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HPC Fingerprints and *In vitro* Antimicrobial Activity of Syringic Acid, Caffeic Acid and 4-hydroxybenzoic Acid against *Ganoderma boninense*

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Abstract: This study discusses the *in vitro* antimicrobial activity and fungitoxicity of syringic acid, caffeic acid and 4-hydroxybenzoic acid which is found in oil palm root. The presence of these phenolics were first confirmed with the injection of standards using HPLC in a gradient system developed with methanol and 0.1% phosphoric acid. Experiments were observed for fourteen days, repeated at least three times and data were recorded daily. The antimicrobial activities and fungitoxicity of the phenolics against *Ganoderma boninense* were expressed in inhibition of radial growth of *G. boninense* on PDA ameliorated with the three different phenolics with a range concentration of 0.5-2.5 mg mL^{-1}. Syringic acid was found to be very fungitoxic to *G. boninense* even at concentration of 0.5 mg mL^{-1}, the lowest concentration tested in this experiment. When the concentration is increase to 1.0 mg mL^{-1} of syringic acid, the pathogen is inhibited. Caffeic acid and 4-hydroxybenzoic acid were having inhibitory effect with the highest concentration tested; 2.5 mg mL^{-1} strongly inhibited the growth of *G. boninense* in comparison to the control.

Key words: *Ganoderma boninense*, syringic acid, caffeic acid, 4-hydroxybenzoic acid

INTRODUCTION

Oil palm (*Elaies guineensis* Jacq.) is truly "a golden crop of Malaysia" since it generates profitable export earning for the country and truly nature's gifts for alleviating poverty in Malaysia (Basiron, 2007). The Malaysian palm oil industry is economically big and diversified. Malaysia is currently the world's largest producer and exporter of oil palm. Areas of oil palm have increased from 54,000 hectares in 1960 to 4.05 million hectares in 2005, reflecting a compound annual growth of 10.06%. Production increased from 94,000 tones in 1960 to 15 million tones in 2005, or almost 160 times within 45 years. This represents a compound annual growth of 11.93% per year (Basiron, 2007). The devastating Basal Stem Rot (BSR) disease caused by *Ganoderma boninense* is considered the most serious disease faced by oil palm in Malaysia (Benjamin and Chee, 1995). Oil palm has an economic life span of 25-30 years. Basal stem rot can kill more than 80% of stands by the time they are half-way through normal economic life (Abdul Razak *et al.*, 2004). In the late 1960s and early 1970s in Sumatra, there was little decline in the yield of oil palm until the surviving stand had fallen to about 115 palm ha^{-1} but in more recent plantings, any loss of palm was associated with a 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 *Ganoderma* yielded between 13 and 21% less than healthy palms at the same age (Nazeeb *et al.*, 2000). *Ganoderma* infection strongly affected the leaf gas exchange of oil palms through a reduction in stomatal conductance which led to a significant reduction in transpiration rates and interacellular CO_2 level (Haniff *et al.*, 2005).

Heavily infected field yielded 26% less at 11 years after planting and 46% less at 15 years by which time incidence was 67% (Singh, 1991). There is currently no effective cure for *G. boninense* infection in an existing stand. Preventive and ameliorative treatments which are commonly carried out show various degrees of effectiveness (Sariah and Zakaria, 2000). Determination of total phenolic content in *G. boninense* infected and healthy oil palm roots showed susceptible palm roots at week four had low phenolic content, whereas week one had high phenolic content. Gallic acids concentrations decreased in the four weeks old roots of infected susceptible palms compared to healthy roots.

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Determination of total phenolic content in infected palm seedlings root (D X P) also showed low phenolic content compared to the non infected palm seedlings root. This indicate phenolic compounds are involved in oil palm resistance against *Ganoderma* (Mohamad Arif *et al.*, 2007). To identify the possibility of oil palm resistance against *G. boninense* in certain circumstances need further investigation. However, if resistance in oil palm against *G. boninense* is possible, it may contribute to tackling the problem. In this study, we present our works which confirmed the present of three important phenolics; Syringic acid, Caffeic acid and 4-hydroxybenzoic acid in oil palm root and their *in vitro* effect to *G. boninense*.

