts have an alternative, better defence mechanism? Any resistant genes which can be introduced from coconut against this pathogen, may lead to better oil palm breeding programmes in South East Asia. The defence mechanisms of coconut against *G. boninense* may need to be further explored in the future. Perhaps the biolistic method, which successfully introgressed the *cry* genes from *Bacillus thuringiensis*, and was effective in conferring resistance towards insect pests into crops such as maize and rice, could be used in oil palm to construct a GM plant which is resistant to *G. boninense* (Lee, Yeun and Abdullah, 2006). Other approaches such as *Agrobacterium*-mediated transformation can also be

considered. But the compatibility of these genes in oil palm and many other factors such as the quality of oil and yield may need further consideration. Ever since the initial discovery of the molecules and genes involved in disease resistance in plants, attempts have been made to engineer durable disease resistance in economically important crop plants (Hain *et al.*, 1993; He and Dixon, 2000; Hipskind and Paiva, 2000; Rommens and Kishore, 2000). Unfortunately, many of these attempts have failed (Hain *et al.*, 1993; He and Dixon, 2000; Hipskind and Paiva, 2000; Rommens and Kishore, 2000), owing to the complexity of disease-resistance signalling and the sheer diversity of infection mechanisms that different pathogens use. Although disease resistant transgenic plants may or may not available commercially, future development seems likely, as our current level of understanding of pathogenesis and plant defence improves (Stuiver and Custers 2001).

In conclusion, the artificial inoculation method developed in this project provided a fast, reliable and reproducible technique for future work. The ergosterol assay and MDSS assisted faster detection and quantification of *G. boninense* infection of oil palm roots. Screening for high content of phenolic acids, especially syringic and caffeic acids, could become a powerful tool in a breeding screen, as it provides results quicker than field trials and symptom development. The application of chitosan to stimulate host defence is also a promising avenue to explore more fully in future research.

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Appendix 1

Alignment Report of 'GB seq alignment.meg' - ClustalW (Slow/Accurate, IUB) : Sunday, July 11, 2010 7:21 PM

```
      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  Majority
      ---------+---------+---------+---------+---------+-
               10        20        30        40        50
      ---------+---------+---------+---------+---------+-
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  G1_5
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  EU841913
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  EU701010
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  AJ627585
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  AY220544
   1  G T A C A C A C C G C C C G T C G C T A C T A C C G A T T G A A T G G C T T A G T G A G G T C T T G  AY569450
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  AY593865

      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  Majority
      ---------+---------+---------+---------+---------+-
               60        70        80        90        100
      ---------+---------+---------+---------+---------+-
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  G1_5
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  EU841913
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  EU701010
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  AJ627585
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  AY220544
  51  G G A T T G G C T T C G G G G A G C C G G C A A C G G C A C C C T G T C G C T G A G A A C T T G A T  AY569450
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  AY593865

      - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - G T T T C C G T A G G  Majority
      ---------+---------+---------+---------+---------+-
               110       120       130       140       150
      ---------+---------+---------+---------+---------+-
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - T C C G T A G G  G1_5
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - C C T T C C G T A G G  EU841913
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - C C T T C C G T A G G  EU701010
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - A A C A A G G T T T C C G T A G G  AJ627585
   1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  AY220544
 101  C A A A C T T G G T C A T T T A G A G G A A G T A A A A G T C G T A A C A A G G T T T C C G T A G G  AY569450
```

```
  1 ----------------------TTGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGG AY593865

    TGAACCTGCGGAAGGATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCC Majority
    ----------+----------+----------+----------+----------+-
              160        170        180        190        200
    ----------+----------+----------+----------+----------+-
  9 TGAACCTGCGGAAGGATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCC G1_5
 12 TGAACCTGCGGAAGGATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCC EU841913
 12 TGAACCTGCGGAAGGATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCC EU701010
 18 TGAACCTGCGGAAGGATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCC AJ627585
  1 ----------------------------TCGAGTTTTGACTGGGTTGTAGCTGGCC AY220544
151 TGAACCTGCGGAAGGATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCC AY569450
 35 TGAACCTGCGGAAGGATCATTATCGAGTTTTGACTGGGTTGTAGCTGGCC AY593865

    TTCCGAGGCATCGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTT Majority
    ----------+----------+----------+----------+----------+-
              210        220        230        240        250
    ----------+----------+----------+----------+----------+-
 59 TTCCGAGGCATCGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTT G1_5
 62 TTCCGAGGCATCGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTT EU841913
 68 TTACGAGGCATYGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTT EU701010
 29 TTCCGAGGCATCGTGCACGCCCTGATCATCCACTCTACACCTGTGCACTT AJ627585
201 TTACGAGGCATTGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTT AY220544
 85 TTCCGAGGCACTGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTT AY569450
                                                        AY593865

    ACTGTGGGTTATGGAT-GTGTGGAGCGAGCTCGTTCGTTTGACGAGCTTG Majority
    ----------+----------+----------+----------+----------+-
              260        270        280        290        300
    ----------+----------+----------+----------+----------+-
109 ACTGTGGGTTATGGATTGTGTGGAGCGAGCTCGTTCGTTTGACGAGTTTG G1_5
112 ACTGTGGGTTATAGATCGTGTGGAGCGAGCTCGTTCGTTTGACGAGTTTG EU841913
112 ACTGTGGGTTATAGATCGTGTGGAGCGAGCTCGTTCGTTTGACGAGTTTG EU701010
118 ACTGTGGGTTACGGAT--CGTGGAGCGGGCTC------TTCGCGGAGCTTG AJ627585
 79 ACTGTGGGTTATAGATGGCGTGGTAAGAGCTCGTTCGTTTAACGAGCTTG AY220544
251 ACTGTGGGTTACGGAT--CGTGGAGCGGGCTC------TTCGCGGAGCTTG AY569450
135 ACTGTGGGTTACGGAC--TGTGGAGCGGGCTC------T--GCGGAGCTCG AY593865
```

