Evalution of enzyme effects of *Bacillus cereus* to degreed mycelium of *Ganomderma*

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Abstract

This study aimed to evaluate the efficiency of endogenous bacteria in inhibiting the growth of Ganoderma fungus the major causal pathogen of Basal Stem Rot (BSR) disease in palm. This endophytic bacteria were isolated from symptomless oil palm roots it is showed inhibitory effect by suppressing the mycelial growth of *G. boninense*. The isolated bacteria were screened in vitro by culture filtrate test for its antagonistic properties towards *G. boninense*. the bacterial endophytes was established to have potential as biocontrol agents based on their percentage inhibition of radial growth (PIRG) more than 70%. Shrinkage of hyphae in bacillus was more apparent in through mycelial growth test. Therefore, in vitro activities of bacillus against *G. boninense* in these studies suggested that these endophytic endophytic bacteria can be used as an effective biological control agent.

Keywords: Entophytic bactria, Bio-Control, β-Glucanase enzyme assay,

1 Introduction

Oil palm is a major commodity crop in Malaysia. It plays an important role in the development of the country's economy. Palm oil is an export-oriented commodity; therefore, Malaysia receives significant earnings from the export activities annually with a contribution of 3% to Malaysia's gross domestic product (GDP) (Basri, 2010). Apart from that, Malaysian oil palm industry also provides employment to 570,000 people. The foundation of the oil palm industry is attributed to M.A. Hallet, a Belgian who planted palms of Deli origin in 1911 in Sumatera (Hartly, 1988). Palm oil has contributed immensely as a vegetable oil in the role it plays in the circle of fats and oil and has similar importance as olive,

corn, soybean and rapeseed oils. Following the remarkable pressure on oil palm industry, oil palm planters are able to achieve a profitable return (Basiron, 2007). Several number of diseases have been proved to cause serious yield losses to oil palm production. However, the most common disease of oil palms is caused by pathogenic fungi (Corley and Tinker, 2003). Although in most cases the diseases are under control, none of them has been as serious as Fusarium wilt or red ring; limited the growth of oil palm in Africa and South America. Another disease, bud rot disease of oil palm are of uncertain causes and pathogenicity; has been linked to *Phytophthora palmivora* as the causal pathogen and this disease was known to destroys oil palm crops, (Torres *et al*., 2010). Nevertheless, basal stem rot disease of palm is caused by Ganoderma spp. in Malaysia (Nasir *et al*., 2012). Ganoderma disease can kill more than 80% of stands by the time they are half-way through normal economic life (Abdul Razak *et al*., 2004).

Endophytic microorganisms are therefore a relatively new field of study in biological disease control. Endophytic bacteria live in the plant tissues without doing substantive harm or gaining benefit other than residency (Kobayashi & Palumbo, 2000; Zaiton *et al*., 2008). Perez-Garcia, (2011) demonstrated the beneficial effects of several Bacillus species against diseases elicited by oomycetes and fungal pathogens. Therefore, the research progress made in the use of Bacillus as biofungicides during the past two decades has been remarkable (Shoda, 2000; Perez-Garcia, 2011).

Plants defend against fungal infection or other biotic and abiotic stresses by physically strengthening the cell walls through lignification, suberization, and producing various pathogenesis-related (PR) proteins such as chitinases, β-1, 3-glucanases (Ebrahim and Singh, 2011). Elicitation of chitinase enzyme by BCAs will degrade the chitin walls of the pathogens and simultaneously slows-down the vigorousness of infection. This feature will aid in defense mechanism against fungal pathogens (Liu *et al*., 2005).

β-1, 3-glucan is a structural component in the cell wall of many yeasts, bacteria, and filamentous fungi (Carsolio and Chet, 1994). In order to counter most pathogenic fungal infections, plants induce β-1, 3-glucanases which are one of the pathogenesis-related (PR) proteins, and belong to the PR-2 family and are believed to play an important role in plant defense responses to pathogen infection

(Ebrahim and Singh, 2011). Apart from that, β-1, 3-glucanases were expressed at a low concentration (basal level) in plants; however, when plants are infected by fungal, bacterial, or viral pathogens, β-1, 3-glucanases concentration increases drastically. In relation to that, induction of β-1, 3-glucanases and other PR proteins in the plant may also be triggered by some components or degraded components of pathogens. Nevertheless, plant β-1, 3-glucanases are not only induced due to pathogen infection, but also by other stress factors such as wounding, drought, exposure to heavy metals, air pollutant ozone, and ultraviolet radiation (Akiyama *et al*., 2004).

