Effect of Phenolic Acids to *Ganoderma* Viability in Oil Palm Tissues and Soil

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Received: *17 Nov 2014*
Revised: *18 Dec 2014*
Accepted: *30 Dec 2014*
Online: *30 Dec 2014*

Keywords:
Caffeic acid, Syringic acid, 4-Hydroxybenzoic acid, *Ganoderma*, Basal Stem Rot

A b s t r a c t

This paper presents the potential of phenolic acids; caffeic acid (CA), syringic acid (SA) and 4-hydroxybenzoic acid (4-HBA) in Basal Stem Rot (BSR) disease suppression of oil palm. Four concentrations of phenolics combinations were tested which were 0.4 g a.i., 0.8 g a.i., 1.2 g a.i. and 1.6 g a.i. of each CA, SA and 4-HBA. Infected palms with similar BSR disease intensity, age, soil topography and condition were selected for this trial. Assessment on *Ganoderma* viability was based on ergosterol content, possible isolation of the fungus on *Ganoderma* Selective Medium (GSM) from treated palm and Colony Forming of *Ganoderma* on GSM from treated soil. No ergosterol was found in healthy palms but in contrast ergosterol was detected in infected oil palm tissues before and after the treatments of phenolic acids. However, the untreated and infected palms showed significantly higher mean difference of ergosterol (0.6395 µg g^{-1}) compared to infected palms but treated with phenolic acids. Combinations of phenolics with 1.6 g a.i. suppressed *Ganoderma* colonization the most (-0.4379 µg g^{-1} of ergosterol), though, the suppression was no significant in comparison to other treatments such as 0.4 g a.i., 0.8 g a.i. and 1.2 g a.i.. *Ganoderma* was isolated on GSM from all oil palm tissues either treated or untreated with the phenolics acids suggesting the pathogen was suppressed but not killed after treated. However, there was no colony of *G. boninense* form on the GSM from the soil samples collected after observation for one month.

Introduction

Oil Palm (OP) cultivation in South East Asia is hampered by the devastating Basal Stem Rot (BSR) disease caused by *Ganoderma boninense*. The losses were estimated up to USD 500 million a year in Malaysia (Ariff *et al*., 2011). Many research have been conducted to reduce damages and losses in replanting and existing estates (Chong *et al*., 2012a), however, no conclusive remedy has been achieved. The most common practice in established plantations is practicing a good sanitation.The more recent study is investigation on the role of phenolic acids during oil palm – *Ganoderma* interaction (Chong *et al*., 2009a; 2009b; 2011a, 2012b). Phenolic compounds are plant secondary natural metabolites that constitute one of the most common and widespread groups of substances in plants. Phenolicsare arising biogenetically from the shikimate-phenylpropanoids-flavonoids pathways, producing monomeric and polymeric phenols and polyphenols (Harborne, 1989). Phenolic compounds are responsible for plants' pigmentation, growth, reproduction, resistance to pathogens and for many other functions. Many of these compounds have been proposed as a control of pathogens of agricultural crops since their accumulation in plant tissues can act as phytoalexins, phytoanticipins and nematicides against soil-borne pathogens and phytophagous insects (Langcake *et al*., 1981; Akhtar and Malik, 2000; Lattanzio *et al*., 2006). To date, although, there are several work

conducted on phenolics in oil palm but very little effort have been conducted to understand their effect to *Ganoderma* in established oil palm estates. In this paper, we report the role of three selected phenolic acids; caffeic acid, syringic acid and 4-HBA in suppressing *Ganoderma* in infected oil palm trees and soil.

Methodology

Collection of oil palm trunk tissues

The field trial was conducted in an oil palm estate severely affected by BSR in Langkon, Sabah, Malaysia. Oil palm trunk tissues were collected for the ergosterol analysis as described by Chong (2012) with little modification, before selecting the homogenous diseased palms prior to application of phenolics. A drill and drill bit were used for the collection of trunk tissues from oil palm trunk. The drill bit was first sterilized by immersing it into 90% of ethanol for five minutes. Approximate 2 cm depth of the oil palm trunk tissues were collected using the drill bit, one meter high from the ground and discarded to remove any unwanted saprophytic fungi which may presence on the outer layer of the trunk. The drill bit was later sterilized again in ethanol before further collection of tissues (approximate 10 cm into the trunk) at the same point for the analysis of ergosterol. The collection steps were repeated three times at different point with 90^{o} to each other with the same palm. Tissues were pooled together and homogenized in a commercial blender in laboratory. Homogenized tissues were taken for ergosterol analysis.

