Full Length Article

The Antagonistic Effect of Phenolic Compounds on Ligninolytic and Cellulolytic Enzymes of *Ganoderma boninense*, Causing Basal Stem Rot in Oil Palm

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Abstract

Ganoderma sp. is a white-rot fungus and the causal pathogen of basal stem rot (BSR) in oil palm. To identify a potential chemical control for *Ganoderma*, ten naturally occurring plant phenolic compounds, namely benzoic acid, curcumin, 2,6-dimethoxyl benzoic acid, 2,6-dimethoxyl phenol, guaiacol, ferulic acid, pyrocatechol, salicylic acid, syringic acid and vanillic acid were assessed for their antagonistic effect on *Ganoderma* as well their effect on the secretion of wood-degrading enzymes. Mycelial inhibition was dose dependent in each experiment and ranged from 4.4% to 100%. All the phenolic compounds except ferulic acid completely inhibited the growth of *Ganoderma* in a 'poisoned food' test using the range of concentrations that were tested. However, liquid media cultures containing low concentrations of phenolic compounds showed increased biomass production. Microscopic observations revealed that mycelia growing on media containing phenolic compounds showed deterioration. A significant decrease in the production of ligninolytic enzymes, as well as cellulose, amylase and xylanase was identified. The *in vitro* tests revealed that benzoic acid had the greatest inhibitory effect. The selected phenolic compounds inhibited the growth as well as the production of wood degrading enzymes indicating their potential use as chemical controls against *Ganoderma* for disease management of BSR. © 2017 Friends Science Publishers

Keywords: Ganoderma; Basal stem rot; Phenolic compounds; Ligninolytic enzymes; Cellulolytic enzymes

Introduction

The oil palm (*Elaeis guineensis*) is native to Africa but was introduced to British colony of Malaya by the British in 1870. Oil palm produces an annual yield of approximately 4 tons of oil per hectare (Sapak *et al.,* 2008). In terms of the world market, Malaysia and Indonesia are responsible for 84% of global palm oil production and, hence, oil palm is known as the 'golden crop' of Malaysia. The global palm oil demand is increasing rapidly year by year. To meet this demand the oil palm plantation areas, are expanding every year. However, oil palm is prone to various diseases such as *Phytophthora*, *Fusarium* wilt and the *Ganoderma* diseases upper (USR) and basal stem rot (BSR, which can greatly reduce the yield of oil palm) (Goh *et al.,* 2015).

BSR disease is caused by *Ganoderma* species (Susanto *et al.,* 2005) belonging to the family Ganodermataceae. BSR is considered to be the most devastating disease affecting the oil palm industry in Malaysia and Indonesia, causing an economic loss of about $500 million USD a year (Ommelna *et al.,* 2012). Fifteen species of *Ganoderma* have been identified from various parts of the world as the causal agent of BSR (Turner, 1981). *Ganoderma boninense* is the most virulent of these species and causes the most damage to the oil palm industry (Susanto *et al.,* 2005). *G. boninense* was first identified in a 30-year-old palm (Thompson, 1931) however, it has since been detected in palms as young as 12 to 24 months old (Ariffin and Idris, 1996). BSR is spread when a healthy oil palm root comes into contact with an infected root; the other mode of infection spread is by the spores (Thompson, 1931).

In general, species belonging to the Ganodermataceae family cause white rot of hardwood tree species by degrading the lignin initially, followed by the degradation of cellulose and related polysaccharides (Hepting, 1971; Liu *et al.*, 2016). *Ganoderma* infection has a lethal effect on oil

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palm by degrading the xylem and tissues of the palm bole, which subsequently restricts water and nutrient uptake from the roots to the upper parts of the plant, which causes stress. This can lead to symptoms such as chlorosis of newly emerged leaves, partially dead or collapsing old fronds, one-sided mottling of the canopy, the appearance of fully elongated but unopened spear leaves at the center of the crown, and flattening of the crown (Goh *et al.,* 2015). Young palms die within 2–3 years of infection, whereas the economic lifespan of mature plants may be prolonged with proper disease management (Corley and Tinker, 2003). The appearance of symptoms and the presence of basidiomata are only exhibited at the final stage of the disease. The progression of the disease is very slow; however, every infected plant will die eventually and, hence, *Ganoderma* is known as the 'silent killer' of oil palm (Naher *et al.,* 2013).

About 90% of the plantations in West Malaysia have BSR and it is said to be almost impossible to keep a field free from *Ganoderma* infection (Sanderson *et al.,* 2000). Although strict clean clearing practices using mechanical, biological and chemical controls are employed to manage the disease to a certain extent the results have not been satisfactory (Möllers *et al.,* 1998). Thus there is a need to find natural compounds possessing antimicrobial activities that can reach the infection site and inhibit the pathogen as well as aiding the defense reaction of the plant. It would also be an advantage if the natural compounds were able to induce resistance in the palms against the pathogen.

Phenols (phenolic compounds) can fulfill the above requirements owing to their biological and chemical properties. When a pathogen attacks a woody plant the metabolic pathways of phenolic compounds are activated (Witzell and Martín, 2008). Phenolic compounds are involved in the synthesis of plant cell walls and lignin and also play a role in plant defense mechanisms against pathogen infection. Phenols can also serve as phytoalexins, acting as signal modulators during pathogen attack and also in pathogen resistance (Hammerschmidt, 2005).

