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Research Article

Evaluation of Antagonistic Activity of Endophytic Bacteria from Dayak Onion Against *Ganoderma boninense*

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Abstract
Basal stem rot (BSR) is one of the key contributors to the decline in palm oil production in Indonesia. BSR is caused by the pathogenic plant fungus *Ganoderma boninense*. Currently, the control of *G. boninense* growth relies on systemic fungicides. However, systemic fungicides have negative impacts as they can harm natural soil microorganisms. Biocontrol agents present an environmentally friendly alternative for *G. boninense* management. The objective of this research was to select and identify endophytic bacterial isolates from Dayak onion [*Eleutherine bulbosa* (Mill.) Urb.] with antagonistic activity against *G. boninense*. The investigation was initiated with the isolation of 34 endophytic bacterial isolates and *G. boninense* isolate. The hemolytic capabilities of endophytic bacterial isolates were evaluated on blood agar media. Antagonism assessments were conducted through the dual-culture method, and the inhibition of *G. boninense* was measured using the percentage inhibition of radial growth (PIRG). The endophytic bacteria exhibiting the highest PIRG values were identified through molecular analysis based on the 16S rRNA gene sequence. Results revealed that 27 isolates demonstrated no ability to hydrolyze blood agar (gamma hemolysis) indicating no pathogenicity. Isolate CED9 demonstrated the highest PIRG value at 70.26%. Genomic identification based on the 16S rRNA gene sequence confirmed that isolate CED9 was classified as *Bacillus subtilis*. The identification of biocontrol agents from Dayak onion represents valuable information and a potential arsenal for future strategies in mitigating (BSR) disease.

Keywords: *Bacillus subtilis*, biocontrol, dayak onion, endophytic bacteria, *Ganoderma boninense*

1. INTRODUCTION

Palm oil (*Elaeis guineensis* Jacq.) is a tropical palm that serves as a major oil-producing crop. Furthermore, palm oils have been manufactured for various products such as soap, wax, and cosmetic [1]. Currently, Indonesia stands as the world's largest producer of palm oil. In 2022, the palm oil plantation area in Indonesia was estimated to cover 15 million hectares, with a total production reaching 48 million tons. Despite yearly expansions in palm oil plantations, the growth in production does not proportionally align [2]. Basal stem rot (BSR) disease poses a significant challenge in palm oil cultivation, particularly in Southeast Asia, notably Indonesia, and is caused by *Ganoderma boninense*. The progression of this disease is relatively slow, with symptoms manifesting in the late stages of infection, earning it the moniker of a "silent killer" [3]. One approach to control BSR disease is using systemic fungicides such as triadimefon, carboxin, carbendazim, methfuroxam, and hexaconazole [4]. However, the application of systemic fungicides is known to have adverse effects on beneficial soil microorganisms such as mycorrhiza [5]. The utilization of biocontrol agents represents an alternative technology that is more environmentally friendly and relatively cost-effective [6]. The use of biocontrol agents has been widely implemented in Indonesian palm oil plantations as a strategy for integrated plant disease management [7]-[9].

Endophytic bacteria can serve as a potential source of biocontrol agents for managing BSR disease. Some endophytic bacterial species are known to inhibit the growth of *G. boninense*, such as *Bacillus subtilis* [10] and *Pseudomonas aeruginosa* from palm oil [11][12], and *Bacillus toyonensis* from betel roots [13], which can hinder the growth of *G. boninense* mycelium. Dayak onion is a traditional medicinal plant in Kalimantan known for its various benefits, including antibacterial and antifungal properties [14][15]. The isolation of endophytic bacteria from Dayak onion allows for the discovery of potential isolates

Table 1. Pathogenicity test of endophytic bacteria from Dayak onion on blood agar.

