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Identification and Characterizations of Potential Indigenous Endophytic Bacteria which Had Ability to Promote Growth Rate of Tomato and Biocontrol Agents of *Ralstonia solanacearum* and *Fusarium oxysporum* fsp. *solani*

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Among Plant Growh Promoting Rhizobacteria (PGPR) groups, endophytic bacteria considered as one of the options to control vascular wilt disease because of its ability to live and colonized internal roots of plants without causing any damages. Our previous research had screened 9 isolates which had best ability to promote growth rate and increase yields of tomato and biocontrol agents of *Ralstonia solanacearum* and *Fusarium oxysporum* f.sp *solani* in planta condition. In order to know its abilities, those isolates need to be characterized. This research purposed to characterize those isolates abilities to produce Indole-3-asetic acid (IAA), phosphate solubilizing, siderophore production, cyanide production, NH_3 production, and ability to colonize endophytically and identified the isolates using 16S rRNA. Result shown that all isolates can produce IAA, where TLE1.1 produce highest IAA concentration (42.5 ppm). Isolates E1AB1.3, TLE 1.1 and TLE2.2 can dissolved phosphate. None of the isolates produced HCN and NH_3. Only TLE 2.3 isolate can produce siderophore. All of 9 isolates were identified using 16S rRNA gene using 27F and 1492R primers. All isolates were identified as different species, i.e. *Bacillus toyonensis* strain BCT-7112 (EPL1.1.3), *Serratia nematodiphila* strain DZ0503SBS1 (TLE2.3), *Bacillus anthracis* strain ATCC 14578 (EPL1.1.4), *Bacillus cereus* ATCC 14579 (TLE1.1), *Bacillus cereus* strain JCM 2152 (SNE2.2), *Enterobacter cloacae* subsp. *dissolvens* strain ATCC 23373 (E1.AB1.2), *Serratia marcescens* strain NBRC 102204 (E1AB2.1), *Klebsiella michiganensis* strain W14 (TLE2.2), and *Chryseobacterium rhizoplanae* strain JM-534 (KLE3.3).

Key words: 16S rRNA, characterization, endophytes, PGPR

Diantara kelompok *Plant Growth Promoting Rhizobacteria* (PGPR), bakteri endofit dianggap sebagai salah satu pilihan untuk mengendalikan penyakit layu karena kemampuannya untuk hidup dan mengkolonisasi perakaran tanaman secara internal tanpa menimbulkan kerusakan. Berdasarkan hasil penelitian sebelumnya, telah didapatkan 9 isolat yang memiliki kemampuan terbaik dalam memacu pertumbuhan dan hasil tanaman tomat serta sebagai agen biokontrol *Ralstonia solanacearum* dan *Fusarium oxysporum* f.sp *solani* pada kondisi in planta. Untuk mengetahui kemampuan isolat-isolat tersebut perlu dlakukan karakterisasi. Penelitian ini bertujuan untuk mengetahui kemampuan isolat untuk menghasilkan asam indol asetat (IAA), pelarut fosfat, produksi siderofor, produksi sianida, produksi NH_3, kemampuan untuk mengkolonisasi internal perakaran (endofit) dan mengidentifikasinya dengan menggunakan 16S rRNA. Semua isolat dapat menghasilkan IAA, dan isolat TLE1.1 menghasilkan IAA dengan konsentrasi tertinggi (42.5 ppm). Isolat E1AB1.3, TLE 1.1 dan TLE2.2 mampu melarutkan fosfat. Tidak terdapat isolat yang menghasilkan HCN dan NH_3. Hanya isolat TLE 2.3 yang mampu menghasilkan siderofor. Semua isolat diidentifikasi dengangen 16S rRNA menggunakan primer 27F dan 1492R. Semua isolat teridentifikasi sebgai spesies yang berbeda, yaitu *Bacillus toyonensis* strain BCT-7112 (EPL1.1.3), *Serratia nematodiphila* strain DZ0503SBS1 (TLE2.3), *Bacillus anthracis* strain ATCC 14578 (EPL1.1.4), *Bacillus cereus* ATCC 14579 (TLE1.1), *Bacillus cereus* strain JCM 2152 (SNE2.2), *Enterobacter cloacae* subsp. *dilolvens* strain ATCC 23373 (E1.AB1.2), *Serratia marcescens* strain NBRC 102204 (E1AB2.1), *Klebsiella michiganensis* strain W14 (TLE2.2), dan *Chryseobacterium rhizoplanae* strain JM-534 (KLE3.3).