MATERIALS AND METHODS

Seed and plant materials: Certified disease free seeds were provided by Borneo Samudera Sdn Bhd. Sabah Malaysia and grown to one year old.

Ganoderma boninense: Cultures were provided by Borneo Samudera Sdn Bhd, Sabah, Malaysia, maintained at 25°C on Potato Dextrose Agar (PDA).

Oil palm root elicitation: Stimulation trials of the defence mechanisms were conducted on seedlings from one year old. Roots of seedlings were dipped into solution of 5% of $CuSO_4$ for 30 min, rinsed under running tap water and incubated for two days for the accumulation of phenolics.

Extraction of phenolic from roots: Roots were later homogenized using 1Ka A11 basic grinder and soaked into methanol for another two days before filtered, dried up in a Buchi® rotavapour and resuspended into milli-Q® ultra pure water. This aliquot was centrifuged with 13K rpm for 15 min before proceed to solid phase extraction.

Solid Phase Extraction (SPE): Strata X 33 μm Polymeric Reversed Phase (200 mg 6mL⁻¹) by phenomenex® were chosen for this experiment. Four mililiter of methanol were 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 analyte. Samples were loaded to introduce the samples to analyte (maximum 6 mL only). The cartridges were washed with four ml of 0.1M of HCL again to remove any impurities from samples and finally the cartridges were eluted with four ml of 0.1 M HCL:MeOH (1:4; v/v). The aliquots were taken to dryness by Buchi® rotavapour. The final concentration was adjusted to 5 g of tissue per 1 mL of milli-Q® ultra pure water.

HPLC analysis for phenolics: An efficient gradient of 0.1% of phosphoric acid and methanol with an Eclipse XDB-C_{18} 4.6 mm×150 mm×5 μm column with Agilent series 1200 HPLC was used. A Variable Wavelength Detector (VWD) with 280 nm was used for the elution with a flow rate of one mL min⁻¹. Phenolics were identified on the basis of retention times and their characteristic spectra in comparison with standards. When necessary, co-injection and elution with standards were done to ensure the identity of the compounds. References compounds used were vanillin, transcinnamic, benzoic, 4-hydroxybenzoic, 3,4-dihydroxybenzoic, gallic, syringic, p-coumaric, caffeic, ferulic and sinapic acids. References compounds were chosen in accordance to soluble phenolics found in date palm by Daayf *et al.* (2003). For quantification, serial dilutions of known concentration of standards were injected into the HPLC column and ran using the same system. Areas under the peaks were integrated with the known concentrations and standard curves were produced for each of the standard.

***In vitro* bioassay:** A series of 0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg mL⁻¹ of syringic acid, caffeic acid and 4-hydroxybenzoic acid were ameliorated into the PDA which the phenolics were first dissolved in Acetone: Water (50:50; v/v). Solvent was served as positive control. The growth of the pathogen was expressed in centimeter of radial growth.

RESULTS

Development of HPLC analysis method for phenolics: Series of gradient system with different ratio of phosphoric acid 0.1% and methanol were tested for the best elution of the phenolics from standards. The best elution was found with the system in Table 1.

With this system, all phenolics from the standards were able to separate with single peak (Fig. 1). The system was later used for identification of the possible phenolics presence in oil palm root.

Confirmation of the presence of syringic acid, caffeic acid and 4-hydroxybenzoic acid in oil palm root from injection with standards: From HPLC chromatograms, several unknown compounds were found commonly presence in both $CuSO_4$ elicited and healthy control (non-elicited) roots (Fig. 2).

