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THE POTENTIAL OF CHITOSAN IN SUPPRESSING *GANODERMA BONINENSE* INFECTION IN OIL-PALM SEEDLINGS

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Abstract: This paper discusses the possible use of chitosan in controlling *Ganoderma* infection in oil-palm nursery. Research was conducted to determine the suitable concentration of chitosan in suppressing Basal Stem Rot (BSR) in oil-palm seedlings caused by *Ganoderma*. Three different concentrations of chitosan tested were 0.5, 1.0 or 1.5% (w/v). In addition to this, a lower concentration (0.1%) was also evaluated at two different intervals of addition to the seedlings. The effectiveness of chitosan was measured through concentration of ergosterol (the fungal sterol) found in oil-palm roots, mean percentage of disease severity, percentage of bole tissue infection and possible isolation of *Ganoderma* on *Ganoderma* Selective Medium (GSM). Results showed the minimum concentration of chitosan; 0.5% (w/v) suppressed the fungal sterol to the minimum and scored the lowest percentage mean disease severity in comparison to other concentrations. However, there was no significant difference shown between all concentrations of chitosan for bole tissue infection as no infection was noted. *Ganoderma* was successfully isolated from seedlings roots of all treatments except healthy control using GSM. *Ganoderma*-infected seedlings that were treated with chitosan (0.1%, w/v) also showed slightly lower disease severity compared to seedlings pre-treated with chitosan and later infected by *Ganoderma* at the same concentration.

KEYWORDS: *Ganoderma*, Chitosan, Ergosterol

Introduction

Malaysia and Indonesia are the major palm-oil producers with Malaysia currently having the world's second largest area of oil palm after Indonesia. Productions of these two countries account for about 84% of total world production and 88% of global exports (Khairil and Hasmadi, 2010). The total exports of oil-palm products from Malaysia, constituting palm oil, palm-kernel oil, palm-kernel cake, oleochemicals, biodiesel and finished products increased by 11.1% or 2.18 million tonnes to 21.75 million tonnes in 2008 from 19.57 million tonnes recorded in 2007. The total export earnings also increased by 44.3% or RM20.02 billion to RM65.19 billion compared to RM45.17 billion in 2007 (MPOB, 2009). The devastating Basal Stem Rot (BSR) disease caused by *Ganoderma boninense* is considered the most serious disease faced by oil palm in Malaysia and Indonesia (Chong *et al*., 2009 a,b; Chong *et al*., 2011 b). The losses that were reported ranges from RM225 Million to RM1.5 Billion a year (Arif *et al*., 2011). Numerous controls have been utilised from conventional cultural control to attempt in developing genetic-resistant variety in combating BSR. However, the most common controls in plantations are practicing better sanitation and application of fungicide such as Hexaconazole. Attempts to control this disease in field with fungicides have been made by various workers, but the results are inconclusive, though some systemic fungicides seem to be promising (Idris *et al.,* 2002). The methods of fungicide application include soil drenching, trunk injection or combinations of these two methods. The applications were, however, very costly, not environmental friendly and the yield reduction and mortality rate was still high. A more effective but sustainable approach to control this disease would be worth exploring. Chitin is the major component of fungal cell walls, and chitin oligosaccharides act as PAMPs in plant and mammalian cells.

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Microbial pathogens deliver effector proteins to suppress PAMP-triggered host immunity and to establish infection (de Jonge *et al*., 2010). Chitosan contains more than 5000 glucosamine units and is obtained commercially from shrimp and crab shell chitin (a *N*-acetylglucosamine polymer) (Han *et al*., 1999). It has wide applications in the cosmetic, food, biotechnology and pharmacology industries. The fungicidal activity of chitosan has been well documented both in *in vitro* and *in situ* studies. Literature generally reports that the level of inhibition of fungi is highly correlated with chitosan concentration, indicating that chitosan performance is related to the application of an appropriate rate (Bautista-Bañosa, 2006). Furthermore, Chong *et al*., (2012) has also reported the efficacy of 0.1% (w/v) chitosan as the soil amendment in suppressing BSR intensity and inducing accumulation of phenolic acids in oil-palm seedlings. Therefore, this paper will discuss the different concentrations of chitosan and also time of chitosan-addition in suppressing *Ganoderma* infection.

