

# The potential of endophytic bacteria from the root of *Eucalyptus pellita* as a biocontrol agent against *Ralstonia solanacearum*

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**Abstract.** Susanti Y, Giyanto, Sinaga MS, Mutaqin KH, Tjahjono B. 2021. The potential of endophytic bacteria from the root of *Eucalyptus pellita* as a biocontrol agent against *Ralstonia solanacearum*. *Biodiversitas* 22: 3454-3462. Bacterial wilt disease (BWD) caused by *Ralstonia solanacearum* is one of the most damaging eucalyptus (*Eucalyptus pellita* F. Muell.) diseases. However, endophytic bacteria can be utilized to control it. This study aims to obtain and screen endophytic bacteria isolated from the root of eucalyptus as a biocontrol agent against BWD. A pathogenic *R. solanacearum* was isolated for artificial inoculation; subsequently, isolation, characterization and screening of the potential endophytic bacteria were conducted. Isolates of *R. solanacearum* demonstrated the pathogenicity for BWD in eucalyptus, with the highest virulence found in Rs 18. In total, 24 isolates of endophytic bacteria were obtained and screened for their biocontrol potential. The endophytic bacteria conveyed the capacity of antibiosis, indole acetic acid (IAA) hormone, nitrogen fixation, phosphate dissolution, protease enzyme and AHL-lactonase, which are the main indicators of bioagent characteristics in endophytic bacteria, as well as being plant growth stimulators. Four isolates were selected with the highest according to the analytic hierarchy process (AHP), identified on the 16S rRNA gene. Most isolates belonged to gram-negative bacteria. The four isolates, namely B33/77 (*Serratia nematodiphila*), B32/77 (*Paenibacillus polymyxa*), B13/497 (*Brevundimonas olei*) and B17/111 (*Bacillus megaterium*), indicated their potential for biocontrol as plant growth promoters. This study is the first report on the isolation of endophytic bacterial from eucalyptus roots with regard to its benefit as a plant growth promoter. It provides basic information about the characteristics of the endophytic bacteria, which showed potential as a biocontrol agent of *R. solanacearum* on eucalyptus.

**Keywords:** Characteristics, endophytic bacteria, *Eucalyptus pellita*, identification

## INTRODUCTION

In Indonesia, eucalyptus is the second main industrial timber commodity (or HTI in Indonesia) after acacia. The type of eucalyptus that is predominantly planted is *Eucalyptus pellita* F. Muell, due to its suitability as a raw material for the pulp and paper industries. *E. pellita* is also an alternative to *Acacia mangium*, which is currently exhibiting high mortality due to root fungi disease (*Ganoderma philippii*, *Phellinus noxius*, *Rigidoporus microporus*) (Yuskianti et al. 2013). Moreover, *E. pellita* has also shown excellent results in terms of its fast growth, straight boles/trunks, good coppice capability, wide adaptation range to various environmental conditions, robustness against pests and disease, and as it is suitable for various wood products (Hung et al. 2015).

The industrial forest of log production was mainly supplied from six tree species, one of which is eucalyptus. After acacia, eucalyptus has the second-highest log production, contributing 19.58% of the total national timber for log production, or 40,628.78 m<sup>3</sup>/year 2018 (BPS 2019). However, the productivity of eucalyptus silviculture has declined recently due to disease, one of the most widespread being bacterial wilt disease (BWD). This is

caused by *Ralstonia solanacearum*, which is also the main pathogen in annual crops. BWD caused by *R. solanacearum* in woody perennial plants has only been recorded only in the eucalyptus species. However, BWD has spread significantly to major eucalyptus-producing countries in Africa, Asia, and America (Ran et al. 2005).

Bacterial wilt disease had caused vast damage in concession areas of the industrial forest. The first incident of BWD in Indonesia was reported by Machmud (1985). An outbreak in a lowland eucalyptus plantation in Jambi was also reported in 2006, which spread to plantations in Riau, Palembang and East Borneo. Siregar et al. (2012) report that the losses caused by BWD during the period 2010-2011 in Riau, from incidences in nurseries affecting the planting blocks, totaled 16 billion rupiahs.

Bacteria *R. solanacearum* infection in eucalyptus could spread from nurseries, even if no wilt symptoms are yet evident. The disease can then progress through the planting fields, triggered by silviculture stresses, in which the infected plants exhibit yellowing leaves, followed by wilting. Incisions on infected stems show an elongated necrotic symptom. When a cut on an infected stem is immersed in clear water, milky white bacterial fluid will ooze out (Coutinho and Wingfield 2017). The wilting

progresses rapidly in the infected plant because the xylem vessels are blocked by *exopolysaccharide* (EPS) from the bacterial cells, thus disturbing water and nutrient transport. The virulent cells of the bacteria produce large amounts of EPS (Arwiyanto 2014).

