The endophytic bacteria of oil palm and areca nut are
beneficial as antagonist of *Ganoderma boninense* and potential
as plant growth promoter

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Abstract. *Ganoderma boninense* is the pathogenic fungi causing Basal Stem Rot (BSR) disease on oil palm. This study aimed to obtain potential endophytic bacteria as antagonist as well as plant growth promoting rhizobacteria (PGPR) agents. This study employed three stepwise as follows: 1) isolation and selection of endophytic bacteria *in vitro*, 2) *in vitro* antagonism and plant growth promoting assays, and 3) molecular identification of the bacteria. Endophytic bacteria isolated from the root tissues of oil palm and areca nut totally produced 88 isolates. Among those, EG17, EG26, EG113, EG215, AC28, AC112, and AC214 were investigated further because they showed negative result on both hypersensitive and hemolytic assays suggesting that these are not plant pathogen and harmless to mammals. Antagonism assay showed that three isolates with highest growth inhibitions to *G. boninense* were exhibited by EG26, EG113 and AC112 with 56.80%, 56.51% and 56.11%, respectively. These isolates also significantly enhanced the growth of rice seedlings as a model plant, particularly AC112 that increase root length and plant height up to 30.77% and 39.57%. Molecular identification using 16S rDNA sequencing showed that EG26, EG113 and AC112 were identical to *Bacillus subtilis* strain VD1, *Bacillus velezensis* strain Bac57, *Bacillus toyonensis* strain JCT-23, respectively.

Key words: *Bacillus velezensis*, *Bacillus toyonensis*, biocontrol, growth promoting

1. Introduction

Oil Palm (*Elaeis guineensis* Jacq.) is an important crop of Indonesia. However, it has an economically serious disease, as a constraint, named the Basal Stem Rot (BSR) that caused by *G. boninense* Pat. [1]. BSR can affect oil palm production caused by death of up to 50% of the entire oil palm population per hectare. Losses arising from every 1% of *G. boninense* attacks on Indonesian oil palm plantations are equivalent to 256 million USD per year [1].

The control mechanisms that usually carried out for BSR are implementing *Trichoderma*-based fungicide as biological control and removing the infected trunks outside the plantation. Nevertheless, these control methods are needs to be strengthened with other control methods to obtain an optimum result. An alternative approach that can be done to control BSR is by using endophytic bacteria. These bacteria can live, develop well, associate in plant tissues without causing symptoms of disease [2].

Endophytic bacteria can inhibit pathogen growth through the mechanism of space and nutritional competition, produce antibiotics, degrade enzymes, siderophores and induce plant resistance through
induced resistance mechanisms [3, 4]. In addition, endophytic bacteria are also able to produce compounds that have the potential to stimulate plant growth known as Plant Growth Promoting Rhizobacteria (PGPR) because it produces plant growth regulators (PGR) to improve nutrition for plants, fix nitrogen and induce plant pathogen resistance [4]. The purpose of this study was to obtain potential endophytic bacterial isolates as biocontrol for G. boninense as well as PGPR agents.

2. Materials and methods
This study was conducted at the Plant Nematology Laboratory of IPB University and the Biotechnology Laboratory of PT SMART Tbk, starting from October 2018 until June 2019. Root tissue samples were collected from E. guineensis grows in PT SMART Tbk Sentul, Bogor and A. catechu grows in Lembaga Alam Tropika Indonesia (LATIN) Situ Gede, Bogor.

2.1. Isolation of endophytic bacteria
The endophytic bacterial isolates tested in this study were obtained from the root tissues of oil palm and areca nut (Areca catechu). Areca nut contains chemical compositions such as alkaloid, arekolin, arekolidine, arekain, guvase and isoguvase, condensed tannins, hydrolyzed tannins, flavan, phenolic compounds, gallic acid, sap, lignin, oil evaporates and does not evaporate, and salt [39]. The endophytic bacterial isolation protocol were followed the method of surface sterilization [5].

2.2. Biosafety assays
These assays comprised of hypersensitive assay on tobacco leaves and hemolytic assay on blood agar to confirm that all tested bacteria are not pathogen on plants and mammals. Hypersensitivity assay was done by observing any of necrotic area in the leaf segment of tobacco that might raise as a result of hypersensitive response of plant in response to the presence of bacterial cells [6]. Hemolytic assay was carried out by observing the formation of clear zones resulting from hemolysis of blood protein by the bacteria surrounding the colony after 24 hours incubation on blood agar medium 28°C.

