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Selection and Characterization of Endophytic Bacteria as Biocontrol Agents of Tomato Bacterial Wilt Disease

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Biological control of bacterial wilt pathogen (*Ralstonia solanacearum*) of tomato using endophytic bacteria is one of the alternative control methods to support sustainable agriculture. This study was conducted to select and characterize endophytic bacteria isolated from healthy tomato stems and to test their ability to promote plant growth and suppress bacterial wilt disease. Among 49 isolates successfully isolated, 41 were non-plant pathogenic. Green house test on six selected isolates based on antagonistic effect on *R. solanacearum* or ability to suppress *R. solanacearum* population in dual culture assays obtained BC4 and BL10 isolates as promising biocontrol agents. At six weeks after transplanting, plants treated with BC4 isolate showed significantly lower disease incidence (33%) than that of control (83%). Plants height was not significantly affected by endophytic bacterial treatments. Based on 16S rRNA sequence, BC4 isolate had 97% similarity with *Staphylococcus epidermidis* (accession number EU834240.1), while isolate BL10 had 98% similarity with *Bacillus amyloliquefaciens* strain JK-SD002 (accession number AB547229.1).

Key words: biological control, endophytic bacteria, *Ralstonia solanacearum*, *Staphylococcus epidermidis*, tomato

INTRODUCTION

Ralstonia solanacearum, the causal agent of bacterial wilt disease, is one of the most damaging pathogens of tomato in Indonesia. Farmers have controlled the disease using several measurements including application of bactericides, use of resistant varieties, and implementation of cultural practices, but fail to obtain disease reduction. Concern on the harmful effects of bactericidal application has led to the development of alternative control method using biocontrol agents.

Several groups of bacteria have been explored for biocontrol of pathogens. Plant Growth-Promoting Rhizobacteria (PGPR) are one of the most studied biocontrol agents. However, PGPR are usually effective at laboratory or green house scale, and often fail to provide effective disease suppression in a field. Another group of bacteria called endophytic bacteria which live inside the plants can be explored as effective biocontrol agents (Reiter *et al*. 2002).

Endophytic bacteria colonize healthy plant tissue without causing symptoms or damages to the host (Hallmann *et al*. 1997). Bacterial endophytes were isolated from sugar beet (Dent *et al*. 2004), prairie plants, agronomic crops (Zinniel *et al*. 2002), potato varieties (Sessitsch *et al*. 2002), *Abelmoschus esculentus* (Vetrivelkalai *et al*. 2010), *Eucalyptus* spp. (Procopio *et al*. 2009), and sugarcane (Magnani *et al*. 2010). Endophytic bacteria can promote the plant growth by producing phytohormones (Feng *et al*. 2006), siderophores (Burd *et al*. 1998), and increasing resistance to pathogens (Reiter *et al*. 2002). These bacteria enter the plant tissue mainly through rootlets. Other plant parts i.e. flower, stem, and cotyledone can also become their entry points (Zinniel *et al*. 2002). Bent and Chanway (2002) hypothesized that host plants can get benefit from endophytic bacteria for nutrition, pollutant catabolism, and elevated defense response to abiotic stress or pathogen's attack.

The objective of these experiments were to select and characterize endophytic bacteria isolated from tomato stems, and to investigate whether they can act as plant growth-promoter or as biocontrol agents of tomato bacterial wilt disease.

MATERIALS AND METHODS

Isolation of Endophytic Bacteria. Tomato plants with no apparent disease symptoms were collected from fields in Bogor, Cipanas, and Lembang , West Java Province of Indonesia. Stems were cut into 5-cm pieces and then surface sterilized by sequential immersion in ethanol 70% for 1 minute, 2% of NaOCl for 3 min, and 70% of ethanol for 30 sec, followed by three washes in distilled water and blotted dry on sterile filter paper. Both ends of each stem were burnt on a flame and fragmented to about 1 cm segments. The success of surface sterilization was checked by rolling the stem pieces on the surface of Nutrient Agar (NA) medium. Succeeded sterilization was indicated by

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no bacterial growth on the medium after three days of incubation. Each piece of stem was macerated in a sterile mortar and re-suspended in 5 ml of phosphate buffer. Aliquots of 50 µl from a serial dilution up to 10^{-5} were plated on NA medium in duplicate. Plates were incubated at room temperature (± 28 °C) for 24-48 h. Bacterial colonies were purified on fresh NA medium. Pure isolates were preserved in 20% glycerol solution and stored at -4 °C.

