Efficacy of Bacterial Biocontrol Agents and Environmental-Friendly Fine Chemicals for Suppression of *Ganoderma Boninense* Growth

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Abstract. Various means are available to control *Ganoderma boninense*, however none of those methods are efficacious. Biological control agents method is one of promising way to control the pathogen, that has eco-friendly-mannered. The objective of this research was to evaluate the effectiveness of bacterial strains and the application of some environmentally friendly fine chemicals including ammonium, calcium, and salicylic acid in suppressing of mycelia growth of *G. boninense*. *In vitro* results showed that among eight strains, EB13, as identified as *B. amyloliquefaciens*, has reduced *G. boninense* growth diameter significantly (P<0.05%) at the highest percentage (81%). It followed by MB31, MB72, MB58 and MB41 74%, 74%,71% and 63%, respectively. Conversely MB20, EB45 and EB53 did not suppress the fungal pathogen growth. The chemical control test indicated that salicylic acid has inhibited the growth of the pathogen significantly at the highest percentage of 52% (P<0.05%). The lowest growth inhibition was by ammonium nitrate (2%). On the contrary, calcium chloride could not inhibit the *G. boninense* growth. Further assessment on pH in PDA media containing of 200mM ammonium was conducted in order to increase the inhibition activity of the ammonium nitrate. The result exhibited that the medium containing ammonium nitrate with pH 8.5 has reduced *G. boninense* growth significantly at the highest percentage (53%).It followed by pH 8.0 and pH 7.5 at 10% and 2%, respectively.

1. Introduction
*Ganoderma* species is a soil-borne fungus caused a major basal stem rot (BSR) disease of oil palm (*Elaeis guineensis* Jacq) in Southeast Asia, particularly in Indonesia and Malaysia [1-4]. Incidence of *Ganoderma boninense* BSR in oil palm plantations continues to increase that caused economic losses in the global oil palm industry. Several means have been done in controlling of *Ganoderma* disease including chemical, cultural, and sanitation by the removal of diseased oil palms. Field trials to control *Ganoderma* disease by using systemic and contact fungicides have been performed by various researchers with no conclusive results although *in vitro* they showed to be effective. Application chemical pesticides resulted in the development of insecticide resistance, extensive damage to the environment, and lethal effects on non-target organisms. Therefore, an alternative eco-friendly application is required to increase the production and to generate healthy agricultural products. The possible approach in controlling the diseases caused by *G. boninense* could be through the bacterial biocontrol agents and supplementation of ammonium, calcium, and salicylic acid.
Interest in biocontrol has increased since public has been concerned over the use of chemical in general [5]. The benefits of biocontrol are 1) potentially self-sustaining, 2) spread on their own after initial establishment, 3) reduced inputs of non-renewable resources and 4) long-term disease suppression in an environmentally manner [6, 7].

Several plant pathogens have been killed at elevated levels of ammonium, such as *Ralstonia solanacearum* [8], *Verticillium dahlia* [9] and *Phytophthora cinnamoni* and *P. parasitica* [10]. Application of calcium (Ca) increased the resistance of tomato to bacterial wilt and decreased the population of *Ralstonia solanacearum* in the xylem [11], suppressed the BSR symptoms on clonal materials [12], decreased disease incidence and delayed the onset of *Phytophthora* stem rot in soybean [13].

Salicylic acid (2-hydroxybenzoic acid, SA) is phenolic compound that induces plant diseases resistance [14]. Induction to pathogen resistance is promising way for controlling of plant diseases. Efficacy of SA in suppression of plant diseases has been studied e.g., *Fusarium graminearum* in wheat [15], *F. oxysporum* in tomato (Fol) [8], and *Penicillium expansum* in apples [16].

The objective of the present research work was to evaluate the effectiveness of bacterial strains and the application of some environmentally friendly fine chemicals including ammonium, calcium, and salicylic acid in suppressing of mycelia growth of *G. boninense*.