In general, the phenols are widely involved in the host response to the pathogen. Previous studies have shown that lower molecular weight phenols such as benzoic acid, cinnamic acid and their derivatives accumulate during the initial stages of infection of various plants (Bolwell *et al.,* 1985; Farmer 1985; Kurosaki *et al.,* 1986; Aist *et al.,* 1988; Niemann *et al.,* 1991). An example is the accumulation of lignin by etherification of ferulic acid which is a host response to infection (Matern and Kneusel, 1988). Coumarin and salicylic acid are involved in signal transduction (Strasser *et al.,* 1983; Strasser and Matern 1986). Many studies have demonstrated that phenols such as vanillic acid, salicylic acid, benzoic acid, benzoic acid derivatives and syringic acid have antimicrobial activity (Amborabé *et al.,* 2002; Chong *et al.,* 2012; Vio-Michaelis *et al.,* 2012).

The white rot fungi are the most efficient ligninolytic microorganisms. The degradation of lignin is the most crucial part of the disease process and is considered to be

the rate limiting step given that *Ganoderma* expends more energy degrading the lignin (Paterson, 2007). In general, white-rot fungi produce enzymes such as lignin peroxidase, manganese peroxidase, and laccase which are capable of producing strong oxidants to break the structure of lignin in the wood (Kirk and Farrell, 1987). White-rot fungi gain energy by degrading lignin and there by gaining access to celluloses in the wood. The lignin-degrading enzymes of white-rot fungus are extracellular small in size and non-specific. The ligninolytic system of *Ganoderma* involves both peroxidase and laccase for the complete oxidation process (Paterson, 2007). Plants accumulate phenols as a first line stage of defense against pathogen infection (Dekker and Barbosa, 2001). However, *Ganoderma* is able to overcome this first stage of defense in oil palm by producing laccase which oxidizes the phenols. Therefore, the aim of the present study was to evaluate the inhibitory effects of phenols on the growth of *G. boninense* (PER 71). In particular, we examined the effect of different phenols on the mycelia of *G. boninense* and on the secretion of ligninolytic and hydrolytic enzymes.

Materials and Methods

Chemicals

Potato dextrose agar (PDA), potato dextrose broth (PDB), 2,6-dimethoxbenzoic acid, 2,6-dimethoxphenol, guaiacol, ferulic acid, p-coumaric acid, pyrocatechol, salicylic acid, syringic acid and vanillic acid were purchased from Friendemann Schmidt. The malt extract agar (Merck), benzoic acid, tannic acid, Congo red, sodium chloride, starch, peptone, yeast extract and Lugol's iodine were obtained from R and M Chemicals and Reagents. Remazol brilliant blue R (RBBR) and birchwood xylane were obtained from Sigma Aldrich. The carboxymethyl cellulose (CMC) was purchased from HiMedia laboratories.

Microorganism, Culture Conditions and Treatments

The *Ganoderma boninense* (PER 71) strain was obtained from the Malaysian Palm Oil Board (MPOB). The culture was maintained by subculturing the fungus at regular intervals (every 11 days) onto PDA and incubating at 28°C.

Ten phenolic compounds namely benzoic acid, 2,6-dimethoxybenzoic acid, 2,6-dimethoxphenol, guaiacol, ferulic acid, pyrocatechol, salicylic acid, syringic acid, vanillic acid and curcumin were utilized to test their ability to inhibit the growth of *G. boninense* and their ability to inhibit the secretion of ligninolytic and cellulolytic enzymes produced by *G. boninense*.

Growth of *G. Boninense* on Agar Medium Containing Phenolic Compounds: the 'Poisoned Food' Test

Each of the ten phenolic compounds were added separately

to heat-sterilized PDA media at 40°C in different quantities to obtain six different concentrations of each of the phenolic compounds i.e. the PDA media plates contained either 0 (control), 1, 5, 10, 15, 20 or 25 mM of a phenolic compound. The amended plates were then allowed to solidify. All the amended media plates were then inoculated with an agar plug of mycelium (3 mm Ø) that was excised from an actively growing seven-day-old culture of *G. boninense* (Srivastava *et al.,* 2013). The plates were then incubated at 28±2°C until the control plate was fully colonized. The radial growth on all the plates was measured at two perpendicular points from the center and the average value of the replicate plates was taken.

The percentage inhibition of radial growth (PIRG) was measured according to Skidmore and Dickinson (Skidmore and Dickinson, 1976) as follows:

$$PIRG\ (\%) = [(C-T)/C] \times 100 \quad (1)$$

Where C is the average increase in the radial growth of mycelia on the control plate and T is the average increase in the radial growth of mycelia on the treatment plate.

Effect of a Dip Test using Phenolic Compounds on *G. Boninense* Growth

Similar concentrations of the phenols were used for the dip test (Bivi *et al.,* 2012). Most of the phenols were dissolved in hot water at 75°C except for coumaric acid, ferulic acid and syringic acid which were dissolved in an acetone:water mixture (50:50 v v^{-1}) (Chong *et al.,* 2009). A 5 mm Ø mycelial disc was excised from a culture of actively growing *G. boninense* mycelium and dipped in one of the ten phenolic solutions. A mycelial disc dipped in water served as the control. In the case of the ferulic and syringic acids the mycelia discs dipped in the mixture of acetone and water were considered to be negative controls. The prepared discs were then air dried in a laminar airflow chamber and placed firmly onto fresh Petriplates containing 15 mL of PDA medium. Plates were then incubated at a room temperature of 28 ± 2°C for about seven days. The PIRG was then calculated using equation (1).