Efforts to control BWD have been made through various approaches; e.g., eradication, using synthetic chemicals; selection of resistant eucalyptus; multi-clone planning; molecular early detection of BWD in nurseries to ensure the planting stock is BWD free; and use of biological agents. However, those efforts have not yet shown effective results. Several attempts to control BWD in eucalyptus have been reported in research (Santiago et al. 2015; Nuri et al. 2016; Asril et al. 2020). Recent reports on *R. solanacearum* show that infection takes place not only through the roots, but also occurs through the eucalyptus seeds (Siregar et al. 2020), and that it has high genetic diversity and a wide host. This will make controlling BWD even more difficult. Nevertheless, efforts continue to develop, one of which is the biocontrol technique using endophytic bacteria.

Endophytic bacteria as a biocontrol agent have advantages over other bioagents due to their presence in plant tissues, thus readily adapting to the familiar environment conditions in order to survive biotic and abiotic stresses (Hong and Park 2016). They are able to colonize the plant tissues where the *R. solanacearum* develops. Moreover, endophytic bacteria could reduce or prevent the negative effects of particular pathogen organisms. The mechanism of endophytic bacteria in controlling pathogens in the host plant occurs directly and indirectly. In the direct mechanism, the bacteria enhance the nutrient (phosphate, nitrogen, iron) uptake and increase the plant hormones (auxin, ethylene, cytokinin, gibberellin). On the other hand, the indirect mechanism involves antibiosis, lytic enzyme, nutrient competition, and defense induction (Afzal et al. 2019). Due to the alarming incidents that have occurred in the industrial forest eucalyptus in Indonesia, it was considered necessary to conduct research to obtain potential endophytic bacteria to control BWD, thus maintaining and enhancing the productivity of the eucalyptus plantations.

## MATERIALS AND METHODS

### Collection of healthy and bacterial wilt disease-infected eucalyptus

To isolate pathogenic *R. solanacearum*, purposive sampling of roots was conducted in the eucalyptus plantation of PT. Arara Abadi Perawang, Riau, on 1-2 year old plants. Root samples from healthy plants were collected from five susceptible clones and one commercial/resistant clone from six plots in the area where eucalyptus was planted. In addition, plant samples infected with BWD were taken from five clones of susceptible plants from five plots. The roots collected from healthy and infected plants were kept in labelled plastic bags and transferred to the laboratory for further isolation processes. The infected plant samples were then isolated to obtain the virulent *R.*

*solanacearum* isolate, while isolates of endophytic bacteria were isolated from the healthy plant samples.

### Isolation of *R. solanacearum*, physiology test and pathogen identification

The pathogen was isolated from the stem of an *R. solanacearum* infected plant. A crosswise-cut stem showing BWD symptoms was immersed in sterile water for 2-3 minutes. Whitish bacterial ooze exuded from the stem into the water. From the water in which the stem was soaked, 1 mL were taken and put into a reaction tube, then 9 mL sterile water added. The bacteria suspension was serial-diluted to  $10^{-3}$  and cultured in a modification of media triphenyl tetrazolium chloride (TZC), a combination of nutrient agar (NA) medium with potato sucrose agar (PSA), known as NAPSA medium (modified by the R&D PT. Arara Abadi). The cultures were incubated at room temperature for 48 hours. The composition of NAPSA medium was 3 g beef extract, 2.5 g glucose, 5 g peptone, 20 g sucrose, 100 g potato extract, 20 g agar, and 1000 mL water, with 4 mL TZC added after the medium had been sterilized. The identification of *R. solanacearum* isolate was made by morphology (the shape and color of the bacterial colony) and a physiology test (hypersensitive response, fermentative oxidative, potato soft root, levan), following the method of Schaad (2001). Molecular identification was based on the Y2/Oli-1 (Ashmawy 2015) and 16S rRNA gene (Jiang et al. 2006). A pathogenicity test was performed by the wound inoculation technique on the roots of eucalyptus seedlings (Fonseca et al. 2015).

### Isolation of endophytic bacteria

Isolation of the endophytic bacteria was conducted in accordance with method of Hallmann et al. (1997). A root sample of eucalyptus was surface-sterilized by washing it under running water and it was cut with sterile scalpel into 1-2 cm pieces 10 g of the root was then soaked in 70% alcohol for two minutes, immersed in NaOCl 1% for two minutes, and then rinsed three times with sterile water. The root cuts were then dried with sterile tissue. The success of the surface sterilization was tested by rubbing the root cut on NA and *tryptone soya agar* (TSA) medium and incubated for two days. Sterilization was deemed successful if there were no bacteria detected growing in the medium. The sterilized roots were macerated using a blender until fine with the addition of sterile water 1:10 (v/v). 1 mL of the bacteria suspension was taken and serially diluted to  $10^{-3}$  dilution. From each dilution, 0.1 mL of suspension was cultured on TSA 10% and NA 10% media, and incubated for 48 hours. The growth of the colony was observed for two days. Each detected colony with different morphologies was then purified in the NA medium.