Table 1: The system for best elution

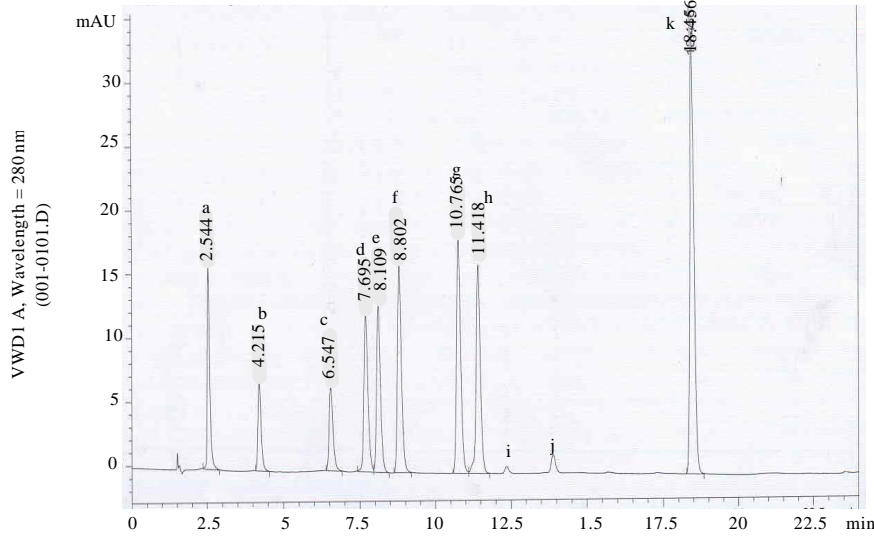| Solvent	Time (min)		
	0	23	24
Acetic acid (0.1%)	80	30	80
MeOH (100%)	20	70	20

Fig. 1: Chromatogram showing the detection of phenolics standard by variable wavelength detector after separation by HPLC. a: Gallic acid. b: 3,4dihydroxybenzoic acid. c: 4-hydroxybenzoic acid. d: Caffeic acid. e: Syringic acid. f: Vanillin. g: p-coumaric acid. h: Sinapic acid. i: Ferulic acid. j: Benzoic acid. k: Transcinnamic acid
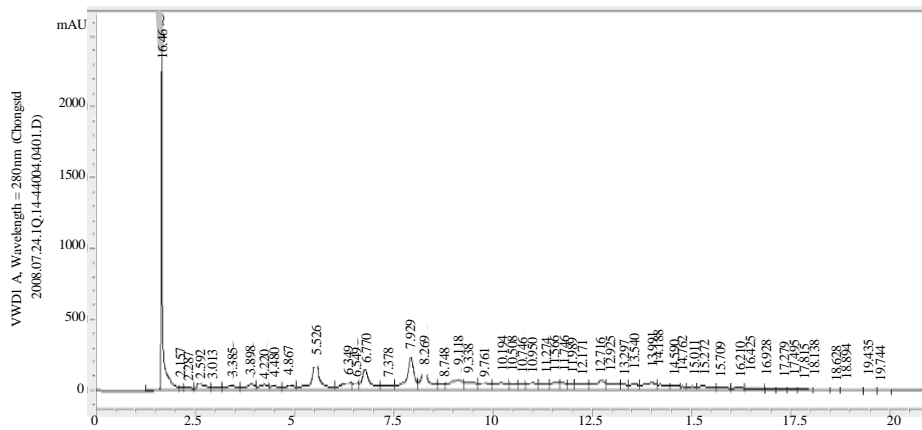

Fig. 2: Chromatogram showing the peaks commonly presence in oil palm root extract both in control and elicited roots detected by variable wavelength detector after separation by SPE and HPLC

The different was mainly on the amount of the compounds being produced, where the elicited roots have slightly higher in the accumulation of those compounds. The focus of the research was later on compounds that presence in higher amount after the elicitation or challenge. Based on the chromatogram as shown in Fig. 2, five standards phenolics; 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, caffeic acid, syringic acid and vanillin were selected in accordance with their retention times to the higher accumulated compounds

after elicitation by $CuSO_4$. Those standards were later injected together with aliquots of elicited oil palm roots for a matching or spiking run. Only three standard phenolics that matched with the compounds of interest from the oil palm roots. Those compounds were later confirmed as 4-hydroxybenzoic acid (Rt: 6.716 min), caffeic acid (Rt: 7.071 min) and syringic acid (Rt: 8.214 min) (Fig. 3).