Isolate	Hemolytic group	Activities	Isolate	Hemolytic group	Activities
AEU1	γ	no activity	CED1	α	greenish zone
AEU2	γ	no activity	CED2	γ	no activity
AEU3	γ	no activity	CED4	γ	no activity
AEU4	γ	no activity	CED5	γ	no activity
AEU5	γ	no activity	CED6	γ	no activity
AED6	γ	no activity	CED7	γ	no activity
AED7	γ	no activity	CED8	γ	no activity
AED8	γ	no activity	CED9	γ	no activity
AED9	β	clear zone	CED10	β	clear zone
CEA1	β	clear zone	CED13	γ	no activity
CEA2	γ	no activity	CEU1	γ	no activity
CEA3	γ	no activity	CEU2	γ	no activity
CEA4	β	clear zone	CEU3	γ	no activity
CEA5	γ	no activity	CEU4	γ	no activity
CEA6	γ	no activity	CEU5	γ	no activity
CEA7	β	clear zone	CEU7	γ	no activity
CEA8	γ	no activity	CEU8	β	clear zone

Note: γ = gamma hemolysis (non-pathogenic), β = beta hemolysis (complete hemolysis), α = alpha hemolysis (partial hemolysis).

capable of producing new antifungal compounds that could inhibit the growth of *G. boninense*. A study by Rizali et al. revealed that endophytic fungi from *Eleutherine palmiflora* inflorescence, a relative of *E. bulbosa*, exhibit antifungal activity against *Fusarium*, a phytopathogenic fungus affecting horticultural crops [16]. Research on the utilization of Dayak onion endophytes for biocontrol is currently limited to endophytic fungi. However, endophytic bacteria from Dayak onion have significant potential as biocontrol agents, given that bacterial isolates tend to grow relatively faster in cultivation media compared to fungi. Therefore, this research aims to select and identify endophytic bacterial isolates from Dayak onion (*Eleutherine bulbosa*) exhibiting antagonistic activity against *G. boninense*. This serves as an initial step in developing biocontrol agents for the effective management of *G. boninense*.

2. MATERIALS AND METHODS

2.1. Materials

Bacterial isolates were obtained from a previous study reporting the antibacterial activity of endophytic bacteria from Dayak onion against clinical pathogens [17]. The bacteria were subcultured on nutrient agar (NA) medium using the streak plate method and then incubated at 30 °C for 48 h prior test. Stock culture of *G. boninense* isolate was sourced from the Soil Science Laboratory at IPB University, originating from Perkebunan Nusantara 7 Company, Rejosari Unit-Pematang Kiwah, Lampung, Indonesia [18]. The fungus was subcultured on potato dextrose agar (PDA) medium and incubated at 28 °C for 7 days prior to experimentation.

2.2. Methods

2.2.1. Hemolytic Test

The safety assessment of bacterial isolates was conducted through a hemolysis test using blood agar. Bacterial isolates, aged 24 h, were inoculated onto the surface of blood agar media and incubated at 35 °C for 24 h. Hemolytic activity was observed through the formation of clear zones surrounding the endophytic bacterial colonies and grouped based on their lytic traits [11].

2.2.2. In Vitro Antagonistic Test against G. boninense

The antagonistic test of endophytic bacteria against *G. boninense* was conducted using the dual-culture method [19]. Indicator fungus, *G. boninense* isolate, approximately 1 cm^2 in size, was placed in the center of a petri dish containing PDA media. Endophytic bacterial isolates were streaked adjacent to the *G. boninense* colony, while a *G. boninense* culture without endophytic bacterial isolates served as the negative control. All plates were incubated at 28 °C for 7 days, accompanied by observations of the radial growth of *G. boninense* colonies. Each treatment was repeated 3 times. The percentage inhibition of radial growth (PIRG) was determined using the formula (1):

$$\text{PIRG} = \frac{\text{R1} - \text{R2}}{\text{R1}} \times 100\% \quad \textbf{(1)}$$

The R1 and R2 are the diameter of radial growth of *G. boninense* mycelium in the control and dual culture plates, respectively.