### Filtering of endophytic bacteria isolates as a bioagent

#### *Test of hypersensitivity reaction (HR)*

An HR test was performed on 173 isolates of endophytic bacteria isolated from the eucalyptus root. An isolate of endophytic bacteria was cultured in a nutrient broth (NB) medium for 12 hours in a shaking incubator at a speed of 120 rpm. 2 mL of bacteria suspension was

infiltrated on the abaxial side of a tobacco leaf, thus wetting the cell inter-spaces. An observation was made 48 hours after the application. Positive reactions were denoted by the emergence of necrotic symptoms on the inoculated leaf, while negative reactions were signified by no occurrence of necrosis. Necrotic symptoms indicate the pathogenic potential of endophytic bacteria on plants (Schaad et al. 2001). Isolates of bacteria with positive reactions were not used in further tests.

#### *Test of hemolysis*

This test aimed to differentiate endophytic bacteria isolated from the tissue of eucalyptus based on the capability of lysis red blood cells, which indicate its potential as a mammal pathogen. An isolate of endophytic bacteria was cultured in a blood agar medium and incubated at room temperature for 18-24 hours. The occurrence of a clear zone around the colony indicated the potential of the isolates as a pathogen for humans and animals (Zimbro et al. 2009). Isolates that showed clear zones in the test were not used in further tests.

#### *Test of pathogenicity on eucalyptus seedlings*

The eucalyptus seedlings used in the test originated from shoot cuttings. These were surface sterilized with Clorox 0.5% for 1 minute and rinsed three times with sterile water. The cuttings were then soaked in 150 mL of the suspension of endophytic bacteria isolated for six hours (Paz et al. 2012). The control plant was treated by soaking it only in sterile water. The shoot cuttings were planted in a pot tray containing a sterile cocopeat medium. Observation for BWD symptoms was conducted three months after planting. Endophytic bacteria that caused symptoms of necrosis, decay, wilting, collapse, and mortality were not used in further tests.

#### *Growth promoter test on eucalyptus seedlings*

The preparation of eucalyptus seedlings and the inoculation of endophytic bacteria isolate followed the pathogenicity test protocol. The growth parameters measured were stem height and diameter. Endophytic bacteria isolate that caused less growth than the control treatment was not chosen for further analysis.

### **Characterization of endophytic bacteria to determine potential isolates for biocontrol**

#### *Antibiosis capacity*

Analysis of the antibiosis capacity of endophytic bacteria on *R. solanacearum* was made in accordance with the cross-streak method (Velho-Pereira and Kamat 2011). The isolate of *R. solanacearum* used for the analysis was BWD 18, which had already been identified as showing the highest virulence. The isolates of endophytic bacteria were cultured in an NA medium and the isolates of *R. solanacearum* were cultured in a NAPSA medium for 48 hours. A loop of *R. solanacearum* was put into 10 mL of sterile water ( $10^8$  cfu mL<sup>-1</sup>) and subsequently vortexed. From the *R. solanacearum* suspension, 0.1 mL was spread using a glass bead on the NAPSA medium in petri disc. In addition, endophytic bacteria were scratched vertically on

the NAPSA medium. The treatment was replicated 3 times. Antibiosis activity was denoted by the formation of a clear zone around the endophytic bacteria colony.

#### *Protease production*

Endophytic bacteria were cultured in skim milk agar (SMA) media. The media composition was 25 g skim milk, 20 g agar and 1000 mL water. The formation of a clear zone around the colony of endophytic bacteria denoted the activity of the bacteria in producing protease enzyme (Hazra and Susanti 2017).

#### *Production of indole acetic acid (IAA)*

The IAA production of the endophytic bacteria was analyzed with a colorimeter, using a Salkowski reagent (150 mL concentrated H<sub>2</sub>SO<sub>4</sub>; 7.5 mL FeCl<sub>3</sub>.6H<sub>2</sub>O 0.5 M; 250 mL sterile water). A loop of 48-hour old endophytic bacteria was inoculated on the NB medium. After 24 hours, 500 µL of the bacteria culture was then inoculated in 50 mL of the NB medium, which contained 0.1% DL-tryptophan, and incubated in a shaker at 180 rpm for 48 hours in dark conditions. It was then centrifuged at 10,000 rpm and 4°C for 10 minutes. The resulting supernatant IAA was then estimated with the colorimetry method. 1 mL of the supernatant was prepared and then mixed with 4 mL of Salkowski reagent. When the supernatant turned pink after 30 minutes, the absorbance was then observed with a spectrophotometer at a wavelength of 535 nm. The pink-colored supernatant conveyed the production of IAA (Ghevariya and Desai 2014).

#### *Phosphate dissolving*

The capability of endophytic bacteria to dissolve phosphate was analyzed in a Pikovskaya medium test. The composition of the medium was glucose 10 g; Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> 5 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.5 g; NaCl 0.2 g; MgSO<sub>4</sub>.7H<sub>2</sub>O 0.1 g; KCl 0.1 g; yeast extract 0.5 g; MnSO<sub>4</sub> 0.5 g; FeSO<sub>4</sub> 0.5 g; agar 15 g; and 1000 mL water at pH 7. The cultures of endophytic bacteria were grown overnight in an NB medium. Subsequently, the bacteria were scratched onto the Pikovskaya medium and incubated for 4 days in room temperature. The presence of a clear zone around the bacteria colony indicated the capability of endophytic bacteria to dissolve phosphate (Ghevariya and Desai 2014).