Growth of *G. Boninense* in Submerged Liquid Medium Containing Phenolic Compounds

0, 1, 5, 10, 15, 20 and 25 mM of each of the phenols were added separately to 250 mL Erlenmeyer flasks containing 40 mL of sterilized Luria-Bertani broth (LB) liquid medium. Each of the flasks was inoculated with three 3-mm Ø mycelial plugs that had been excised from an actively growing seven-day-old culture of *G. boninense*. The flasks were incubated at 28°C constant shaking table (200 rpm) (PROTECH, SI 50) for ten days. The mycelium in each flask was then recovered gravimetrically by centrifuging at 1250 rpm (Eppendorf 5804 R amd 5810) for 30 min and then dried at 70°C for 48 h to a constant weight.

Effect of Phenolic Compounds on *G. Boninense* Morphology

The effect of each of the ten different phenolic compounds at each of the different concentrations (0, 1, 5, 10, 15, 20 or 25 mM) on the morphology of *G. boninense* was evaluated using the slide culture technique with minor modifications (Riddell, 1950). An agar plug square amended with a phenolic compound was placed in the center of a slide on a v rod in a Petridish containing wet filter paper. A needlepoint inoculum of *G. boninense* mycelium was placed at the four corners of the agar gel and covered with a cover slip. This setup was maintained for five days and observed under the light microscope at a 40× magnification.

Effect of Phenolic Compounds on the Production of Ligninolytic Enzymes by *G. Boninense*

To detect the production of ligninolytic enzymes by *G. boninense*, the culture was grown on a medium containing RBBR dye and tannic acid in the presence of a phenolic compound. A Petri plate containing 2% MEA amended with 0.05% of RBBR and one of the ten phenols (0, 1,5,10,15,20 or 25 mM) was inoculated with a 3-mm Ø agar plug excised from a seven-days-old *G. boninense* culture. The Petriplates containing MEA amended only with RBBR and inoculated with/without *G. boninense* were used as control plates. All the plates were incubated at room temperature for about 10 days until the control plate was fully colonized. A decolorization of the dye would confirm the production of ligninolytic enzymes (Machado *et al.,* 2005).

G. boninense cultures were grown on medium containing 5 g of tannic acid, 15 g of malt extract and 20 g of agar l^{-1} and amended with one of the ten phenolic compounds (0, 1, 5, 10, 15, 20 or 25 mM). Medium that was not amended with phenol was used as a control. The prepared media were inoculated with a mycelial disc (3-mm Ø) excised from a seven-day-old culture of *G. boninense* and incubated in the dark for 10 days. The production of a dark-brown color under the colony would indicate the production of ligninolytic enzymes (Okino *et al.,* 2000).

Screening Phenolic Compounds for their Effect on the Production of Hydrolytic enzymes by *G. Boninense*

Effect of phenolic compounds on the cellulase activity of *G. Boninense*: To evaluate the endoglucanase activity, a 3-mm Ø plug excised from an actively growing *G. boninense* culture was cultured on CMC agar media comprising 20 g of CMC and 15 g of agar L^{-1} amended with one of the ten phenols (0, 1, 5, 10, 15, 20 or 25 mM). Medium that was not amended with a phenol served as a control. All plates were incubated in the dark for 10 days. After 10 days, the plates were flooded with Congo red (1 mg L^{-1}) for 15 min, followed by the addition of 1mM NaCl to visualize the degradation of cellulose. A clear yellow or red background

would indicate that the cellulose had been degraded (Pointing, 1999).

Effect of phenolic compounds on the amylase activity of *G. Boninense*: The media was prepared using 2 g of starch, 1 g of peptone, 1 g of yeast extract, 20 g of agar L^{-1} and amended with one of the ten phenols (0, 1, 5, 10, 15, 20 or 25 mM) (Paterson and Bridge, 1994). The media plates were inoculated with a *G. boninense* plug (3-mm Ø) excised from an actively growing *G. boninense* culture and then incubated in the dark for 10 days. The plates were then flooded with Lugol's iodine (0.25% w v^{-1}) for a few seconds. A clear zone around the colony or a purple–black background would indicate the production of amylase.

Effect of phenolic compounds on the xylanase enzyme activity of *G. Boninense*: A medium containing 1g of birchwood xylan, 0.1 g of peptone, 0.01 g of yeast extract and 20 g of agar L^{-1} was used to measure the presence of xylanase activity. The medium was amended with one of the ten phenols (0, 1,5,10,15,20 or 25 mM) (Bucher *et al.,* 2004). The media plates were inoculated with a *G. boninense* plug (3-mm Ø) excised from an actively growing *G. boninense* culture and were incubated in the dark for 10 days. The plates were then flooded with Lugol's iodine (0.25% w v^{-1}). A clear zone around the colony or purple–black background would indicate the production of xylanase.

Statistical Analysis

Data were analyzed using SAS statistical software (PC-SAS software V8.2, SAS Institute, Cary, NC and USA). A *P* value of ≤ 0.05 was considered significant. All the experiments were repeated twice with six replicates of each treatment. The means were compared using Tukey's (HSD) test. A *P* value of ≤ 0.05 was considered significant.

Results

Growth of *G. Boninense* on Agar Medium Containing Phenolic Compounds

All the phenols that were tested significantly inhibited the radial growth of *G. boninense* mycelium at various different concentrations (Fig. 1). The minimum concentration at which each of the phenolic compounds inhibited the growth of *G. boninense* mycelia ranged from 5–15 mM depending on the phenolic compound. Mycelia growth on plates amended with 5 mM benzoic acid or syringic acid or with 15 mM coumaric acid, guaiacol, salicylic acid or vanillic acid was completely inhibited. All the phenolic compounds except ferulic acid completely inhibited the mycelial growth of *G. boninen*se at concentrations of 20 mM and 25 mM. At a concentration of 25 mM ferulic acid only inhibited mycelial growth by 43.4% compared with the growth shown on the control plates. Generally syringic acid and benzoic acid were the most effective phenolic compounds and mycelial growth was significantly more inhibited by

syringic acid even at the lowest concentration compared with the other phenols tested.