***In vitro* bioassays:** *In vitro* bioassays were conducted to test the fungitoxicity and antimicrobial activities of those
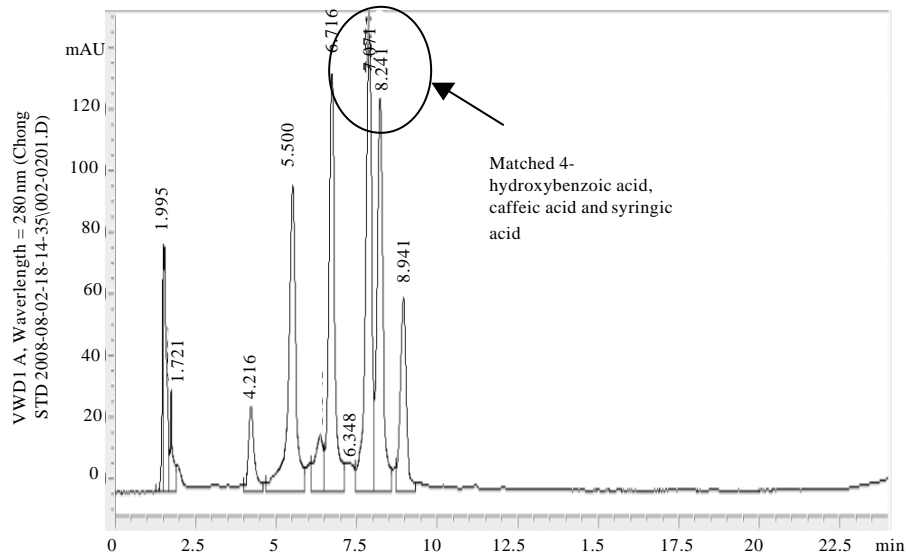
Fig. 3: Chromatogram showing injection with sample and the most potential standards that may match the compound of interest. Arrowed: 4-hydroxybenzoic acid, caffeic acid and syringic acid
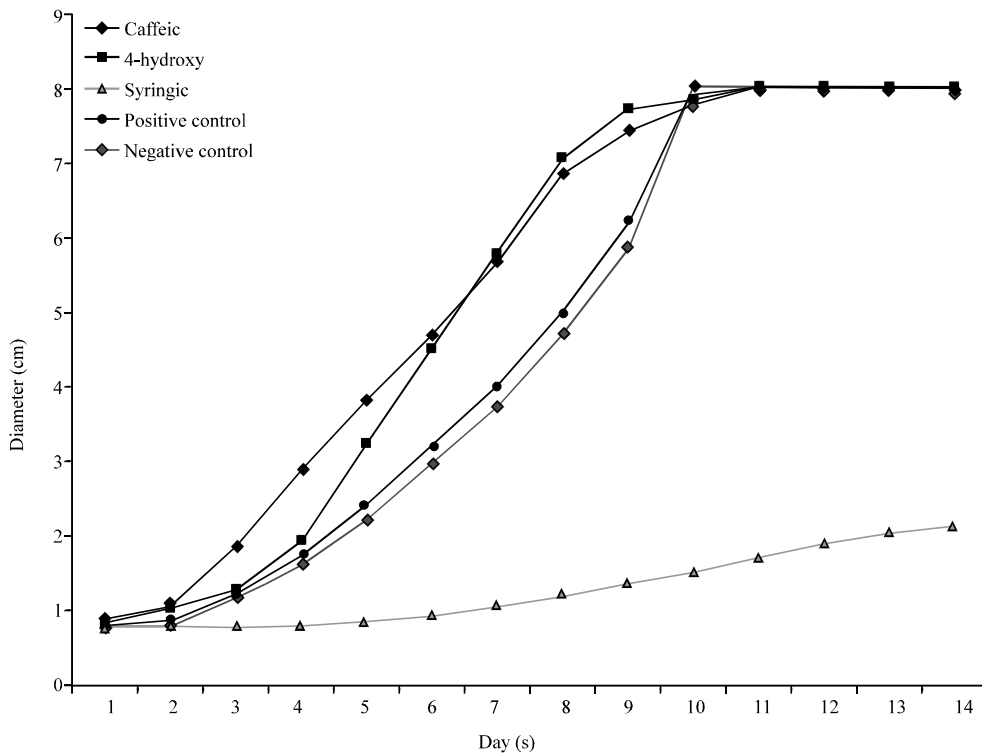