#### *Nitrogen fixation*

Analysis of nitrogen fixation was made in a semi-solid nitrogen-free medium. The medium composition was DL-malic acid 5 g, K<sub>2</sub>HPO<sub>4</sub> 0.5 g, MgSO<sub>4</sub>.7H<sub>2</sub>O 0.2 g, CaCl<sub>2</sub> 0.02 g, NaCl 0.1 g, FeSO<sub>4</sub>.7H<sub>2</sub>O 0.5 g, Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O 2 mg, MnSO<sub>4</sub>.H<sub>2</sub>O 10 mg, 0.5% bromothymol blue in 0.2 N KOH- 2 mL, 1.64% FeEDTA solvent 4 mL, agar 2 g, and 1000 mL water at pH 6.8. Endophytic bacteria were incubated for 5 days at 30 °C. The bacteria colonies with evidence of a thin layer (pellicle) were considered positive as a nitrogen fixer (Ghevariya and Desai 2014).

#### *Detection of AHL-lactonase molecularly*

The detection of gene coding AHL-lactonase was initiated by extracting the total DNA of the endophytic

bacteria using a DNA extraction kit (Geneaid blood and cultured cells). The amplification of lactonase genes was performed using a specific primer that was *aiiA1* forward (5'-ATGACAGTAAARAARCTTTATTTC-3') and *aiiA2* reverse (5'-TCACTATATATAYTCMGGGAACTC-3), with a 753 pb target size (Pan et al. 2008). The PCR cocktail with a total volume of 25  $\mu$ L consisted of 5  $\mu$ L PCR buffer 5x; 0.5  $\mu$ L dNTP 10 mM; 2.5  $\mu$ L MgCl 25 mM; 0.25  $\mu$ L Taq polymerase 5  $\mu$ L, each of primer forward and reverse was 0.1  $\mu$ L; template DNA 4  $\mu$ L and water 12.55  $\mu$ L. The PCR was conducted using a 2720 Thermal Cycler Applied Biosystem machine, with a PCR program that covered pre-denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1.5 minutes, extension at 72 °C for 2 minutes, and with a final extension at 72 °C for 5 minutes. Loading electrophoresis of the total volume of the PCR results was made in agarose 1%. The PCR results of the bacteria isolates that formed an amplicon with a designated size indicated the capacity of the bacteria to produce AHL lactonase.

#### Selection of potential endophytic bacteria isolate

The results of the endophytic bacteria characterization, which included antibiosis capacity, protease enzyme production, IAA, phosphate dissolving, nitrogen fixation and AHL lactonase, were further analyzed using the analytic hierarchy process (AHP) method, in accordance with Saaty (2008). The chosen bacteria were those with the highest scores, ranked 1-4.

#### Identification of endophytic bacteria based on the 16S rRNA gene

The four chosen isolates of endophytic bacteria based on the results of the AHP analysis were then further identified molecularly. Extraction of the total DNA of the endophytic bacteria was performed using a DNA extraction kit (Geneaid blood and cultured cells). The isolated DNA was then amplified using a bacteria universal primer consisting of primer forward 27F 5'-AGAGTTTGATCCTGG CTCAG-3' and primer reverse 1492R 5'-GGTTACCTGTTACGACTT-3', with a target 16S rRNA gene which was approximately 1500 pb in size (Jiang et al. 2006). The resulting PCR products were subsequently sent for sequencing to paid analytic institution, First Base (Selangor, Malaysia). Sequent analysis was conducted following several steps: editing sequents with the Bioedit program, and construction of a phylogeny tree using the Mega X program.

## RESULTS AND DISCUSSION

#### Characterization of pathogenic bacteria

The identification of bacteria isolates collected from eucalyptus with BWD symptoms showed similar morphology traits to *R. solanacearum* bacterium. The isolates reacted positively in fermentative-oxidative, hypersensitive and potato soft rot tests. On the other hand, the isolate reacted negatively in gram, stain, and levan tests. Colony of isolates bacteria, milky white in color, with fluidal pinkish colony center in the NAPSA media. Five pathogenic bacteria isolates were collected from five eucalyptus clones. Molecular detection on isolates Rs 10, Rs 15, Rs 16, Rs 18, and Rs 19 showed DNA amplicons matched the pathogen *R. solanacearum* species complex (RSSC) and electrophoretic visualization showed amplified of the Oli-1/Y2 primer pair resulting in DNA bands with a length of 288 pb. Alignment of the sequence DNA fragments generated from PCR with BLAST using 16S rRNA primers showed the results of the bacterial species *Ralstonia solanacearum* (Table 1).