2.2.3. Molecular Identification of Antagonistic Isolate

The bacterial isolates with the highest PIRG were molecularly identified based on the 16S rRNA gene. DNA amplification was performed using the forward primer 27F (5’-AGAGTTTGATCMTGGCTCAG-3’) and 1492R (5’-TACGGYTACCTTGTTACGACTT-3’) [20]. The amplification process took place in a polymerase chain reaction (PCR) machine. The resulting 16S rRNA gene amplicons were purified and sequenced using the Sanger sequencing method with primers 785F (5’-GGATTAGATACCCTGGTA-3’) and 907R (5’-CCGTCAATTCMTTTRAGTTT-3’). The sequencing results were aligned with accessions in GenBank, National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLASTn) feature to identify bacterial isolates. The 16S rRNA gene sequences of the samples and several isolates from NCBI GeneBank data were aligned through Multiple Sequences Alignment (MSA) using the MEGA XI program. The MSA results were saved as a compatible file and used as the basis for constructing a phylogenetic tree using MEGA11 [21]. Phylogenetic analysis was conducted using the Neighbor-Joining method and Kimura’s 2-parameter model with a bootstrap value of 1000×.

2.2.4. Statistical Analysis

Numerical data consisting of *G. boninense* colony diameter were expressed in mean ± standard deviation (SD) and tested using one-way ANOVA and continued with Duncan's Multiple Range (DMRT) test at the level of 5% in Minitab ver. 19.0. Percentage inhibition of radial growth (%) was expressed in the mean for each bacterial isolate.

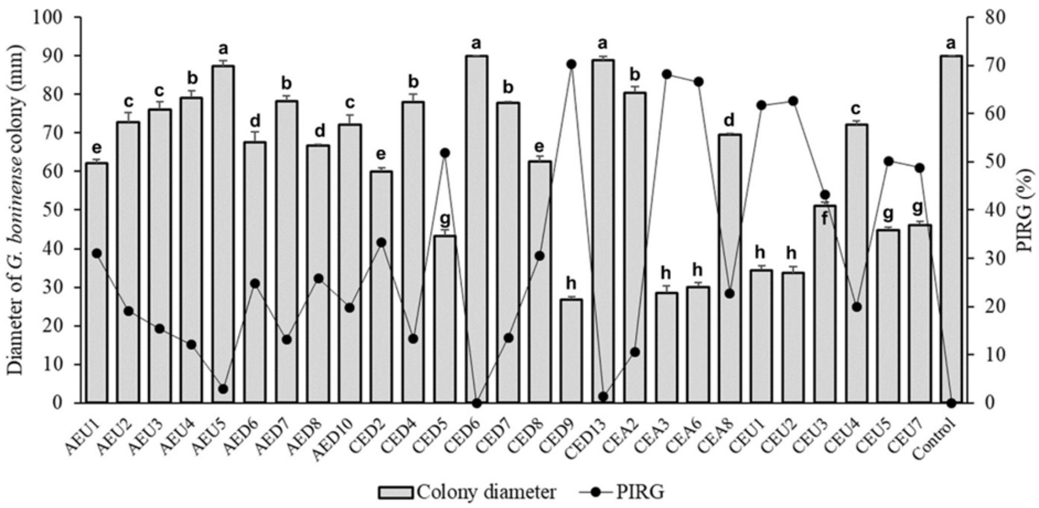

Figure 1. Antagonistic profile of endophytic bacteria from Dayak onion against *Ganoderma boninense*.

