Antifungal Activity of Methanol-Extracted Secondary Metabolites of Rhizobacteria Isolated from Rhizosphere of Oil Palm Trees Against Ganoderma Boninense Pat.

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Abstract. Several rhizobacteria were isolated from the rhizosphere of healthy oil palm trees showed prospective ability as biocontrol agent against pathogenic fungi Ganoderma boninense Pat. The fungus is the causal agent of basal stem rot (BSR) disease can cause yield reduction up to 80%. Therefore, searching for effective compound to control the disease is a continuous process. Rhizosphere bacteria isolates were grown on ISP2 agar media and incubated for 14 days at room temperature. The media with fully grown cultures were cut into cubes and added with methanol. It was shaken and the bacterial cells were then removed through centrifugation and series of filtration. The final filtration was conducted using 0.2 µm to sterilize the filtrate. The filtrates were subjected for antifungal activity against G. boninense using agar well diffusion. The result showed that the crude metabolites produced by the rhizosphere bacteria demonstrated the ability to inhibit the growth of G. boninense in the agar diffusion method. The highest inhibition reached 51.63%. Furthermore, G. boninense mycelia were also experienced malformation by the presence of the secondary metabolites. The isolated rhizosphere bacteria showed promising ability to produce antifungal compounds which were able to inhibit the growth of G. boninense as well as causing the morphological changes of its mycelia.

1. Introduction

Ganoderma boninense Pat. is one of important fungal phytopathogens of woody plants especially oil palm trees. The fungal pathogen causing the decay of the oil palm basal stem which in severe infection showing the woody fan-like basidiocarp [1]. The Basal Stem Rot (BSR) disease caused by the fungal is often incurable. Symptoms will only appear when the plant has been severely infected. Young oil palm tree will die within 1 or 2 years following the emergence of the symptoms whilst at older plant, it will die within 3 or so years [2]. At this point no available control measures are effective in controlling the BSR disease [3]. Systemic fungicides are thought to be the immediate short-term solution to control BSR. Together with the application of culture practices methods and the use of biological control agents, systemic fungicides are commonly used to slow down the development of the disease [4]. However, the use of fungicide has not been without problems. One of most serious problems arise due to the use of fungicide is the adverse effects on the environment. Therefore, finding alternative antifungal compounds which are effective to control G. boninense as well as save to the environment is necessary to be done.

Antifungal compounds have been found produced by microorganisms. More than 22,000 microbial origin biologically active compounds have been reported [5]. Microbial metabolites have been targeted as source for searching agro-active compounds. This is due to their novel properties including their structure and activities. It has been reported that antifungal produced by microbes cause less pollute and less stress to the environment [6].
Rhizobacteria is one of microbes that reported able to produced antifungal compounds. Bacteria inhabitant soil surrounding the plant roots (rhizosphere) plays important role not only as plant growth promoters but also providing protection against phytopathogens in the form of biocontrol through competition and antibiosis [7], [8] showed that pyrrole type of compound produced by Pseudomonas protegens strain W45 were able to inhibit the growth of Sclerotinia sclerotiorum. Crude bioactive metabolites produced by a plant growth promoting rhizobacteria, Pseudomonas aeruginosa PM 105, demonstrated noticeable antimicrobial activities toward several plant pathogens ie Colletotrichum gloeosporioides, Fusarium oxysporum, Pencillium expansum, nad Rhizoctonia solani [9].

Attempts to isolate antagonistic rhizobacteria from oil palm trees have resulted several potential biocontrol isolates namely rhizobacteria BARK4, BARK5, BARK6, BARK7, BARK8, BARK9, BARK15 and BARK16 (reported elsewhere). In vitro testing demonstrated that those isolates were able to inhibit the growth of G. boninense as well as alter its mycelia morphology. The ability of the crude metabolites of these isolates in inhibiting the growth of G. boninense is reported here.

2. Materials and Methods

2.1 Preparation of culture filtrates of rhizosphere bacteria

The rhizobacteria isolates were isolated from rhizosphere of healthy oil palm trees and showed antifungal activity against G. boninense on dual culture assay (reported elsewhere). The bacteria were grown and maintained on HPDA (half strength Potato Dextrose Agar, Oxoid) media. To obtain secondary metabolites from the rhizosphere bacteria isolates, ISP2 media (malt extract 10 g, yeast extract 4 g, glucose 4 g, 1 l RO water, pH 7.2 ± 0.2) [10] was used to grow all of isolates. The rhizobacteria isolates were inoculated into the ISP2 media covering all of media surface. It was then incubated at room temperature for 14 days. ISP2 media fully covered with the rhizobacteria isolate was cut into cubes of 5 x 5 mm and submerged with absolute methanol in a 50 ml centrifuge tube. It was shaken overnight to extract the secondary metabolites secreted into the ISP2 agar media. The agar and bacterial cells were removed through Whatman filter paper no 1. The filtrate was filter sterilized using 0.22 µm membrane filters (Millipore). It was then subjected for further test for detecting its ability to inhibit the growth of G. boninense.