Extraction of Ergosterol

Homogenized tissues (100 mg) from each infected and uninfected palms were extracted in methanol using bead beating to physically crush the sample. The extract was centrifuged at 13,000 rpm for 5 minutes and the supernatant was made up to 1.5 ml before being filtered through a 0.45 µm acetate syringe tip filter.

Analysis and Quantification of Ergosterol

The Eclipse XDB-C_{18} 4.6mm x 150mm x 5µm column was utilized with an Agilent Series 1200 HPLC system for this analysis. The wavelength of UV detector was set at 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 pure standard (20 µg mL^{-1}) from Sigma at the flow rate of 1.5 mL min^{-1}. The system was run isocratically with 100% methanol. 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. The developed standard curve was then used for further ergosterol quantification for oil palm tissues extracts.

Application of Phenolic Acids to Infected Palms

Three phenolic acids; caffeic acid, syringic acid and 4-HBA were prepared in a final concentration of 0.4 g, 0.8 g, 1.2 g and 1.6 g of each of the individual phenolic acid per Liter of aqueous alcoholic solution. Four holes were dug for each infected palm with depth of 15 cm and a distance of 30 cm from the infected palm, and 90^{o} to each other. The combinations of phenolics were applied by pouring the well-mixed phenolic acids into the holes surrounding the infected palms.

Assessment on Ganoderma Viability based on Ergosterol Content

The viability of *Ganoderma* was assessed six months after treatments using the same technique for ergosterol quantification and analysis as described by Chong *et al*. (2012c).

Ganoderma Colony Forming Unit

The number of *Ganoderma* that are capable of forming colonies on GSM after exposure of the phenolics to soil was determined by performing the spread plate and plate count assessments. Knowing the dilution factor, volume plated, and number of colonies on the plate (or average from the duplicate plates), the equation as described by Ahmed and Carlstrom (2003) was used to calculate count of microorganisms in the treatment.

Isolation of Fungus on Ganoderma Selective Media (GSM)

Trunk tissues from infected palms were collected using drill and drill bit as described earlier and cultured on GSM. The preparation of GSM was as described by Ariffin and Idris (1992). The identity of the fungus was later identified using molecular technique as described by Chong *et al*. (2011a).

Statistical Analysis

Completely Randomized Design (CRD) was chosen as the experimental design for this field trial. One-Way ANOVA was calculated at 5% significance level by using SPSS 17.0 (Statistical Package for Social Science software) for the data analysis. Data was subjected to analysis variance, differences was compared using a Tukey test at a significance of $P<0.05$.

Result and Discussion

Ergosterol Analysis

The content of ergosterol is a direct representation of the presence of fungi. In this study, no ergosterol was found in healthy palms but in contrast ergosterol was detected in infected tissues before and after the treatments with varies concentrations of phenolics (as in Table 1). There was a significant difference between the control palms (infected palms; without treatment) and those treated with different concentrations of phenolics.

Table 1: The difference in ergosterol concentration (µg g^{-1}) of trunk tissues before and after five months in infected oil palms treated with different concentrations of phenolics acids.

Treatments (g a.i.* of phenolics)	**Control** (no phenolic added)	**0.4**	**0.8**	**1.2**	**1.6**
Ergosterol content (in µg g^{-1}) of trunk tissues	0.6395 a	-0.2623 b	-0.3930 b	-0.3649 b	-0.4395 b

*a.i. Active Ingredient(s)

Means tagged with the same letter are not significantly different using the Tukey test (P >0.05). The sign of '-' means reduction of ergosterol content compared to original.