2 Location of experiment

The experiments were conducted at Laboratory of Plant Pathology, Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia (UPM).

3 MATERIALS AND METHODS

3.1 Culture filtrate test

Culture filtrate test were conducted based on the method described by Bivi *et al*. (2010). UPM15 bacterium culture was inoculated in 250 mL of nutrient broth (NB) and this treatment was maintained at 28±2ºC in the dark for seven days with eight replications. The mixture was later centrifuged at 1000 rpm for five minutes, then the supernatant was collected and the pellet was discarded. The supernatant was filtered through a 0.25 µm membrane filter in sterile conditions for UPM15 samples and a Whatman® Grade. The filtrate was later incorporated into sterilized double strength PDA in the ratio of 2:1, 20 mL of the amended agar, and poured into each petri dish and allowed to solidify. Subsequently, *G. boninense* mycelia plug (five millimeters) was centrally placed in each of the plate containing amended agar. In addition, non-amended PDA was used as the control. The diameter of the mycelia growth spread of *G. boninense* was measured after seven days of incubation. The antagonistic activity was expressed as PIRG in relation to the mycelia growth of *G. boninense* in the control plate. The PIRG was calculated according to Equation 1. Six individual replicates were prepared for each treatment and this experiment was repeated thrice.

3.2 Preparation of biological control microbe suspensions

Twenty five mL of NB was prepared in each 150 mL conical flask as per instructions on the label and sterilized in an autoclave at 121oC at 1.05kg/ cm2 for

about 15 minutes. After sterilization, the broth was left to 45°C and all the procedures were carried out under aseptic conditions to eliminate contamination. Subsequently, one loop of UPM15 bacterium pure culture was transferred into each conical flask and shaken for 48 hours at 160 rpm. Five replications were prepared for both treatments.

3.2.1 Mycelia growth test

Mycelia growth test was conducted based on method by Bivi *et al.* (2010). UPM13 (*G. boninense*) seven days old culture mycelium plug was cut from the growing edge of the PDA plate using a cork borer and dipped into the BCA suspensions prepared for 30 minutes and air dried in the laminar airflow. Subsequently, the treated mycelia plugs were placed onto the petri dish containing PDA media individually. Five replicates were prepared for each treatment. In addition, UPM13 plug dipped in sterile distilled water served as a control. The PDA plates were then incubated at 28±2°C for seven days. Hyphae strands at the end of the fungal growth spread were removed and examined under a compound light microscope (Olympus) for abnormalities.

3.3 β-Glucanase enzyme assay

3.3.1 Preparation of dinitrosalicylate reagent (DNS)

Five g of 3, 5-dinitrosalicylic acid, 91 g Rochelle salt (sodium potassium tartrate), 1 g phenol, 0.25 g sodium sulphate (Na2SO4) and 5 g of sodium hydroxide (NaOH) were dissolved in 400 mL distilled water and final volume was made up to 500 mL. The reagent was stored in the refrigerator at 4˚C in a brown coloured bottle.

3.3.2 Preparation of sodium acetate buffer

27.22 g of sodium acetate was dissolved in 321.5 ml of distilled water to get 0.1M of volume. 6 ml of acetic acid was added to 178.5 ml of distilled water in order to get 0.1M of volume. 0.1M of acetic acid was combined with 0.1M of sodium acetate. After that, the solvent was dissolved in 994 ml of water to get pH 5.0 and it should be maintained.