2.2 Agar well diffusion assay of rhizobacterial crude metabolites

The antifungal activity of the metabolites was conducted on PDA media using agar well diffusion method (Balouiri et al., 2016). A 6 mm diameter well was made using a sterile cork borer by which 30 µl of the filtrate prepared earlier was inoculated into the well. A 6 mm plug of G. boninense was inoculated at 3 cm away across the well. The antifungal activity of the metabolites was detected by the growth inhibition of G. boninense. Percentage inhibition of radial growth (PIRG) was calculated based on the reading of G. boninense radial colony at the direction of filtrate containing well (R2) against the G. boninense radial colony in the control plate (R1) [11].

\[
\text{PIRG} = \frac{R_1 - R_2}{R_1} \times 100\% \quad (1)
\]

Morphology of the G. boninense following the agar well diffusion assay was observed under microscope. Mycelia of G. boninense facing the filtrate-containing well were collected at several spots to observe the effect of the secondary metabolites on the G. boninense. The fungal colony diameter was measured and used to calculate the Area Under Growth Colony Curve (AUCGC) using modified formula of Area Under Disease Progress Curve (AUDPC) [12].

\[
\text{AUCGC} = \sum_{t=1}^{n} \frac{y_{t+1} + y_t}{2} \times (t_{t+1} - t_t) \quad (2)
\]
Where \( y_i \) is the fungal colony growth diameter at the \( i \)th observation, \( t_i \) is the time of the \( i \)th observation taken and \( n \) is the total number of observations taken. All data was analyzed using ANOVA and Duncan Multiple Range Test on SPSS version 17.0.

3. Result and Discussion

Agar well diffusion assay demonstrated the ability of the crude metabolites secreted by the rhizobacterial isolates in inhibiting the growth of \( G. boninense \) (Table 1; Fig 1). The diameter colony of \( G. boninense \) was measured in three days interval. At most of the observation times, the colony of \( G. boninense \) in plates containing crude metabolite-wells was significantly shorter than the diameter colony of \( G. boninense \) in control plates, except at the 9th day after inoculation. At the 9th day after inoculation, there were no significant differences of the means of diameter colony of \( G. boninense \) among treatments. Although in general, the colony of \( G. boninense \) at control plates was wider than in the crude metabolites treatments. On the control plate, \( G. boninense \) was able to fully cover media surface at 12 days after inoculation whilst at treated plates, \( G. boninense \) colony growth was inhibited.

| Treatment | Means diameter colony of \( G. boninense \) (cm) |
|-----------|---------------------------------------------|
| BARK4     | 1.14 a** 1.51 a 2.79 a 2.93 a           |
| BARK5     | 1.12 a 2.29 ab 3.84 a 4.49 a            |
| BARK6     | 1.10 a 1.99 ab 3.63 a 4.04 a            |
| BARK7     | 1.22 a 1.52 a 2.77 a 3.22 a            |
| BARK8     | 1.08 a 1.48 a 3.02 a 3.28 a            |
| BARK9     | 1.11 a 2.00 ab 3.09 a 3.32 a           |
| BARK15    | 1.17 a 1.70 a 3.09 a 3.49 a            |
| BARK 16   | 1.10 a 2.05 ab 3.13 a 3.37 a           |
| Control   | 1.67 b 3.15 b 4.76 a 9.00 b          |

* DAI = day after inoculation
** Means following with different lowercase letters in the same column indicate significant difference at \( P<0.05 \) level by Duncan Multiple Range Test (\( P<0.05 \))

Table 2. Area under colony growth curve (AUCGC) and percentage of growth inhibition of \( G. boninense \) following crude metabolites treatments.

| Treatment | AUCGC* | Growth inhibition (%) |
|-----------|--------|-----------------------|
| BARK-4    | 6.34 b | 51.63                 |
| BARK-5    | 8.94 b | 32.73                 |
| BARK-6    | 8.19 b | 38.46                 |
| BARK-7    | 6.50 b | 51.09                 |
| BARK-8    | 6.69 b | 50.23                 |
| BARK-9    | 7.30 b | 43.57                 |
| BARK-15   | 7.12 b | 46.29                 |
| BARK 16   | 7.43 b | 43.81                 |
| Control   | 13.25 a| -                     |

*Means following with different lowercase letters in the same column indicate significant difference at \( P<0.05 \) level by Duncan Multiple Range Test (\( P<0.05 \))

Mycelia of \( G. boninense \) after treated with the crude secondary metabolites experienced morphological alteration (Fig 1A-I). Visually, the colony growth of \( G. boninense \) was suppressed. The mycelia treated
The progress growth of *G. boninense* was observed through AUCGC (Table 2). It can be seen that the colony of *G. boninense* on plates without crude metabolite treatment grew normally as shown by highest AUCGC value compared to its growth on plates with crude metabolite treatments. The data also demonstrated the lower AUCGC value, the better effect of crude metabolites against *G. boninense*. *G. boninense* growth was significantly (*P* < 0.05) inhibited by the presence of crude metabolites produced by the rhizobacteria isolates. The growth inhibition of *G. boninense* ranged from 43.57% to 51.63%. With crude metabolites showed centered growth in the middle of the plates. The mycelia avoided the crude metabolites-containing well on each it side. This showed by its upward and denser growth which was concentrated on the center of the plate. Thick white threads were also found on several treatments. Furthermore, mycelia thinning were observed at the edge of the colony where it got closer to the well containing crude metabolites.