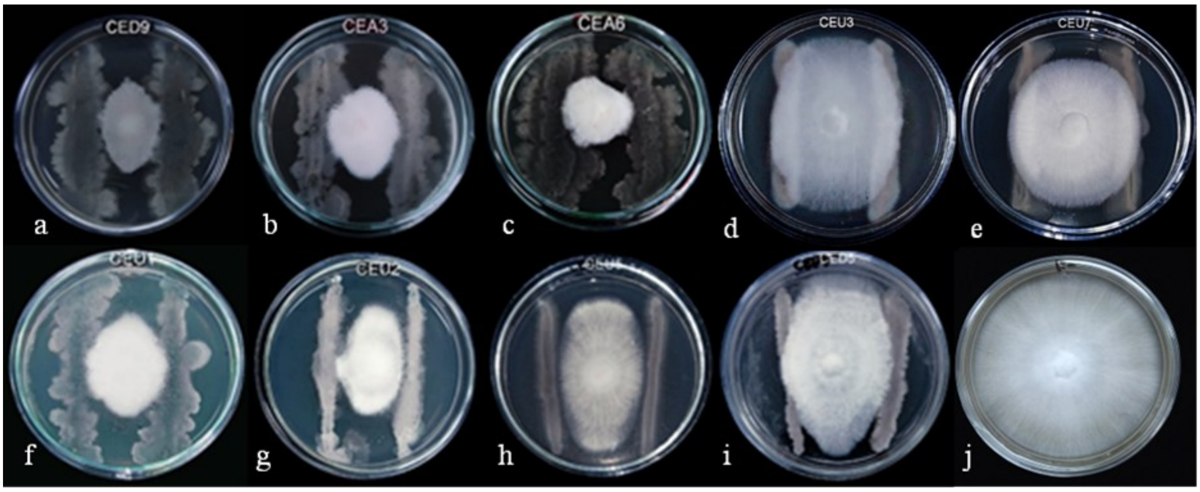

Figure 2. The results of the dual-culture test of Dayak onion endophytic bacteria against *G. boninense* on PDA medium after 7 days of incubation at 28 °C: treatment with CED9 (a), CEA3 (b), CEA6 (c), CEU3 (d), CEU7 (e), CEU1 (f), CEU2 (g), CEU5 (h), CED5 (i), and negative control (j).

3. RESULTS AND DISCUSSION

3.1. Hemolytic Traits as Pathogenicity Indicator

Based on the lytic performance, it was observed that 1 isolate exhibited α-hemolysis (CED1), 6 isolates displayed β-hemolysis (AED9, CEA1, CEA4, CEA7, CEU8, and CED10), while the remaining 27 isolates demonstrated γ-hemolysis. The results of the hemolysis test for endophytic bacterial isolates are provided in Table 1. Hemolysis involves the breakdown of erythrocyte membranes by extracellular proteins, such as enzymes or toxins produced by bacteria, indicating their pathogenicity [22]. Blood agar media is commonly employed to distinguish the hemolytic activity of bacteria, with sheep blood agar being a commonly used standard medium for this purpose, facilitating optimal bacterial growth. Alpha-hemolysis is characterized by a change in the media to greenish color. This phenomenon occurs due to the hydrogen peroxide produced by bacteria oxidizing hemoglobin, resulting in green-colored oxidized methemoglobin. Alpha-hemolysis is referred to as the partial lysis of erythrocytes. Beta-hemolysis occurs due to the complete lysis of erythrocytes by bacteria, forming a clear zone around the media. In contrast, gamma-hemolysis implies no lysis of red blood cells, indicating that the endophytic bacteria are non-pathogenic [23].

3.2. In Vitro Growth Inhibition of G. boninense by Endophytic Bacteria

We documented 26 endophytic bacterial isolates that exhibit varying inhibitory activities to the colony of *G. boninense*. Nine isolates demonstrate strong inhibitory potential, namely CED9, CEA3, CEA6, CEU2, CEU1, CED5, CEU5, CEU7, and CEU3. Three isolates exhibit moderate inhibitory activity, specifically CED2, CED8, and AEU1 while fourteen isolates show weak inhibitory activity, including AED8, AED6, CEA8, AEU2, CEU4, AED10, AEU3, CED7, CED4, AED7, AEU4, CEA2, AEU5, and CED13. The highest inhibition is demonstrated by isolate CED9 at 70.26%. The results of the antagonism test of endophytic bacteria against *G. boninense* are presented in Figure 1. Meanwhile, the antagonistic profile of endophytic bacteria from Dayak onion against *G. boninense* is shown in Figure 1. The inhibitory activity of endophytic bacteria is categorized into 4 groups based on the PIRG: strong (>40%), moderate (30–40%), weak (<30%), and no inhibition (0%) [24]. Isolate CED9 displays the highest PIRG, indicating its ability to produce stronger or larger amounts of antifungal compounds compared to other isolates in inhibiting the growth of *G. boninense*. According to Rahma et al., the quantity of antifungal compounds excreted into the media affects the magnitude of the resulting inhibitory effect [25]. Higher concentrations lead to increased inhibitory activity. Endogenously generated secondary metabolites in microorganisms have the capacity to trigger antibiosis, functioning as a defensive strategy for survival and competitive advantage [26]. The potential of secondary metabolites produced by endophytic bacterial isolates to influence the growth of *G. boninense* could be harnessed for controlling basal stem rot in oil palm. Endophytic bacteria employ several mechanisms as biocontrol agents, including the production of antibiotics, siderophores, and lytic enzymes; competition for substrates and habitats; and activation of the plant's systemic resistance system [27]. Endophytic bacteria are known to produce bioactive compounds capable of degrading fungal cell walls. Moreover, these bioactive compounds can influence the permeability of fungal cell membranes, disrupting the transport of substances required for metabolism. Endophytic bacteria can also produce compounds that act as inhibitors of enzymes produced by fungi. Additionally, there is another mechanism involving the production of compounds that can suppress protein synthesis in fungi. Consequently, disruptions in protein synthesis can lead to a deficiency of specific proteins affecting fungal growth [28]. The dual-culture test of Dayak onion endophytic bacteria against *G. boninense* on PDA medium after 7 days of incubation at 28 °C with different treatments is depicted in Figure 2.

