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The Potential of Palm Oil Mill Effluents and *Bacillus amyloliquefaciens* EB13 in Reducing of *Ganoderma boninense* Disease in Oil Palm

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Abstract. Indonesia is the largest palm oil producing country in the world. *Ganoderma boninense* disease is the most destructive that cause limiting factor in production of oil palm. It is a crucial to discover an effective management strategy for a serious threat of basal stem rot disease of *G. boninense* in oil palm. The objective of this research was to assess the possible use of palm oil mill effluents (POME) and *Bacillus amyloliquefaciens* EB13 in reducing of oil palm disease caused by *G. boninense*. In greenhouse experiment has shown that POME and *B. amyloliquefaciens* EB13 significantly reduced disease index (DI) of oil palm disease from 4.0 to 2.3 and from 4 to 1.8 respectively. Biocontrol efficacy of *B. amyloliquefacient* and POME were 70.9 and 70.4 respectively. The addition of POME into soil has significantly reduced population of fungus from 40.0 to 1.8×10^4 CFU g⁻¹ fresh soil, and the addition of *B. amyloliquefaciens* reduced population of fungus from 40.0 to 2.3×10^4 CFU g⁻¹ fresh soil. Amount of bacteria population increased from 13.8 to 19.0×10^6 CFU g⁻¹ fresh soil by addition of POME, even the increasing was not significant. While addition of *B. amyloliquefaciens* significantly increased bacteria population from 13.8 to 56.8×10^6 CFU g⁻¹ fresh soil. Laccase activity in soil inoculated only with *G. boninense* would significantly highest compared with combined treatment of POME and *G. boninense*, and the combined treatment of *B. amyloliquefaciens* and *G. boninense*, their activities were 2991.2 U mL⁻¹, 1140.6 U mL⁻¹, and 609.6 U mL⁻¹ respectively. Lignin peroxidase activity in soil inoculated only with *G. boninense* and soil with combined treatment of POME and *G. boninense* higher than the combined treatment of soil with *B. amyloliquefaciens* and *G. boninense*, their activities were 666.4 U mL⁻¹, 655.3 U mL⁻¹, and 492.4 U mL⁻¹ respectively. The highest activity of mangan peroxidase was detected in soil inoculated only with *G. boninense* compared with combined treatment of POME and *G. boninense*, and the combined treatment of *B. amyloliquefaciens* and POME, although the differences were not significant, their activities were 202.3 U mL⁻¹, 146.3 U mL⁻¹, and 148.9 U mL⁻¹.

1. Introduction

Oil palm (*Elaeis guineensis* Jacq.) is one of the most important crop plantations in Indonesia as it produces the highest vegetable oil in the world. The average production per year can approach as much as 36 million tones [1]. A crucial problem in oil palm (OP) plantation is the diseases, such as the most important and destructive disease of basal stem rot (BSR) caused by *Ganoderma boninense*. In few decades BSR has been spreading rapidly, and it leads to losses as much as 50% after repeated planting cycles (25 years) in North Sumatera,



Indonesia [2]. The disease not only affect the old OP, but also affect the nursery and upper stem [3,4]. Incidence of BSR continuously increase, the most severe losses occur in Indonesia and Malaysia, and lower incidence found in Africa, Papua New Guinea, and Thailand [5]. The control effort of BSR using cultural techniques, chemical, mechanical, still did not show significant results. The use chemical method, through application of fungicides did not show effective results yet in controlling soilborne diseases [6]. This may be due to the fact that by the time fungicides are applied, OP may already have the disease. Antifungal producing bacteria such as *Bacillus amyloliquefaciens* is a promising biocontrol agent (BCA) to overcome BSR. The BCA does not necessarily be remedy for the disease but to slowing or even to stop the disease spread by protecting or enhancing the OP defense.

