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

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Manuscript received: 23 May 2019. Revision accepted: 14 August 2020.

ABSTRACT

Characterization of eggplant endophyte bacteria and rhizobacteria as well as their antagonistic ability against Ralstonia solanacearum. Bacterial wilt caused by Ralstonia 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, Ralstonia 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. solanacearum 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% H₂O₂ solution, bubbles formed were observed. In addition, microscopic observations were also conducted by using a NIKON Binocular Xsz-107 microscope with magnifications of 10³ 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. solanacearum. 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 (Priatiningsih 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. solanacearum 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.](image)

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

| Characters              | 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 members of the 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 and dry 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, ellipsoidal 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.

| Characters                  | Isolated *R. solanacearum* 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 antibacterial 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.

![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.](image)

| 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      | Medium            |
| 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.
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. solanacearum*. TK isolates collected from rhizosphere showed best ability to inhibit the growth of *R. solanacearum*.

ACKNOWLEDGMENTS

We like to acknowledge the Head of the Plant Protection Laboratory and Screen House Experimental Farm, Faculty of Agriculture, Jenderal Soedirman University for the facilities and infrastructure provided.

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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.

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