Kata kunci: 16S rRNA, endofit, karakterisasi, PGPR

Endophytic bacteria are groups of bacteria that capable to live within the inner tissues of healthy plants without causing any disease symptoms (Golinska, *et al.* 2015). Endophytic bacteria had advantage over any rhizospheric inhabiting bacteria, because this bacteria can live within tissue plants, had opportunity to always contact with plant cells and have a better chance to exert its beneficial effects (Santoyo *et al.* 2016). They can contribute an important role in agriculture by conferring advantages for plant (Mengoni *et al.* 2003).

Many studies had demonstrated that endophytic bacteria can produce beneficial effects on host plants, such as growth promoting substances, or prevent the negative impact caused by pathogens (Bulgari *et al.* 2009). As plant growth-promoting bacteria (PGPB), endophytic bacteria can also enhance plant growth by activating a number of similar mechanisms, including

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indole-3-acetic acid (IAA) production, phosphate solubilization, siderophores production and nitrogen fixation (Lugtenberg *et al.* 2013).

From previous research, we had screened 9 potential endophytic bacteria which had ability to promote growth of tomato and control both *Ralstonia solanacearum* and *Fusarium oxysporum* fsp. *Lycopersici* in *in planta* conditions (Yanti *et al.* 2017). Although their ability in *in planta* conditions had been shown good result, their PGPR traits must be characterized and identified to know its mechanisms to control pathogens and promote growth of tomato. This research purposed to identify isolates and characterized the ability of the 9 potential endophytic bacteria isolates to control *R. solanacearum* and *F. oxysporum* fsp . *lycopersici* and promote growth rate of tomatoes in in vitro conditions.

MATERIALS AND METHODS

This research has been done in Microbiology Laboratory, Department of Agriculture, Universitas Andalas, Padang, Indonesia from March to June 2017.

Indole Acetic Acid Production. Indole acetic acid (IAA) productions was determined using quantitative methods of Patten and Glick (2002). Isolates cultured in King's B broth supplemented with the 1g mL^{-1} of l-tryptophan. After 42 h of incubation, the density of each culture was measured spectrophotometrically at 600 nm, and then the bacterial cells were removed from the culture medium by centrifugation (5500 × *g*, 10 min). A 1-mL aliquot of the supernatant was mixed vigorously with 4 mL of Salkowski's reagent (150 mL of concentrated H_2SO_4, 250 mL of distilled H_2O, 7.5 mL of 0.5 M $FeCl_3 \cdot 6H_2O$) and allowed to stand at room temperature for 20 min before the absorbance at 535 nm was measured. The concentration of IAA in each culture medium was determined by comparison with a standard curve.

Phosphate Solubilizing Activity. Phosphate solubility was assayed using Pikovskaya agar based on methods of Wahyudi *et al.* (2011). Each isolate was streaked to the surface of Pikovskaya agar contain tri-calcium phosphate and the phosphate solubilizing activity was estimated after 5 d of incubation at room temperature. Phosphate solubilization activity was determined by the development of the clear zone around bacterial colony.

Siderophore Production. Siderophore productions was determined using Chrome Azurol Sulphonate (CAS) agar medium described by Alexander and Zuberer (1991). Each isolate was streaked on the surface of CAS agar medium and incubated at room temperature for 3 d. Siderophore production was indicated by orange halos around the colonies after the incubation.

Hydrogen Cyanide Production. Hydrogen Cyanide (HCN) production was assayed using methods of Lorck (1948). Isolates cultured on nutient agar supplemented with glycine (4 g L^{-1}). The production of cyanide was detected 48 h after inoculation, using picrate/Na_2CO_3 paper fixed to the under side of the Petri-dish lids which where sealed with parafilm before incubation at 28 °C. A change from yellow to orange, red, brown, or reddish brown was recorded as an indication of cyanide production.