### 2. Materials and Methods

Pure fungal cultures of *G. boninense* was obtained from Laboratory of Microbiology, Indonesian Research Institute for Biotechnology and Bioindustry, Bogor, Indonesia. Bacterial biocontrol agents ammonium nitrate (NH$_4$NO$_3$) (Fisher scientific), salicylic acid (C$_7$H$_6$O$_3$) (Merck), calcium chloride dehydrate (CaCl$_2$2H$_2$O) (Merck) were taken from the Laboratory of Environmental Microbiology, Research Center for Biology, LIPI.

#### 2.1. Regrowth of *G. boninense* and bacterial biocontrol agents (BCAs)

The pure culture of the *G. boninense* fungi was sub-culture into a potato dextrose agar (PDA, brand) medium in test tube, and incubated for about 5-7 days. The seven days old of the fungi taken from the test tube was then inoculated on the sterile PDA plates. For the BCAs, eight of the bacterial strains, namely, EB13, EB45, EB53, MB20, MB31, MB41, MB58, and MB72 were regrowth in to nutrient agar (NA) medium in test tube, and incubated for about 2 days at room temperature.

#### 2.2. Screening of bacterial biocontrol agents for suppression of *G. boninense* growth

Eight of bacteria strains (EB13, EB45, EB53, MB20, MB31, MB41, MB58, and MB72) were examined of their ability to inhibit *G. boninense* mycelia growth. A hundred (100) µl of each of the fifth days of the bacterial culture in No. 3 medium (i.e. 10 g polypepton (Becton, Dickinson and Company), 10 g glucose (Merck), 1 g KH$_2$PO$_4$, 0.5 g MgSO$_4$$\cdot$ 7H$_2$O (Merck), pH 6.8, 1 L distilled water) was pipetted into sterile plates. An autoclaved-PDA agar in an erlenmeyer flask was poured into the sterile plates. The tested plates were rotated to mix the bacterial culture and the medium. After the media solidified, in the center of the plates were inoculated with the previously prepared-plug of 5-7 days of *G. boninense* mycelia growth in PDA plates as described in a section 2.1 (the plug diameter size was about 5 mm). For the control were used 100 µl of sterile distilled water. This experiment was performed in a clean bench, in four replicates. Finally, the plates were incubated at room temperature. The diameter of the mycelia growth were measured and recorded at first, fifth, seventh, eleventh, and thirteen days incubation time. Percentage of *G. boninense* inhibition growth was calculated, using the following formula;

$$\text{Inhibition growth} = \frac{\phi_C - \phi_T}{\phi_C} \times 100\%$$

Where is $\phi_C$ = diameter of control  
$\phi_T$ = diameter of treatments
2.3. Screening of environmentally friendly fine chemical compounds for suppression of G. boninense growth

Three kinds of the chemical compounds: ammonium nitrate (NH$_4$NO$_3$), salicylic acid (C$_7$H$_6$O$_3$) and calcium chloride dehydrate (CaCl$_2$.2H$_2$O) were assessed their possibility to suppress the mycelia growth of the fungus. Each compound of 200 µL of 25 mM of NH$_4$NO$_3$, 16 mM of C$_7$H$_6$O$_3$, and 15 mM of CaCl$_2$.2H$_2$O was pipetted into plates. Then PDA medium was poured into the plates and rotated to mix. After the medium was solidified, the mycelia of G. boninense sized 5 mm in diameter was inoculated in the center of the tested plates. The plates were incubated at room temperature, the diameter growth of the fungus mycelia was observed and measured on first, seventh, ninth, thirteenth day incubation time. The experiment was performed in four replication.