2.3. In vitro antagonism assay against G. boninense
All isolates that showed negative results in the biosafety assays were tested further for their ability to inhibit the growth of G. boninense in vitro using a method described by [7]. A mycelia plug (Ø 5 mm) of G. boninense was placed in the middle of a Petri dish (Ø 90 mm) containing Potato Dextrose Agar (PDA) medium. Whilst, endophytic bacterial isolates were scratched on two sides of the plate. This plate was incubated at 25-29°C for 6 days. The percentage of inhibition of endophytic bacteria against G. boninense was calculated using the percentage inhibition formula [8] R1-R2 / R1 x 100%; where R1 is the radius of the pathogenic fungus away from the colonies of endophytic bacteria and R2 is the radius of the pathogenic fungi that approach the colonies of endophytic bacteria.

2.4. Plant growth promoting assay
A growth booster test was carried out to determine the potential of bacterial isolates as plant growth promoter (PGP) agent. In this experiment, rice seed cv. Situ Bagendit was used as a plant model following the method used by [9]. Twenty rice seeds were surface-sterilized through soaking in NaOCl 2% solution for 2 minutes and rinsed with sterile water for 3 times. Subsequently for each treatment, the seeds were soaked in a bacterial suspension with concentration 10^8 of 12-hours-old culture for 24 hours then sown on sterile soil media in the planting tray [10]. Observation variables included plant height and root length, after 21 days of incubation from each isolate. Seeds were soaked in a sterile water as control treatment that did not applied any endophytic bacteria.

2.5. Molecular identification of endophytic bacteria
Molecular identification of endophytic bacteria was carried out to determine the genus and species of the bacteria based on the 16S rDNA gene which is widely used as a fingerprint method to identify bacteria. Bacterial genomic DNA was extracted following the Qiagen Protocol Handbook DNA
isolation protocol. The amplification of the 16S rRNA gene from the bacterial genome was carried out using a universal primer pair using the Polymerase Chain Reaction (PCR) technique. The primer pair were comprised of forward 27F primer (AGAGTTTGATCCTGGCTCAG) and reverse 1429R primer (GGTTACCTTGTTTACGACTT) [11]. PCR amplicon of each sample was purified with QIAquick PCR purification system (Qiagen, Germany) prior to sequencing analysis at the FirstBASE Laboratories, Singapore. Resulted 16S rRNA sequences were analyzed using NCBI's Basic Local Alignment Search Tool (BLAST) for identification purposes.

2.6. Statistical analysis
Statistical analysis was done for antagonism and PGP assay at the 95% confidence level. Significantly different results were tested further by the Tukey test at the 5% level using SAS 9.1.

3. Results

3.1. Isolation and selection of endophytic bacteria in vitro
Isolation of endophytic bacteria produced 88 isolates, 45 of which came from the roots of oil palm and 43 from the roots of areca nut. Bacterial density was ranged from $10^4$-10$^5$ colonies g$^{-1}$. Based on the morphological characters, those isolates were varied in colors, edges and elevation of the cell’s colonies. However, most isolates were dominated with cloudy white, round/irregular, and flat colonies.

These isolates were tested for biosafety assays to confirm its safety aspect as plant pathogens or mammals. Hypersensitivity assay exhibited 11 isolates with negative results whereas no necrotic reaction was occurred on the injected leaves. These 11 isolates were carried on hemolytic assay and produced remaining 7 isolates that shown negative result (table 1). These were used for further investigation.

3.2. Antagonism assay test
A total of 7 endophytic bacterial isolates passed the biological safety assay. These were then tested for their ability to inhibit pathogenic fungi *G. boninense* with a dual culture method. Antibiotic endophytic bacteria isolates showed varying percentage of inhibitory power towards *G. boninense* on PDA medium, the highest inhibition percentage were showed by EG26, EG113 and AC112 (table 2). These isolates were used further in the PGP assay to uncover their ability in promoting plant growth.