Ammonia Production. Ammonia (NH3) production was assayed according to methods of Cappuccino and Sherman (2004) using peptone water (10mL), incubated for 48-72 h at 28 $\pm$ 2 °C and Nesseler's Reagent (0.5 mL) added after incubations. The color change to brownish yellow indicated positive result.

Root Colonization. Root colonization ability of endophytic bacteria were assayed by reisolations of resistance isolates mutated with rifampicin from root of tomato. Bacterial isolates were mutated with sequentially streaked on TSA with concentration of 0, 10, 20, 50 and 100 ppm of rifampicin each for 24-36 h. The mutant isolates growth from 100 ppm rifampicin culture then regrowh on the same media for 24-36 h, then were suspensed with sterile aquadest (10^6 cell mL^{-1}). Seedlings of tomato introduced with the mutant strains by dipped the seedlings for 5 min into the bacterial solutions before planted in sterilized soil. Roots of tomatoes were harvested after 4 weeks and surface sterilized with NaOCl 2%. Sterilized roots were macerated and diluted to 10^{-4}, 0.1 mL of each homogenized with TSA contained 100 ppm rifampicin and plated to petri dishes for 48 h. Bacterial colonies growth on the medium with the same morphologies as its mutant culture then were counted.

Bacteria Identification Using 16S rRNA. Endophytic bacterial isolates was identified by 16S rRNA gene. Bacterial DNA was extracted using the protocol of PureLink Genomic DNA mini Kit (Invitrogen, Thermo Scientific Inc. USA). The extracted DNA then used as PCR template and amplified using universal primer 27F (5' AGA GTT TGA TCM TGG CTC AG'3) and 1492R (5'CGG TTA CCT TGT TAC GAC TT '3). The PCR conditions were following Xiong *et al.* (2014), denaturation at 94 °C for

1 min, annealing at 54 °C for 30 s and extension at 72 °C for 1 min for 30 cycles and final extention for 30 min. The 16S rRNA fragment then assayed qualitative and quantitative by electrophoresis on agarose gel 1%. DNA fragment sequenced in Macrogen Inc. (Korea). The 16S rRNA sequence data then compared with the sequences from the GenBank using BLAST analysis (http://ncbi.nlm.nih.gov).

RESULTS

Growth Promotion Characters of Endophyte Isolates. We aimed to characterize selected endophytic bacteria associated with the roots of tomato from previous reearch which have potential as biocontrol of *R. solanacearum* and *F. oxysporum* f.sp *solani* and promote growth of tomato in field conditions. All isolates shown varies ability to produce IAA, produce NH3 and solubilize phosphate (Table 1). All isolates can produce IAA. Isolate TLE 1.1 had shown highest IAA productions, 42.5 ppm, then E1AB2.1, 38.9 ppm and TLE2.2, 36.5 ppm. However, not all isolates such as EPL1.1.3, TLE2.3, EPL1.1.4, SNE2.2, E1AB2.1 and KLE3.3 can solubilize phosphate. Only isolates TLE1.1, E1AB1.2, and TLE2.2 had shown ability to solubilize phosphate. All isolates were also found could not produce NH3 which one the main source of Nitrogen for plants.

Biocontrol Characters of Endophyte Isolates. Besides the growth promotion ability by productions of hormone indole acetic acid (IAA) and phosphate solubility, the endophytic bacteria isolates also had ability as biocontrol *R. solanacearum* and *F. oxysporum* f.sp *solani* which characterized by siderophore production, cyanide production and the ability of bacteria to colonize roots of tomato. From all isolates characterized, only TLE2.3 can produce siderophore, and no isolates can produce cyanide. All isolates can persistent in the roots tissue until 4 weeks after introduction.

R. solanacearum is one of the most devastating pathogens that attack plants from roots and invade the vascular tissues of plants. The good ability of the endophytic bacteria isolates to inhabit and persistent in roots tissue may prevent the attack of *R. solanacearum* both from direct mechanisms such as competitions and antimicrobial substance productions, or from indirect mechanisms such as Induced Systemic Resistance (ISR).