Figure 1. Representative colony growth of *G. boninense* on plates containing crude metabolite-well produced by BARK4 (A), BARK5 (B), BARK6 (C), BARK7 (D), BARK8 (E), BARK9 (F), BARK15 (G), BARK16 (H) and control (I). Macroscopic of *G. boninense* mycelia on control plate (J) and representative of crude metabolites effect on *G. boninense* (K, L and M), magnification 400x.

Under the influence of crude metabolites, morphological alterations were detected on the *G. boninense* mycelia (Fig 1K-M). Mycelia of *G. boninense* were observed lysed (Fig 1K) and in several spots observed to be more compact compared to control (Fig1L, M). An important feature of mycelia is the ability to grow forward at its apex to explore the surrounding environment. Hyphae plays important role in recognizing the plant surface thus trigger infection [13]. Therefore, when its growth was inhibited, it will affect the virulence of the fungal pathogen. Antimicrobial compounds produced by the rhizobacteria secreted during stationary phase [14]. These can act against the fungal pathogens through various mechanisms including ceased the fungal growth, inhibited conidia germination, lysed of fungal mycelia and/or trigger the fungal death [15]. The rhizobacteria isolates demonstrated biocontrol potential to be developed as biocontrol agents on *G. boninense* of oil palm tree. However, the antimicrobial compounds need to be optimized to achieve maximum production. Indigenous

Conclusion
Control of pathogen by indigenous biocontrol agents is enviromentally advantageous. The rhizobacteria isolates demonstrated promising ability to control basal stem rot pathogen, *G. boninense*. 
The isolates were able to produce antifungal compounds which were capable to inhibit the growth of G. boninense and triggered mycelia morphological alteration.

Acknowledgement
The research was finacially supported by internal fundamental research grant (contract no 855/UN6.3.1/PL/2017) provided by Universitas Padjadjaran.

References
[1] Chong KP, Dayou J, Arnnyitte A 2017 Detection and Control of Ganoderma boninense in Oil Palm Crop. Springer Briefs in Agriculture.
[2] Hushiarian R, Yusof NA, Dutse SW 2013 Detection and control of Ganoderma boninense: strategies and perspectives SpringerPlus 2 555.
[3] Susanto A, Sudharto PS, Purba RY 2005 Enhancing biological control of basal stem rot disease (Ganoderma boninense) in oil palm plantation. Mycopathologia 159 153-7.
[4] Bivi MSHR, Paiko AS, Khairulmazmi A, Akhtar MS, Idris AS 2016 Control of basal stem rot disease in oil palm by supplementation of calcium, copper, and salicylic acid The Plant Pathology Journal 32 396-406.
[5] Qin S, Xing K, Jiang J-H, Xu L-H, Li W-J 2011 Biodiversity, bioactive natural products and biotechnological potential of plant-associated endophytic actinobacteria Applied Microbiology and Biotechnology 89 457-73.
[6] Tanaka Y, Omura S 1993 Agroactive compounds of microbial origin Annual Review of Microbiology 47 57-87.
[7] Ahemad M, Kibret M 2014 Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective Journal of King Saud University - Science 26 1-20.
[8] Bajpai A, Singh B, Joshi S, Johri BN 2018 Production and characterization of an antifungal compound from Pseudomonas protegens strain W45 Proceedings of the National Academy of Sciences, India Section B: Biological Sciences 88, 1081-9.
[9] George E, Kumar SN, Jacob J, Bommasani B, Lankalapalli RS, Morang P, Kumar BSD 2015 Characterization of the bioactive metabolites from a plant growth-promoting rhizobacteria and their exploitation as antimicrobial and plant growth-promoting agents Applied Biochemistry and Biotechnology 176 529-46.
[10] Kiranmayi MU, Sudhakar P, Sreenivasulu K, Vijayalakshmi M 2011 Optimization of culturing conditions for improved production of bioactive metabolites by Pseudonocardia sp. VUK-10 Mycobiology 39 174-81.
[11] Skidmore AM, Dickinson CH 1976 Colony interactions and hyphal interference between Septoria nodorom and phylloplane fungi Transactions of the British Mycological Society 66 57-64.
[12] Simko I, Piepho H-P 2012 The area under the disease progress stairs: Calculation, advantage, and application Phytopathology 102 381-9.
[13] Mendgen K, Hahn M, Deising HB 1996 Morphogenesis and mechanisms of penetration by plant pathogenic fungi Annual Review of Phytopathology 34 364-86.
[14] Trivedi P, Pandey A, Palni LMS 2008 In vitro evaluation of antagonistic properties of Pseudomonas corrugata Microbiological Research 163 329-36.
[15] Gould WD, 1990. Biological control of plant root diseases by bacteria. In: Nakas JP, Hagedorn C, eds. Biotechnology of Plant-Microbe Interactions. New York: McGraw-Hill Inc., 287-317.