2.4. Influence of pH of PDA medium containing ammonium nitrate

In order to enhance the ammonium nitrate in suppression of G. boninense mycelia growth, the effect of various pH of PDA medium containing ammonium nitrate was investigated. The pH of PDA medium containing 0.2M of NH$_4$NO$_3$ was adjusted to 7.5, 8.0, and 8.5 by using 1N NaOH, and 1N HCl. Subsequently, the medium was autoclaved, and pour into sterile plates. The solidified medium was inoculated with a plug of G. boninense sized 5mm in diameter, and incubated for about fourteen (14) days. The mycelia growth of the fungus was observed and recorded on first, fourth, seventh, eleventh, and fourteen days. The experiment was performed in four replication.

2.5. Data analysis

Data were statistically calculated using analysis of variance (ANOVA) with Minitab 16 software. The significance of mean differences was determined using the Duncan’s test. The responses were judged significant at 5% level.

3. Results and Discussion

3.1. Screening of bacterial biocontrol agents for suppression of G. boninense growth

Significantly, the highest suppression effect of the bacterial biocontrol agents against G. boninense mycelia on day seventh was observed in application of EB13 strain, followed by MB31, 72, 58 and 41 strains (P<0.05) (Figure 1, Figure 2, and Figure 3). The suppression effect of EB13 strain increased from 78.43% on day 5 to 90.41% on day 13 (Figure 2). The MB72 strain has also increased its suppression effect from 72% on day 5 to 87.14% on day 13 (Figure 2). Similarly, the MB31 strain has increased its effect from 78.43% to 86.74% (Figure 2). Degrees of G. boninense growth inhibition on day 5 to day 13 after application of MB41 and MB58 strains were almost the same. Respectively, they increased from 37% to 81% and from 47% to 82%. In contrary, application of the EB45, EB53 and MB20 strains have not reduced the mycelia growth of fungal pathogen (Figure 1). On day eleventh of incubation time, the EB13 strain showed the greatest effect on suppression of the fungal mycelia growth consistently (Figure 1b). Our previous research showed that based on 16S rRNA identifications, the EB13 strain was identified as Bacillus amyloliquefaciens. B. amyloliquefaciens has been reported in increased survivability of tomato against Ralstonia solanacearum and Rhizoctonia solani significantly [17]. Suppression growth of G. boninense due to antifungal compounds produced by B. amyloliquefaciens has been reported in Yuliar et al. [17]. The author reported that B. amyloliquefaciens produced an antifungal of iturin and antibacterial of surfactin. The other bacterial biocontrol reduced G. boninense growth has been reported by Azahar et al. [2, 18] who found that Pseudomonas aeruginosa changed the hyphal tips of G. boninense, and caused a great malformation of the hyphae. The culture broth and culture filtrate of P. aeruginosa reduced the radial growth of the fungal pathogen by 93% and 96%, respectively.
Figure 1. Suppressive ability of the biocontrol agents to inhibit *G. boninense* growth on days seventh (a) and eleventh (b) of incubation time. Means with different letters are significantly different (P<0.05)

Figure 2. Percentage of the biocontrol agents ability to inhibit *G. boninense* growth during thirteen days of incubation time

Figure 3. Growth inhibition of *G. boninense* at seventh day incubation by biocontrol agents of EB13 (a), MB20 (b), MB31 (c), MB41 (d), EB45 (e), EB53 (f), MB58 (g), and MB72 (h), Negative control (i)
3.2. Screening of environmentally friendly fine chemical compounds for suppression of G. boninense mycelia growth

*G. boninense* growth was significantly inhibited by salicylic acid (SA) at the highest rate (48%) compared by ammonium nitrate at 12% (Figure 4 and Figure 5). In contrast, calcium chloride did not inhibit the fungal pathogen growth. Similar results on the effects of SA on plant pathogens were also observed by Saikia *et al.* [19]. The author reported application of *P. fluorescence* pfl4-92 with SA has resulted in the highest suppression of chickpea seedling wilting compared to acetyl salicylic acid, DL-norvaline, indole-3-carbinol and lichenan. The presence of increasing concentration of SA in liquid and solid media has significantly inhibited mycelial growth and conidia germination of *Fusarium graminearum*, and finally halted head blight in wheat [15]. Further application of 200 mM SA through root feeding and foliar spray of tomato could induce resistance against *F. oxysporum* f. sp *Lycopersici* (Fol) [14]. Similarly, the germination of *Penicillium expansum* was completely inhibited by the application of 2.5 mM salicylic acid [16].

