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ANTIMICROBIAL ACTIVITY OF CHITOSAN-INDUCED PHENOLIC ACIDS IN OIL-PALM ROOTS AGAINST *GANODERMA BONINENSE*

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Abstract: This paper discusses the accumulation of phenolic acids in three different varieties of oil palm and *in vitro* antimicrobial activity against *Ganoderma boninense* after elicitation with chitosan. The phenolic acids detected in oil-palm root tissues were syringic acid (SA), caffeic acid (CA) and 4-hydroxybenzoic acid (4-HBA). The *in planta* accumulation were monitored for four weeks and the *in vitro* antimicrobial activities of the phenolics against the pathogen for eleven days. The radial growth of the pathogens on 10% PDA and on oil-palm root agar (OPRA), ameliorated with the three phenolics, with range concentration of 50-110 µg mL^{-1} were recorded daily. AVROS variety was reported to have the highest amount of the three phenolic acids compared to Ekona and Calabar varieties. SA was found to be the most active in inhibiting the pathogen, followed by CA and 4-HBA. The latter was virtually inactive, both in 10% PDA and OPRA. Antifungal activity of SA and CA was detected, which was consistent with those detected in oil-palm roots.

KEYWORDS: *Ganoderma boninense*, phenolics, syringic acid, caffeic acid, 4-hydroxybenzoic acid

Introduction

Basal Stem Rot (BSR), caused by *Ganoderma boninense*, is considered the most serious disease faced by the oil-palm industry in Malaysia and South East Asia (Chong *et al*., 2009 a, b). 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 *et al*., 1996). In the latest incidence, *G. boninense* was found to attack seedlings or immature palms less than one year old in the nursery (Susanto, 2009). There are two kinds of losses generated by *G. boninense*: direct and indirect loss. The direct loss refers to reduced 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). 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 attempt to develop varieties with enhanced resistance (Sariah and Zakaria, 2000; Abdul Razak *et al*., 2004; Idris *et al*., 2006; Khairudin and Chong, 2008; Jayanthi *et al*., 2009; Shamala *et al*., 2009). To date, no method gives good control of *Ganoderma* infection in established plantations and some have technical limitations in application.

Phenolics serve dual functions, 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, fungal and bacterial pathogens (Ravin *et al*., 1989; Dakora and Phillips, 1996; Lattanzio *et al*., 2006). Phenolics which are active as allelochemicals are mainly volatile terpenoids, toxic water-soluble hydroquinones, hydroxybenzoates, hydroxycinnamates and the 5-hydroxynapthoquinones (Bhattacharya

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et al., 2010). A number of simple and complex phenolics accumulate in plant tissues and act as phytoalexins, phytoanticipins, nematicides against soil-borne, foliar pathogens and phytophagous insects (Akhtar and Malik, 2000; Lattanzio *et al*., 2006; Chong *et al*., 2011 b). To date, although there are several published articles on the role of phenolics in different plants effective against different pathogens, there is limited information on phenolics related to oil palm. Some earlier research conducted on young oil palms reported that 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 decreased by four weeks. These preliminary findings 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 as a defence mechanism of oil palm against *G. boninense* invasion or the roles of specific phenolic compounds against the pathogen.

Chitosan is a cationic polymer, a deacetylated chitin (Muzzarelli, 1997). The fungicidal activity of chitosan has been well documented both *in vitro* and *in situ* studies. Literature generally reports that the level of inhibition of fungi is highly correlated with the chitosan concentration (Bautista-Baños *et al*., 2006). The role of the elicitation of several defence-related enzymes has also been reported. Chitosan and chitin oligomers have also been reported to stimulate wheat's resistance (Bohland *et al*., 1997; Vander *et al*., 1998). Despite the vast knowledge on the efficacy of chitosan in plants' defence mechanisms and its antifungal activities, none have been reported in oil palm. This paper describes the *in planta* accumulation and *in vitro* antifungal activity and degradation of specific phenolics from oil palm to *G. boninense* mediated by chitosan addition to the growing medium.