The results of the confirmation on the pathogen bacteria based on sequencing of the 16S rRNA gene showed similarities with *R. solanacearum*. Bacteria *R. solanacearum* has a high diversity of genotypes and phenotypes, and is thus called the *R. solanacearum* species complex (RSSC). The results of the virulence test showed that isolate Rs 18 caused the highest level of wilting in the eucalyptus seedlings, reaching 90%, with average latent period of 6.6 days. Even though the pathogen isolates used was the same, the virulence levels and pathogen malignancy causing disease symptoms on the plants were diverse. Based on the results, isolate Rs 18 was used for further analysis.

#### Endophytic bacteria morpho-physiological characteristics

Total bacteria that were isolated successfully from the eucalyptus root comprised 173 isolates. Of these, 24 passed the screening for the bioagent safety test. The screening results are shown in Table 2.

**Table 2.** Screening of endophytic bacteria isolated from eucalyptus for *Ralstonia solanacearum* bioagent

Bioagent safety test	Screening of endophytic bacteria			
	Number of bacteria	Reactive reaction	Positive reaction	Negative reaction
HR Test	173	83	83	90
Hemolysis test	90	48	48	42
Pathogenicity test	42	8	8	34
Growth test	34	10	10	24

**Table 1.** Alignment results and virulence level of *Ralstonia solanacearum* isolate based on sequencing of the 16S rRNA gene

Host eucalyptus	Isolate code	Accession number	Homolog with sequencing of GenBank		Homology (%)	Virulence test	
			Species	Query cover (%)		Incidence (%)	Average incubation period (days)
Clone ep A	Rs 10	CP052129	<i>R. solanacearum</i>	99	99.28	60	8.2
Clone ep B	Rs 15	MT367730	<i>R. solanacearum</i>	100	99.64	60	13.8
Clone ep C	Rs 16	MK825301	<i>R. solanacearum</i>	99	98.74	80	8.6
Clone ep D	Rs 18	KP017467	<i>R. solanacearum</i>	99	98.26	90	6.6
Clone ep E	Rs 19	MT367730	<i>R. solanacearum</i>	100	99.71	50	8.2

An HR test was designated as the preselection method to determine whether or not the bacteria were pathogenic on plants. Necrotic symptoms on the tobacco leaves indicated the pathogenicity of the endophytic bacteria. A reactive reaction was denoted by necrotic symptoms, while no apparent necrosis indicated a negative reaction. HR will take place in plants as a response to pathogen infection (Balint-Kurti 2019). Moreover, it is essential to ensure the safe use of the endophytic bacteria in the environment, especially in relation to mammals. The capability of the endophytic bacteria lysis erythrocytes would classify the endophytic bacteria as a mammal pathogen or show a positive reaction (Suardana et al. 2014).

Even though the endophytic bacteria passed the HR selection, this did not fully demonstrate the pathogenicity and bioagent capacity of the endophytic bacteria for all plant species, as there are other bacteria that are pathogenic to tobacco; for example, *Pseudomonas syringae* (Krzymowska et al. 2007). Therefore, further analysis was required; for instance, a pathogenicity test and bacteria impact on plant growth. In the pathogenicity test, a negative reaction was represented by a 100% plant survival rate. On the contrary, a positive reaction was indicated by disease symptoms (e.g. wilt, necrosis, stunted growth, rotting, and collapse with survival rate of less than 100%). Growth analysis of the eucalyptus seedlings was made to ensure that the endophytic bacteria did not impede growth, even though it showed no signs of pathogenicity. Screening of all the isolated endophytic bacteria isolates through HR, hemolysis, pathogenicity and a growth test resulted in 24 candidate isolates of endophytic bacteria from the eucalyptus root (Table 2).

The selected endophytic bacteria were then macroscopically characterized and analyzed in a Gram test. The morphology of the endophytic bacteria was observed based on color, shape of the top and edge, elevation, and surface of the colony. Analysis of the colony's morphology and of the Gram test showed diversity amongst the isolates,

and after the results of the growth and AHP analysis, several isolates displayed apparent potential species of endophytic bacteria (Table 3). These selected potential species were then subsequently identified.

### Endophytic bacteria as biocontrol agents and plant growth promoters

Analysis of the characteristics and capacity as plant growth promoters was made on the 24 screened isolates. The characterization resulted in 18 isolates with antibiosis capacity, nine with IAA hormone, nine as nitrogen fixers, 14 with phosphate dissolving capacity, 15 with protease enzyme and nine with AHL lactonase (Figure 1).

### Selection of potential isolates based on the Analytic Hierarchy Process (AHP) and 16S rRNA analysis

The AHP method selected endophytic bacteria that exhibited excellence in several previously conducted tests. The results from these tests on the 24 isolates were tabulated and scored in terms of the excellence of each isolate. From this method, isolates with the highest scores were then chosen, namely B33/77, B32/77, B13/497, and B17/111 (Table 4).