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     C G A A G C G C G T C T G T G C C T G C G T T T T - A T C A C A A A C A C T A T A A A - G T A T T A  Majority
     ----------------+-----------------+-----------------+-----------------+-----------------+-
                     310               320               330               340               350
     ----------------+-----------------+-----------------+-----------------+-----------------+-
159  C G A A G C G C G T C T G T G C C T G C G T T T T T A T C A C A A A C A C T A T A A A - G T A T T A  G1_5
162  C G A A G C G C G T C T G T G C C T G C G T T T T - A T C A C A A A C A C T A T A A A - G T A T T A  EU841913
162  C G A A G C G C G T C T G T G C C T G C G T T T T - A T C A C A A A C A C T A T A A A - G T A T T A  EU701010
161  T G A A G C G C T T C T G T G C C T G C G T T T T - A T A A C A A A C A C T T T A A A A A G T A T T A  AJ627585
129  C G A A G C G C G T C T G T A C C T G C G T T T T - A C C A C A A A C A C T A T A A A - G T A T T A  AY220544
294  T G A A G C G C T T C T G T G C C T G C G T T T T - A C A A C A A A C A C T T T A A A A A G T A T T A  AY569450
176  T G A A G C G C G T C T G T G C C T G C G T T T T - A T T A C A A A C A C T A T A A A - G T C T T A  AY593865

     G A A T G T G T A T T G C G A T G T A A C G C A T C T A T A T A C A A C T T T C A G C A A C G G A T  Majority
     ----------------+-----------------+-----------------+-----------------+-----------------+-
                     360               370               380               390               400
     ----------------+-----------------+-----------------+-----------------+-----------------+-
208  G A A T G T G T A T T G C G A T G T A A C G C A T C T A T A T A C A A C T T T C A G C A A C G G A T  G1_5
210  G A A T G T G T A T T G C G A T G T A A C G C A T C T A T A T A C A A C T T T C A G C A A C G G A T  EU841913
210  G A A T G T G T A T T G C G A T G T A A C G C A T C T A T A T A C A A C T T T C A G C A A C G G A T  EU701010
210  G A A T G T G T A T T G C G A T G T A A C G C A T C T A T A T A C A A C T T T C A G C A A C G G A T  AJ627585
177  G A A T G T G T A T T G C G A T G T A A C G C A T C T A T A T A C A A C T T T C A G C A A C G G A T  AY220544
343  G A A T G T G T A T T G C G A T G T A A C G C A T C T A T A T A C A A C T T T C A G C A A C G G A T  AY569450
224  G A A T G T G T A T T G C G A T G T A A C G C A T C T A T A T A C A A C T T T C A G C A A C G G A T  AY593865