3.3.3 β-Glucanase enzyme assay

This assay was carried out according to Miller *et al.* (1999). One gram of solute (barley powder) was dissolved in a suitable solvent and dilute with solvent to a total volume of exactly 100 ml in order to get the desired concentration 1 %

(weight/ volume). β-glucanase activity was assayed by the reducing-sugar method with β-glucan as the substrate. The assay system consisted of 50 µl of 1 % (wt/vol) β-glucan dissolved in 100 mM sodium acetate buffer, pH 5.0, and 50 µL enzyme sample (bacterial suspension). The reaction of two replications of each bacterium was allowed to proceed for 30 minutes. The reaction of another two replications of each bacterium was allowed to proceed for 1 hour. The reaction of one replication for each bacterium was allowed to proceed for 1 hour at 50°C. All the reactions were stopped by adding of 300 µL dinitrosalicylate reagent (prepared above). The absorbance of the reaction mixture was determined at 550 nm using a spectrophotometer. The amount of reducing sugar produced was determined using a standard curve, which was constructed with glucose.

4 Statistical analysis:

The experiments culture filtrate testnd Mycelium growth test were conducted in Completely Randomized Design (CRD) with four replicates. Recorded data were analyzed with SAS® Software. The significant data was determined using Duncan's Multiple Range Test at 5% probability level. The percentage data were transformed into Arcsine transformation before subjected to ANOVA.

5 Results

Culture filtrate test

In this culture filtrate test was selected on this entophytic bacteria showed maximum percentage inhibition 72.9% of radial growth value against *G.boninese* at seven days of incubation with respect to control. Hance, *B.cereus* was suggested as effective biological control agent *G.boninese* in vitro. Sine shoed effective result in culture filtrate test (Figure 3.1). Generly, entophytic bacteria exhibited greater activity in the culture filtrate test due to entophytic bacteria has potential as bio control agents against *G.boninese*.

Table 3.1: PIRG value of biocontrol candidates against *Ganoderma boninense* mycelium growth grown on potato dextrose agar for culture filtrate test

Microbes	Average value of percentage inhibition of radial growth [PIRG (%)]
UPM15 (Bacillus cereus)	72.9b

Means within columns with same letters are not significantly different with LSD at P≤0.05 n=8

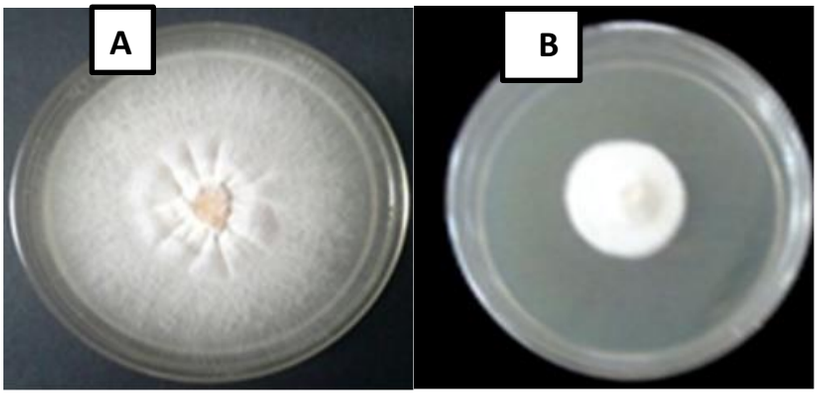

Plate 3.2: Effect of (B) UPM15 (Bacillus cereus) on the radial growth of *G. boninese* in culture filtrate test at seven days incubation. (A) *G. boninense* in control plate.

Mycelium growth test

The damage observed was the malformation of hyphae on *G. boninense* plug treated with B. cereus suspension (Figure 3.3) Mycelia growth test: The effect of the suppression of potential endophytic bacteria against *G. boninense* was further investigated using compound microscope. The changes of hyphal tips of G. boninense treated with *B.cereus* in mycelium growth test.