Pathogenicity Test of Endophytic Bacteria. The endophytic bacteria were cultivated on King'B Agar for 48 h at 28 °C and suspended in sterilized distilled water. Bacterial suspensions were adjusted to 10^8-10^9 cfu/ml using spectrophotometer. Suspension of each endophytic bacteria isolate was infiltrated into the lamina on abaxial/ adaxial side of tobacco leaves using a disposable syringe. Inoculated tobacco plants were incubated for 24 h. Isolates inciting the development of chlorotic to necrotic zone on the leaf area (hypersensitive reaction, HR) were potent plant pathogenic and were excluded from subsequent screening as biocontrol agents.

Screening of Endophytic Bacteria for Antibiosis and Competition Mechanisms *In Vitro*. The antibiosis and competition mechanism test was conducted to investigate the ability of endophytic bacteria to suppress the *in vitro* growth of *R. solanacearum* in dual culture assay. One milliliter suspension of *R. solanacearum* (10^7-10^8 cfu/ml), prepared by suspended one loopfull of bacteria growing on KB agar for 48 h at 28 °C into 10 ml of sterilized distilled water, was added into 10 ml of melted KB agar (± 55 °C). The agar medium was allowed to solidify and air dried. A piece of sterile filter paper (diameter 0.5 cm) was placed on the center of the agar and then dripped with 20 µl suspension of endophytic bacteria (10^7-10^8 cfu/ml). Filter papers dripped with sterile distilled water were used as controls. Treatments were replicated three times. After incubation at room temperature (± 28 °C) for 48 h the diameter of inhibition zone produced by endophytic bacteria was measured.

Endophytic bacteria which did not show inhibition zone activity were tested for their competitiveness ability to grow with *R. solanacearum* in KB broth medium. One milliliter suspensions of *R. solanacearum* (10^8-10^9 cfu/ml) and 1 ml suspension of the endophytic bacteria (10^8-10^9 cfu/ml) were added into 50 ml of 10% KB broth in 250 ml Erlenmeyer flasks. One ml of sterile distilled water and 1 ml of *R. solanacearum* suspension added into KB broth in Erlenmeyer flasks were provided as control. Flasks were incubated on a shaker (100 rpm) at room temperature for 24 h. After a serial dilution, 100 µl aliquots from each dilution were spread on KB agar plate in duplicate. *R. solanacearum* colonies were counted after incubation at 28 °C for 24 h.

Effect of Endophytic Bacteria on Tomato Plant Growth. Six endophytic bacteria isolates consisting of three isolates which produced the widest inhibition zones and three isolates which did not produce inhibition zones were tested for their effects on tomato plants growth in green house. Each isolate was cultivated on King'B Agar for 48 h at 28 °C and suspended in sterilized distilled water. Bacterial suspensions were adjusted to 10^8-10^9 cfu/ml using spectrophotometer. Tomato seeds (cv. Arthaloka) were sown in a pot tray (30 x 50 cm) consisted of 60 holes and filled with a 1:1 mixture of steam-sterilized soil and compost. One week after sowing, seedlings were uprooted, thoroughly rinsed in water to dislodge soil from the root system, and subsequently dipped in the suspension of each endophytic bacterium (10^7-10^8 cfu/ml) for 12-14 h. Seedlings roots of tomato plants dipped in sterile distilled water were used as control. Seedlings were then transplanted to polybags (diameter 20 cm) filled with 500 g of sterile mixture of soil and compost (1:1). Treatments were replicated three times. Two weeks after treatment (WAT), plant height was measured weekly until 6 WAT.

