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CHARACTERIZATION OF EGGPLANT ENDOPHYTE BACTERIA AND RHIZOBACTERIA AS WELL AS THEIR ANTAGONISTIC ABILITY AGAINST *Ralstonia solanacearum*

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ABSTRACT

***Characterization of eggplant endophyte bacteria and rhizobacteria as well as their antagonistic ability against* Ralstonia solanacearum**. Bacterial wilt caused by *Ralsonia solanacearum* is one of important diseases causing severe loses in eggplant production. Various strategies were used to manage bacterial wilt, including planting resistant varieties, soil amandement, and soil solarization. However, management of *R. solanacearum* in eggplant by using endophytic bacteria and rhizobacteria were not been done that much. The objective of this study was to: (1) characterization of endophytic and rhizobacteria; (2) determines the inhibition ability of endophytic and rhizobacteria isolates against *R. solanacearum* pathogen on eggplant. The laboratory experiment was arranged in completely randomized design with 5 treatments and 5 replications. The double layer method using yeast peptone glucose agar (YPGA) medium was used in vitro test. Based on the morphological characteristics these isolates were suspected as a member of genus Bacillus. Among the isolates used in this study, TK isolate showed the best capability to inhibit growth of *R. solanacearum*.

Key words: endophytic bacteria, *Ralsonia solanacearum,* rhizobacteria

INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* is one of the important diseases of eggplant. Dewi *et al*. (2014) stated that wilting in horticultural crops can reduce yields up to 90%. The *R. solanacerum* has a wide range of hosts including tomato, potato, legumes, several monocots, trees, and shrubs such as mulberries, olives, cassava, and eucalyptus, as well as certain ecotypes such as *Arabidopsis thaliana* (Genin & Boucher, 2002).

According to Ramesh & Phadke (2012), diversity in *R. solanacearum* strains could cause difficulties in the management of bacterial wilt in eggplant and other plants. This is due to the ability of *R. solanacearum* to survive in poor soil conditions, wide hosts range including asymptomatic hosts, and efficient in host attack mechanisms. Pathogen controls for *R. solanacearum* usually done by applying synthetic pesticides, however, excessive use of pesticides in long term could cause pathogen resistance. Therefore, there is a need for environmentally friendly disease control (Dewi *et al*., 2014).

Ramesh *et al*. (2009) stated that endophytic bacterial colonies have a specific relationship with plant pathogens, especially the one that causes withering in vascular vessels, which may be used as potential candidates as biological agents. Endophytic bacteria that live in plant tissue is not causing any substantial damage, not giving any benefit nor causing any symptoms of disease in plants (Reinhold-Hurek & Hurek, 2011).

Endophytic bacteria have been reported to have the ability as biocontrol agents against bacterial wilt disease (Achari & Ramesh, 2014). Based on the results of in vitro test, the endophytic bacteria proved to be antagonistic towards *R. solanacearum*. These bacteria were able to produce volatile compounds and inhibiting compounds, including HCN, ammonium, acetoin, and siderophore. This study was aimed to examine the characteristics of endophytic and rhizosphere bacteria as well as their ability against *R. solanacearum*.

MATERIALS AND METHODS

Research Site. This research was conducted at the Laboratory of Plant Protection, Universitas Jenderal

Soedirman, Purwokerto, Banyumas Regency. The study began in May 2018 and finished in February 2019.

Isolation of Endophytic Bacteria. Endophyte bacteria were isolated from the root tissue of healthy eggplant. Plant root samples were collected and washed in tap water after that the root was cut ± 2 cm and sterilized by soaking in 70% alcohol for ± 60 seconds then washed in sterile water twice. The root sample then crushed using porcelain mortar and put it into a test tube containing sterile water. Root samples that have been mixed with sterile water were diluted to 10^{-3} (Pranoto *et al*., 2014). After that, the suspension at the 10^{-3} dilution was grown on tauge extract agar (TEA) medium (200 g bean sprouts; 20 g agar; 20 g glucose; 1000 mL water) in a 9 cm sterile petri dish by using quadrant streak method and incubated for 2 days at room temperature. A single bacterial colony then transferred onto a new TEA medium to be purified until single culture were obtained (Afizar & Parlina, 2017). Endophytic bacterial isolates then observed for its morphological characteristics of the colony and cells including shape, edge, and color of the colony, cell shape, as well as cell wall properties (gram type).