Materials and Methods

Seed and plant materials

Certified disease-free, pre-germinated seeds, variety of AVROS were purchased from Borneo Samudera Sdn Bhd and grown to four-months old in Peat Vriezenveen Substrate, in field house of Universiti Malaysia Sabah.

Ganoderma boninense

Fruiting bodies of *G. boninense* were collected from oil-palm trees in Borneo Samudera Langkon Estate in Sabah. Internal tissues of fruiting bodies was excised and cultured on *Ganoderma* Selective Medium (GSM). GSM was prepared as described by Ariffin and Idris (1991). Once the pure culture of *Ganoderma* was isolated, it was transferred and maintained at 25°C on Potato Dextrose Agar (PDA) for normal growth, Furthermore, isolate of *G. boninense* was also subjected to molecular identification as described by Chong *et al*., (2011 a) later, *G. boninense* was re-inoculated into oil-palm seedlings after 12-days fully grown on PDA. The pathogen was later re-isolated from infected oil-palm seedlings to GSM. Procedures were repeated throughout the project to maintain the pathogenicity of *G. boninense.*

Preparation of Chitosan

Different concentrations of chitosan were prepared by adding chitosan (R & M Chemicals, Essex, UK) at 1g, 5 g, 10 g and 15 g respectively into 50 mL of HCL (38%) before top up to a final volume of 1L with double-distilled sterilised water.

Treatments with Chitosan and Ganoderma

Seedlings of oil palm aged four-months old, except healthy control, were carefully uprooted, and sprayed with 20 mL of different concentrations of chitosan. The amount of the liquid chitosan was first calibrated using an atomiser and a measuring cylinder. Seedlings were later re-planted and watered daily up to two weeks before being uprooted again and sprayed with the suspension of *Ganoderma* mycelia for infection purpose. Mycelia suspension of *Ganoderma* was prepared and introduced to seedlings as described by Chong *et al*., (2012). These seedlings were further incubated with normal agronomic practices for four weeks before being harvested and rinsed under running tap water prior to extraction. Meanwhile, for treatment with chitosan 0.1% (w/v), a group of seedlings was first introduced with this suspension, incubated for two weeks prior to inoculation of *Ganoderma*. After the inoculation, the seedlings were further incubated for four weeks before roots were excised for analysis. For a separate group of seedlings, *Ganoderma* was introduced to the seedlings' roots, incubated for two weeks before being introduced with chitosan (0.1%, w/v) and further incubated for four weeks. Finally, the roots were harvested for further analysis.

Extraction of ergosterol from infected oil-palm roots

All infected roots and uninfected roots (as control) were extracted as described by Genney (2000). Roots with 100 mg of fresh weight were extracted in methanol using bead beating to physically crush the sample at the same time. Polyvinylpyrrolidone

(PVPP) was added (10% w/v) to the methanol to precipitate phenolic compounds. The extract was centrifuged and supernatant was made up to 1.5 mL before being filtered through a 0.45µm acetate syringe tip filter.

Ergosterol analysis and quantification

The Eclipse XDB-C_{18} 4.6 mm x 150 mm x 5 µm column was utilised with an Agilent Series 1200 HPLC system for ergosterol studies. The wavelength of UV detector was set to 282 nm, and the isolated peak elution at about 5.5-5.8 min retention time was identified as ergosterol based on its co-chromatography and identical absorption spectrum with 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%, v/v methanol.

Percentage of disease severity

The percentage of disease severity was assessed according to the modified Horsfall and Barratt (1945) scale which consists of 12 scores ranging from 0-100% of infection.

Isolation of Ganoderma on GSM

To confirm the presence of *Ganoderma* in infected roots after different treatments, the pathogen was further isolated on GSM. The media was prepared as described by Ariffin and Idris (1991).

Percentage of bole tissue infection

The percentage of bole tissue infection was evaluated based on Teh and Sariah (1999). The bole of infected seedlings and control were cut longitudinally for assessment of percentage infection, expressed as (d/e) x 100, where d: the lesion length (mean of two measurements) and e: the bole diameter.