Molecular Identifications. DNA amplificons shown that all sequences were compatible with 27F and 1492 primers. All fragments shown on parallel with aproximately 1500 bp markers, as expected results of 27F and 1492R primers amplificons (Fig. 1). Electrophoresis results shown all amplicons acquired were a single DNA band indicated that the primers can specifically amplified the expected fragment. Sequence analysis from the 16S rRNA gene of endophytic bacteria shown a variable species of bacteria (Table 3). All isolates were identified as different species as seen in table 3. Out of 9 isolates only 3 isolates shown high similarities between 97% to 100% with the databases in GeneBank. Another isolates shown 96% (3 isolates), 95% (1 isolates) and 94% (2 isolates) isolates of similarity with those in genebank.

DISCUSSION

R. solanacearum and *F. oxysporum* f.sp *solani* are the most devastating pathogens that attack plants from roots and invade the vascular tissues of plants. The good ability of the endophytic bacteria isolates to inhabit and persistent in roots tissue may prevent the attack of *R. solanacearum* and *F. oxysporum* f.sp *solani* both from direct mechanisms such as competitions and antimicrobial substance productions, or from indirect mechanisms such as Induced Systemic Resistance (ISR). In this study we have characterized and identified endophytic bacteria which are shown potential activity to control *R. solanacearum* from tomato roots.

Some of strains were characterized (TLE1.1, E1.AB1.2 and TLE2.2) also had ability to solubilize phosphate which is one of essential substance for plant growth. Moreover, all isolates also produce IAA. Endophytic bacteria can enhance of plant growth through synthesis of plant auxin IAA (Chen *et al.* 2010). IAA can stimulate growth by cell elongations or cell division (Patten and Glick 2002). However, ability to produce NH_3 which also essential substance of growth tested negative for all isolates. This can be concluded that the ability of the endophyte bacteria to promote growth rate of tomato are mainly by IAA productions and phosphate solvent ability.

Endophytic bacteria has been found inhabit most plant species and have been isolated from variety of plants. We found that all isolates were well estabilished in plant root tissues. The capability of bacteria to colonize plant tissues is desirable because its greater chance of influencing development of host plants (Kuklinsky *et al.* 2004). The colonizations of the

Table 1 IAA productions, phosphate solubility and NH_3 productions of endophytic bacteria isolates

Isolates	IAA Production (ppm)	Phosphate solubility	Ammoniac (NH3) Production
EPL1.1.3	34.6	-	-
TLE2.3	29.8	-	-
EPL1.1.4	36.7	-	-
TLE1.1	42.5	+	-
SNE2.2	28.7	-	-
E1.AB1.2	26.8	+	-
E1AB2.1	38.9	-	-
TLE2.2	36.5	+	-
KLE3.3	30.9	-	-

Table 2 Characterizations of endophytic bacteria isolates to produce siderophore, cyanide (HCN), Ammonia (NH_3) and ability to colonize tomato plant root

Isolates	Siderophore Production	Cyanide (HCN) Production	Endophyte Colonization (10^5) 4 weeks after introduction
EPL1.1.3	-	-	15
TLE2.3	+	-	20
EPL1.1.4	-	-	17
TLE1.1	-	-	35
SNE2.2	-	-	28
E1.AB1.2	-	-	16
E1AB2.1	-	-	1.7
TLE2.2	-	-	20
KLE3.3	-	-	9

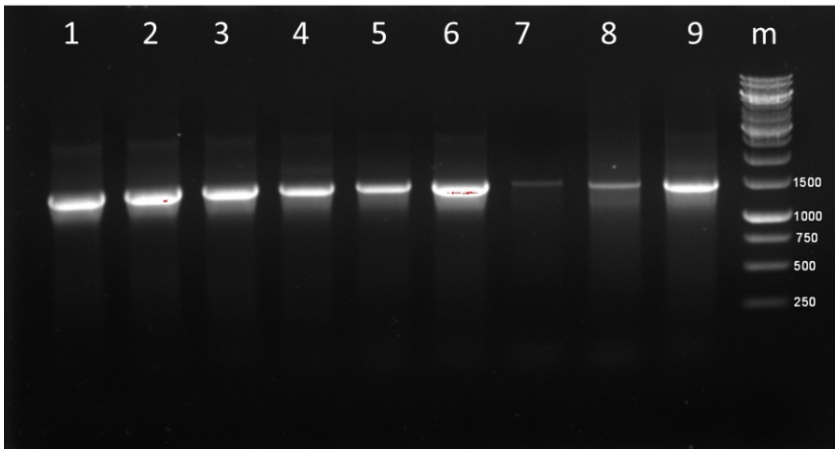

Fig 1 Results of DNA amplification of 165 rRNA gene from endophytic bacteria using 27F and 1492R primers.