Fig. 4: Radial growth of *Ganoderma boninense* on PDA ameliorated with 0.5 mg mL^{-1} of three different phenolics

phenolics found in oil palm roots; syringic acid, caffeic acid and 4-hydroxybenzoic acid to *G. boninense*. Syringic acid was found to be very toxic to *G. boninense*. Although at the lowest concentration tested (0.5 mg mL^{-1}), *G. boninense* was fully inhibited up to day five. There was slight non significant increase in the diameter measured of the pathogen at day six up to day 15 (Fig. 4).
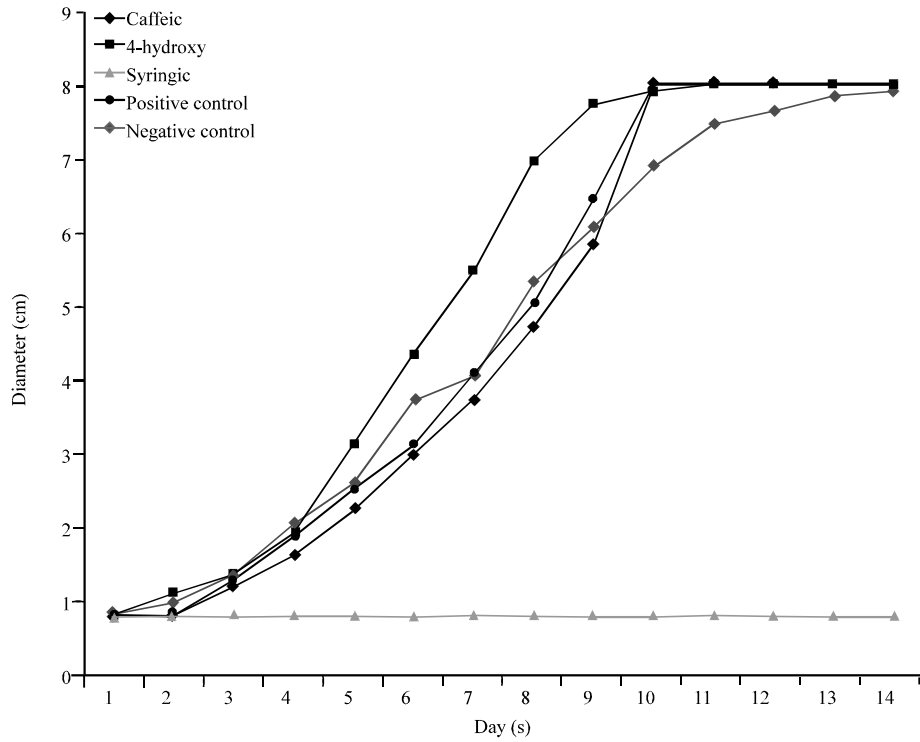
Fig. 5: Radial growth of *Ganoderma boninense* on PDA ameliorated with 1.0 mg mL^{-1} of three different phenolics
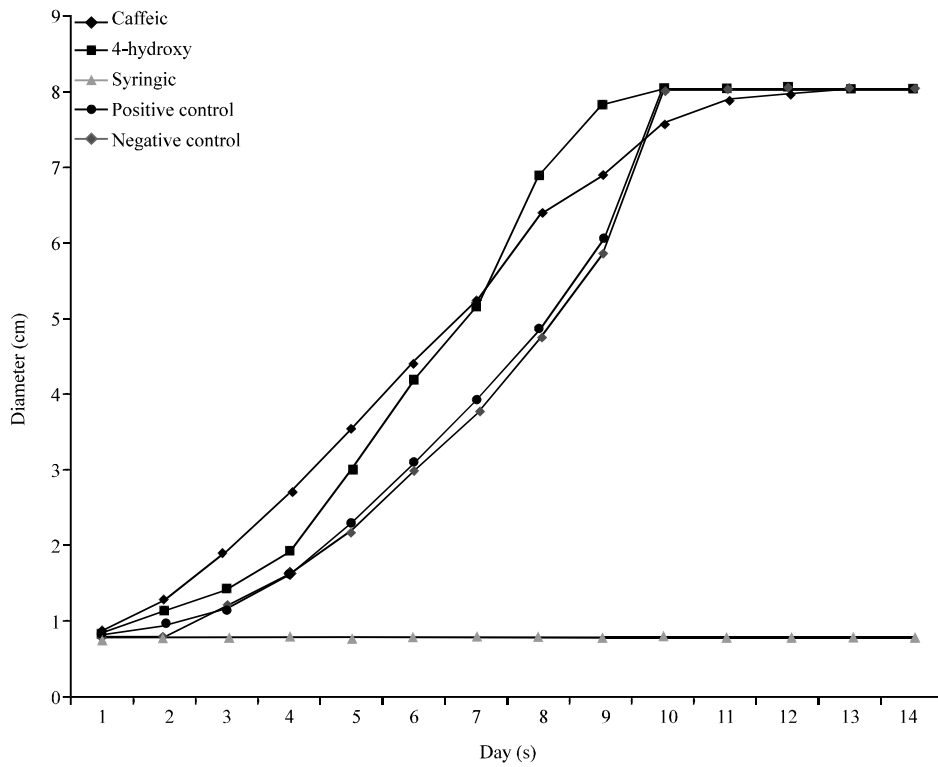