Statistical analysis

Data were statistically analysed by one-way analysis of variance and significant differences between treatments were tested by a DMRT. Analyses used the Statistical Package for Social Sciences (SPSS) version 17.

Results and Discussion

Ergosterol analysis and quantification

Chitosan with the lowest concentration (T1:0.5%) appeared as the best treatment in suppressing *Ganoderma* infection (0.29 µg g^{-1} of tissue). This was shown with the lowest ergosterol, the fungal sterol accumulated in roots treated with this concentration (Figure 1). However, the content of the ergosterol was not significant ($P>0.05$) in comparison with roots treated with 1.0% chitosan (T2: 0.42 µg g^{-1} of tissue). Surprisingly, the fungal biomass which directly related to the ergosterol content was not fully inhibited with the highest concentration of chitosan tested (T3), but T3 (0.66 µg g^{-1} of tissue) performed significantly better ($P<0.05$) compared to untreated seedlings (T4) which accumulated the highest fungal sterol at the end of the experiment (0.88 µg g^{-1} of tissue).

Chitosan suppressed the growth of *Ganoderma* more if added after the seedlings were infected by the pathogen (Figure 2). There was significant difference in the fungal sterol accumulated between seedlings to which chitosan was added (0.1%, w/v) before (T6: 0.73 µg g^{-1} of tissue) in comparison to after (T7: 0.59 µg g^{-1} of tissue) the infection of *Ganoderma*. The difference of 0.14 µg of the fungal sterol per g of root tissue between the T6 and T7 proposed chitosan served well as an ameliorative measure instead of preventive. Roots from the untreated seedlings accumulated the highest ergosterol content (0.9 µg g^{-1} of root tissue).

Chitosan possibly plays an important role in preventing the BSR development. Chitosan may activate an early defense mechanism in the seedlings. However, whether chitosan acts as an elicitor of resistance mechanism in oil-palm roots or is directly fungitoxic to *G. boninense* needs further investigation. In general, induced defence reactions in plants are highly correlated with enzymatic responses. Several studies have demonstrated that chitosan is an exogenous elicitor of host defence responses, including accumulation of chitinases, β-1,3-glucanases and phenolic compounds, induction of lignification, synthesis of phytoalexins by the infected host tissue and inhibition of host tissue maceration enzymes (Arlorio *et al.,* 1992; Zhang and

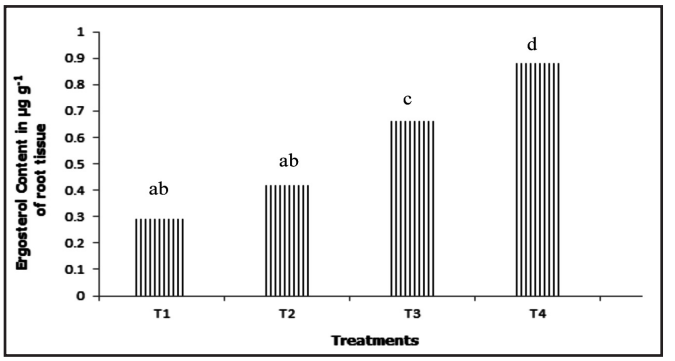

Figure 1: Accumulation of ergosterol in *Ganoderma*-infected oil-palm roots after different treatments. T1: Treated with 0.5% chitosan, T2: Treated with 1.0% chitosan, T3: Treated with 1.5% chitosan, T4: Infected but untreated seedlings. Note: Ergosterol not found in all control healthy seedlings (T5, not shown). Means tagged with the same letter are not significantly different using the DMRT test (P=0.05).