Table 3 Identification results of endophytic bacteria using 16S rRNA sequence

Isolates	Sequence Analysis R esults	% of Similarity	Accession Number
EPL1.1.3	*Bacillus toyonensis* strain BCT-7112	96	NR_121761.1
TLE2.3	*Serratia nematodiphila* strain DZ0503SBS1	94	NR_044385.1
EPL1.1.4	*Bacillus anthracis* strain ATCC 14578	97	NR_041248.1
TLE1.1	*Bacillus cereus* ATCC 14579	97	NR_074540.1
SNE2.2	*Bacillus cereus* strain JCM 2152	97	NR_113266.1
E1.AB1.2	*Enterobacter cloacae* subsp. *dissolvens* strain ATCC 23373	96	NR_118011.1
E1AB2.1	*Serratia marcescens* strain NBRC 102204	94	NR_114043.1
TLE2.2	*Klebsiella michiganensis* strain W14	95	NR_118335.1
KLE3.3	*Chryseobacterium rhizoplanae* strain JM-534	96	NR_134711.1

endophytes will barrier the pathogen attack to plants and increase the competence in the rhizosphere which lead to lower the possibility of pathogens infection. Out of all isolates, only TLE2.3 (*Serratia nematodophilla*) can produce siderophore. Siderophore are organic molecules that had high affinity for Fe ions, prevent another pathogenic microbe to get Fe as one of nutrient source.

We had successfully identified all 9 isolates using 16S rRNA sequences. From the *Bacillus* genus, we have identified 3 species, i.e *B. toyonensis,* 2 strain of *B. cereus* and *B. antrachis*. *B. toyonensis* could be a valuable strain for further studies because of its ability to promote growth and control pathogens. There are not much publications of *B. toyonensis* ability as biocontrol agents and growth promoting agents. Rocha et al. (2017) found 9 isolates of *B. toyonensis* which can control *Fusarium oxysporum* f. sp. Lycopersici. *B. toyonensis* also reported had antagonistic activities against *Meloidogyne incognita* (Xiang *et al.* 2017).

B. cereus is one of the well studied species for plant growth promotions and biocontrol activity among of *Bacillus* genera. *B. cereus* known to promote plant growth by produce growth hormone such as gibberellin (Joo *et al.* 2004), produce antibiotic compounds (Silo-suh *et al.* 1994) and induce systemic resistance (Niu *et al.* 2011).

We also found two isolates from *Serratia* genera which are *S. marcesens* and *S. nematodiphila*. Another rarely studied strains we found in this research are *S. nematodiphila*. Although *Serratia* spp. have been classified as opportunistic human pathogens (Grimont and Grimont 2009), *S. nematodiphila* had reported as growth promotor, gibberellin producer and biocontrol agents of chili (Kang *et al.* 2015), enhanced heavy metal phytoremediation (Wan *et al.* 2012), and control *Xanthomonas oryzae* pv. *oryzae*.

The isolates E1AB1.2 was identified as *Enterobacter cloacae* subsp. *Dissolvens*. This species were not a common plant growth promoting species, but had been reported can promote plant growth by regulatory of antifungal compounds, phenolic compounds and IAA (Slininger *et al.* 2004).

All isolates showed various characters which led to growth promotions of plants and biocontrol activity. We had identified all 9 isolates from genera *Bacillus, Serratia, Klebsiella, Enterobacter,* and *Chrysobacterium*. This research found new PGPR strains and further research need to be done related to *B. toyonensis, K. michiganensis* and *C. rhizoplanae.* However, all isolates shown the similarity under 99% compared to Genebank Database indicated that these bacteria are likely to be a novel strains or species. Bosshard et al. (2003) defined ≥99% similarity from 16S sequence can be considered as new species. Turenne *et al.* (2001) also designated 16S sequence similarity range under 0.8 to 2% can might be suggested as new species. We suggest further genomic research to confirm the novelty of these isolates.

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