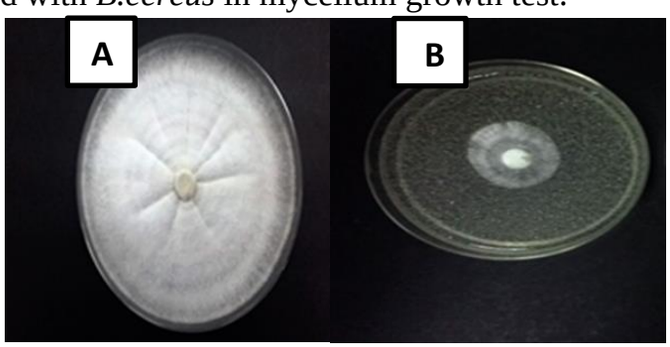

Plate 3.3: *Ganoderma boninense* mycelium as control plate (A), Inhibition of *Ganoderma boninense* growth in UPM15 (*Bacillus cereus*) Mycelum growth test amended with PDA (B) after seven days of incubation.

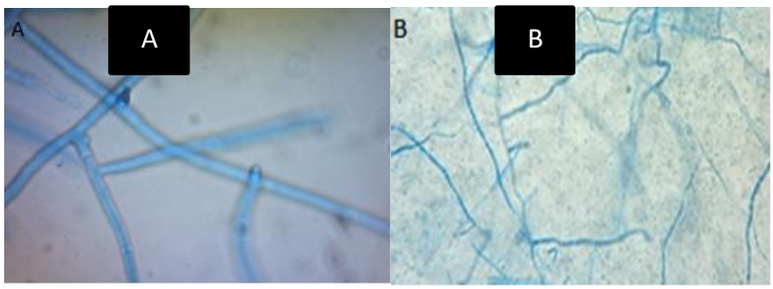

Figure 3.2: Observation of hyphae abnormalities of *G. boninense* at 400 magnifications as treated with *B.cereus*. (A) Normal hyphae in control treatment. (B) Shrinkage of hyphae by (*B.cereus*).

Hydrolytic cell degrading enzymes

β-Glucanase enzyme production

In the present study, the BCA candidates; *B. cereus* was able to hydrolyze barley β-glucan substrate by 1,3-1,4-β-glucanase activity which was assayed by the reducing sugar method (Miller *et al*., 1959). Table 3.6 shows the absorbance reading of each treatment mixture whereby, the higher the absorbance reading, the more glucan has been hydrolyzed by β-glucanase enzyme produced by the BCAs. Entophytic bacteria *B.cereus* was significantly more efficient in hydrolyzing β-glucan substrate.

Table 3.6: Hydrolysis of β-glucan substrate by candidate BCAs. Δ Abs 550 nm represents the net absorbance of the reaction mixture after incubation for 0.5 h with the enzyme at 50°C.

Microbes	Δ Abs 550nm
UPM15 (Bacillus cereus	0.573 ± 0.33b

Means within columns with same letters are not significantly different with LSD at P≤0.05 n=5

6 Discussion

Through this present study, entophytic bacteria isolated in a prelimanry study from oil palm soil plantionn, to evaluate the BCAs in culture filtrate and mycelia growth test were used as a mothed to detemain the antagonistic activitiy of entophytic bacteria against *G.boninense* which cauces basal stem root on palm. Furthermore,

culture filtrate test gave more convincing resultnoting a PIRG value toward *G.boninense* myceliu combered to control.

Some studies have shown esponse of endophytic bacterial communities in potato, suggested that disease may be inhibited by a diverse endophyte community maintaining the pathogen population below the threshold for expression of pathogenicity Reiter et al. (2002). Furthermore, that functioning communities of endophytes in plants contribute to their resistance to pathogens e.g., microbial endophytes in potato controlling bacterial soft rot (Sturz *et al*., 1999).

This isolated endophytic bacteria was known as *B.cereus* showed inhibitory effect against the mycelium of G.boninense. From this potential entophytic bacteria gave PIRG greater than 50% in culture filtrate test. Endophytic bacteria produced very high inhibitory effect on microbs.

In this method culture filtrate test gave more convincing result noting a PIRG value towards G. boninense mycelium. This could be due to the ability of BCAs in secreting more volume of non-volatile metabolites in liquid media compared to solid media. Work by Montealegre et al., (2003) demonstrated, bacteria that inhibit fungal mycelial growth were able to produce antibiotics that functioned to inhibit fungal activity by chemical toxicity and penetration through cell wall.