3.3. Molecular Identification of Antagonistic Isolate CED9

Identification based on the 16S rRNA gene revealed that isolate CED9 exhibited an identity percentage of 99.60% and a query coverage of 100% with *Bacillus subtilis*. The BLAST results for isolate CED9 on NCBI are presented in Table 2. Identity percentage is utilized to denote the similarity between the species of isolate CED9 and those present in the database. The value obtained by isolate CED9 is 99.60%, signifying a relatively high similarity at the species level. The similarity obtained is categorized into two groups: one with a sufficiently high identity percentage (>97%) and another with a low identity percentage (97%-93%). Bacteria with a sufficiently high identity percentage share similarities at the species level. Conversely, a low identity percentage indicates similarity only at the genus level based on the 16S rRNA gene sequence. Query coverage represents the percentage of the sequence length that matches the database. The query coverage value for isolate CED9 is 100%, indicating a high sequence similarity between isolate CED9 and *B. subtilis* R8. Higher query coverage values correspond to greater sequence similarity. *B. subtilis* is a bacterium capable of thriving in water, soil, air, and decaying plant remnants [29]. Previous study has demonstrated that endophytic *B. subtilis* from wheat plants, can inhibit the growth of the pathogenic fungus *Blumeria graminis* on wheat [30]. Another study on *B. subtilis* strain E-1, isolated from the rhizosphere of oil palm, exhibits antifungal activity against *G. boninense* [31]. *B. subtilis* is known for its capability to inhibit the growth of its pathogens by secreting antibiotic compounds and producing enzymes that are toxic to pathogenic fungi. Antibiotic compounds produced by *B. subtilis* include streptavidin, basitrasin, surfactin, fengycin, iturin A, mycobacillin, subtilin, subtilosin, polymyxin, and difisidin. Surfactin, fengycin, and iturin A are lipopeptide compounds; basitrasin is a polypeptide effective in inhibiting the growth of the cell walls of pathogenic fungi; subtilosin is an antimicrobial protein; and subtilin is a peptide [32]. The species can produce enzymes that degrade fungal cell walls, such as xylanase, β-levan sucrase, chitinase, α-amylase, β-glucanase, and protease.

Table 2. BLASTn result of isolate CDE9 based on 16S-rRNA database in GenBank.

Isolate	Species assemble	Accession number	Query coverage	Identity percentage
CED9	*Bacillus subtilis* R8	MH371779.1	100%	99.60%
	Bacillus subtilis SB-1	OR342736.1	99%	99.67%
	Bacillus subtilis ST-15	MK511833.1	99%	99.67%
	Bacillus subtilis ZB	KX450400.1	99%	99.67%
	Bacillus subtilis RSP2	OQ726228.1	99%	99.67%