BCAs have more value, since they are potentially self-sustaining, spread on their own after initial establishment, and long-term disease suppression in an environmentally manner [7,8]. Various strain of *B. amyloliquefaciens* produce potent antibiotics and others secondary metabolites for biocontrol of plant pathogens, such as surfactin, fengycin, iturin [9,10], macrolatin, difficidin, and bacillaene [11,12]. BCA have been applied in many ways and on many crop species such as by pouring [13-15], mixed with soil [10,16,17], and stem injection [18]

Palm oil mill effluent (POME) is a thick brownish liquid waste with high biochemical oxygen demand (BOD) and chemical oxygen demand (COD) resulted from the process of oil extraction in palm oil industry [19]. Initially the application of POME into soil for fertilizer is considered as harmful effluent for environment, lead to an aerobic condition, decreasing plants ability in nutrients absorption. However currently POME used as fertilizer, because it is not a toxic waste, since no chemical is added during process of the oil extraction, however it will pose environmental issues due to a high content of BOD and COD. A high organic matter content of the effluent due to the presence of different sugars such as arabinose, galactose, glucose, manose, and xylose. Its supended solids are mainly oil bearing cellulosic materials from the fruits. Furthermore POME contains nutrient in a significant amount such as ammonia nitrogen ($450\text{--}590\text{ mg L}^{-1}$), P ($92\text{--}104\text{ mg L}^{-1}$), K ($1246\text{--}1262\text{ mg L}^{-1}$) and Mg ($249\text{--}271\text{ mg L}^{-1}$) [20]. Therefore POME is non toxic, it is a good source of nutrients for microorganisms. Besides as nutrient source, ammonia contents of POME may use for suppressing of plant pathogens growth, for instance ammonia killed *Verticillium dahliae* [21], and suppressed the growth of *Fusarium oxysporum* f. sp. lactuceae and *Ralstonia solanacearum* [22]

The aim of this research work was to evaluate the impact of POME and a bacterial biocontrol agent of *B. amyloliquefaciens* EB13 in reducing incidence of *G. boninense* disease in oil palm.

2. Materials and Methods

2.1. Preparation of oil palm germinated, soil, and *G. boninense*

Oil palm germinated was obtained from Research Center of Oil Palm, Medan, North Sumatera, Indonesia and pure fungal cultures of *G. boninense* was gotten from Laboratory of Microbiology, Indonesian Research Institute for Biotechnology and Bioindustry, Bogor, Indonesia. Soil was collected from in the experimental area of Research Center for Biology, Indonesian Institute Science, Cibinong, West Java Province, Indonesia. Some of the characteristics of the Cibinong soil were as follows: pH (H_2O) 5.7; total C and N 22.6 and 2.1 g kg^{-1} ; texture, silty clay (sand 7%, silt 50%, and clay 43%); soil taxonomy Latosol.

The soil was dried and sieved through a 2-mm mesh sieve and then used for the following experiments.

2.2. Preparation of culture broth of *B. amyloliquefaciens* EB13

B. amyloliquefaciens EB13 was preincubated in 5 mL sterilized LB medium (10 g polypepton, 5 g yeast extract, 5 g NaCl, 1 L distilled water) at 30°C with shaking (124 rpm) overnight. Forty mL of a sterile No.3 medium (10 g polypepton, 10 g glucose, 1 g KH_2PO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 6.8, 1 L distilled water) in a 200 mL Erlenmeyer flask was inoculated with 400 μL of the bacterial strain preincubated in LB and incubated at 30°C with shaking (124 rpm) for 5 days. After incubation, 30 mL of a 5 day-culture of the bacterial strains in No.3 medium was added to the pot of the experiment and mixed.

2.3. Preparation of *G. boninense* inoculum.

G. boninense isolate was used for infection of the oil palm seedlings. 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, and incubated for 7-10 days.

2.4. Influence of POME and *B. amyloliquefaciens* EB13 in oil palm diseases caused by *G. boninense* under greenhouse condition

Two hundreds and fifty grams of dried soil was mixed with 60 mL of POME, 10 mL of steril distilled water and inoculated with a ½ part of *G. boninense* mycelia grown in a petri dish with 9 cm in diameter containing PDA at room temperature. After mixing, it was transferred to a palstic pot (Hammy production, Indonesia: 9.5 cm in diameter, 6.5 cm in height). Germinated of oil palm was transplanted to the pots, and were grown for 2-3 month in greenhouse. The treatments in this experiment were as follows;

- Control negative (no inoculation of *G. boninense*, and *B. amyloliquefaciens* EB13)
- Control positive (inoculation with *G. boninense* only)
- POME + *G. boninense* (oil palm germinated in pot experimnets were inoculated with *G. boninense* and supplemented with POME
- EB13 + *G. boninense* (oil palm germinated in pot experiments were inoculted with *G. boninense* and supplemented with 30 mL of a 5 day- culture broth of *B. amyloliquefaciens* EB13)

The disease index was categorized as follows: 0; no chlorotic leaves symptoms, 1; 0 to 25% plant showing chlorotic leaves, 2; 26 to 50%, 3; 51 to 75%, 4; 76 to 100%.

