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Original Article

Screening of plant growth promoting rhizobacteria (PGPR) to promote growth of soybean

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Abstract

Rhizobacteria is an essential component for the maintenance of soil fertility and plant growth. The objective of the study was to characterize plant growth promoting rhizobacteria (PGPR) activity such as phosphate solubilization, nitrogen-fixing, IAA production, Acc-deaminase and cellulase activity isolated from the rhizosphere and nodules of soybean and evaluate their effectiveness in stimulating soybean seedlings growth. Greenhouse assay was conducted by completely randomized design with three replications per treatment and two controls (K1-control without inoculation of bacteria and Nitrogen, K2-control inoculated with *Bradyrhizobium japonicum*. Pot experiments were conducted using sterile sand and supplemented with water and Muller solution, periodically. The results showed that nine rhizobacteria isolates were efficacious as potential PGPR. Greenhouse assay results showed that the highest vegetative growth yield was obtained in soybean inoculated with SWNC1 isolate, it was characterized as *Bradyrhizobium* spp. which gave the total nodules of 29.67; fresh weight of nodules of 0.61 g; nodules dry weight of 0.09 g; number of flowers of 23; plants dry weight of 5.57 g and plant height of 46 cm.

Keywords: Bradyrhizobium, PGPR, rhizobacteria, rhizosphere, soybean

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Introduction

Soybean [*Glycine max* (L.) Merr.] is one of valuable oil seed crops due to high oil (18-22%), protein (17 to 42%), minerals (calcium, zinc, and iron) and vitamins (folic acid and vitamin B including riboflavin, thiamin and niacin) contents (Ali, 2010). Because of its importance, more research related to the production of soybean in different parts of the world is being done. This includes utilization of plant growth promoting rhizobacteria (PGPR) to increase soybean yield and to aid in soybean adaptation in extreme environments. Recently, USA, Brazil, Argentina, China, and India dominate the production of soybean worldwide (Pagano and Miransari, 2016).

Root nodulation in legumes is a key determination of plant growth and productivity, because it provides biological nitrogen (N) fixation through symbiosis with rhizobacteria. Plant growth promoting rhizobacteria (PGPR) composed of several genera of soil bacteria such as Acetobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Beijerinckia, Burkholderia, Serratia, Enterobacter, Erwinia, Flavobacterium, Herbaspirillum, Pseudomonas, Azospirillium, Azotobacter, Klebsiella (Sudhakar et al., 2000; Glick, 2012) and Rhizobium, of which capable in stimulating the growth and development of plants of their hosts (Noel et al., 1996). The PGPR association with their hosts may either be restricted to the rhizosphere or endophyte (while some actually reside within apoplastic spaces inside the host plant with or without forming specialized structures such as nodules) (Vessey, 2003).

Mechanism of PGPR in improving plant growth is not fully elucidated. Several mechanisms include direct effect such as improvement of nutrient availability to the plant such as fixation of atmospheric nitrogen, production of iron chelating siderophores, solubilization of insoluble phosphates, production of plant growth hormones (IAA, cytokinine), and the stress regulating hormone 1-aminocyclopropane-1-carboxylate (ACC) deaminase. Other mechanisms such as hydrolysis of molecules released by pathogens, synthesis of enzymes that hydrolyze fungal cell walls, improvement of symbiotic relationships with rhizobia and mycorrhizal fungi, and insect pest control are considered as indirect mechanisms (Das et al., 2013).

Application of bacterial inoculants such as PGPR and organic amendments (e.g. compost) has recently been emerged as useful tool to improve plant growth and soil quality in various soil conditions (Shahzad et al., 2013). The aim of this study was to isolate PGPR from Cibinong Science Center area and characterize their plant growth promoting activity to the soybean seeds. Nodulation improvement in soybean through is also examined.