Data were plotted into the plant height growth curve with time (days after treatment) as abscissa (X) and plant's height as coordinates (Y). Total area under the curve (AUPHGC) of each isolate was calculated using the formula reported by Cooke (1998):

$$\text{AUPHGC} = \sum_{i=1}^{n} [(X_{i+1} + X_i)/2] \times [t_{i+1} - t_i]$$

Effect Selected Endophytic Bacteria on Bacterial wilt Incidence. Tomato seedlings (cv. Arthaloka) were raised in green house and treated with the six endophytic bacteria using the methods as described previously. Tomato seedlings dipped in sterile distilled water were used as controls. Seedlings were transplanted to polybags (diameter 20 cm) filled with 500 g of a mixture of sterile soil and compost (1:1). The top of soil was covered with 250 g of soil infested with *R. solanacearum* which was then covered with another 250 g of sterile soil. Infested soil was prepared by pouring an amount of suspension of *R. solanacearum* to sterile soil and the population of the bacteria was adjusted to 10^7-10^8 cfu/g soil. Treatments were replicated three times. Four weeks after treatment infected plants were counted weekly until 6 WAT. Percentage of Disease Incidence (PDI) was calculated using formula:

$$\text{PDI} = (n/N) \times 100\%,$$

where: n = number of infected plants, and N = total number of observed plants.

Data were plotted into the disease progress curve with time (days after treatment) as abscissa (X) and PDI as coordinates (Y). Total area under the curve (AUDPC) of each isolate was calculated using the formula reported by Cooke (1998) and Bowen (2004):

$$\text{AUDPC} = \sum_{i=1}^{n} [(Xi+1 + Xi)/2] \times [ti+1 - ti]$$

Characterization and Identification Selected Endophytic Bacteria. Four isolates of the endophytic bacteria were characterized based on conventional methods including microscopic and colony appearances, and physiological and biochemical properties, following the methods of Schaad *et al*. (2001). Two isolates were identified to species levels by comparing sequence analysis of their 16S rRNA gene. DNA was extracted from log phase culture using phenol-chloroform extraction

procedure (Sambrook & Russel 2001). The 16S rRNA gene was amplified using the forward primer 16 SF (5'-CAGGCCTAACACATG-CAAGTC-3') and reverse primer 1387 R (5'-GGGCGGWGTGTA-CAAGGC-3'). Total volume reaction for PCR was 20 µl containing 1x of Ex-Taq buffer 2 µl, pNTP 1.6 µl or 10-20 ng DNA, Ex-Taq 0.1 µl. PCR was perform under the following conditions: one cycle of predenaturation at 94 °C for 4 min, annealing at 64 °C for 30 sec, and extension at 72 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 64 °C for 30 sec, and extension at 72 °C for 30 sec. The reaction was terminated with a 7-min final extension at 72 °C. The PCR products were sent to MACROGEN Inc., Geumchun-gu, Seoul, South Korea, for sequencing. BLAST searches were performed for sequences obtained to find the similarity with sequence data in GeneBank.

Data Analysis. Plant growth, PDI, AUDPC, and AUPHGC data were statistically analyzed by ANOVA as completely randomized block design. Treatment means were compared using the DMRT test at 5% level of significance. All statistical analyses were done using SAS Program version 9.0.

RESULTS

A total of 49 endophytic bacteria isolates were successfully recovered from tomato stems. Nearly equal number of isolate was obtained from each location: 17 isolates from Bogor, 18 from Cipanas, and 14 from Lembang. Forty-one isolates not plant pathogenic as it did not cause HR on tobacco leaves. Further test on these isolates obtained 17 isolates which showed antibiosis activities to *R. solanacearum* by producing inhibition zone from 1 to 5 mm diameter (Table 1). The widest inhibition zone was produced by BC4 isolate. The remaining 23 isolates which had no antibiosis activity were tested for their ability to suppress *R. solanacearum* population in liquid medium. Based on these initial in vitro screening, six isolates (AC1, BC4, BC5, BC10, BL10, and BL17) were selected and tested for their ability to promote plant growth and to suppress bacterial wilt incidence in green house. Isolate BC4, BC5, and BC10 isolates were selected on the basis of wide inhibition zones, where as AC1, BL10, and BL17 isolates were selected for their positive *in vitro* growth characteristics including ability to grow fast, easy to be re-cultured, and ability to maintain colony characteristics during sub culturing.