Biocontrol efficacy was calculated using the following formula;

$$\text{Biocontrol efficacy} = \frac{\text{disease incidence in control} - \text{diseases treatment}}{\text{Disease incidence in control}} \times 100 \quad (1)$$

2.5. Monitoring of laccase, mangan peroxidase, and lignin peroxidase activity in the soil.

One gram soil was taken from each treatment and put into an Erlenmeyer flask with 50 mL of sterile distilled water, incubated at room temperature with shaking 150 rpm for one hour. After that 2 mL of its supernatant were pipetted into a small tubes and centrifuged 12.000 rpm for 10 minutes, and its supernatant was collected. The supernatant was used for measurement of laccase, mangan peroxidase, and lignin peroxidase activity.

Laccase activity was measured by monitoring the oxidation of 2,2'-azino-bis (3 ethybenzthiazoline)-6-sulfonate (ABTS) as described by Papinutti [23]. The reaction mixture consisted of 0.1 mL of 2,2'-azino-bis (3 ethybenzthiazoline)-6-sulfonate (ABTS) 1mM, 0.5 mL citrate buffer, pH 6.0, 0.4 mL the supernatant. The ABTS oxidation was monitored by the rise in absorbance at 420 nm. In addition mangan peroxidase (MnP) activity was detected by observing the oxidation of guaiacol spectrometrically [24]. The reaction mixture were 0.1 mL guaiacol 4mM, 0.2 mL MnSO₄ 1mM, 0.1 mL of lactate buffer 50 M, pH 4.5, 0.1 mL H₂O₂, 0.3 mL of distilled water, and 0.2 mL of the supernatant. Guaiacol oxidation was monitored by the rise in absorbance at 465 nm. Then lignin peroxidase (LiP) activity was measured as described by Tien and Kirk [25]. The measurement based on the oxidation of veratryl alcohol to veratryl aldehyde in the presence of H₂O₂. The reaction mixture consisted of 0.1 mL of 8 mM veratryl alcohol, 0.05 H₂O₂ 5mM, 0.2 mL of acetate buffer 50 mM, pH=3.0, 0.2 mL of the supernatant. The rising of absorbance was observed at 310 nm, with four replication.

2.6. 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 Influence of POME and *B. amyloliquefaciens* EB13 in oil palm disease caused by *G. boninense* under greenhouse condition

The result of disease occurrence in oil palm showed that POME and *B. amyloliquefaciens* EB13 significantly efficacy reduced disease index (DI) of oilpalm disease, the DI were 2.3 and 1.8 (Figure 1a). This may be due POME contains ammonia that can kill the fungal pathogen of *G. boninense* caused the oil palm disease. Some of possibilities disease suppression mechanisms by using POME are the ammonia content in the POME kill plant pathogens [21, 22], organic and nutrient content of POME enhance plant growth [26,27]. Furthermore Dordas [28] stated that disease incidence are influenced by soil nutrients. Tengoua [27] reported that the double combination of micronutrient of B with Cu, B with Mn, and Cu with Mn reduced the incidence and severity of BSR better than single one.

Degree of biocontrol efficacy did not differ between *B. amyloliquefaciens* and POME as high as 70 (Figure 1b). POME addition reduced population of fungi significantly from 40 to 1.8×10^4 CFU g⁻¹ fresh soil, and tend to increase population of bacteria even though was not significant (Figure 2a and 2b). In addition *B. amyloliquefaciens* EB13 addition significantly reduced fungi population and increased bacteria population (Figure 2a and 2b). Therefore, the decrease of DI was supported by result of the decrease the fungi population. The increasing of bacteria population significantly by addition *B. amyloliquefaciens* EB13, its may due the biocontrol agent grown well and colonize the oil palm root and thereby decreased the fungal population, resulted lower DI compared with the positive control. Yuliar *et al.*, [10] also reported that the lower amount of fungal pathogens cause the decrease of disease incidence.