Isolation of Rhizosphere Bacteria. Rhizosphere bacteria were isolated from soil around the roots of healthy eggplants. As much as 10 g of soil were suspended in 90 mL of aquadest (Nawangsih *et al*., 2014) then homogenized using vortex mixture. The results of 10^{-1} dilution were incubated in oven at 80 °C for 30 minutes (Mukamto *et al*., 2015). Then 10^{-2} dilution was carried out for bacterial isolation on TEA medium using streak method. Rhizosphere bacterial isolates were characterized by observing the colony characteristics such as shape, edge, elevation or height and color, as well as cellular characteristics such as gram properties by using 3% KOH, and catalase tests which were carried out by taking 1 ose of bacteria and then dripping with 10% H_2O_2 solution, bubbles formed were observed. In addition, microscopic observations were also conducted by using a NIKON Binocular Xsz-107 microscope with magnifications of 10^3 including the grams stain, cell shape, and endospores.

Isolation of *R. solanacearum* from Wilted Eggplant. *R. solanacearum* was isolated from the roots of eggplant plants which showed symptoms of wilting. The root tissue was taken ± 1 cm then cut using a sterile knife (Kuswinanti *et al*., 2014). The root section then surface sterilized using 70% alcohol for 5 seconds by immersed and rinsed with sterile water 3 times (Setyari *et al*., 2013). The root pieces then crushed using a mortar and added with 1 mL of sterile water (Kuswinanti *et al*., 2014). Single culture of *R. solanacearum* was isolated from the suspension by quadrant streaking in specific casamino peptone glucose (CPG)-tryphenyl tetrazolium chloride (TTC) medium agar and incubated for 72 hours at room temperature. The virulent *R. solanacearum* colonies which were characterized by its irregularly shaped, fluidal, and pink in color then selected for further purification and incubation using TEA medium at room temperature (Setyari *et al*., 2013). *R. solanacearum* isolate was inoculated in Mustang F1 eggplant by sprinkling it on injured roots at 14 days after planting to ensure its pathogenicity (Rahmawanto *et al*., 2015).

Ability of Endophyte and Rhizosphere Bacteria Against *R. solanacerum*. The study was conducted using a completely randomized design with 5 treatments and 5 replications. In vitro test of endophytic bacteria ability inhibiting the cause of bacterial wilt was carried out using yeast pepton glucose agar (YPGA) with a double layer method with 0.6% water agar (Ghosh *et al*., 2007; Priatiningsih & Djatmiko, 2016). *R. solanacearum* that grew on the YPGA slant were harvested after 2 days by adding 10 mL of sterile water to the test tube (Prihatiningsih *et al*., 2017). After that, the endophytic and rhizosphere bacteria were cultured on a petri dish containing 10 mL of YPGA medium and incubated for 48 hours at room temperature. The petri dish then turned and dropped with 0.5 mL of chloroform on the lid and left for 3–4 hours until the chloroform was completely evaporated. Then the petri dish was turned back to its original position. After that, the surface of the medium was poured with 0.2 mL of *R. solanacearum* suspension in 4 mL of 0.6% water agar at 45 °C and incubated for 24 hours at room temperature (Djatmiko *et al*., 2007).

Growth inhibition of *R. solanacerum* by endophyte and rhizosphere bacteria was characterized by the formation of clear zones around endophyte and rhizosphere bacterial colonies. Observations were made on the criteria for the strength of antibacterial ability based on Davis & Stout (1971), with the formation clear zone diameter as follows: < 5 mm= weak; 5–10 mm= medium; 10–20 mm= strong; > 20 mm= very strong.