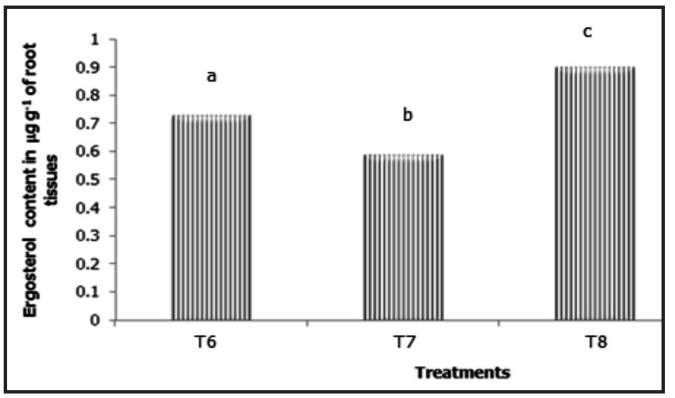

Figure 2: Accumulation of ergosterol in *Ganoderma*-chitosan added oil-palm roots. T6: Chitosan (0.1%) added two weeks prior to inoculation of *Ganoderma*, T7: Chitosan (0.1%) added after infected by *Ganoderma*, T8: No addition of chitosan. Note: Ergosterol not found in all control healthy seedlings (T9, not shown). Means tagged with the same letter are not significantly different using the DMRT test (P=0.05).

Quantick, 1998; Bhaskara Reddy *et al.,* 1999). It is believed that the polycationic nature of this compound is the key to its antifungal properties and that the length of the polymer chain enhances its antifungal activity (Hirano and Nagao, 1989). An additional explanation includes the possible effect that chitosan might have on the synthesis of certain fungal enzymes (El Ghaouth *et al.,* 1992). Recent studies have shown that chitosan is not only effective in halting the growth of pathogens, but also induces marked morphological changes, structural alterations and molecular disorganisation of fungal cells (Ait Barka *et al.,* 2004). Ganeson and Supramaniam (2009) reported higher concentrations of chitosan gave a reduction in *G. boninense* mycelia growth *in vitro* and the highest percentage inhibition of radial growth was observed with chitosan at a concentration of 2% (w/v). However, the changes to microbial activities in some plant species may not be obvious after exposure to a higher concentration of chitosan when it reached a stable inhibition stage (Shin *et al*., 2001).

Percentage of disease severity

The trend observed in the percentage of BSR severity is similar to that in ergosterol content. Seedlings treated with the lowest concentration of chitosan T1 showed significantly the least infected in comparison to other concentrations of chitosan ($P<0.05$). However, the index suggested T3 with higher concentration of chitosan performed better than T2. Roots from seedlings untreated by chitosan were severely infected (Figure 3). Observation on the morphology of the *Ganoderma*-infected oil-palms roots suggested application of the chitosan (0.1%, w/v) after the infection suppress the disease more than if applied before the infection occurred (Figure 4). However, the difference was not significant ($P>0.05$). Both the chitosan- treated seedlings appeared significantly less infected in comparison to untreated seedlings ($P<0.05$). The disease index is mainly based on visualisation and may be very subjective between different observers. However, the similar trend between the ergosterol results and the index suggested that

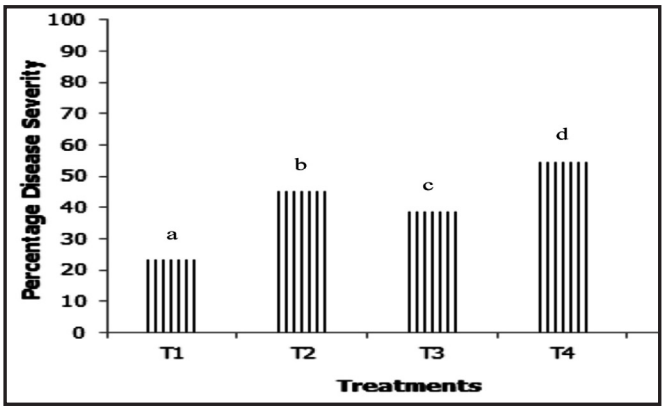

Figure 3: Percentage disease severity of *Ganoderma*-infected oil-palm roots after different treatments based on modified Horsfall and Barratt (1945) scale. T1: Treated with 0.5% chitosan, T2: Treated with 1.0% chitosan, T3: Treated with 1.5% chitosan, T4: Infected but untreated seedlings. Note: No score was given to all control healthy seedlings (T5, not shown). Means tagged with the same letter are not significantly different using the DMRT test (P=0.05).