The selected isolates of endophytic bacteria based on the AHP method were then molecularly identified. Homology analysis revealed that isolates B33/77, B32/77, B13/497 and B17/111 had the highest nucleotide homolog, at 91.62 - 99.63 % for *Serratia nemadophila* (MN691588), *Paenibacillus polymyxa* (KF439806), *Brevundimonas olei* (MT341805), and *Bacillus megaterium* (CP049296). The endophytic bacteria were identified using BLAST and compared to similar bacteria in the NCBI GenBank. When the homology is close to 100% or above 97% (Table 4), it can be confirmed as the same species. On the contrary, if the homology is less than 97%, then the species could be an entirely new or unconfirmed one (Vetrovsky and Baldrian 2013).

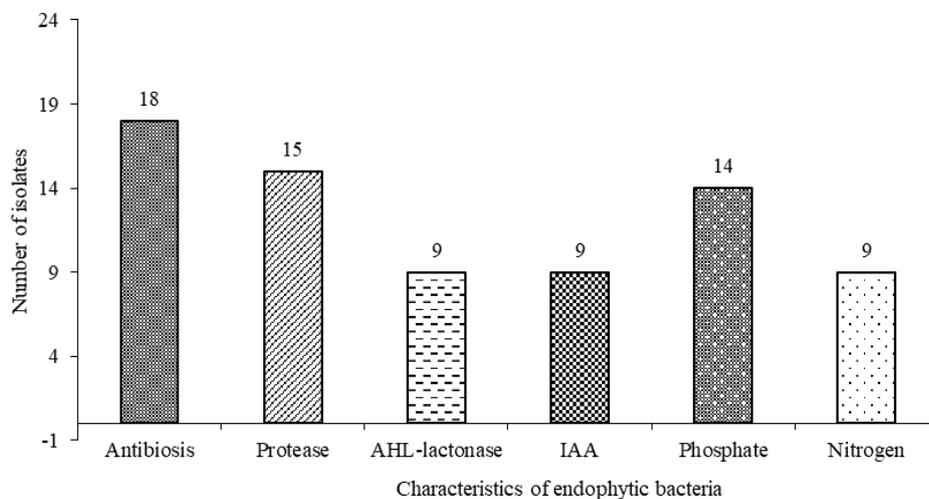


Figure 1. Characteristics of endophytic bacteria as bioagents and plant growth promoters

**Table 3.** General characteristics of endophytic bacteria from the eucalyptus root

Isolate code	Color	Shape of		Elevation	Surface	Gram
		Colony form	Margin			
B19/497	White	Round	Entire	Convex	Glistening	Positive
B8/497	Beige	Round	Rhizoid	Umbonate	Not glistening	Negative
B30/497	Beige	Round	Entire	Umbonate	Glistening	Negative
B13/497	White	Round	Entire	Umbonate	Not glistening	Negative
B4/497	Beige	Round	Entire	Umbonate	Glistening	Negative
B32/77	White	Round-Concentric	Filamentous	Convex	Glistening	Positive
B1/77	White	Round	Entire	Convex	Glistening	Positive
B34/77	White	Round	Entire	Convex	Glistening	Negative
B36/77	White	Round	Entire	Convex	Not glistening	Positive
B33/77	White	Round	Lobate	Umbonate	Glistening	Negative
B15/77	White	Round	Rhizoid	Umbonate	Not glistening	Negative
B37/77	White	Round-Concentric	Rhizoid	Raised	Glistening	Positive
B20/77	Beige	Round	Entire	Convex	Glistening	Negative
B3/5147	White	Filamentous	Filamentous	Umbonate	Glistening	Positive
B37/5147	White	Round-Concentric	Filamentous	Umbonate	Not glistening	Negative
B15/5147	Beige	Round	Lobate	Convex	Glistening	Positive
B14a/5147	Beige	Round	Entire	Plat	Glistening	Negative
B25/5147	White	Round	Entire	Umbonate	Glistening	Positive
B14/5147	Beige	Round	Entire	Umbonate	Glistening	Positive
B17/111	White	Round	Entire	Raised	Glistening	Negative
B15/111	Milky white	Irregular	Irregular	Convex	Glistening	Positive
B4/111	White	Round	Lobate	Convex	Glistening	Negative
B22/111	White	Round-Concentric	Lobate	Raised	Glistening	Negative
B13/111	White	Round	Irregular	Umbonate	Not glistening	Positive

**Table 4.** Scoring of endophytic bacteria based on excellence from several tests

Isolate code	Criteria						Result of AHP analysis	Rank	Identification of the endophytic bacteria isolates based on the 16S rRNA gene			
	A	B	C	D	E	F			Accession	Species	Query cover (%)	Identity (%)
B33/77	4	3	2	1	1	1	0.3264	1	MN691588	<i>Serratia nematodiphila</i>	100	98.45
B17/111	3	1	1	2	1	2	0.3139	2	CP049296	<i>Bacillus megaterium</i>	100	91.62
B13/497	3	1	2	2	1	2	0.3133	3	MT341805	<i>Brevundimonas olei</i>	99	98.42
B32/77	3	2	1	3	1	1	0.2853	4	KF439806	<i>Paenibacillus polymyxa</i>	100	99.63