Inhibition index was calculated using the following formula (Nafiah *et al*., 2017):

$$\text{Inhibition index} = \frac{\text{Clear zone diameter}}{\text{Colony diameter}}$$

Data Analysis. The data obtained were analyzed by ANOVA using DSAASTAT program and followed by least significant difference (LSD) test at significant level 5%.

RESULTS AND DISCUSSION

Endophyte and Rhizosphere Bacteria Isolation. Three endophyte bacteria isolates (AKa, AKb, dan AKc) and one rhizosphere bacteria isolate (TK) were obtained. The bacterial colonies found were white, dull white, and cream. Two isolates (AKa and AKc) had smooth edges, while the other two isolates (AKb and TK) had wavy edges (Figure 1; Table 1).

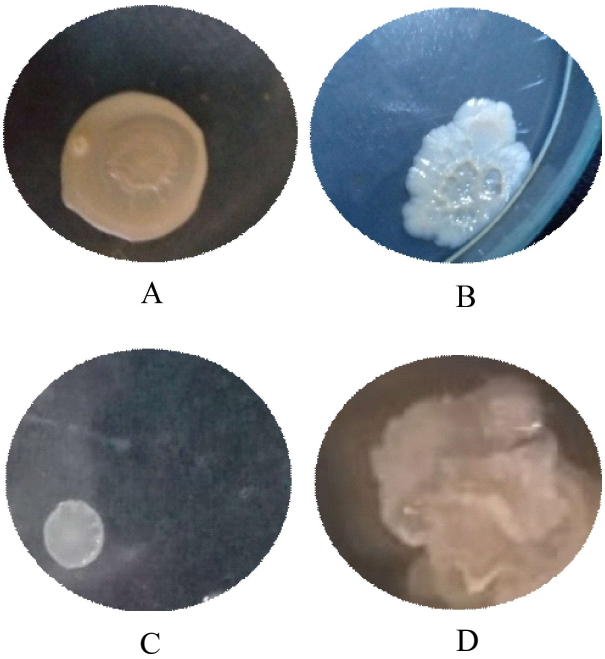

Figure 1. Colony of isolated bacteria. (A) AKa: root endophyte bacteria; (B) AKb: root endophyte bacteria; (C) AKc: root endophyte bacteria; (D) TK: rhizosphere bacteria.

Table 1. Characteristics of endophyte and rhizosphere bacteria isolated from eggplant

Characters	Isolates			
	AKa	AKb	AKc	TK
Colony form	Irregular	Irregular	Irregular	Irregular
Colony edge	entire	undulate	entire	undulate
Elevation	Flat	Flat	Flat	Flat
Color	Cream	Dull white	White	Cream
Surface	Smooth	Rough	Rough	Rough
Consistency/ texture	Buttery	Buttery	Buttery	Buttery
Cell shape	Rod	Rod	Rod	Rod
Gram	+	+	+	+
KOH3%	+	+	+	+
Catalase production	+	+	+	+
Endospore	+	+	+	+
R. solanacerum inhibition	Medium	Medium	Strong	Strong

(A) AKa: root endophyte bacteria; (B) AKb: root endophyte bacteria; (C) AKc: root endophyte bacteria; (D) TK: rhizosphere bacteria.

Characterization results showed that all the isolates were gram positive and produce endospores (Table 1). These results indicated that all four isolates were thought to be member of genus Bacillus. Hatmanti (2000) stated that *Bacillus* spp. had different colony forms on the TEA medium. Bacterial colonies were generally white to yellow or gloomy white, the edges of the colony were vary but generally undulate, rough withdry surface, and some even tend to powdery, large colonies and not shiny. Breed *et al*. (1957) reported that Bacillus was characterized as a gram positive, rod shaped capable of producing endospores which were cylindrical, ellipsodial or spherical, and which were located in the center of the cell, subterminally or terminally. Some species of Bacillus were capable of growth at 55 °C. single-cell, and size around (0.5–2.5) x (1.2–1.0) µm, produce catalase enzyme, form endospores that can survive in hot, dry and other damaging environmental conditions (Soesanto, 2013).