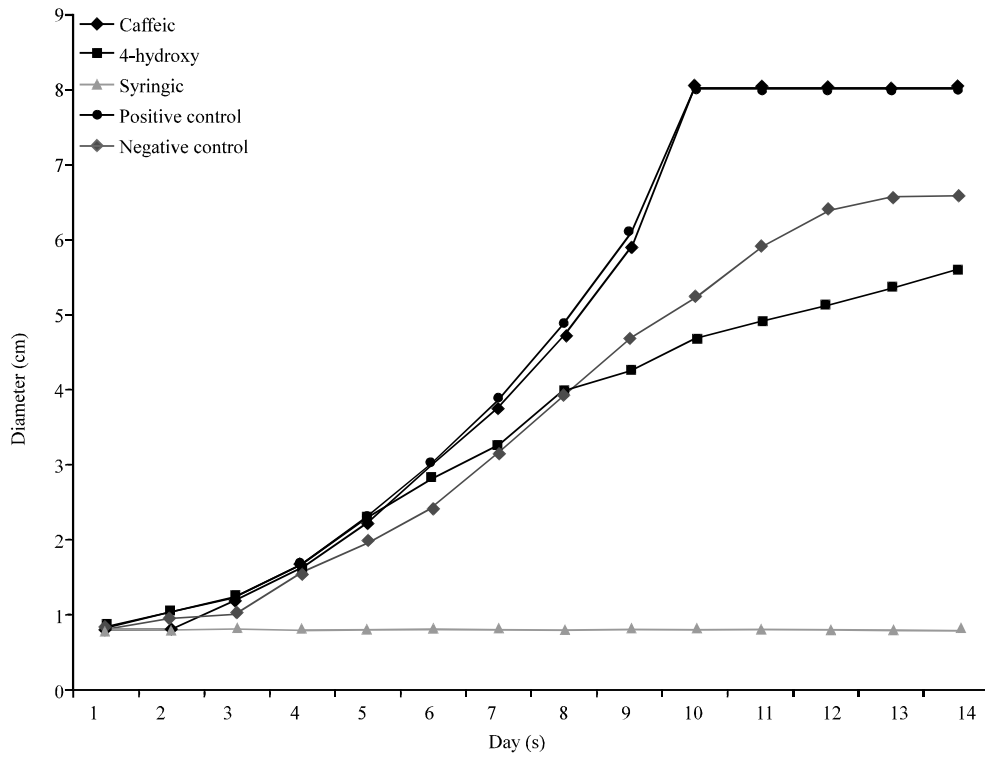Fig. 6: Radial growth of *Ganoderma boninense* on PDA ameliorated with 1.5 mg mL^{-1} of three different phenolics