Apart from that, the effect on the morphological structure of G. boninense hyphae due to the inhibition of B. cereus was further investigated in mycelia growth test using light compound microscope. Malformation and shrinkage of hyphae structure was observed in B. cereus suspension treated G. boninense plugs, which was used to subculture G. boninense mycelium on PDA as compared with hyphae in the control plate respectively.

1,3-1,4-β-Glucans are polysaccharides, present as a cell wall component of plant pathogenic fungi. Thus, to select a good BCA of plant pathogenic fungi, traits in ability to produce β-glucanase enzyme is crucial. However, this trait; production of β-glucanases has been less studied in candidate microbes for biological control. Therefore, the purpose of this experiment was to characterize this trait the candidate BCAs. In this study, barley was used as the glucan substrate to assess β-glucanase enzyme assay of the BCAs. According to a study by Celestino et al. (2006), glucan from barley was best degraded by endophytic microbes studied compared to other glucan substrates such as laminarin, xylan, and manan. Based

on the results obtained in the present study, both BCAs showed the ability in producing β-glucanase enzyme. On the other hand, we found that the Δ Abs obtained for the BCAs was low compared to other studies which gave ≥0.826 (Celestino et al., 2006) for the same substrate. This could be due to the barley powder may be coated with some proteins, which make them more resistant to lytic enzymes produced by B. cereus. However, in culture filtrate test and mycelial growth test conducted, good inhibition rates was obtained against G. boninense growth and its physical structure damage that could be concluded here, that β-glucanase enzymes produced by both BCAs studied more efficiently degrade the type of glucan substrate present in G. boninense cell wall. The in vitro assessment of the BCAs successful demonstrated its ability to inhibit G. boninense growth via varies antagonistic mechanism.

Hence, the complexity of the pathogenic (G. boninense) and non-pathogenic (BCAs) microbe interactions with oil palm warrants an in vivo investigation to confirm the level of BCAs in promoting palm growth and most importantly in suppressing Ganoderma infection and the effects in the oil palm rhizosphere.

REFERENCES

Abdul Razak, J., H. Ahmad, K. Ramdhan, A.S. Idris, S. Abdul Rahim, R. Aminul and I. Fauzi. 2004. Mechanical trunk injection for control of Ganoderma. MPOB Information Series. MPOB TT No. 215.

Akiyama T., Pillai M. A., Sentoku N. (2004). Cloning, characterization and expression of OsGLN2, a rice endo-1,3-β-glucanase gene regulated developmentally in flowers and hormonally in germinating seeds. Planta.; 220: 129-139.

Basiron, Y. (2007). Palm oil production through sustainable plantations. European Journal of Lipid Science and Technology, 109(4), 289-295.

Basri, M.W. (2010). Overview of the Malaysia oil palm industry 2009 Malaysian Palm Oil Board.htt://mpob.gov.my/economy/Overview 2009.pdf (2 May 2019).

Celestino, S. M. C., Maria S. de Freitas, F. Javier Medrano, M. Valle de Sousa, and E. X. F. Filho, "Purification and characterization of a novel pectinase from Acrophialophora nainiana with emphasis on its physicochemical properties," Journal of Biotechnology, vol. 123, no. 1, pp. 33–42, 2006.

Dorworth, C.E. and B.E. Callan. 1996. Manipulation of endophytic fungi to promote their utility as vegetation biocontrol agents. 1996. Pp. 209-219 in: S.C. Redlin and L.M. Carris, eds. Endophytic fungi in grasses and woody plants: systematics, ecology, and evolution, St. Paul, MN. APS Press, St. Paul, MN.

Dong, Xu and Jean-Charles, Coˆte (2003) Phylogenetic relationships between Bacillus species and related genera inferred from comparison of 39 end 16S rDNA and 59 end 16S– 23S ITS nucleotide sequences. International Journal of Systematic and Evolutionary Microbiology. 53: 695–704

Carsolio, C., Gutiérrez, A., Jiménez, B., Van Montagu, M., & Herrera-Estrella, A. (1994). ation of ech-42, a Trichoderma harzianum endochitinase gene expressed during mycoparasitism. Proceedings of the National Academy of Sciences, 91(23), 10903-10907.