Materials and Methods

Fungal isolates and growth

G. boninense isolate GB1_5 was used in infection studies. GB1_5 was obtained from a basidiocarp at the base of a diseased oil palm from Langkon Estate, Sabah, Malaysia. Internal tissues of fruiting bodies were excised and cultured on *Ganoderma* Selective Medium (GSM). The medium was prepared as described by Ariffin and Idris (1992). GSM 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. The fungi which successfully grew on the GSM after 5 days were further examined under the Nikon YS100 microscope with magnification of 400x for the presence of clamp connection, a common characteristic of basiodiomycetes. Once a pure culture of *Ganoderma* was isolated, it was transferred and maintained on Potato Dextrose Agar (PDA) at 25^{o}C. The pathogen was subjected to further molecular identification as described by Chong *et al*., (2011 a). 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, a procedure which was repeated throughout the project to maintain the pathogenicity of *G. boninense.*

Oil-palm seedlings

Oil-palm seedlings used for infection studies are the varieties of Ekona, Calabar and AVROS, supplied by Borneo Samudera (M) Sdn Bhd, Sabah, Malaysia. In Universiti Malaysia Sabah (UMS), seedlings were grown in 30 x 30 cm black polythene bags in Peat Vriezenveen Substrate, Product of Holland. The seedlings were grown in the nursery of UMS and watered

twice a day. Liquid 15/15/15 NPK fertiliser at 5 L per 100 seedlings was applied every three months until the seedlings were one year old.

Oil-palm root phenolic elicitation with chitosan

Chitosan stimulation of the defence mechanisms was conducted on one year-old seedlings. Chitosan (5 g) was incorporated into 5 kg of Peat Vriezenveen Substrate by mixing to homogeneity. Roots were then grown and watered daily for three, four, five and six weeks before harvesting, rinsing under running tap water and analysis. Untreated seedlings served as a control.

Artificial inoculation technique

The artificial inoculation method developed for inoculating oil-palm roots with *G. boninense* was as described by Chong *et al*., (2012). In brief, the mycelium of *G. boninense* which covered the entire surface of a 9 cm Petri dish was scraped off and suspended in 20 mL Potato Dextrose Broth (PDB), blended and 50 µL of Tween 20 was added. The oil-palm roots were sprayed with this suspension of *G. boninense* fragments, with approximately 17.5 mL of the inoculum per seedling, before the seedlings were re-planted in growth medium and grown-on as described above.

Extraction of phenolic from roots for SPE and HPLC analysis

Roots (100 g) were homogenised using IKa A11 basic grinder and soaked in 500 mL of methanol for 2 days before filtering through Whatman No. 1 filter paper, dried with a Buchi rotary evaporator and resuspended in milli-Q ultra-pure water. Samples were centrifuged at 15,000 x g for 15 min before solid-phase extraction (SPE).

Solid-phase extraction (SPE)

Strata X 33 µM Polymeric Reversed Phase (200 mg 6 mL^{-1}) by Phenomenex cartridges were chosen for this work. 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 to optimise interaction with the analyte. Samples were then loaded onto the catridges. 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.

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

All standards, obtained from Sigma, were prepared at a concentration of 0.02 mg mL^{-1} and 20 µL of each of the standard was injected into the HPLC system. Specific phenolics were compared with the standards based on the basis of retention times and their characteristic spectra. The three specific phenolics; Syringic acid (SA), Caffeic acid (CA) and 4-hydroxybenzoic acid (4-HBA) were chosen for further quantification due to their presence in infected root after detected by LCMS (data not presented).

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.

In vitro antifungal activity of phenolics against G. boninense

A series of concentrations of 0, 50 µg 70 µg, 90 µg, and 110 µg mL^{-1} of SA, CA and 4-HBA were incorporated into 10% PDA and Oil Palm Root Agar (OPRA). These are the common concentrations of phenolics that were present in infected root quantified by HPLC. The

phenolics were first dissolved in acetone: water (50:50; v/v) before incorporation into the media. 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 root in 500 mL of SDW for approximately 1 hour. 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 root, 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* taken from the edge of 7-8 day-old cultures, using a sterile micropipette tip, and introduced to the middle of the plates. The mycelium was excised from these plugs with a sterile scalpel. The growth of the pathogen was expressed as radial growth (cm).