Fig. 7: Radial growth of *Ganoderma boninense* on PDA ameliorated with 2.0 mg mL^{-1} of three different phenolics
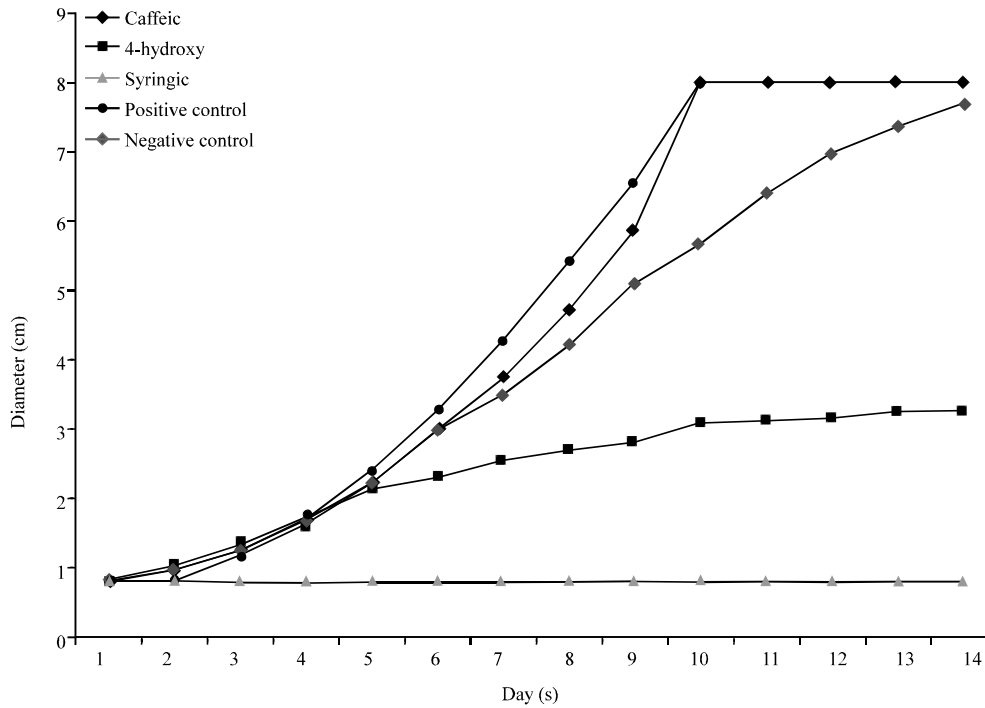

Fig. 8: Radial growth of *Ganoderma boninense* on PDA ameliorated with 2.5 mg mL^{-1} of three different phenolics

Higher concentrations of syringic acid tested (1.0, 1.5, 2.0 and 2.5 mg mL^{-1}) totally stopped the growth of the pathogen (Fig. 5-9). Growths of *G. boninense* both in positive and negative control were not significant
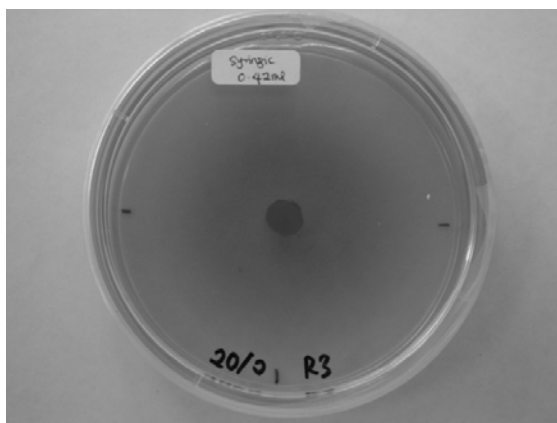
Fig. 9: Ganoderma boninense failed to grow after incubated for 14 days in 1.0 mg mL^{-1} of syringic acid ameliorated in PDA and killed in this concentration

to each other indicate the toxicity was coming from the phenolic and not from the solvent. Maximum growths (9cm-maximum size of petri dish) were achieved by both positive and negative control within 10 days after incubation.