Note: Antibiosis (A), Protease (B), Phosphate (C), IAA (D), Nitrogen (E), AHL lactonase (F)

## Discussion

Eucalyptus plants infected with BWD in the field showed symptoms of partial to complete wilting, starting with yellowing and finally drying of the leaves. Eucalyptus stems that are attacked will turn blackish in color (discoloration). If this part of the plant is cut, it will become brown rot vertically. If the stems or branches of the plant are attacked, and are then cut and left for a while, they will release a milky white liquid, which is a bacterial colony. According to Ferreira et al. (2017) and Coutinho and Wingfield (2017), eucalyptus plants infected with BWD show symptoms of wilting; yellowing of the leaves, which fall and leave bare plant stems without leaves; and when the stems are cut, the color of the xylem is changed and bacterial fluid is secreted. If the symptoms are severe, the tree will easily collapse due to infection and a defective root system. The results of the isolation of disease causes in eucalyptus show the same morphological and physiological characteristics as *R. solanacearum* (Schaad et al. 2001). The results of molecular pathogen identification also show

the same species as *R. solanacearum species complex* (RSSC). This proves that the pathogenic bacteria used in this study are *R. solanacearum* isolates, which are the cause of BWD in eucalyptus in the concession area of PT. Arara Abadi in Perawang, Riau.

The results of the isolation of endophytic bacteria from the eucalyptus roots obtained 24 isolates that passed the biosafety test. These bacteria had different morphological characteristics, which showed their diversity in the roots of eucalyptus plants. Endophytic bacteria can be isolated from all plant organs, such as leaves, roots, stems and fruit (Firdous et al. 2019), and one plant can acquire different types of them (Gaiero et al. 2013). In addition, they have different physiological characteristics, such as Gram bacteria. There are several endophytic bacteria that are Gram-negative and positive. In general, Gram-positive bacteria have a cell wall layer that is thicker than the Gram-negative type, while Gram-negative bacteria contain a higher percentage of lipids than positive ones (Wulandari et al. 2012). Although in this study most of the endophytic

bacteria obtained were Gram-negative, some were proven to be biological agents against plant pathogens, such as *Brevundimonas olei*, which can inhibit the development of *Fusarium* wilt disease in tomato plants (Islam et al. 2019).

Endophytic bacterial isolates from eucalyptus showed characteristics of antibiosis ability, protease enzymes, AHL lactonase, IAA hormone, phosphate solvent, and nitrogen-fixing. These characteristics have the potential to trigger plant growth and can suppress the development of plant diseases. Eighteen isolates showed antibiosis activity (Figure 1). The majority of endophytic bacteria produce various types of antibiotics, such as ecomycins, pseudomycins, and kakadumycins, which have an antibacterial function against pathogens (Singh et al. 2017). The endophytic bacteria *Bacillus amyloliquefaciens* has been shown to be antagonistic against various plant pathogenic bacteria, namely *R. solanacearum*, *Pseudomonas syringae*, and *Xanthomonas campestris*, and has been shown to be effective in biological control of Capsicum bacterial wilt in pot and field experiments (Hu et al. 2010). In addition, antibiosis-producing endophytic bacteria can act as an inducing agent (elicitor) for plant resistance to disease (Fadiji and Babalola 2020).

Fifteen endophytic bacteria isolated from the eucalyptus plants showed the capability to produce protease enzyme (Figure 1). Protease is a proteolytic enzyme that catalyzes the breaking down of peptide bonds in protein into simpler molecules (Rori et al. 2020). Bacteria with protease enzyme can hydrolyze casein and break down the peptide bonds of the pathogenic bacteria. The enzyme breaks protein down into peptides and amino acids, which are utilized for the growth of the bacteria. The endophytic bacteria with protease enzyme can also protect the roots of tomato and chili from *R. solanacearum* infection (Amareesan et al. 2012). Some endophytic bacteria, e.g. *Pseudomonas*, *Burkholderia*, *Bacillus*, *Serratia*, and *Paenibacillus*, acknowledged for their secondary metabolites, including antibiotic and extracellular enzymes, have shown the ability to fight plant pathogens (Khan et al. 2017).