Corley R, Tinker P (2003) Vegetative propagation and biotechnology. The oil palm 4:201–215.

Ebrahim, S. and Singh, K.U. Pathogenesis related (PR) proteins in plant defense mechanism. In: MÉNDEZ-VILAS, A. (Ed.) Science against microbial pathogens: communicating current research and technological advances. Extremadura: Formatex Research Center, v. 1, 2011, p. 1043-1054.

Freeman, S., Minzm O., Kolesnik, I., Barbul, O.,Zveibil, A., Maymon, M., Nitzani, Y., Kirshner,B., Rav-David, D., Bilu, A., Dag, A., Shafir, S.and Elad, Y. 2004. Trichoderma biocontrol of Colletotrichum acutatum and Botrytis cinerea and survival in strawberry. Eur. J. Plant Pathol.,110:361-370.

Hartly, C.W.S. (1988). The oil palm. Third edition. New York. John Wiley &Sons, Inc.761p.

Kim, G. Y., Lee, G. J., Ha, M. G., Lee, T. H., & Lee, J. D. (2000). Intrageneric relationships of Trichoderma based on internal transcribed spacers and 5.8 S rDNA nucleotide sequences. Mycobiology, 28(1), 11-16.

Montealegre, J.; Reyes, R.; Perez, L.M.; Herrera, R.; Silva, P. and Besoain, X (2003). Selection of antagonists to be used in biological control of Rhizoctonia solani in tomato. Electronic Journal of Biotechnology 2:2.

Naher, L., Ho, C. L., Tan, S. G., Yusuf, U. K., & Abdullah, F. (2011). Cloning of transcripts encoding chitinases from Elaeis guineensis Jacq. and their expression profiles in response to fungal infections. Physiological and molecular plant pathology, 76(2), 96-103.

Nasir, I. A., Jamal, A. R. S. H. A. D., Rahman, Z. I. A. U. R., & Husnain, T. A. Y. Y. A. B. (2012). Molecular analyses of gladiolus lines with improved resistance against fusarium wilt. Pak. J. Bot, 44(1), 73-79.

Nawani NN, KapadnisBP, Das AD, Rao AS, Mahajan RJ, (2002), Purification and characterization of a thermophilic and acidophilic chitinase from Microbispora sp.V2., Journal of Applied Microbiology, 93(6), pp 965–975

Pérez-García, A., Romero, D., and de Vicente, A. (2011). Plant protection and growth stimulation by microorganisms: Biotechnological applications of bacilli in agriculture. Current Opinion in Biotechnology, 22(2), 187-193.

Paterson, R. R. M. (2007). Ganoderma disease of oil palm—A white rot perspective necessary for integrated control. Crop protection, 26(9), 1369-1376.

Shoda, M. (2000) Bacterial control of plant diseases. Journal Biosci. Bioeng 8: 515–521

Soepena, H., Purba, R. Y., & Pawirosukarto, S. (2000). A Control Strategy for Basal 6. Ganoderma diseases of perennial crops, 83.

Someya, M. Nakajima, K. Hirayae, T. Hibi, K. Akutsu (2001). Synergistic antifungal activity of chitinolytic enzymes and prodigiosin produced by biocontrol bacterium, Serratia marcescens strain B2 against gray mold pathogen Botrytis cinerea. J Gen Plant Pathol, 67 (4), pp. 312–317.

Torres, G.A, Sarria, G.A. Varon, F., Coffey, M.D., Elliott, M.L. and Martinez, G. (2010) First report of Bud Rot caused by Phytophthora palmivora on African oil palm in Colombia. Volume 94 (9): 1163.1 -1163

Yeoh, K. A., Othman, A., Meon, S., Abdullah, F., & Ho, C. L. (2012). Sequence analysis and gene expression of putative exo-and endo-glucanases from oil palm (Elaeis guineensis) during fungal infection. Journal of plant physiology, 169(15), 1565-1570.

Zaiton, S., M. Sariah, M.A. Zainal Abidin, (2008). Effect of entophytic bacteria on growth and suppression of Ganoderma infection in oil palm. Int. J. Agric. Biol., 10: 127–132.