3.2 Influence of POME and *B. amyloliquefaciens* EB13 on oil palm height

The highest oil palm was observed in applicatioan of POME and or application of *B. amyloliquefaciens* EB13. The increasing of oil palm height by application of POME (Figure 3), it is probably because of contents large amount of organic material and nutrients in POME such as N,P,K, Mg, and Ca [29] that are essential elements for plant growth. Since POME contains fertilizer properties, it can be used as fertilizer [26,30]. While the reason why *B. amyloliquefaciens* (Figure 3) also tend to enhance the oil palm height was *B. amyloliquefaciens* produced phytohormones such as gibberellins, abscisic acid, indole acetic acid and its phosphate solubilization ability may also contribute for enhancement of plant growth [31,32].

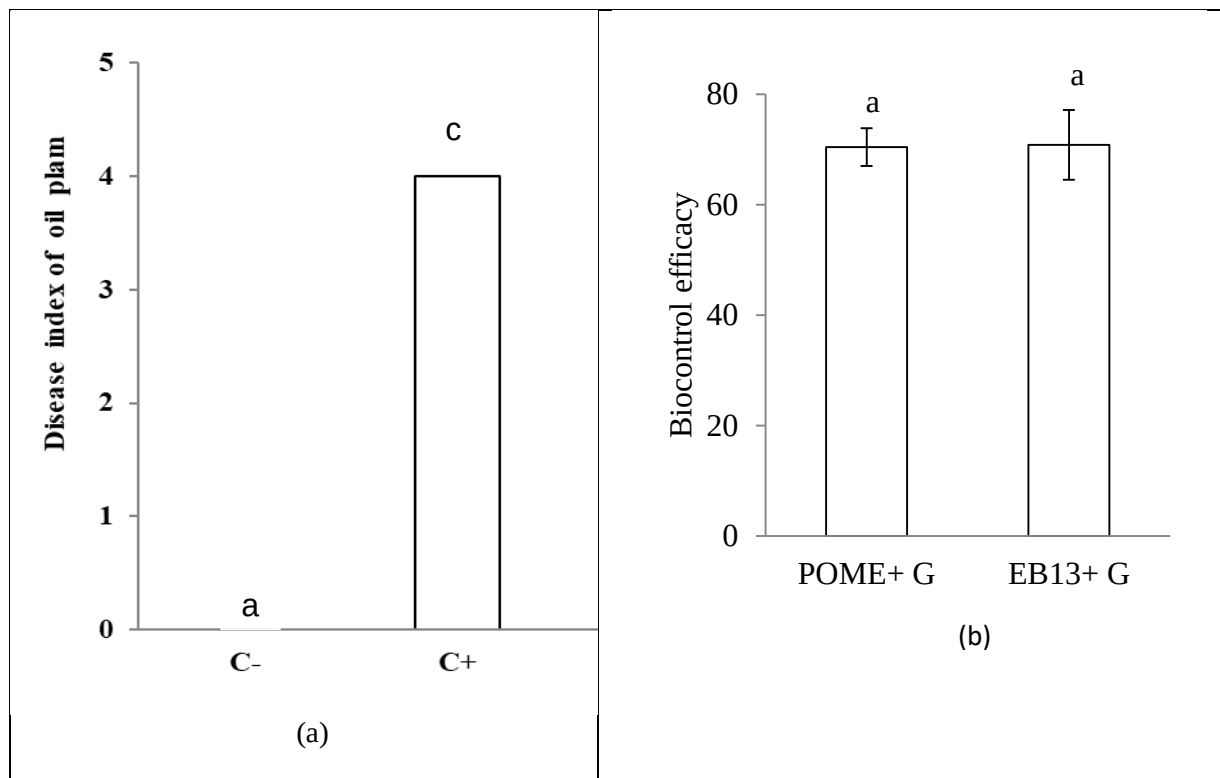


Figure 1. Influence of POME and *B. amyloliquefaciens* EB13 on disease index of oil palm (a) and biocontrol efficacy of oil palm (b). Means with different letters are significantly different ($P < 0.05$). G= *G. Boninense*