The control palms had significantly higher mean difference of ergosterol (0.6395 µg g^{-1}) at $p<0.05$ compared to palms treated with different concentration of phenolics. The combinations of phenolics with 1.6 g a.i. suppressed the colonization of *Ganoderma* to minimum, followed by 1.2 g a.i., 0.8 g a.i. and 0.4 g a.i.. Although palms treated with phenolics (1.6 g a.i.) reduced *Ganoderma* ergosterol the most (-0.4395 µg g^{-1}) but there was no significant difference between this treatment with other concentrations of phenolics at $p>0.05$. The control palms showed the highest accumulation of ergosterol (0.6395 µg g^{-1}), compared to infected palms treated with different concentration of phenolics. This may due to the progress of *G. boninense* infection if the palms left untreated. This is in correlation with the finding by Idris *et al*. (2004) which reported infected palms left untreated will not survive after a certain period of time. The result shown in Table 1, clearly supports the potential phenolics application as a field treatment to palms infected by *Ganoderma*. This is also in accordance to reports by Chong *et al*. (2009a & b) which reported the three phenolic acids from oil palm root; caffeic acid, syringic acid and 4-hydroxybenzoic acid to be toxic to *G. boninense* while the synergy effect of the combinations of them against this pathogen were also reported to have higher toxicity effect against the pathogen. However, the ANOVA analysis showed that there was no significant difference among the different concentrations of phenolics applied; this indicated the different concentrations give the similar effect in suppressing *Ganoderma*. If cost of application is concerned, planters may consider using the lower concentration of phenolics such 0.4 g a.i. as it found to be as effective as other higher concentrations tested.

Isolation of Ganoderma on Ganoderma Selective Media (GSM)

Fungus was successfully isolated on the GSM after 15 days of incubation from all infected tissues either from treated or untreated with phenolic acids (Figure 1). The identity of the fungus was later identified using molecular technique as described by Chong *et al*. (2011a) to be *G. boninense*.

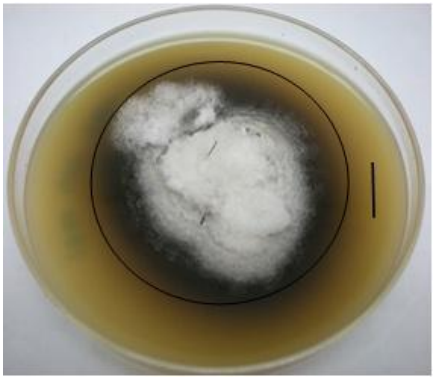

Figure 1: Mycelia of *Ganoderma*(in circle) from infected tissues grown on GSM. Similar result obtained from tissues treated with different concentrations of phenolic acids. Bar: 0.8 mm.

GSM was employed in this project as a tool for isolation of *Ganoderma* from infected tissues. This is an alternative method beside the biochemical analysis of ergosterol from the tissues. There was no *Ganoderma* isolated from healthy tissues, suggesting this method is reliable for isolating this devastating pathogen. In contrast, *Ganoderma* was successfully isolated from palms infected with this pathogen. *In vitro* studies on the morphological characteristics of *G. boninense* by Idris *et al*. (2000) 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. Since 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 economical solution for the elimination of other unwanted bacteria and saprophytic fungi from oil palm infected areas (Ariffin and Idris, 1992). However, GSM is unable to differentiate fungi among the basidiomycetes, and identification up to species level may not possible. Therefore, a more appropriate identification using PCR and sequence homology as described by Chong *et al*. (2011a) was employed in this study to confirm the identity of the *Ganoderma* isolated from the infected tissues.

Ganoderma Colony Forming

There was no colony of *G. boninense* form on the GSM for both soil treated and untreated with the combination of the phenolics after incubation of one month. The soil samples were taken from 15 cm in depth from the ground which reported the most active area of *Ganoderma*. However, no colony was form on media in this experiment, this may due to little amount of *Ganoderma* presence in the soil during the sampling process. Therefore, this parameter may not reliable in assessing the colonization of *Ganoderma* in other future study related to *Ganoderma*-soil.

Conclusion

Combinations of phenolics; caffeic acid, syringic acid and 4-HBA with the concentrations from 0.4 g to 1.6 g are able to suppress the colonization of *Ganoderma* in infected oil palm. The percentage of

Ganoderma colonization is reduced to 50% with only a single application of these phenolics. Future experiments may need to consider multiple applications of these combinations which may potentially reduce the colonization to lower.