Results and Discussion

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 CA and SA (Fig. 1). 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.

HPLC analysis and quantification of phenolics

Statistical analysis revealed several differences in accumulation of SA among the treatments throughout the four weeks of study (Fig. 2). Examination of this figure demonstrates that SA accumulated constitutively in control, untreated seedling 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 SA; indeed later in the time course there was evidence of degradation by the pathogen. This metabolism was greatly reduced in the presence of chitosan. There was a little difference in the accumulation of SA between the varieties tested, although the variety AVROS produced a small but significant increase in the level of this compound. Accumulation of CA in oil-palm roots showed a similar trend to SA (Fig. 3). 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 and that CA was also metabolised by *G. boninense*, as evidenced by the reduction in accumulated levels observed between four and six weeks after inoculation. Once again there was a little evidence of varietal differences, although AVROS produced small but significantly higher levels of CA. 4-HBA is the most abundant phenolic acid detected in oil- palm roots among the three phenolics that were monitored in this study (Fig. 4). In all three different varieties, 4-HBA accumulated almost two-fold in comparison to SA and CA either in control or challenged seedlings. The pattern of elicitation and metabolism of this compound, however, followed similar trends to those described for SA and CA.

In vitro antifungal activity

All concentrations of SA tested failed to stop completely the growth of this pathogen within the range of 50 to 110 µg mL^{-1} (Fig. 5a). 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} SA. OPRA was used to provide an alternative and possibly more representative condition to the environment of

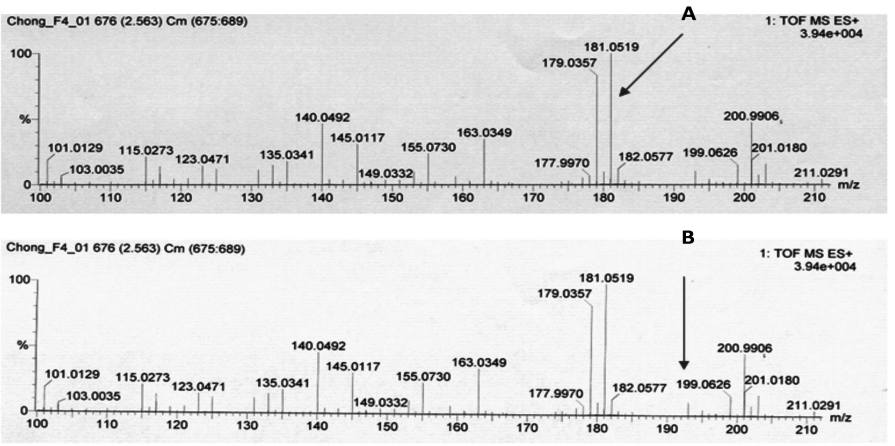

Figure 1: Electro spray mass spectrum of caffeic acid (181.05) and syringic acid (199.06). x-axis shows m/z ratio and y-axis shows the percentage. Arrowed: Caffeic acid (**A**) and syringic acid (**B**).