The possible mechanism of SA in suppressing the plant fungal pathogens was related to its behaviour as dissociating agent. It caused a change in the trans-membrane pH gradient in membranes of organelles and in the plasma membrane, possibly caused cellular energy loss [20]. In addition, SA caused leakage of the pathogen’s proteins to the medium, lipid damage, and intracellular disorganization [16,21].

*G. boninense* was suppressed by ammonium nitrate (Figure 4). It was probably because ammonium could kill the fungal pathogen. This finding agrees with the findings of Tenuta and Lazarovit [9] and Amari *et al.* [8] who reported that ammonia are toxic to *Verticillium dahliae*, and *Fusarium oxysporum* and *Ralstonia solanacearum* respectively.

The fungal growth was not inhibited by supplementation of calcium chloride (Figure 4). However, different result was obtained by Bivi *et al.* [22] who revealed that the mixture addition of calcium, copper, and salicylic acid had potential to suppress growth of *G. boninense in vitro*. The contradictory of this result was probably due to addition of calcium in different way of application and concentration. In this study, the calcium chloride was applied as a single addition instead of a mixed addition.

![Figure 4](image1.png)

**Figure 4.** Suppressive ability of ammonium nitrate, calcium chloride, and salicylic acid to inhibit *G. boninense* growth. Means with different letters are significantly different (P<0.05)

![Figure 5](image2.png)

**Figure 5.** Growth inhibition of *G. boninense*; distilled water as negative control (a), salicylic acid (b), calcium chloride (c), and ammonium nitrate (d)
3.3. Influence of pH of PDA medium containing ammonium nitrate on G. boninense growth

The fungal pathogen grew at pH 8.5 of the medium PDA containing ammonium was highly suppressed from day fourth to day seventeenth consistently (Figure 6). The growth inhibition of G. boninense was significantly at the highest at pH 8.5 compared to pH 8.0 and pH 7.5 in PDA medium containing ammonium nitrate (Figure 7). An explanation for this results is probably because of increasing of pH affected the increasing of ammonium uptake rates [9]. Furthermore, the increasing of ammonium uptake leads to increase of the toxicity and finally impacted on the higher reducing of the diameter growth of G. boninense.

Figure 6. Influence of pH on PDA medium containing ammonium nitrate in suppression of G. boninense growth. Means with different letters are significantly different (P<0.05)

Figure 7. Growth inhibition of G. boninense at thirteenth day incubation on PDA medium containing ammonium nitrate at various of pH range. (a) pH 8.5, (b) pH 8 and (c) pH 7.5

4. Conclusions

To summarize, the highest suppression of G. boninense growth was observed in the application of B. amyloliquefaciens (EB13 strain). It followed by MB31, 41, 58, and 72 strains. On the contrary, EB45, EB53, and MB29 strains have not suppressed growth of G. boninense.

G. boninense growth was inhibited significantly by salicylic acid with the highest percentage (48%) in comparison to ammonium nitrate (12%). Conversely, the growth of fungal pathogen was not inhibited by calcium chloride. Furthermore, the pH of media containing ammonium nitrate influenced its ability in suppressing the growth of G. boninense. Its suppressing ability was increased by increasing its pH from 7.5 to 8.5.
Acknowledgment
This research work was partly supported by project of DIPA (Issuance of spending authority) of Research Center for Biology, Indonesian Institute of Sciences. Our gratitude to Mrs. Ety Suryati for her technical assistance during research activities.

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