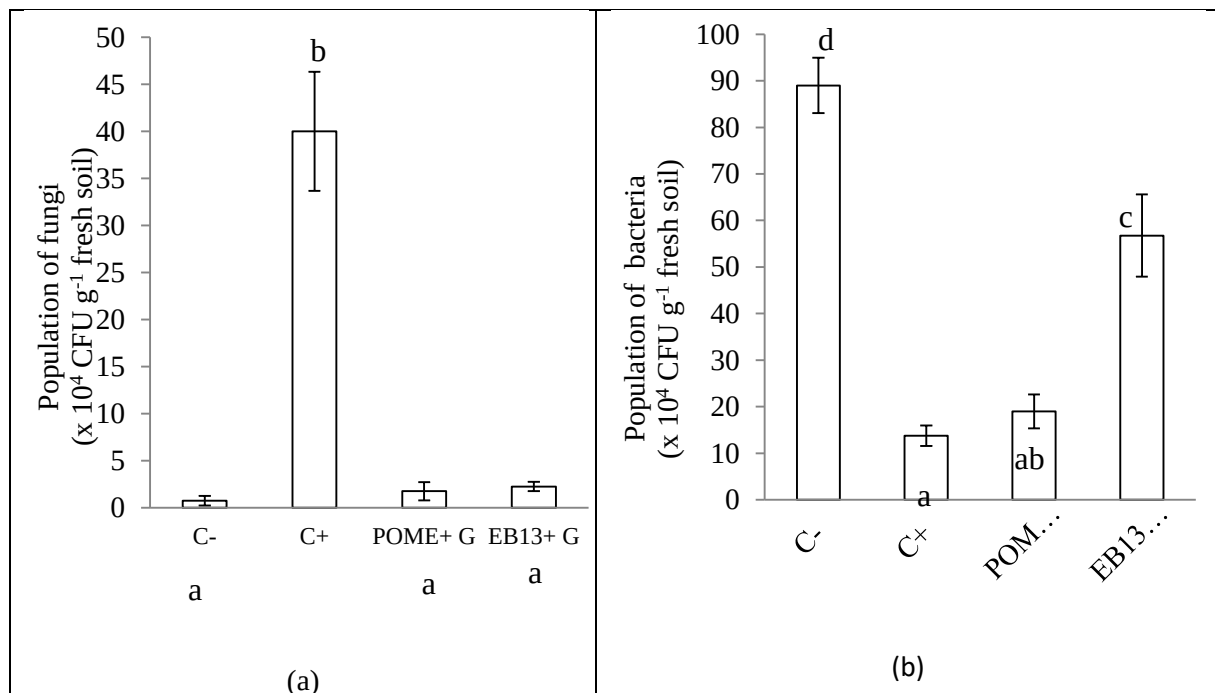


Figure 2. Influence of POME and *B. amyloliquefaciens* EB13 on population of fungi (a) and bacteria (b). Means with different letters are significantly different ($P < 0.05$). G= *G. boninense*