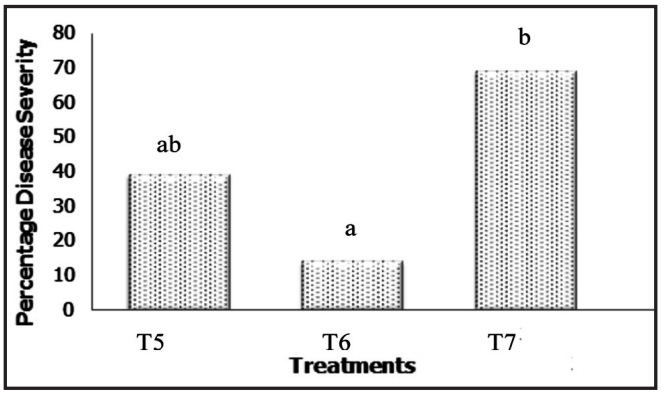

Figure 4: Percentage disease severity of *Ganoderma*-infected oil-palm roots based on modified Horsfall and Barratt (1945) scale after addition of chitosan (0.1%) at different intervals. T5: chitosan added before *Ganoderma* infection, T6: chitosan added after *Ganoderma* infection, T7: untreated roots but infected with *Ganoderma*. Note: No score was given to all control healthy seedlings (T8, not shown). Means tagged with the same letter are not significantly different using the DMRT test (P=0.05).

Figure 5: Mycelia of *Ganoderma* (in circle) from infected roots grown on GSM. Similar results obtained from roots treated with different concentrations of chitosan. Bar: 6cm

this scale could be utilised in future *G. boninense*-BSR research. The index, which is mainly based on visual changes on the roots, provides a faster reference for the oil-palm planters in comparison to some other complex indices that depend on slow development of foliar symptoms such as described by Teh and Sariah (1999); Sariah and Zakaria (2000).

Isolation of Ganoderma on GSM

Ganoderma which infected AVROS seedlings roots in this experiment were successfully isolated from all roots using GSM though treatment with different chitosan concentrations (Figure 5), suggesting none of the seedlings were free from infection. No *Ganoderma* was isolated from non-infected healthy roots. This simple test proved the artificial inoculation success in introducing disease to the targeted seedlings with chitosan only able to suppress the disease intensity but not fully eliminate the pathogen. GSM was developed to isolate and confirm *Ganoderma* (Ariffin and Idris, 1991). This medium is very convenient to isolate the fungus from diseased tissue and provides a useful tool for isolating *Ganoderma*,

free from other contaminants. The content of fungicides and antibiotics is optimal to control growth of bacteria and other contaminating fungi, while allowing *Ganoderma* to thrive. It does not, however, differentiate between different species within the genus. It was reported that, using GSM, it is possible to detect *Ganoderma* in 5 to 16% of oil palms, not showing any obvious external symptoms of infection, or detected using a drilling technique (Idris *et al*., 2009).

Percentage of bole tissue infection

No bole tissue infection was observed within all the seedlings either treated with chitosan or not. As the infection was initiated via the artificial inoculation from roots, bole tissue infection would only be detectable when the disease progresses further from the roots. The *Ganoderma* infection rate is slow in nature and may be unable to reach the bole within the observation period (four months). However, heavily injured roots could influence the speed of *G. boninense* infection in oil palm. The rate of movement of *G. boninense* within infected roots was 1.62 to 2.12 cm month^{-1}, and the average was 1.83 cm month^{-1} (Idris, 2009). This rate is slower than the speed of *G. lucidum* at 2.3 cm month^{-1} in roots of grape (Adaskaveg and Gilberstson, 1987). At the rate of approximately 1.8 cm month^{-1}, *G.. boninense* would take about four years to reach the bole in a mature palm if, hypothetically, a root of one metre was infected through contact with disease debris.

Conclusion

Lower concentration of chitosan in this experiment, 0.5%, (w/v) successfully reduced the BSR severity in oil-palm seedlings. Results obtained from the ergosterol content and percentage of disease severity confirmed the potential of this concentration to suppress the BSR disease. Chitosan with concentration of 0.1% (w/v) performed better if applied after *Ganoderma* infected the oil-palm seedlings in comparison to applying before the infection.

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