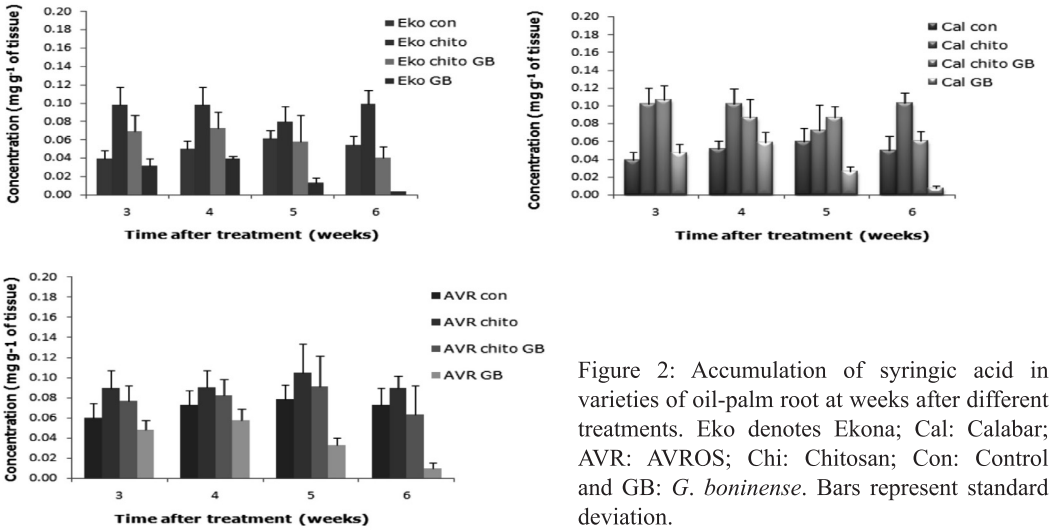

Figure 2: Accumulation of syringic acid in varieties of oil-palm root at weeks after different treatments. Eko denotes Ekona; Cal: Calabar; AVR: AVROS; Chi: Chitosan; Con: Control and GB: *G. boninense*. Bars represent standard deviation.

the *G. boninense*-oil palm root interaction (Fig. 5b). 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 in CA but with less antifungal activity compared to SA (Figs 6a and 6b). By day 11, in all CA 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 (Figs 7a and 7b). The pathogen grew steadily, except in the highest 4-HBA tested concentration. In summary, antifungal activity of SA and CA was detected even at the lowest concentration tested but it was weak for 4-HBA. SA was the most antifungal molecule tested. With the highest tested concentration (110 µg $mL^{-1)}$ of SA, there was no significant growth of the pathogen ($p \geq 0.05$) up to day-9.

Higher concentrations of SA, such as 90 and 110 µg mL^{-1}, possibly play a role in limiting the growth of *G. boninense*, but the concentration of this phenolic, which was detected in the oil-palm roots may not be sufficient to fully inhibit the pathogen. Higher concentrations may have

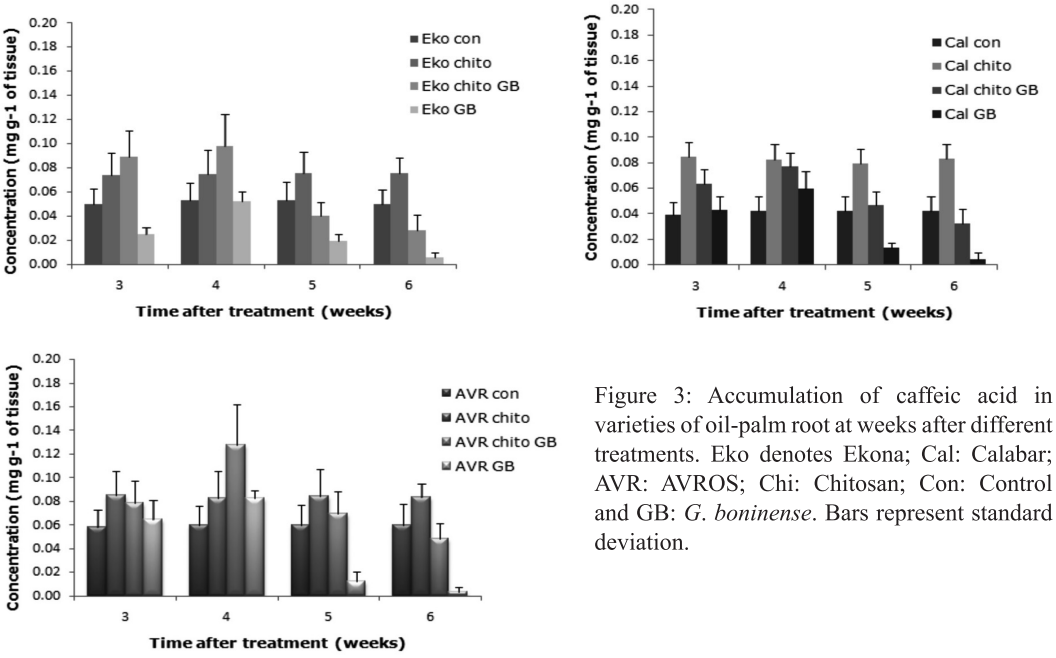

Figure 3: Accumulation of caffeic acid in varieties of oil-palm root at weeks after different treatments. Eko denotes Ekona; Cal: Calabar; AVR: AVROS; Chi: Chitosan; Con: Control and GB: *G. boninense*. Bars represent standard deviation.