     C T C T T G G C T C T C G C A T C G A T G A A G A A C G C A G C G A A A T G C G A T A A G T A A T G  Majority
     ----------------+-----------------+-----------------+-----------------+-----------------+-
                     410               420               430               440               450
     ----------------+-----------------+-----------------+-----------------+-----------------+-
258  C T C T T G G C T C T C G C A T C G A T G A A G A A C G C A G C G A A A T G C G A T A A G T A A T G  G1_5
260  C T C T T G G C T C T C G C A T C G A T G A A G A A G A A C G C A G C A G G                          EU841913
260  C T C T T G G C T C T C G C A T C G A T G A A G A A G A A C G C A G C A G G                          EU701010
260  C T C T T G G C T C T C G C A T C G A T G A A G A A C G C A G C G A A A T G C G A T A A G T A A T G  AJ627585
227  C T C T T G G C T C T C G C A T C G A T G A A G A A C G C A G C G A A A T G C G A T A A G T A A T G  AY220544
393  C T C T T G G C T C T C G C A T C G A T G A A G A A C G C A G C G A A A T G C G A T A A G T A A T G  AY569450
274  C T C T T G G C T C T C G C A T C G A T G A A G A A C G C A G C G A A A T G C G A T A A G T A A T G  AY593865

     T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T G A A C G C A C C T T G C G C T  Majority
     ----------------+-----------------+-----------------+-----------------+-----------------+-
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                460                 470                 480                 490                 500
    ----------------+-------------------+-------------------+-------------------+-------------------+-
308 T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T G A A C G C A C C T T G C G C T  G1_5
297                                                                                                    EU841913
297                                                                                                    EU701010
310 T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T G A A C G C A C C T T G C G C T  AJ627585
277 T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T G A A C G C A C C T T G C G C T  AY220544
443 T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T G A A C G C A C C T T G C G C T  AY569450
324 T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T G A A C G C A C C T T G C G C T  AY593865

    C C T T G G T A T T C C G A G G A G C A T G C C T G T T T G A G T G T C A T G A A A T C T T C A A C  Majority
    ----------------+-------------------+-------------------+-------------------+-------------------+-
                510                 520                 530                 540                 550
    ----------------+-------------------+-------------------+-------------------+-------------------+-
358 C C T T G G T A T T C C G A G G A G C A T G C C T G T T T G A G T G T C A T G A A A T C T T C A A C  G1_5
297                                                                                                    EU841913
297                                                                                                    EU701010
360 C C T T G G T A T T C C G A G G A G C A T G C C T G T T T G A G T G T C A T G A A A T C T T C A A C  AJ627585
327 C C T T G G T A T T C C G A G G A G C A T G C C T G T T T G A G T G T C A T G A A A T C T T C A A C  AY220544
493 C C T T G G T A T T C C G A G G A G C A T G C C T G T T T G A G T G T C A T G A A A T C T T C A A C  AY569450
374 C C T T G G T A T T C C G A G G A G C A T G C C T G T T T G A G T G T C A T G A A A T C T T C A A C  AY593865

    C T A C A A G - - C T T T T T A A X G G X T T T G T A G G C T T G G A C T T G G A G G C T T G T C G  Majority
    ----------------+-------------------+-------------------+-------------------+-------------------+-
                  560                 570                 580                 590                 600
    ----------------+-------------------+-------------------+-------------------+-------------------+-
408 C T A C A A T - - C T C T T T G C G G T T C T T G T A G G C T T G G A C T T G G A G G C T T G T C G  G1_5
297                                                                                                    EU841913
297                                                                                                    EU701010
410 C T A C A A G - - C T T T T T A A T G G G T T T G T A G G C T T G G A C T T G G A G G C T T G T C G  AJ627585
377 C T A C A A T - - C T C T T T G C G G T T T T T G T A G G C T T G G A C T T G G A G G C T T G T C G  AY220544
543 C T A C A A G - - C T T T T T A A T G G G T T T G T A G G C T T G G A C T T G G A G G C T T G T C G  AY569450
424 C T A C A A G G T C T T T G T A A A G G C T T T T T A G G C T T G G A C T T G G A G G C T T G T C G  AY593865