3.3. Plant growth promoting assay
In this assay, EG26, EG113, and AC112 were tested for their ability to promote the growth of rice seedlings. The result showed that only AC112 that gave significant root length elongation up to 30.77% compared to control. Meanwhile, all isolates gave significant results on plant height compared to untreated control, and the best percentage that reached 39.56% was shown by AC112 (table 3). This isolate apparently has an important role in promoting plant growth in rice seedlings.

| Plant                | Total Isolate | Hypersensitive Assay | Hemolytic Assay |
|----------------------|---------------|----------------------|-----------------|
|                      |               | Positive | Negative | Positive | Negative |
| *Elaeis guinensis* (EG) | 45            | 37       | 8        | 4        | 4        |
| *Areca catechu* (AC)    | 43            | 40       | 3        | 0        | 3        |
| Total                | 88            | 77       | 11       | 4        | 7        |

3.4. Statistical analysis
Statistical analysis was done for antagonism and PGP assay at the 95% confidence level. Significantly different results were tested further by the Tukey test at the 5% level using SAS 9.1.
Table 2. Antagonistic of endophytic bacteria in dual culture test against *G. boninense in vitro*.

| Isolates | Inhibition Percentage (%) |
|----------|---------------------------|
| EG26     | 56.80 a                   |
| EG215    | 24.44 b                   |
| EG17     | 14.43 bcd                 |
| EG113    | 56.51 a                   |
| AC112    | 56.11 a                   |
| AC28     | 5.56 cd                   |
| AC214    | 16.7 bc                   |
| Control  | 0 d                       |

*Number followed by different letter in the same column are significantly different at Tukey Test P=0.05

Table 3. Effect of endophytic bacteria on root length and height of rice plant at 21 days after treatment.

| Isolates | Root Length | Percentage compared to control (%) | Plant Height | Percentage compared to control (%) |
|----------|-------------|------------------------------------|--------------|-------------------------------------|
| EG26     | 4.99 b      | -0.49                              | 17.42 ab     | 34.88                               |
| EG113    | 5.00 b      | -0.39                              | 15.89 b      | 23.03                               |
| AC112    | 6.56 a*     | 30.77                              | 18.02 a*     | 39.56                               |
| Control  | 5.02 b      | 0.00                               | 12.91 c      | 0.00                                |

*Number followed by different letter in the same column are significantly different at Tukey Test P=0.05

3.4. Molecular identification of endophytic bacteria

Three DNA genomes of potential endophytic bacterial isolates were identified using 16S rDNA sequence’s gene. The PCR results of three potential endophytic bacterial isolates were traced to their base arrangement with 1st BASE DNA Sequencing Services and sequences were analyzed using NCBI’s Basic Local Alignment Search Tool (BLAST). EG26 was identified 99.93% similar to *Bacillus subtilis*, EG113 was 99.79% similar with *Bacillus velezensis*, and AC112 was 99.58% similar to *Bacillus toyonensis* (table 4).

Table 4. Molecular identification using 16S rDNA.

| Isolates | Identity          | Identity matrix (%) | Query cover (%) |
|----------|-------------------|---------------------|----------------|
| EG26     | *Bacillus subtilis* | 100                 | 99.93          |
| EG113    | *Bacillus velezensis* | 100                | 99.79          |
| AC112    | *Bacillus toyonensis* | 100               | 99.58          |

4. Discussion

The diversity of endophytic bacteria isolated from the roots of oil palm and areca nut was similar. Endophytes population is mainly influenced by the factors of plant organs, plant varieties, genotypes, cultivation techniques, and other environmental conditions [12]. Lower ambient temperature increases the density and the quality of physiological processes, especially those which producing IAA.
hormones [13]. Colonies of endophytic bacteria in plant roots can reach 10^6 colonies g^-1, while in the stem the density of endophytic bacteria tends to decrease in the range of 3.3 x 10^5 - 1.9 x 10^3 colonies g^-1, even decreasing in leaves [14].

Biological safety assay is important to ensure that the bacterial isolate harmless for plants and mammals. Biocontrol agents are not only focus on their effectiveness in suppressing pathogen development, but also on their safety for human, animal and environmental health [15]. In the hypersensitivity assay, plant pathogen organisms will interact with tobacco hypersensitive response and thus forming a necrotic zone at the injected leaf area. Hypersensitive reactions are triggered by pathogens that have the Avr-gene. Local lesions will occur as a hypersensitivity reaction if the test plant has an R gene that will recognize the Avr-gene from each pathogenic bacterium. Local lesions symptoms are the response of plants to the presence of pathogens to inhibit the spread of pathogenic microorganisms in plant tissues. In hemolytic assay, the clear zone on blood agar indicating microbial protein activity in producing hemolysin that can degrade the red blood cell membrane [16].