Table 1. Maximum diameter of the inhibition zone produced by the isolates of the endophytic bacteria from tomato stems

Isolates	Inhibition zone (mm)	Isolates	Inhibition zone (mm)	Isolates	Inhibition zone (mm)
AB2	1.0	BB7	1.0	BC7	4.0
AB4	1.0	AC2	3.0	BC9	3.0
AB9	2.0	AC3	4.0	BC10	4.0
AB10	3.0	AC8	4.5	BL14	2.0
BB1	2.0	BC4	5.0	BL32	1.5
BB5	1.0	BC5	4.5		

Effect of Endophytic Bacteria to the Height of Tomato Plants and Disease Incidence. Six isolates of endophytic bacteria were tested for their growth promotion activity and the effectiveness to control the bacterial wilt disease in the green house. Three isolates positively produced inhibition zones, i.e. BC4, BC5, and BC10 isolates and three isolates negatively produced inhibition zone, i.e. AC1, BL10, and BL17 isolates. Disease incidence on plants treated with BC4 and BC10 were suppressed on six weeks after planting (Table 2). Averages of disease incidence on both treatments were up to 33.33 and 40.00%, respectively, and there were significantly different compared with those on control treatment, i.e. 83.33%.

Total effects of the endophytic bacteria to the disease incidence during observation were shown by the calculation of total area under disease progress curve (AUDPC). Based on the AUDPC values of each isolate on Table 2, it can be stated that all of the endophytic bacteria were able to suppress the disease progress compared with control (840.0 units). The highest suppression, shown by the lowest value of AUDPC, was caused by BC4 isolate (361.7 units). The second highest suppression was performed by BC10 isolate with AUDPC value was up to 478.3 units.

Effects of the endophytic bacteria to the height of the plants were shown on Table 3. Two weeks after treatment, the tallest tomato plants were shown by tomato seedlings treated with the endophytic bacteria isolate AC1 and BL10, i.e. 5.68 and 5.70 cm, while the shortest were shown by the seedlings treated with BC4 and BC5 isolates, it was 4.92 and 4.82 cm, respectively. Four weeks after treatment, growth promotion was shown by AC1, BC4, and BC10 isolates. Seedlings treated with this isolate produced the highest tomato plants, it was up to 34.70 cm, and significantly different compared with those on control treatment which up to 28.58 cm. Heights of the plant were continuously increased, but on five and six weeks after treatment, heights of the plants treated with most of the

Table 2. Effects of the endophytic bacteria on tomato bacterial wilt disease suppression

Isolates	Percentage of disease incidence (%)*			AUDPC***
	4 WAT**	5 WAT	6 WAT	
Control	3.33 ± 5.77a	70.00 ± 17.32a	83.33 ± 5.77a	840.0
AC1	0.00 ± 5.77a	50.00 ± 10.00ab	53.33 ± 11.55ab	595.0
BC4	0.00 ± 0.00a	33.33 ± 28.87ab	33.33 ± 28.87b	361.0
BC5	0.00 ± 0.00a	50.00 ± 10.00ab	53.33 ± 11.55ab	356.7
BC10	10.00 ± 17.32a	23.33 ± 25.17b	40.00 ± 10.00b	478.3
BL10	6.67 ± 11.55a	36.67 ± 30.55ab	43.33 ± 35.12ab	525.0
BL17	6.67 ± 11.55a	36.67 ± 32.15ab	46.67 ± 25.17ab	536.7

*Means followed by the same letter are not significantly different according to Duncan's multiple range tests at the 5% level, *WAT = Week After Treatment, **Area under disease progress vurve.