***Characteristics R. solanacearum* Isolate.** The isolated *R. solanacearum* on TEA medium showed an irregular, white and mucoid colony, whereas on CPG-TTC medium the isolate showed an irregular shape, white with a pink, and mucoid at the center (Figure 2). Nasrun *et al*. (2007) reported that *R. solanacearum* isolates that grew on YPA medium plus TTC with 24-hours incubation, will form white colony with mucoid and pink center. This characteristics was specific for virulent type of *R. solanacearum*.

According to Moorman (2011), *R. solanacearum* was a gram-negative, rod-shaped bacteria with a size of 0.5–1.5 µm, moved with one or more flagella, aerobic, able to reduce nitrates and produced ammonia. These

Table 2. Characteristics comparison of *R. solanacerum* isolated from wilted eggplant and identified culture collection

Characters	Isolated *R. solancearum* from wilted eggplant	Identified *R. solanacearum* from culture collection (Nasrun *et al*., 2007)
Colony form	Mucoid, irregular	Mucoid
Colony color	White	White
Gram	-	-
Catalase	+	+
Fluorescent	-	-

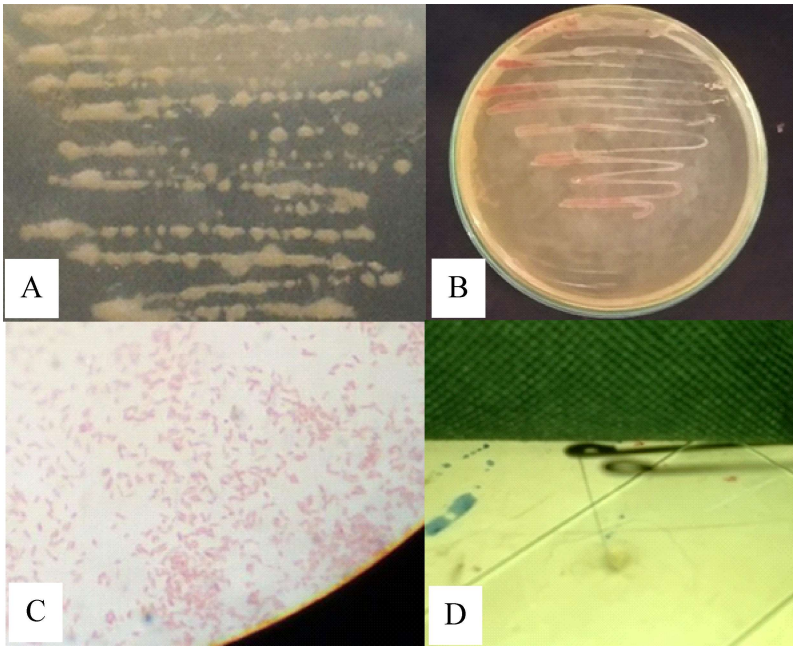

Figure 2. (A) Colony of *R. solanacearum* on TEA medium; (B) Colony of *R. solanacearum* on CPG-TTC medium; (C) Cell morphology of *R. solanacearum*; (D) KOH test of *R. solanacearum*.

bacteria were classified into several races based on different host ranges and Biovar based on biochemical properties (carbon sources). Another characteristic of *R. solanacearum* was not forming any fluorescent pigments, catalase and kovac's oxidase positive, chemo organotroph, unable to grow at 4 °C or 40 °C, grows on medium containing 1% NaCl, but does not grow on medium containing 2% NaCl (OEPP/EPPO, 2004).