This study showed that endophyte isolates could inhibit the growth of G. boninense whereas the inhibition was ranged from 5.56 until 56.80%. The highest inhibition was shown by EG26 with 56.80%. Endophytic bacteria isolated from oil palm plants have an inhibition of G. boninense with an inhibitory percentage value greater than 50% [17]. The criteria for selecting biocontrol agents for fungi and bacteria are different. This is due to the growth properties of bacteria which do not expand the colonies through the entire plate as in fungi. Fungi are considered as biocontrol agents when they produce an inhibitory power greater than 80%, whereas for bacteria is only greater than 40% [18]. Accordingly, EG26, EG113 and AC112 are potential candidate as biocontrol agents. Endophytes have several beneficial effects on plants, including the antibiotic production for pathogen, growth promoting, inducing plant resistance, disturbing the signals formation in pathogen for its pathogenicity (quorum sensing) [19]. Some endophytic bacteria in soybean such as Enterobacter, Acinetobacter, Pseudomonas, Ochrobactrum, and Bacillus were associated with the nodules formation, promoted growth, and inhibited the pathogenicity of Phytophora sojae [20].

Some endophytic bacteria are reported to be able to stimulate plant growth by producing plant growth-promoting hormones such as IAA, nitrogen-fixing bacteria and producing phosphates that support plant growth [21]. Besides, it can also produce growth regulators such as cytokinin, gibberellin, and activity of 1-aminosiklopropan-1-carboxylic (ACC) deaminase [22]. In this study, rice seedling was used as a model to indicate if EG26, EG113 and AC112 have a capability to enhance growth. All of the isolates showed enhancement of rice seedling growth. The results of this study obtained isolates capable of increasing the growth of rice seeds. Increased plant height can be influenced by growth hormones produced by endophytic bacteria [38]. Growth hormone-like IAA has a large role in regulating root length and plant height. The existence of essential nutrients such as N and P can also affect plant growth [39]. The results of other studies also showed that endophytic bacteria isolated from mangrove plants could increase the growth of rice seeds and tomatoes [23]. Treatment of endophytic bacteria from rice plants can increase the total growth of upland rice seedlings compared to controls [12]. Endophytic bacteria isolated from Arecaceae plants can increase the growth of rice seeds [9].

Based on the 16S rDNA sequencing, EG26, EG113 and AC112 are Bacillus group. Bacillus spp. produces a variety of antimicrobial substances, with most of them being low molecular weight antimicrobial peptides and some protein antagonists [24]. Their ability to form endospores allows them to survive in a wide range of environmental conditions [25]. Bacillus species is an effective biocontrol agent against Botrytis cinerea infecting rose [26]. B. subtilis inhibits the development of pathogens through mechanisms of nutritional competition, antibiosis and growth promoters. The effectiveness of B. subtilis can inhibit the reproduction of pathogenic fungi in controlling diseases in various plants, such as Fusarium graminearum [27], Ralstonia solani, Colletotrichum panacicola, and Pseudomonas siringae [28]. Fegicin antibiotics produced by B. subtilis strain B-FS01 play an important role as antifungal Fusarium verticillioides [29]. Antifungal Bacilysocin from B. subtilis 168 inhibited the growth of Candida and Aspergillus niger [30]. Besides antibiotics, B. subtilis also
produces protease, amylase and chitinase enzymes which can break down the walls of pathogenic cells. *B. subtilis* is a PGPR bacterium that can increase the percentage of seed germination, plant vigor, root growth and plant biomass [31]. *B. subtilis* can inhibit the reproduction of pathogenic fungi through the effects of competition and antibiotics [32]. *B. velezensis* AR1 supernatant could inhibit the growth of *Glomerella cingulata* by 50% at 50% concentration rate [33]. Another study showed that *B. velezensis* exhibited antagonistic activity against major plant fungal pathogens, namely *Fusarium oxysporum*, *F. graminearum*, *Botrytis cinerea*, *Alternaria alternata*, *Fulvia fulva*, and *Ustilaginoidea virens* [34]. *B. cereus* have been reported to produce antifungal molecules, which are mostly found in the rhizosphere [35]. *B. cereus* ATCC 53522 produced antifungal *zwittermicin* [36] which suppressed the growth of *Fusarium* and *Rhizoctonia solani* [37].

5. Conclusion
This study revealed some endophytic bacteria that are beneficial as potential candidate for biocontrol agents of *G. boninense* and potential candidate as plant growth promoter (PGP) agent. The best inhibiting bacteria against *G. boninense* was *B. subtilis*, while the best stimulating bacteria in case of rice seedling’s growth was *B. toyonensis*. This research is expected to be continued on a field scale and the mode of action of endophytic bacteria can be studied more deeply.

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