Table 3. Effects of the endophytic bacteria to the growth of tomato plants

Endophytic bacteria (code of isolates)	Height of plants (cm)					AUHPGC***
	2 WAT*	3 WAT	4 WAT	5 WAT	6 WAT	
Control	5.29abc**	12.32ab	28.58c	53.90a	58.30a	886.17
AC1	5.68a	13.00ab	34.70a	54.75a	58.25a	940.91
BC4	4.92bc	12.73ab	32.15ab	56.92a	60.35a	941.05
BC5	4.82c	12.11ab	31.17bc	49.00a	53.90a	851.66
BC10	5.48ab	13.25a	34.29ab	55.75a	58.55a	947.31
BL10	5.70a	11.30b	28.90c	51.95a	56.25a	861.78
BL17	5.35abc	11.70ab	25.08cd	38.50b	41.58b	687.02

*WAT = Week after treatment, **Means followed by the same letter are not significantly different according to Duncan's multiple range tests at the 5% level, ***Area under height of plant growth curve.

Table 4. Physiological and biochemistry characters of the endophytic bacteria

Characters	Bacterial isolates code			
	AC1	BC4	BL10	BL17
Gram reaction	–	–	+	+
Cells shape	Rod	Rod	Rod	Rod
Motility	Non-motile	Non-motile	Dubious	Motile
Spore formation	–	–	–	–
Un-aerobic growth	–	–	–	–
Catalase	+	+	+	+
Oksidase	+	+	–	–
Urea	–	–	–	–
VP	–	–	–	–
Reduction of nitrate	–	–	–	–
Starch	+	+	+	+
Glucose	+	+	+	+
Mannitol	+	+	+	+
Lactose	+	+	+	+
Maltose	+	+	+	+
Trehalose	–	–	–	–
Xylose	–	–	–	–
Salicin	+	+	+	+
Gelatin	–	–	–	–
Aesculin	Dubious	Dubious	Dubious	Dubious

endophytic bacteria were not significantly different compared with control. One isolate of the endophytic bacteria which suppressed the height of the plants and significantly different compared with control was BL17 isolate.

Effect of each isolate to the total plant height during observation was shown by the value of AUHPGC. The highest AUHPGC value was shown by BC10 isolate while the second highest was shown by isolate BC4, i.e. 947.31 and 941.05 units, respectively (Table 3).

Characterization and Identification of Selected Endophytic Bacteria. Four isolates (AC1, BC4, BL10, and BL17 isolates) with significant differences on colony appearances (morphology, color, and growth characters) and disease suppression ability, were selected for physiological and biochemical characterization. With the exception of Gram reaction, motility, and oxidase enzyme activity, physiological and biochemical properties were identical for all isolates. Isolates of AC1 and BC4 were non-motile Gram negative bacteria, and positive for oxidase enzyme activity, whereas BL10 and BL17 isolates were Gram positive bacteria, motile or dubious, and did not show oxidase enzyme activity (Table 4).

One representative isolate from each group of inhibiting and non-inhibiting zone producers was selected for molecular identification. These isolates were BC4 isolate which showed the widest inhibition zone towards in vitro growth of *R. solanacearum* and BL10 isolate which significantly different colony morphology from the rest of the group members. Based on the sequence of 16S rRNA, BC4 isolate had 97% similarity with *Staphylococcus epidermidis* (accession number EU834240.1), whereas BL10 isolate had 98% similarity with *Bacillus amyloliquefaciens* strain JK-SD002 (accession number AB547229.1).