The growth of the *G. boninense* mycelium was influenced by the presence of phenols at different concentrations in the media (Table 1). The growth rate of *G. boninense* on the control treatment was 0.16–0.20 cm day^{-1}, while the growth rate of the cultures growing on media containing a phenol ranged from 0.01 to 0.04 cm day^{-1}.

Effect of a Dip Test using Phenolic Compounds on *G. Boninense* Growth

The dip test results revealed that the total inhibition of mycelial growth was only achieved by four of the phenolic compounds and that phenolic concentrations ranging from 15 to 25 mM were required to achieve total inhibition (Fig. 2). Syringic acid and salicylic acid completely inhibited hyphal growth at a concentration of 15 mM, which was significantly greater than the percentage of inhibition achieved at a concentration of 10 mM ($P \leq 0.05$). Benzoic acid and dimethoxybenzoic acid completely inhibited hyphal growth at concentrations of 20 mM and 25 mM respectively. However, the other phenolic compounds inhibited mycelial growth by less than 50%. Dimethoxyphenol and vanillic acid inhibited hyphal growth by up to 42% and 43% respectively at 25 mM however, at lower concentrations (15 and 20 mM) the percentage of hyphal inhibition achieved by the different treatments were not significantly different from each other. Guaiacol, ferulic acid and pyrocatechol 25 mM treatments inhibited hyphal growth by between 30–38% coumaric acid inhibited hyphal growth the least. The growth of *G. boninense* in the positive and the negative controls was significantly different indicating that the inhibition of hyphal growth was caused by the phenols i.e. syringic acid and vanillic acid and not by the solvent.

Growth of *G. Boninense* in Submerged Liquid Medium Containing Phenolic Compounds

Significantly more *G. boninense* biomass formed when the fungus was grown in media containing 1 mM of curcumin, 2,6-dimethoxy benzoic acid 2,6-dimethoxy phenol, guaiacol, ferulic acid, salicylic acid or vanillic acidcompared with the biomass produced in the control flasks. However, at higher phenol concentrations the phenols inhibited biomass production (Fig. 3). All ten phenols used in this study were able to inhibit mycelial growth in liquid culture at concentrations of 20 and 25 mM. Furthermore, media containing 5 mM syringic acid or benzoic acid completely inhibited hyphal growth and media containing 10 mM coumaric acid, guaiacol or salicylic acid significantly inhibited hyphal growth ($P \leq 0.05$).

Effect of Phenolic Compounds on *G. Boninense* Morphology

Microscopic examination of the mycelium grown in media

Table 1: The growth rate of *Ganoderma boninense* in the phnenols incorporated with PDA media observed for 11 days at 32°C
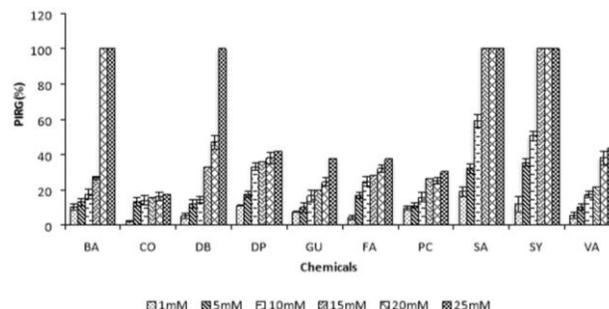
Concentration	BA (cm)	CO (cm)	DB (cm)	DP (cm)	GU (cm)	FA (cm)	PC (cm)	SA (cm)	SY (cm)	VA (cm)
Control	16.49±2.14	18.24±2.57	12.91±3.13	4.45±1.70	9.04±1.14	4.83±1.56	19.50±1.56	19.30±3.33	33.60±2.60	20.07±2.31
1 mM	0.36±0.01	0.32±0.01	0.32±0.01	0.4±0	0.31±0	0.40±0	0.28±0.01	0.33±0.05	0.30±0.01	0.33±0.01
5 mM	0	0.32±0.01	0.282±0.01	0.4±0	0.30±0	0.40±0.01	0.22±0.02	0.20±0.01	0	0.32±0.02
10 mM	0	0.28±0.01	0.19±0.01	0.38±0.01	0.22±0.01	0.38±0	0.19±0.01	0.03±0	0	0.29±0.02
15 mM	0	0	0.11±0.01	0.28±0	0	0.38±0	0.12±0	0	0	0
20 mM	0	0	0	0	0	0.37±0	0	0	0	0
25 mM	0	0	0	0	0	0.35±0	0	0	0	0

BA- BA- benzoic acid, CO - Curcumin, DBA - 2,6- Dimethoxy benzoic acid, DP - 2,6- Dimethoxyl phenol, Gly – Guaiacol, FA - Ferulic acid, PC- pyrocatechol, SA- Salicylic acid, Syg- Syringic acid, VA – Vanillic acid
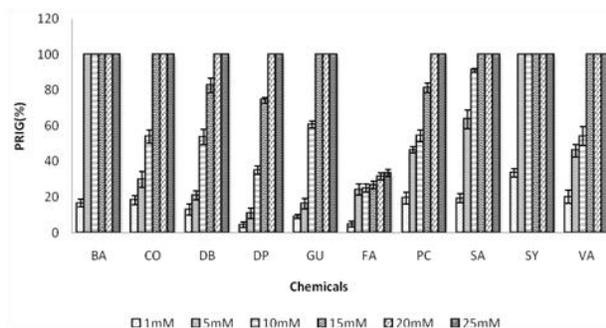
Fig. 1: The percentage inhibition of radial growth (PRIG) of *Ganoderma boninense* in the 'poisoned food'assays with different phenols incorporated into the PDA medium. A vertical bar indicates the standard error of the mean of two experiments each with six replicates (n = 10) according to Tukey's (HSD) test at $P \leq 0.05$. Abbreviations: BA, benzoic acid;CO, curcumin; DB, 2,6-dimethoxy benzoic acid; DP, 2,6-dimethoxy phenol; GU, guaiacol; FA, ferulic acid; PC, pyrocatechol; SA, salicylic acid; SY, syringic acid; VA, vanillic acid