The characteristic of endophytic bacteria of producing the AHL lactonase enzyme can prevent pathogenic bacterial infections in plants. Such bacteria from eucalyptus have the ability to produce the enzyme. Virulence factors of plant pathogenic bacteria are toxins, enzymes and exopolysaccharides (Prasannath 2013). The expression of virulence factors of plant pathogenic bacteria is controlled by the quorum sensing (QS) mechanism. Efforts that can be used to inhibit QS include the use of endophytic bacteria which produce the enzyme AHL lactonase. This enzyme can degrade and inactivate the QS signaling molecule. The mechanism of plant disease control using AHL-lactonase-producing bacteria has been widely reported, such as *R. solanacearum* (Achari and Ramesh 2015). 3-hydroxy palmitic acid methyl ester (3OH-PAME) is a QS molecule that regulates virulence factor expression in *R. solanacearum*. *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, *Rhodococcus corynebacterioides* are able to degrade 3OH-PAME, thereby reducing the expression of virulence factors (exopolysaccharides and endoglucanase)

from *R. solanacearum* in vitro and reducing the incidence of wilt in eggplant seedlings in greenhouses (Achari and Ramesh 2015). In this study, nine isolates showed endophytic bacteria with the AHL lactonase enzyme.

Endophytic bacteria with the characteristic of *Indole-3-acetic acid* (IAA) production play a role in nutrient absorption and promote plant growth; nine isolates exhibited this trait (Figure 1). IAA is the main auxin in plants that is involved in various physiological processes, such as cell signaling, regulation of plant development, and the induction of defense systems (Fu et al. 2015). The bacteria can promote plant growth through the synthesis of IAA, cytokinin and gibberellin hormones, or through the regulation of hormone internal level in plants. IAA produced by endophytic bacteria within plants can increase the number of lateral and adventive roots, thus better-facilitating nutrient access and improving root exudation, which in turn will enhance the height and biomass of the plant (Kandel et al. 2017; Olatunji et al. 2017; Guan et al. 2019). Auxin is involved in various aspects of plant growth and development. It also plays a role as a microbe-signaling molecule, directly influencing the biology of several pathogens (Kunkel and Harper 2018).

The phosphate-dissolving character of endophytic bacteria supports plants' nutrient provision. Phosphorous (P) is an essential micronutrient involved in enzymatic reaction and is accountable in most plant physiological processes (Ahemad 2014). P is the second mineral nutrient after nitrogen (N) that often limits plant growth. The average soil P is around 0.05%, but the available P that can be utilized by plants is only 0.1% of that finite amount (Alori et al. 2017). Endophytic bacteria can increase the available P by dissolving precipitated phosphates through mechanisms such as oxidation, chelation, ion exchange and organic acid production (Walia et al. 2017). This study found fourteen isolates of endophytic bacteria with the phosphate-dissolving characteristic (Figure 1).

The ability of endophytic bacteria to fix nitrogen can help provide macronutrients for plants. Nitrogen is an essential macronutrient because it is a component of proteins and enzymes, the nucleic acids that make up DNA, and chlorophyll, which makes the photosynthesis process in plants possible. In the atmosphere, much of the nitrogen is in an unavailable form (N<sub>2</sub>), which cannot be used by plants. In order for plants to use N<sub>2</sub>, nitrogen must be fixed in the form of nitrate (NO<sub>3</sub><sup>-</sup>) and ammonium (NH<sub>4</sub><sup>+</sup>) (Doty 2017; Puri et al. 2018). Endophytic bacteria can supply nitrogen from the atmosphere to their host plants by expressing nitrogenase activity. Most of the genera of endophytic bacteria, including *Achromobacter*, *Azoarcus*, *Burkholderia*, *Enterobacter*, *Gluconoacetobacter*, *Herbaspirillum*, *Klebsiella* and *Serratia*, have been reported to increase plant growth due to their ability to fix nitrogen (Hallmann et al. 1997). Some endophytic bacteria have nitrogen fixation (nifH gene) and denitrification genes. The *Paenibacillus polymyxa* P2b-2R isolates obtained from lodgepole pine tissue was able to colonize the rhizosphere and endosphere niches of maize plants and encourage their growth (Liu et al. 2017). Only nine isolates from this study showed this N fixation capacity (Figure 1).

The filtering, characterization, selection and identification of the endophytic bacteria based on the sequence of the 16S rRNA gene from the eucalyptus resulted in *Serratia nematodiphila*, *Paenibacillus polymyxa*, *Brevundimonas olei*, and *Bacillus megaterium* species. The collected isolates of *S. nematodiphila* showed antibiosis, protease enzyme, phosphate dissolving and nitrogen fixation capacity, while *P. polymyxa* showed characteristics of antibiosis, protease enzyme and IAA hormone. *B. olei* revealed traits of antibiosis, phosphate dissolving, IAA hormone, nitrogen fixation and AHL lactonase enzyme, while the isolates of *B. megaterium* exhibited capacity for antibiosis, IAA hormone and AHL lactonase enzyme. The results of this study are in accordance with previous ones that have reported these species of endophytic bacteria as bioagents to control plant diseases (Timmusk et al. 2019; Soenens and Imperial 2020; Vasconez et al. 2020).

Therefore, endophytic bacteria collected from eucalyptus plants showed significant potential as bio-control agents against BWD in eucalyptus both in nurseries and fields. The collected endophytic bacteria that have already been successfully characterized could be further explored for other plant disease controlling mechanisms; for example, to attest the defense induction, plant growth promoter and pathogen suppression mechanisms.

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