Acknowledgements

The authors wish to thank Ministry of Education Malaysia for the financial support via grant PRGS0001-STWN-1/2012and also to Sawit Kinabalu Sdn. Bhd. for their technical assistance in this project.

References

Ahmed, E. Y. & Carlstrom, C. 2003. Food Microbiology: A Laboratory Manual. John Wiley & Sons, Inc. Hoboken, New Jersey.

Akhtar, M. & Malik, A. 2000. Roles of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: a review. *Bioresource Technology,* 74: 35-47.

Arif, M. S., Roslan, A., Idris, A. S. & Ramle, M. Economics of oil palm pests and *Ganoderma* disease and yield losses. Proceedings of the 3rd MPOB-IOPRI International Seminar: Integrated Oil Palm Pests and Diseases Management. 14 November 2011, Kuala Lumpur Covention Centre, Kuala Lumpur, Malaysia.

Ariffin, D. & Idris, A. S. 1992. The Ganoderma Selective Medium (GSM).*PORIM Information Series,* ISSN 0128-5726.

Chong, K. P. 2012. An evaluation of *Ganoderma* fungal colonisation using Ergosterol analysis and quantification. *Planter,* 88(1034): 311-319.

Chong, K. P., Atong, M. & Rossall, S. 2012a. Antimicrobial activity of chitosan-induced phenolics in oil palm roots against *Ganodermaboninense*. *Journal of Sustainable Science and Management*, 7(2): 170-178.

Chong, K. P., Atong, M. &Rossall, S. 2012b. The role of syringic acid in the interaction between oil palm and *Ganoderma boninense*, the causal agent of Basal Stem Rot. *Plant Pathology*, 61: 953-963.

Chong, K. P., Atong, M. & Rossall, S. 2012c. The roles of syringic, caffeic and 4-hydroxybenzoic acids in *Ganoderma* oil palm interaction. *Asian Journal of Biotechnology, Microbiology and Environmental Science,* 14(2): 157-166.

Chong, K. P., Lum, M. S., Foong, C. P., Wong, C. M. V. L., Atong, M. & Rossall, S. 2011a. First identification of *Ganoderma boninense* isolated from Sabah based on PCR and sequence homology. *African Journal of Biotechnology,* 10(66): 14718-14723.

Chong, K. P., Rossall, S. & Atong, M. 2011b. HPC Fingerprints and *in vitro* antimicrobial activity of syringic acid, caffeic acid and 4-hydroxybenzoic acid against *Ganoderma boninense*. *Journal of Applied Science,* 11(13): 2284-2291.

Chong, K. P., Rossall, S. & Atong, M. 2009a. *In vitro* antimicrobial activity and fungitoxicity of syringic acid, caffeic acid and 4-hydroxybenzoic acid against *Ganoderma boninense*. *Journal of Agricultural Science*,1(2): 15–20.

Chong, K. P., Rossall, S. & Atong, M. 2009b. *In vitro* synergy effect of syringic acid, caffeic acid, 4-hydroxybenzoic acid against *Ganoderma boninense*. *International Journal of Engineering Technology*, 1(4): 282–284.

Harborne, J. B. 1989. Methods in Plant Biochemistry. *In*: DeyP. M. & Harborne J. B. (eds.) *Plant Phenolics, Vol. 1*. Academic Press, London.

Idris, A. S., Ariffin, D., Swinburne T. R. & Watt, T. A. 2000. The identity of Ganoderma species responsible for BSR disease of oil palm in Malaysia-Morphological characteristics. *MPOB Information Series,* MPOB TT No. 77a.

Idris, A. S., Ismail, S., Ariffin, D. & Ahmad, H. 2004. Prolonging the productive life of Ganoderma-infected palms with Hexaconazole. *MPOB Information Series,* MPOB TT No. 214.

Langcake, P., Irvine, J. A. & Jeger, M. J. 1981. Alternate chemical agents for controlling plant disease. *Philosophical Transactions of the Royal Societyof London Series B: Biological Sciences,* 295: 83-101.

Lattanzio, V., Lattanzio, V. M. T. & Cardinali, A. 2006. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. *In*: Imperato F. (eds.) *Phytochemistry Advances in Research, pp: 23-67*. Research Signpost, India.