Fig. 2: The percentage inhibition of radial growth (PIRG) of *Ganoderma boninense* by phenolic compounds using the dip test method.The fungal plugs were dipped in different concentrations of each of the ten phenolic compounds, air dried and cultured on PDA plates. A vertical bar indicates the standard error of the mean of two experiments each with six replicates according to Tukey's (HSD) test at $P \leq 0.05$ (n=10). Abbreviations: BA, benzoic acid; CO, curcumin; DB, 2,6-dimethoxy benzoic acid; DP, 2,6-dimethoxy phenol; GU, guaiacol; FA, ferulic acid; PC, pyrocatechol; SA, salicylic acid; SY, syringic acid; VA, vanillic acid

without phenols (control) revealed that the mycelium was smooth, dense and healthy with long, clear and separated hyphae with clamp connections (Fig. 4A). By contrast, the hyphae of *G. boninense* grown in media with phenols were less dense, distorted, intertwined and empty more branches were observed at the tips of the hyphae (Fig. 4). The hyphae treated with benzoic acid and curcumin were thin, lysed and fragmented (Fig. 4B). In the case of dimethoxybenzoic acid, ferulic acid and pyrocatechol, the hyphae were thin with more branches at the tips (Fig. 4C). The hyphae treated with dimethoxybenzoic acid were much thinner when compared to the hyphae subjected to the other two phenol treatments. The hyphal density was reduced in the dimethoxyphenol treatment and split hyphae were also observed. Hyphae treated with guaiacol or vanillic acid showed similar effects i.e. thin and fewer hyphae that were intertwined with one another. At the minimum concentration (1 mM) of salicylic acid the hyphae were densely packed, whereas at higher concentrations the hyphal density was reduced and the hyphae were fragmented (Fig. 4D).
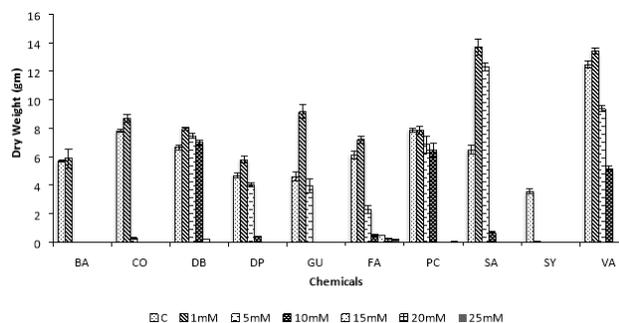
Effect of Phenolic Compounds on the Production of Ligninolytic Enzymes by *G. Boninense*

The *G. boninense* cultures grown on media containing RBBR dye and the phenol treatments showed significantly less decolorization ($P \leq 0.005$) compared with the control (Fig. 5). During the observation period the culture growing on the control plate fully colonized the plate and decolorized the RBBR dye completely. The decolorization of the dye indicated the production of ligninolytic enzymes. Media containing phenols at concentrations ranging from 5 to 20 mM were able to inhibit the growth of *G. boninense* completely, which in turn means that ligninolytic enzymes were not produced by the fungus and hence the media was not decolorized. *G. boninense* growth on media containing 5 mM benzoic acid or salicylic acid was completely inhibited ($P \leq 0.05$) these two phenols were considered to be more inhibitory than the other phenols.

Similarly, the *G. boninense* cultures grown on agar

Fig. 3: The effect of phenolic compounds on the growth of *Ganoderma boninense* in liquid culture, which was observed for ten days. A vertical bar indicates the standard error of the mean of two experiments each with five replicates according to Tukey's (HSD) test at $P \leq 0.05$ (n=10). Abbreviations: BA, benzoic acid; CO, curcumin; DB, 2,6-dimethoxy benzoic acid; DP, 2,6-dimethoxy phenol; GU, guaiacol; FA, ferulic acid; PC, pyrocatechol; SA, salicylic acid; SY, syringic acid; VA, vanillic acid; C, control
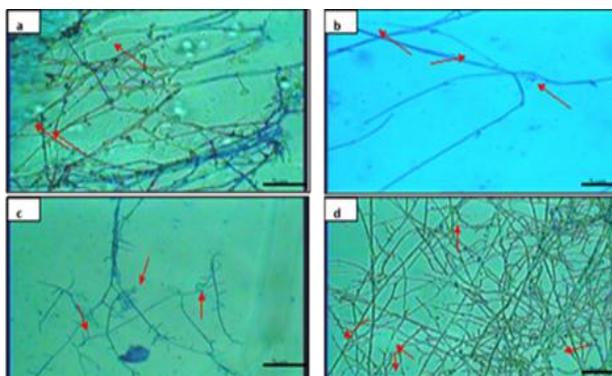