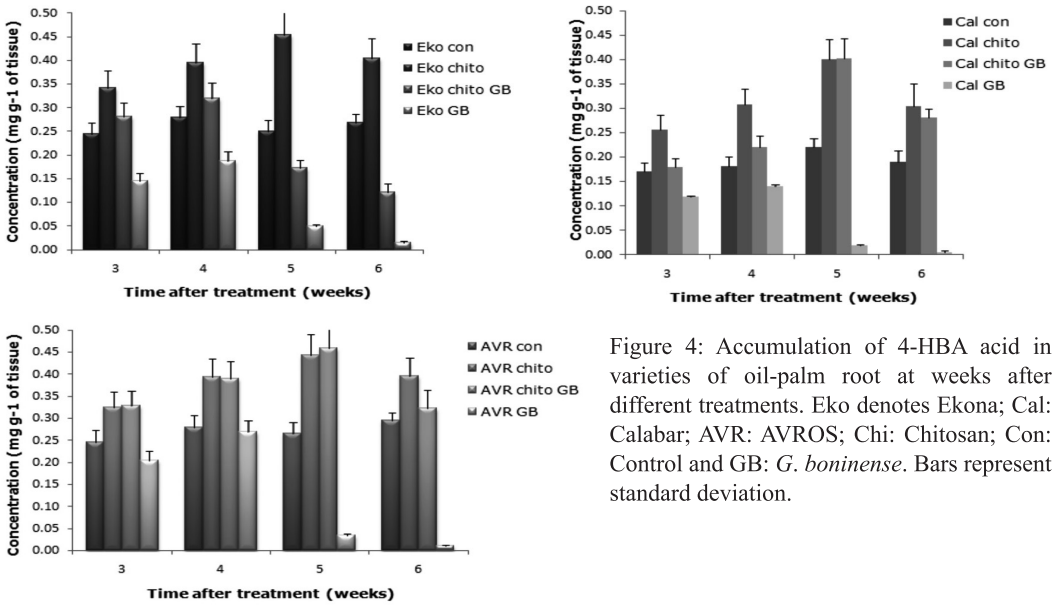

Figure 4: Accumulation of 4-HBA acid in varieties of oil-palm root at weeks after different treatments. Eko denotes Ekona; Cal: Calabar; AVR: AVROS; Chi: Chitosan; Con: Control and GB: *G. boninense*. Bars represent standard deviation.

greater effect on this pathogen. Numerous papers have been published on the effect of SA in other plants but not, to date, in oil palm. Work on the resistance of raspberry to the fungus *Didymella* showed SA accumulated in the bordering zone of lesions forming a barrier to the fungus. The *in vitro* fungitoxicity of SA was confirmed to be very high at low concentrations (Kozlowska and Krzywanski, 1994). Although CA in oil-palm root may not be as inhibitory as SA, the fungitoxicity of the highest concentration, 110 µg mL^{-1}, during the *in vitro* tests should not be ignored. CA is ubiquitously present in plants and has potent phytotoxicity affecting plant growth