In another hand, pathogen inoculated into agar containing 0.5 mg mL^{-1} of caffeic acid and 4-hydroxybenzoic acid showed a significantly increased throughout the observation, to a maximum size of the plate at day 11. Surprisingly, in 1.0 mg mL^{-1} and 1.5 mL mg^{-1} of caffeic acid and 4-hydroxybenzoic acid the growth of this pathogen is greater than control and reached a maximum at day 10 (for 4-hydroxybenzoic acid) but grow slower in 1.0 and 1.5 mg mL^{-1} of caffeic acid after day 10 in comparison to control. Both caffeic acid and 4-hydroxybenzoic acid failed to fully inhibit *G. boninense* when the concentration was raised up to 2.0 mg mL^{-1}. But the pathogen managed only to grow up to 5.58 cm (in 4-hydroxybenzoic acid) and 6.57 cm (in caffeic acid) at last day of observation.

But the effect of 4-hydroxybenzoic acid was obviously inhibitory with the highest concentration tested in this experiment (2.5 mg mL^{-1}) which caused the *G. boninense* reached only 3.28 cm in diameter after 14 days. If this concentration is achievable in oil palm root, 4-hydroxybenzoic acid may play a role in the real *G. boninense*-root interaction. But in caffeic acid the growth of this pathogen continuously to increase significantly after day 6 although in the same concentration as 4-hydroxybenzoic acid, gave a maximum growth of 7.68 cm at day 14. But the growth rate of this pathogen was inhibited by this concentration in comparison to control.

DISCUSSION

In vitro bioassays from this work demonstrated the fungitoxicity effect of syringic in correlation to many works on other plants. In resistance raspberry to fungus *Didymella*, syringic acid was found accumulated in the bordering zone of lesion forming a barrier to the fungus. The *in vitro* fungitoxic of syringic acid was later confirmed to be very toxic at low concentration (Kozlowska and Krzywanski, 1994). In sugar cane, cultivar Mayari 55-14 which is highly resistant to smut disease showed a major accumulation pattern of syringic acid when interact with the pathogen (De Armas *et al.*, 2007). Besides the inhibitory effect found to *G. boninense* of BSR, the role of 4-hydroxybenzoic acid has also been demonstrated 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-hydroxybenzoic acid at IC$_{50}$ concentrations of 100-170 μg mL^{-1} (Cho *et al.*, 1998).

Caffeic acid found in oil palm roots may play a role in resistance of oil palm to *G. Boninense*. Many research also showed caffeic acid is ubiquitously present in plants and a potent phytotoxin affecting plant growth and their physiology (Singh *et al.*, 2009). In a report on sweet potato, caffeic acid was also found inhibiting the growth of four sweet potato pathogenic fungi and germination of proso millet seeds in bioassays. Inhibitory activity in the bioassays reported also suggests high periderm caffeic acid levels contribute to the storage root defense chemistry of some sweet potato genotypes (Harrison *et al.*, 2003). The findings of these phenolics in oil palm roots suggest the potential of them in resistance to *G. boninense*.

CONCLUSION

In this study, we presented the result of *in vitro* experimental investigation on the antimicrobial activity and fungitoxicity of syringic acid, caffeic acid and 4-hydroxybenzoic acid to *G. boninense* inoculated on PDB ameliorated with different concentration of the phenolics, respectively. We found syringic acid is the most fungitoxic phenolic among the three, with the lowest tested concentration; 0.5 mg mL^{-1} can inhibit the growth of *G. boninense* while 1.0 mg mL^{-1} totally killed the pathogen. On another hand, the highest tested concentration of caffeic acid and 4-hydroxybenzoic acid; 2.5 mg mL^{-1} only inhibited the growth of *G. boninense* in comparison to the control.

ACKNOWLEDGMENTS

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