Fig. 4: Different types of *Ganoderma boninense* hyphal damage observed in the slide culture. (a) The arrows indicate the presence of clamp connections in the healthy hyphae. (b) *G. boninense* treated with 1 mM of curcumin: thin and fragmented hyphe were observed. (c) *G.boninense* treated with1 mM dimethoxy benzoic acid: the arrows indicate the ends of thin and multi-branched hyphae. (d) Hyphae of *Ganoderma boninense* treated with 1 mM salicylic acid were densely packed and slightly fragmented

media containing tannic acid and the phenol treatments showed significantly less growth and produced a significantly smaller dark-brown area under the colony compared with the control plates ($P \leq 0.005$) (Fig. 6). The formation of a dark-brown area under the colony signified the secretion of ligninolytic enzymes. Media containing a1mM concentration of benzoic acid, syringic acid or dimethoxybenzoic acid completely inhibited mycelial growth media containing a 5 mM concentration of salicylic
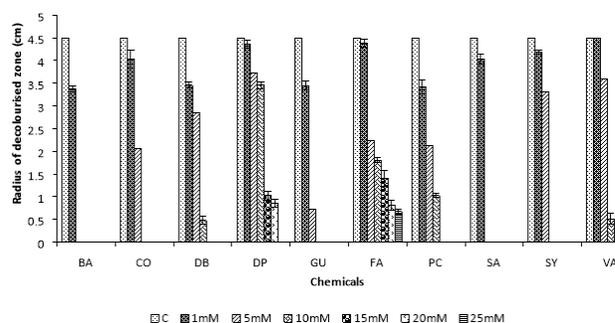
Fig. 5: The effects of phenolic compounds on the decolorization of RBBR dye by *Ganoderma boninese*, indicating the production of ligninolytic enzymes. A vertical bar indicates the standard error of the mean of two experiments each with five replicates according to Tukey's (HSD) test at $P \leq 0.05$ (n =10). Abbreviations: BA, benzoic acid; CO, curcumin; DB, 2,6-dimethoxy benzoic acid; DP, 2,6-dimethoxy phenol; GU, guaiacol; FA, ferulic acid; PC, pyrocatechol; SA, salicylic acid; SY, syringic acid; VA, vanillic acid; C, control
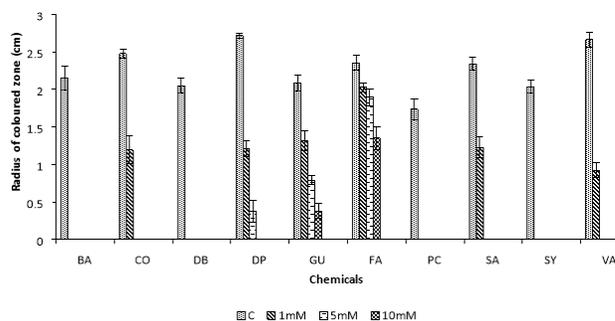

Fig. 6: The enzymatic degradation of tannic acid by *Ganodermaboninese* in the presence of phenolic compounds. A vertical bar indicates the standard error of the mean of two experiments each with five replicates according to Tukey's (HSD) test at $P \leq 0.05$ (n = 10). Abbreviations: BA, benzoic acid; CO, curcumin; DB, 2,6-dimethoxy benzoic acid; DP, 2,6-dimethoxy phenol; GU, guaiacol; FA, ferulic acid; PC, pyrocatechol; SA, salicylic acid; SY, syringic acid; VA, vanillic acid; C, control

acid, vanillic acid or dimethoxy benzoic acid also completely inhibited mycelial growth.

Screening Phenols for their Effect on the Production of Hydrolytic Enzymes by *G. Boninense*

Effect of phenolic compounds on the cellulase enzyme activity of *G. Boninense*: A clear yellow zone of about 2.5 cm was produced in the media by the *G. boninense* cultures growing on the control plates. The *G. boninense* cultures growing on media containing 1mM benzoic acid, pyrocatechol and syringic acid developed significantly
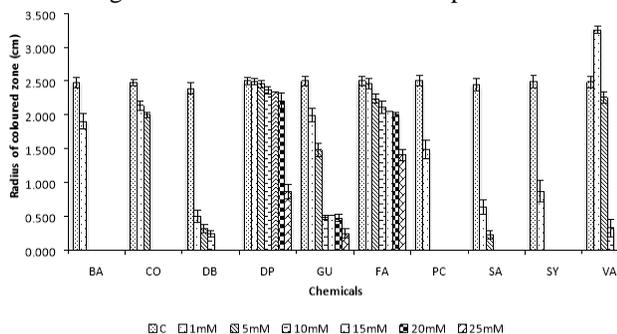
Fig. 7: Radius of the clear yellow zone in the *Ganoderma boninese* culture media. This phenomenon represents the ability to degrade cellulose. A vertical bar indicates the standard error of the mean of two experiments each with five replicates. According to Tukey's (HSD) test at $P \leq 0.05$ (n =10). Abbreviations: BA, benzoic acid; CO, curcumin; DB, 2,6-dimethoxy benzoic acid; DP, 2,6-dimethoxy phenol; GU, guaiacol; FA, ferulic acid; PC, pyrocatechol; SA, salicylic acid; SY, syringic acid; VA, vanillic acid; C, control
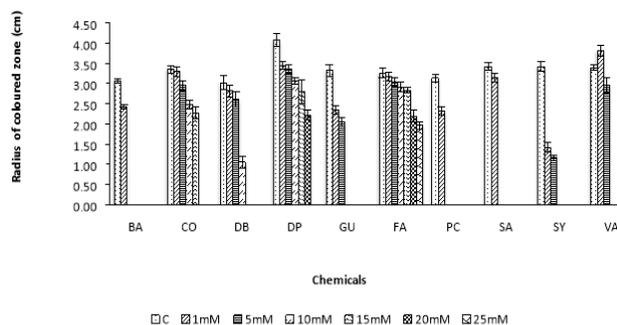
Fig. 8: Radius of the clear zone with a purple–black background in the *Ganoderma boninese* culture media. This phenomenon represents the production of amylase by the fungus. A vertical bar indicates the standard error of the mean of two experiments each with five replicates according to Tukey's (HSD) test at $P \leq 0.05$ (n = 10). Abbreviations: BA, benzoic acid; CO, curcumin; DB, 2,6-dimethoxy benzoic acid; DP, 2,6-dimethoxy phenol; GU, guaiacol; FA, ferulic acid; PC, pyrocatechol; SA, salicylic acid; SY, syringic acid; VA, vanillic acid; C, control