DISCUSSION

Endophytic bacteria could become better biocontrol agents compared with rhizosphere bacteria because they do not compete for nutrition and/or niche in apoplast (Reiter *et al*. 2002). Our laboratory and greenhouse studies obtained two promosing isolates of endophytic bacteria (BC4 and BL10 isolates) for biocontrol of tomato bacterial wilt. At 6 WAT, BC4 isolate significantly decreased disease incidence up to 33%, whereas BL10 isolate reduced disease incidence up to 43%. Based on the AUDPC values, BC4 and BC10 isolate have the lower values compared with control, and the lowest values among the endophytic isolates while BL10 isolate was on the third position. Both isolates of BC4 and BC10 also caused the highest value of AUHPGC that means both of them were the best isolates in controlling the disease and promoting the growth of tomato plants.

As revealed from dual culture assays, the mechanism of disease suppression by BC4 isolate was antibiosis. In contrast, BL10 isolate was not antagonistic to *R. solanacearum*. Hallmann *et al*. (2000) divided endophytic bacteria into two groups: (i) strains that extensively colonize the inner side of plant tissue and suppress the development of pathogens by colonizing the niche, antibiosis, or both; and (ii) strains which from the beginning colonize root cortex tissue and stimulate plant defense or general resistance mechanisms. One important character for endophytic bacteria in order to become successful biocontrol agent is fast colonization of host xylem vessel. It may be that BL10 isolate is the fast colonizer strain that it could minimize *R. solanacearum* colonization of host tissue. Our *in vitro* assays showed that BL10 isolate was a fast growing strain that was able to suppress *R. solanacearum* growth.

Staphylococcus epidermidis (BC4 isolate) and *Bacillus amyloliquefaciens* (BL10 isolate) could be new species of tomato endophytic bacteria that can be used as biocontrol agents of *R. solanacearum*. To our knowledge, *S. epidermidis* has never been reported anywhere as endophyte of tomato. The endophytic bacteria which have been reported are: *Agrobacterium, Bacillus, Bradyrhizobium, Cellulomonas, Clavibacter, Corynebacterium, Enterobacter, Erwinia, Eschericia, Klebsiella, Microbacterium, Micrococcus, Pseudomonas, Rothia,* and *Xanthomonas* from corn and barley (Zinniel *et al*. 2002), *A. tumefaciens, P. fluorescens, Flavobacterium*, and *Enterobacter cloacae* from potato (Reiter *et al*. 2002), *Rhizobium/Agrobacterium* from barley (Sharma *et al*. 2005), *Bacillus, Burkholderia, Clavibacter, Curtobacterium, Eschericia, Micrococcus, Pantoea, Pseudomonas, Serratia,* and *Stenotrophomonas* from the leaves and fruit stem of coffee (Vega *et al*. 2005). Similarly, Zinniel *et al.* (2002) and Sharma *et al.* (2005) isolated *Bacillus* from corn and barley but did not identify this endophytes to species level nor describe its role in biocontrol of pathogens. Future studies are needed to improve the effectiveness of both species for biocontrol of tomato bacterial wilt disease. These includes: (i) studies on the extent to which endophytes can colonize tomato tissue, (ii) studies on the effective antibiotic compound produced by isolate BC4, (iii) studies on compatibility of both species to each other and to other class of biocontrol agents, and (iv) studies to develop effective formulation of endophytic bacteria to increase the capability of bacterial wilt disease suppression.

Staphylococcus epidermidis and *Bacillus amyloliquefaciens* can be used together with other biocontrol agents to give more effectifeness in disease control to support the sustainable of agriculture. Colonization of this bacteria in the plant tissue need to be observed for further experiment in order to get the information wether they will be able to compete with the pathogen or not. Success in colonization of plant tissue, especially xylem vessel, is one of the important character of biocontrol agents to control *R. solanacearum* endophytically. Fast colonization of the endophytic bacteria will minimized the chance of pathogen to develop their population in the xylem vessel.

Isolates of AC1, BL10, and BL17 did not produced inhibition zone but they were able to suppress the disease incidence in week 5th. Mechanism of suppression could be competition in nutrition or/and niche. Competition of niche supported by the fast colonization will cause the occupation of infection site that mean the pathogen will not be able to keep contact with host cells. The durable of suppression could be improved by the formulation or combination of the bacteria with the other biocontrol agents.

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