    G T C T T T - A T T G G T C G G C T C C T C T C A A A T G C A T T A G C T T G G T T C C T T - G C G  Majority
    ----------------+-------------------+-------------------+-------------------+-------------------+-
                  610                 620                 630                 640                 650
    ----------------+-------------------+-------------------+-------------------+-------------------+-
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456 G T C T T T T A T T G A T C G G C T C C T C T C A A A T G C A T T A G C T T G G T T C C T T T G C G  G1_5
297                                                                                                  EU841913
297                                                                                                  EU701010
458 G T C T T T - A T T G G T C G G C T C C T Y T C A A A T G C A T T A G C T T G G T T C C T T - G C G  AJ627585
425 G T C T T T T A T T G A T C G G C T C C T C T C A A A T G C A T T A G C T T G G T T C C T T T G C G  AY220544
591 G T C T T G - A T T G G T C G G C T C C T C T C A A A T G C A T T A G C T T G G T T C C T T - G C G  AY569450
474 G T C T T T - A T A G G T C G G C T C C T C T T A A A C G C A T T A G C T T G A T T C C T T - G C G  AY593865

    G A T C G G C T T G T C G G T G T G A T A A T - - - G T C T A C G C C G C G A C C G A G A A G C G T  Majority
    ----------------+----------------+----------------+----------------+----------------+-
                    660              670              680              690              700
    ----------------+----------------+----------------+----------------+----------------+-
506 A A T C G G C T - G T C G G T G T G A T A A T - - - G T C T A C G C C G C G A C C G T G A C G C G T  G1_5
297                                                                                                  EU841913
297                                                                                                  EU701010
506 G A T C G G C T T G T C G G T G T G A T A A T - - - G T C T A C G C C G C G A C C G A G A A G C G T  AJ627585
475 A A T C G G C T T G T C G G T G T G A T A A T A A T G T C T A T G C C G C G G C T G A A A C G C G T  AY220544
639 G A T C G G C T T G T C G G T G T G A T A A T - - - G T C T A C G C C G C G A C C G A G A A G C G T  AY569450
522 G A T C G G T T T G T C G G T G T G A T A A T - - - G T C T A C G C C G C G A C C G T G A A G C G T  AY593865

    T T T - - G G C G A G C T T C T A A C C G T C T C T G T A T X G A G A C A A X X C T T A T G A C C T  Majority
    ----------------+----------------+----------------+----------------+----------------+-
                    710              720              730              740              750
    ----------------+----------------+----------------+----------------+----------------+-
552 T T - - - G G C G A G C T T C T A A C C G T C C C G T T A T T G G G A C A A C G C T T A T G A C C T  G1_5
297                                                                                                  EU841913
297                                                                                                  EU701010
553 T T T T G G G C G A G C T T C T A G T C G T C T C T G T A T A G A G A C A A T - C T T A T G A C C T  AJ627585
525 T T - - - G G C G A G C T T C T A A C C G T C C C G T T A T T G G G A C A A C T C T T               AY220544
686 T T T T G G G C G A G C T T C T A G T C G T C T C T G T A T A G A G A C A A T - C T T A T G A C C T  AY569450
569 T T T - - G G C A A G C T T C T A A C G G T C T C T G T A T G G A G A C A A A G C T T A T G A C C T  AY593865

    C T G A C C T C A A A T C A G G T A G G A C T A C C C G C T X X X X X X X X X X X X X X X X X X X X  Majority
    ----------------+----------------+----------------+----------------+----------------+-
                    760              770              780              790              800
    ----------------+----------------+----------------+----------------+----------------+-
599 C T G A C C T C A A A T C A G G T A G G A C T A C C C G C T G A A C T T A A G C A T A T C A A T A A  G1_5
297                                                                                                  EU841913
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297                                                                                                  EU701010
602  C T G A C C T C A A A T C A G G T A G G A C T A C C C G C T                                     AJ627585
564                                                                                                  AY220544
735  C T G A C C T C A A A T C A G G T A G G A C T A C C C G C T G A A C T T A A G C                 AY569450
617  C T G A C C T C A A A T C A G G T A G G A C T A C C C G C T G A A C T T A A G C A T A T C A A T A A AY593865

     X X X X X X X X                                                                                 Majority
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649  G C G G A                                                                                       G1_5
297                                                                                                  EU841913
297                                                                                                  EU701010
631                                                                                                  AJ627585
564                                                                                                  AY220544
774                                                                                                  AY569450
667  G C G G A G G A                                                                                 AY593865
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