smaller yellow areas of 1.9, 1.4 and 0.6 cm respectively, compared with the control plates ($P \leq 0.05$) (Fig. 7). At higher phenol concentrations the growth of *G. boninense* was completely inhibited and hence, a clear yellow zone did not develop. Mycelial growth on media containing 10mM coumaric acid or salicylic acid was also completely inhibited.

Effect of phenolic compounds on the amylase enzyme activity of *G. Boninense*: A clear zone in the medium around the colony or a purple–black background indicated the degradation of starch. Media containing 5 mM benzoic

acid, pyrocatechol or salicylic acid not only inhibited the degradation of soluble starch but also the growth of *G. boninense* was significantly reduced ($P \leq 0.05$) compared with the control (Fig. 8). A similar effect was observed on media containing 10 mM syringic acid, guaiacol or vanillic acid.

Effect of phenolic compounds on the xylanase enzyme activity of *G. Boninense*: The generation of a clear colony or a purple–black background after flooding the plate with Lugol's solution was confirmation of the production of xylanase. The growth of *G. boninense* completely ceased on media containing 1 mM benzoic acid, guaiacol or salicylic acid or 15 mM coumaric acid or syringic acid. By contrast the control plates were completely colonized by *G. boninense* and showed significantly different growth when compared with the treatment plates ($P \leq 0.05$) (Fig. 9).

Discussion

Numerous methods are being considered to control BSR disease however, none of them has the ability to cure or eradicate the disease completely. The degradation of lignin is the most important aspect of an infection by a white-rot fungus. White-rot fungi degrade lignin to carbondioxide and water, making the cellulose available as a carbon source (Paterson *et al.,* 2008). Therefore, one potential way that could be considered to control the BSR disease of oil palm is to identify potential inhibitors of ligninolytic enzymes (Paterson *et al.,* 2008).

The purpose of this investigation was to elucidate potential inhibitors of ligninolytic enzymes that could be used to manage BSR disease. All the tested phenols showed a stronger inhibitory effect on hyphal growth when applied using the 'poisoned food' method as compared to the dip test. This result is contradictory to the findings of Bivi *et al.* (Bivi *et al.,* 2012) who obtained similar results when investigating the effect of salicylic acid on hyphal growth using the 'poisoned food' method and the dip test. The phenols tested in this study showed fungicidal activity at the higher concentrations and a fungistatic effect at the lower concentrations. Media containing benzoic acid, salicylic acid or syringic acid had the greatest inhibitory effects on mycelial growth and media containing ferulic acid was the least inhibitory. A previous study reported that syringic acid was the best inhibitor of *G. boninense* at the lowest concentration (0.5 mg/mL) followed by 4-hydroxybenzoic acid (1 mg/mL) and caffeic acid (1.5 mg/mL) (Chong *et al.,* 2009).

In this study, the biomass of *G. boninense* increased when growing in media containing low concentrations of phenols, which could be because the white-rot fungus was able to metabolize these aromatic compounds (Buswell *et al.,* 1979; Ander *et al.,* 1980; Eriksson *et al.,* 1984; Buswell and Eriksson, 1994). At higher concentrations the phenols acted as a toxin and inhibited the growth of *G. boninense* completely, which agrees with observations reported by
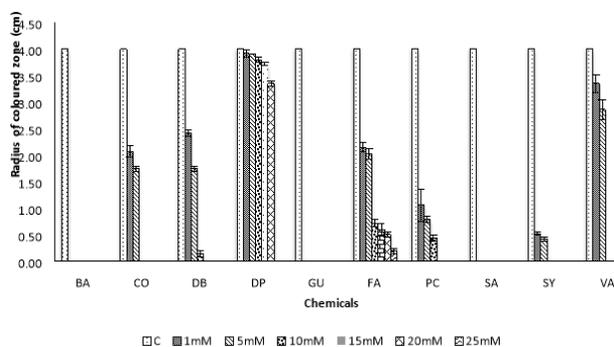
Fig. 9: Radius of the clear zone with a purple–black background in the *Ganoderma boninese* culture medium. This phenomenon represents the degradation of xylane by the fungus. A vertical bar indicates the standard error of the mean of two experiments each with five replicates according to Tukey's (HSD) test at $P \leq 0.05$ (n = 10). Abbreviations: BA, benzoic acid; CO, curcumin; DB, 2,6-dimethoxy benzoic acid; DP, 2,6-dimethoxy phenol; GU, guaiacol; FA, ferulic acid; PC, pyrocatechol; SA, salicylic acid; SY, syringic acid; VA, vanillic acid; C, control

previous studies that were conducted on *Botryosphaeria* sp. (Buswell and Eriksson, 1994; Dekker *et al.,* 2002; Srivastava *et al.,* 2013). Cupul *et al.,* also reported that a low concentration of phenols increased the growth of ligninolytic macro fungi and that aromatic compounds such as atrazine induced the growth of *Pleurotus* sp. and *Caenorhabditis elegans* (Cupul *et al.,* 2014).