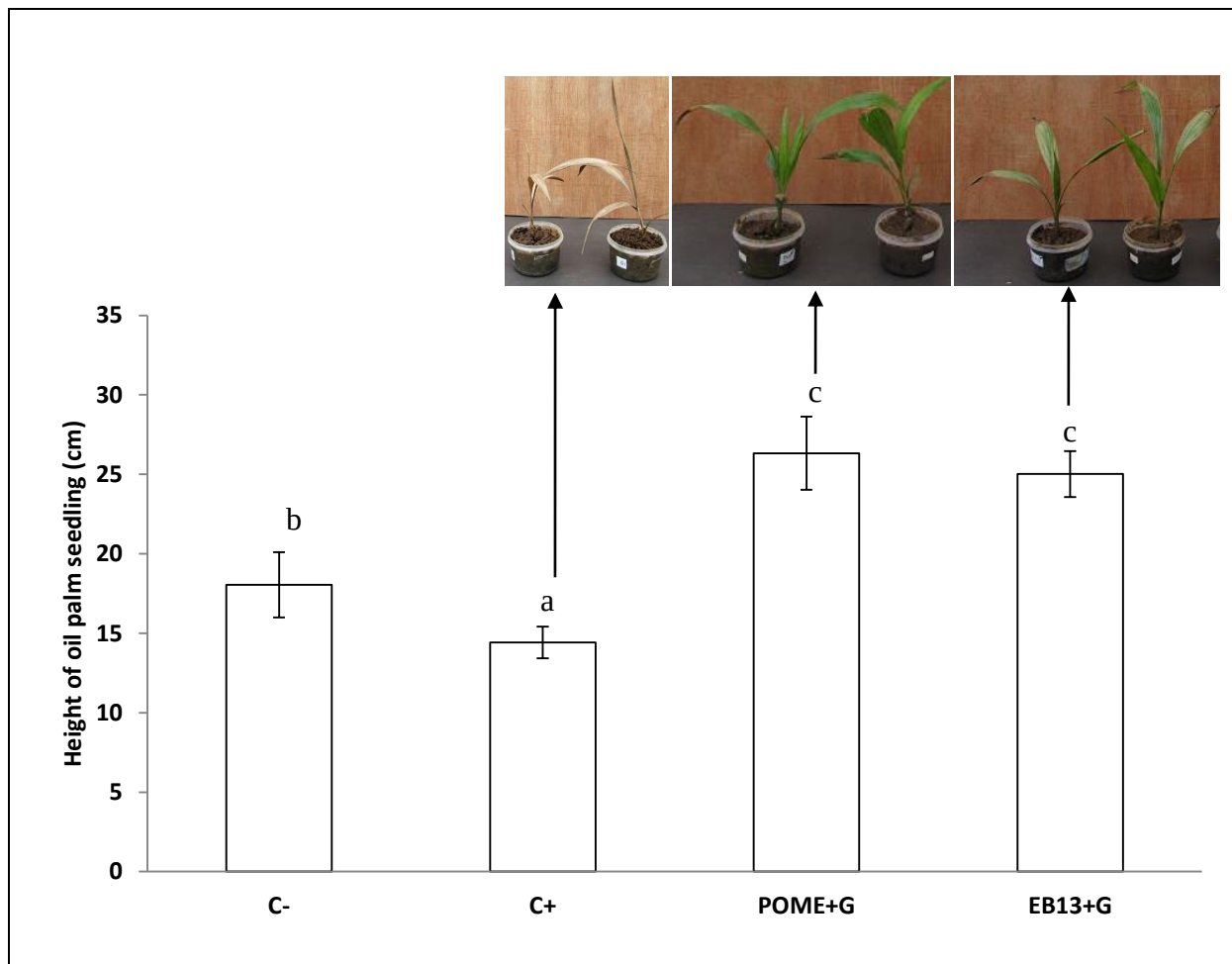


Figure 3. Influence of POME and *B. amyloliquefaciens* EB13 on height of oil palm . Means with different letters are significantly different ($P < 0.05$). G= *G. boninense*

3.3 Monitoring of laccase, mangan peroxidase, and lignin peroxidase activity in the soil.

Measurement of the enzymes activity of laccase, mangan peroxidase (MnP), and lignin peroxidase (LiP) showed that the positive control has the highest one, followed by the addition of POME and *B. amyloliquefaciens* EB13 (Figure 4). The positive control was only inoculated with a fungal pathogen of *G. boninense*, where shown the most severe disease was occurred. This result is one of proof which this an oil palm disease is caused by *G. boninense*.

As explained by Rees *et al.*, [33] a basal stem rot disease of oil palm involved lignolytic enzymes such as laccase, mangan peroxidase, and lignin peroxidase that are produced by *G. boninense*. Therefore, the highest of these enzyme activity was supported by result of the highest of DI (Figure 1a). Mechanism of *G. boninense* infection in oil palm seedlings are as follows; a) the first stage is biotrophic phase where the fungal pathogen infect root cortex or basal stem in which intracellular layers of oil palm greatly colonized by hypae of *G. bononinse*; b) the second is necrotict phase that involve oil palm cell wall degradation through the production of the series of cell wall degrading enzymes, such as laccase, MnP, and LiP [33,34]. Moreover laccase also produce by *B. amyloliquefaciens* as reported by Lončar [35] who found and characterized the enzyme from that bacteria.