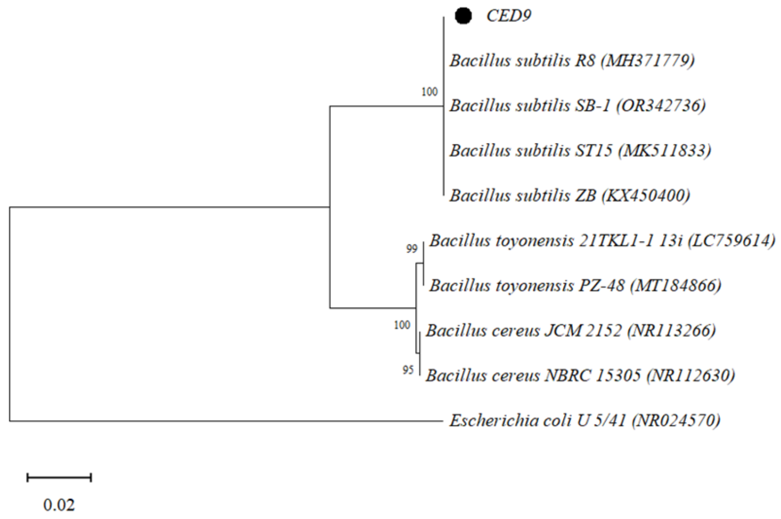

Figure 3. Phylogenetic tree of isolate CED9 based on 16S-rRNA gene sequences using the Neighbor-Joining method with a bootstrap of 1000×.

The production of these enzymes led to the lysis of target cell walls, inhibiting their growth, and consequently, the production and secretion of extracellular enzymes of targets were also inhibited [33]. The biocontrol activity mechanism of *B. subtilis* against plant pathogens may also include biofilm formation, plant growth stimulation, competition for nutrients and colonization sites, as well as inducing systemic resistance [34]. *B. subtilis* was capable of forming biofilms on plant roots, aiding in the production of lipopeptides, enhancing antimicrobial activity in the soil, and increasing tolerance to antimicrobial agents [35]. *B. subtilis* is known to suppress plant diseases by colonizing the host and competing for nutrients. Research indicates that direct colonization of plant roots or leaves by *B. subtilis* contributes significantly to effective biological control against soil-borne pathogenic fungi [36]. Some *B. subtilis* strains also possess the ability to stimulate plant defense responses through the induction of jasmonic acid and ethylene, and under certain conditions, the induction of salicylic acid, which serves as a systemic resistance signal triggered by pathogenic fungi attacks [37]. Puspita et al. stated that the endophytic *B. subtilis* has the ability to induce resistance to *G. boninense* by producing salicylic acid [38]. *B. subtilis* has distinct advantages as a biocontrol agent compared to other microorganisms. One key advantage is its ability to produce endospores, providing resistance to extreme conditions and ensuring prolonged survival in the environment [32]. In addition, the saprophytic nature of *B. subtilis* facilitates easy propagation in cheap and affordable cultivation media such as coconut water and liquid waste from tofu production, supplemented with additional nutrient sources like carbon, nitrogen, and other microelements. The cost of cultivation in such media is more economical compared to standard laboratory media like Luria Broth, Kings B, and Trypticasein Soy Agar [39]. Although *B. subtilis* is considered a helpful organism, it can have a deleterious impact on the endophytic and rhizospheric microbiota by decreasing their numbers and altering their genetic makeup. It is determined by soil texture, composition, pH, and bacterial concentrations in the rhizosphere. As a result, it is critical to do a feasibility assessment before using microbial inoculants in the soil [40]. The phylogenetic tree of isolate CED9 based on 16S-rRNA gene sequences using the Neighbor-Joining method with a bootstrap of 1000× is shown in Figure 3.

4. CONCLUSIONS

Based on our findings, it can be concluded that 27 endophytic bacterial isolates were unable to hemolyze blood agar or declared as safe for in plant application. As many as 9 isolates exhibited strong inhibition based on dual culture assay against *G.*

boninense, with isolate CED9 displaying the highest PIRG value at 70.26%. Molecular identification confirmed that isolate CED9 belongs to *Bacillus subtilis*. Given its potential, isolate CED9 can be considered as a promising biocontrol agent against *G. boninense* in the future.

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Conflicts of Interest

The authors declare no conflict of interest.

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