The phenolic compounds were screened to determine whether they could inhibit the production of enzymes involved in the degradation of wood (lignin and holocellulose). Among all the wood-degrading enzymes laccase production is considered to be one of the most crucial pathogenicity factors. Laccase plays an important role in fungal morphogenesis, lignin degradation and fungal plant-pathogen–host interactions (Baldrian, 2006). Hence, this supports the idea that the inhibition of ligninolytic enzymes could stop or slow down the infection process (Nun *et al.,* 1988). The secretion of ligninolytic enzymes varies depending on the fungal species and their epidemiology (Elisashvili *et al.,* 2008) as well as the substrate composition and the nitrogen content *Invitro* production of laccase depends on the culture medium however, a medium that supports the maximum production of biomass does not favor the production of ligninolytic enzymes (Jong *et al.,* 1994). The role of laccase is to detoxify the phenols via oxidative coupling and polymerization but this is applicable only up to a certain point and there is evidence that phenols have the ability to inhibit laccase at higher concentrations (Souza *et al.,* 2004). The decolorization of RBBR dye and the tannic acid tests are widely used to examine the production of ligninolytic enzymes. Benzoic and salicylic acids are effective in

completely inhibiting the production of ligninolytic enzymes. In this study, the efficacy of different phenolic compounds on the inhibition of enzymes varied greatly and our results are in agreement with previous studies conducted on *Botryosphaeria* sp. (Srivastava *et al.,* 2013).

The salient feature of *Ganoderma* is its degradation of lignin, which leaves the cellulose exposed (Paterson *et al.,* 2009). In oil palm wood, as well as lignin and cellulose, starch and hemicelluloses (xylan) are also present in considerable amounts. Many fungi utilize starch and hemicelluloses as carbon sources; however, starch and hemicelluloses are considered to be the least important carbon sources for a white-rot fungus (Naidu *et al.,* 2015). The *invitro* study demonstrated that *G. boninense* has the ability to degrade cellulose, starch and xylene, which agrees with the findings reported by Naidu *et al.,* (2015) who investigated the production of cellulolytic enzymes by a white-rot fungus in a Petriplate containing xylane and cellulose. During the infection process the production of amylase, xylanase and cellulase helps the fungus to colonize the oil palm, eventually leading to a loss in mass in the oil palm trunk. Hashim *et al.,* reported that oil palm trunks have high-quality holocelluloses (cellulose and hemicelluloses). Even though lignin degradation is a rate-limiting step in the infection process the production of cellulolytic enzymes plays a crucial role in the degradation of the trunk.

There is an increasing amount of evidence that phenols are associated with plant–pathogen interactions (Nicholson and Hammerschmidt, 1992). As the pathogen invades the plant cell the plant activates the hypersensitive response (HR) with the help of low molecular weight phenolic compounds. Matern and Kneusel (Matern and Kneusel, 1988) have proposed that plants have two types of defensive strategy. The first is the accumulation of a phenolic substance at the pathogen-infected site, which slows down the growth of the pathogen by acting as an antimicrobial compound or by necrotizing the cells. Amborabé *et al.* (Amborabé *et al.,* 2002) demonstrated that salicylic acid and benzoic acid were capable of inhibiting *Eutypalata in vitro* when they mimicked the plant cell environment.

The stimulation of resistance in the plants depends on an interconnected signal transduction pathway in which phenols play a key role. The accumulation of lignin occurs at the early stages of infection and also acts as a marker for induced resistance. This in turn acts as a physical barrier and alters the chemical nature of the lignin and converts it into lignin, which is more resistant to biological decay (Pakusch *et al.,* 1989). A study conducted on cucumber plants reported that when the plants were inoculated with *Cladosporium cucumerinum* lignin deposition was greater in the resistant plants than in the susceptible plants (Hammerschmidt *et al.,* 1984).

Oil palm wood consists of 41.2% cellulose, 34.4% hemicelluloses, 17.1% lignin, 0.5% ash, extractives, and 2.3% is ethanol soluble (Sun and Tomkinson, 2001). The lignin unit syringaldehyde contains 65.6–68.5% of the total

phenolic monomers in the lignin and vanillin is the second most abundant phenol in the lignin. The ligninolytic enzymes are capable of oxidizing phenolic compounds via lipid peroxidase reactions(Ruiz-Dueñas *et al.,* 1999). However, ligninolytic enzymes can degrade the lignin only when the phenolic content is less than the threshold limit (Srivastava *et al.,* 2013). The threshold limit and the priorities of the enzymes have not yet been studied (lignin). However, our study suggests that phenols could have a potential application as a chemical control for BSR disease in oil palm.

Conclusion

In summary, we investigated the effects of various phenols at concentrations ranging from 1 to 25mM on the growth and the biomass production of *G. boninense*, along with the secretion of ligninolytic enzymes such as cellulose, amylase and xylanase. In this *in vitro* study the phenolic substances were found to be toxic to *G. boninense* (PER 71) reducing the production of both the ligninolytic enzymes and the wood degrading enzymes. Benzoic acid, salicylic acid and syringic acid have potential as chemical controls that could be applied to control BSR. To our knowledge this is the first report on the use of phenolic compounds (secondary metabolites) to inhibit the wood-degrading enzymes produced by *G. boninense.* Based on the results obtained *in vitro*, we conclude that the use of phenolic compounds to control BSR disease of oil palm may be a promising avenue of research. Further studies are underway to assess the ability of the selected phenols to control *G. boninense* in a field environment.

Acknowledgments

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