Inhibition Ability of Endophyte and Rhizosphere Bacteria. The results showed that endophyte and rhizosphere bacteria isolated from eggplant significantly affected the growth of *R. solanacearum* compared to controls. This was indicated by the formation of clear zones around endophyte and rhizosphere bacteria isolates (Figure 3). However, between these bacteria, the inhibition ability were not significantly different (Table 3). The isolate TK had the highest inhibitory index (3.0) and followed by AKa isolate (2.94) (Table 3). Inhibitory zones formed by antagonistic bacteria against *R. solanacearum* were caused by the presence of secondary metabolites which have anti bacteria activity. The difference in diameter of the inhibition zone was probably due to the differences in the types of antibacterial compounds produced by each bacterial isolates (Kusumawati *et al*., 2014). The mechanism of antibacterial compounds was by disrupting the peptidoglycan component of bacterial cells so that the cell wall layer were not intact and causing cell death. The antibacterial compound could react to several targets in the bacterial membrane, causing damage or autolysis and also stunted growth or even death (Sukmawaty *et al*., 2016). According to Iqlima *et al*. (2017), the effectiveness of antibacterial activity were due to the physical properties of the compound. This can be seen through the length of the chain, the ability to penetrate the cell wall, the integrity of the molecules in the cell and their hydrophilic or lipophilic properties.

The results showed that all isolates had bacteriostatic mechanisms in inhibit the growth of *R. solanacerum*. These antagonistic bacteria were unable to kill *R. solanacearum*. This is indicated by the change of NB medium to become turbid (Figure 4). According to Pratiwi (2017), antibiotics that plays a role as bacteriostatic can inhibit bacterial development and allow the host immune system to take over inhibited bacterial cells. Bacterial protein synthesis inhibitors have a bacteriostatic effect by interfering with protein synthesis without disrupting normal cells and inhibiting the stages of protein synthesis, changing cell membrane permeability by removing cell membrane permeability thereby causing cells to become lysis.

A B C D E

Figure 3. Inhibition ability of isolated bacteria against *R. solanacearum* in vitro. (A) control; (B) Aka: root endophyte bacteria; (C) AKb: root endophyte bacteria; (D) AKc: root endophyte bacteria; (E) TK: rhizosphere bacteria.

Table 3. Screening result of antagonist bacteria and inhibition ability against *R. solanacerum*

Isolates	Clear zone (mm)	Inhibitory index	Inhibition mechanism	Inhibition ability
Control	0.0 a	0.0	-	Neutral
AKa	9.5 b	2.94	Bacteriostatic	Medium
AKb	9.4 b	2.37	Bacteriostatic	Mediaum
AKc	11.8 b	2.83	Bacteriostatic	Strong
TK	14.4 b	3.00	Bacteriostatic	Strong

(A) AKa: root endophyte bacteria; (B) AKb: root endophyte bacteria; (C) AKc: root endophyte bacteria; (D) TK: rhizosphere bacteria.

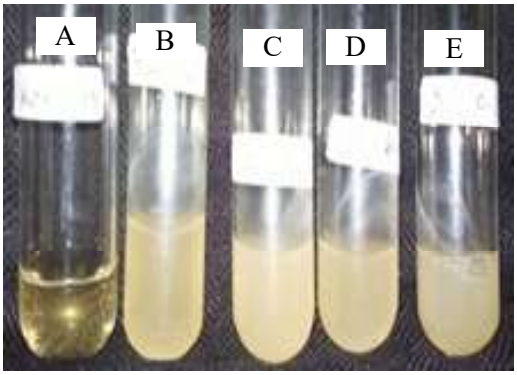

Figure 4. Inhibition ability of isolated bacteria against *R. solanacearum* in NB medium. (A) control; (B) TK: rhizosphere bacteria; (C) AKa: root endophyte bacteria; (D) AKb: root endophyte bacteria; (E) AKc: root endophyte bacteria.

CONCLUSION

In total, four bacterial isolates were collected from root endophyte and rhizosphere of eggplant. Based on the morphological characteristics these isolates were suspected as a member of genus Bacillus. All isolated bacteria had ability inhibiting growth of *R. solanacerum*. TK isolates collected from rhizosphere showed best ability to inhibit the growth of *R. solanacearum*.

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