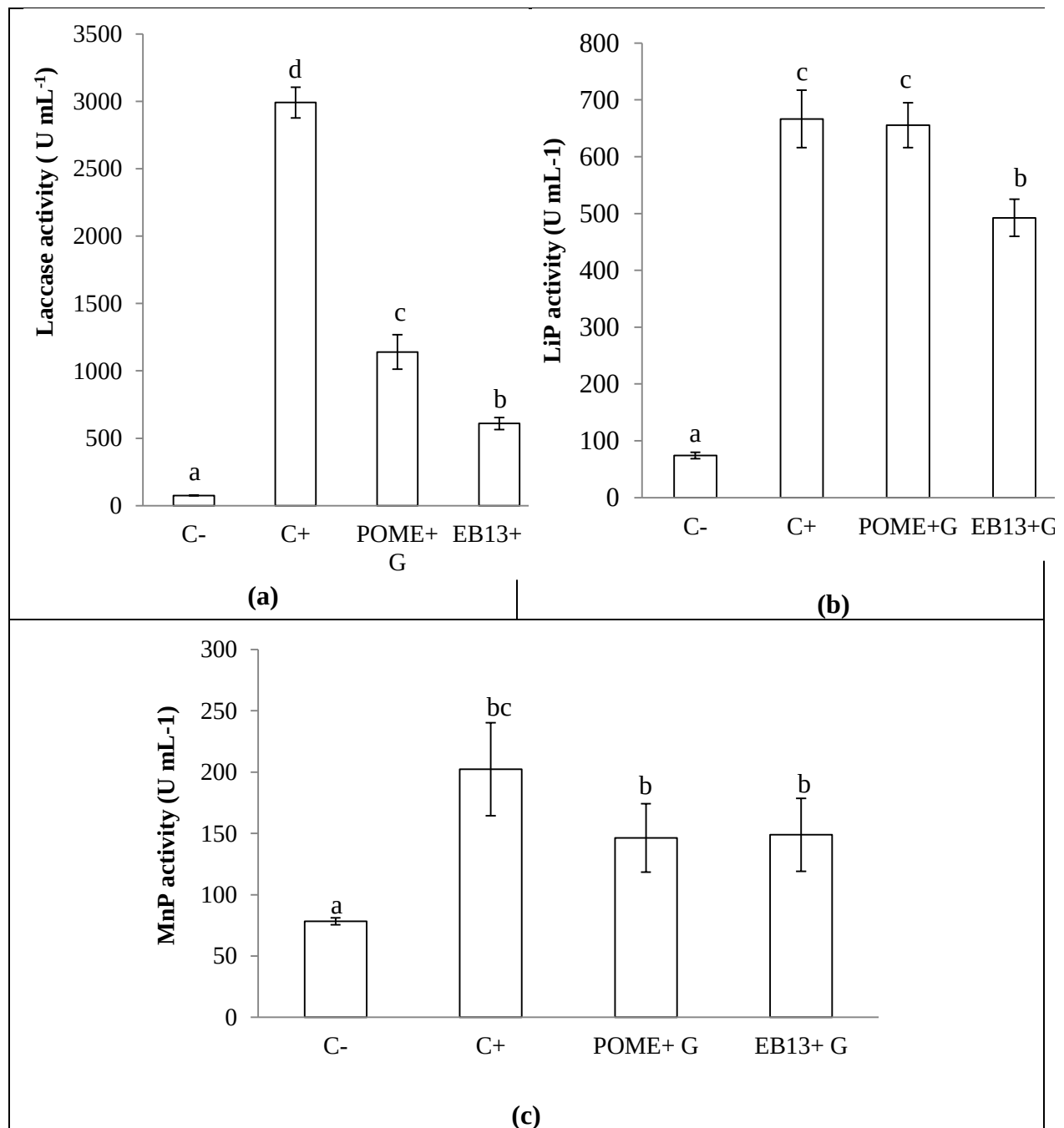


Figure 4. Influence of POME and *B. amyloliquefaciens* EB13 in Laccase (a), LiP (b), and MnP(c) activity in the soil . Means with different letters are significantly different (P<0.05). G= *G. boninense*

4. Conclusions

Conclusions to this reserach work are as follows;

- POME and *B. amyloliquefaciens* EB13 reduced disease index of oil palm disease and its biocontrol efficacy was about 70.

- POME reduced population of fungi from 40 to 1.8×10^4 CFU g⁻¹ fresh soil, and tend to increase population of bacteria. *B. amyloliquefaciens* EB13 addition also decreased fungi population and increased bacteria population
- POME and *B. amyloliquefaciens* addition increased height of oil palm
- The highest of laccase, mangan peroxidase and lignin peroxidase activity was observed in the positive control, since *G.boninense* produced such kinds of lignolytic enzymes to infect of oil palm.

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