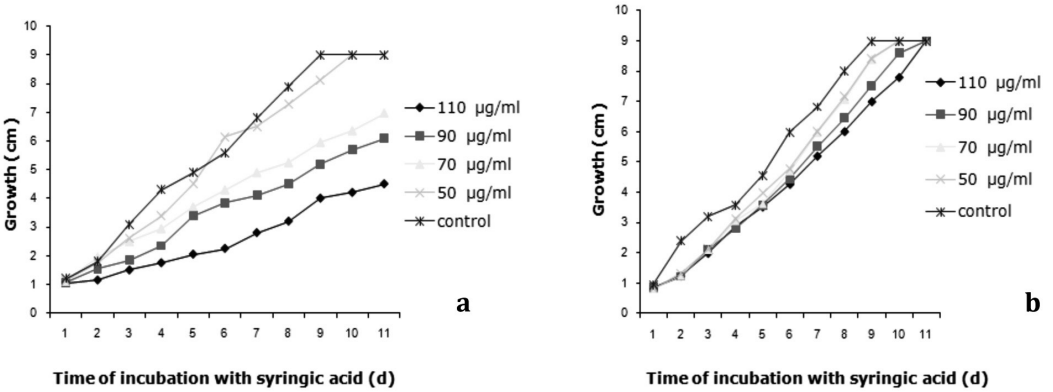

Figure 5: Radial growth of G. boninense on 10% PDA (a) and OPRA (b) with different concentrations of syringic acid incorporated into the agar.

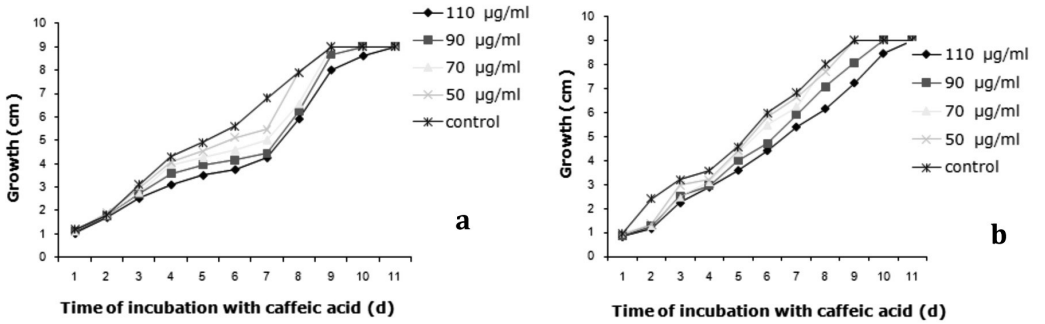

Figure 6: Radial growth of G. boninense on 10% PDA (a) and OPRA (b) with different concentrations of caffeic acid incorporated into the agar.

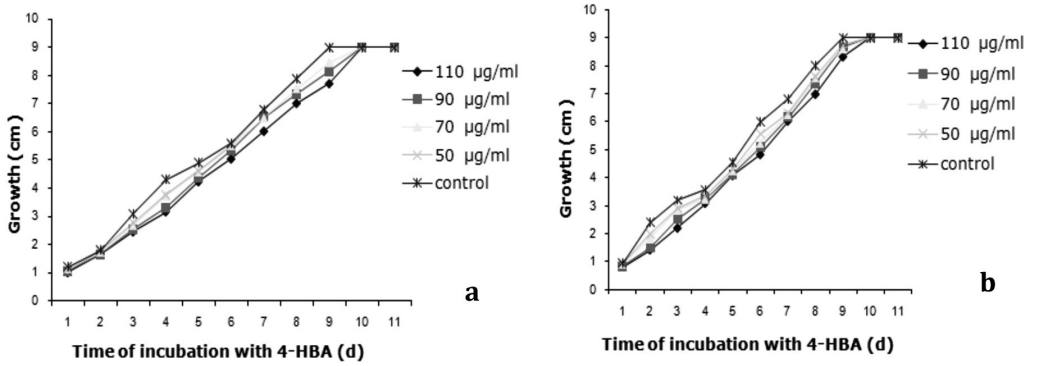

Figure 7: Radial growth of G. boninense on 10% PDA (a) and OPRA (b) with different concentrations of 4-HBA incorporated into the agar.

and physiology. CA was found to be inhibitory to the growth of four sweet potato pathogenic fungi. Inhibitory activity bioassays also suggested high periderm CA 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 less. 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 root situation, 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.

Conclusion

In summary, SA was found to be the most active phenolic against *G. boninense*, followed by CA, with 4-HBA being the least toxic to this pathogen. Inhibition could occur *in planta* with higher levels of SA and CA especially in 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.

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