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Methods

Soil Sampling and Bacterial Isolation

Soil samples were randomly collected from rhizopshere, non-rhizosphere and nodules of soybean (*Glycine max* L.) at Cibinong Science Center, Cibinong, West Java, Indonesia, during April 2016. All soil samples were transferred into plastic bags. Soil samples from the sampling point at the same location were mixed. Soils samples were air dried and sieved (<2 mm or mesh no. 10). 10 g of sieved soils were dissolved in an Erlenmeyer

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flask containing 90 mL of 0.8% sterile saline solution and shaken at 120 rpm for 60 mins. Serial dilution of 10^{-1} to 10^{-7} was made from each sample (Vincent, 1982; Thomas, 2015). 0.2 mL of soil extract from each dilution was spread onto three media: 1) YMA (10 g mannitol; 0.5 g K_2HPO_4; 0.2 g $MgSO_4.7H_2O$; 0.1 g NaCl; 1.0 g yeast extract, 20 g agarose; 1 L distilled water; 2.5 mL Congo red of a 1% solution) (Graham, 1969). On this medium, colonies of Rhizobia stand out as white, translucent, glistening and elevated, with entire margins (Rao, 1977); 2) Ashby's Mannitol Agar (20 g mannitol; 0.2 g K_2HPO_4; 0.2 g $MgSO_4.7H_2O$; 0.1 g NaCl; 0.1 g K_2SO_4; 5 g $CaCO_3$; 20 g agarose; distilled water 1L) (Rao, 1977), *Azotobacter* colony produced slimy, glistening, smooth, whitish, weakly convex, colonies; 3) Semi-solid agar of Caceres [(0.5 g K_2HPO_4; 0.2 g $MgSO_4.7H_2O$; 0.1g NaCl; 0.5 g Yeast extract; 0.02 g $CaCl_2$; 0.01 g $FeCl_3.6H_2O$; 5.09 g DL Malic Acid; 4, 8 g KOH; 20 g Agar; 0.25 % Congo red 15 mL (sterilized separately and added before medium used); Aquades 1L)] (Caceres, 1982). On this medium, colonies of *Azospirillum* stand out as pink to red, round, glistening, and flat edges. For nodule-associated bacteria, the nodule was surface-sterilized with 70% ethanol for 2 mins, followed by treatment with 3% aqueous sodium hypochlorite (NaOCl) for 30 mins. The nodule was washed 10 times in sterile water, cut with a sterile blade, and placed on modified YMA medium containing 0.5 g of yeast extract, 10 g of mannitol, 0.5 g of K_2HPO_4, 0.2 g of NaCl, 0.2 g of $CaCl_2.2H_2O$, 0.1 g of $MgSO_4.7H_2O$, and 20 g agarose per liter (pH 7.0). All petridishes were incubated at room temperature for 5-7 days. The strain was purified by several subcultures.

All strains obtained were selected by inoculating into half strength NFB (Nitrogen Free Bromthymol blue) medium in tubes. The medium contains: 0.5% malic acid, 0.4% KOH, 0.05% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, 0.005% $MnSO_4.H_2O$, 0.002% NaCl, 0.001% $CaCl_2$, 0.005% $FeSO_4.7H_2O$, 0.0002% $Na_2MoO_4.2H_2O$, 0.175% agarose, and 2 mL of 0.5% Bromthymol blue) (Dobereiner, 1995). The tubes were incubated at room temperature for 5-7 days. N-fixing bacteria indicated by ring-like appearance under the medium.

Nitrogen Fixing Activity

Isolates were planted into NFB media (1.25 g malic acid, 0.125 g K2HPO4, 0.05 g MgSO4.7H2O, 0.025 g NaCl, 0.005 g CaCl2, 0.5 ml micro elements, 0.5 ml bromtymol blue, 1 ml Fe EDTA was 1.64%, 1 g KOH, 0.25 vitamins and 0.5 agarose in 250 mL of distilled water). Bacteria that can fix nitrogen (nitrogenase activity) are indicated by the formation of lubricants under the surface of the media after incubation for 3 days (Baldani et al., 1980). These indications concluded that bacteria carried out nitrogenase activity were motyl.

Phosphate Solubilization and PME-ase Activity

Screening of phosphate (P) solubilization activity was conducted on Pikovskaya's agar (PKA) medium consisting of constituents: 10 g glucose; 5 g $Ca_3(PO_4)_2$ (TCP); 0.5 g yeast extract; 0.5 g $(NH_4)_2SO_4$; 0.2 g KCl; 0.2 g NaCl; 0.1 g $MgSO_4.7H_2O$; small amount of $MnSO_4$ trace and $FeSO_4$ trace; 20 g agarose, and 1 L distilled water. The pH was adjusted to 7.0 before sterilization (Pikovskaya, 1948). After 48 h of incubation at room temperature, P-solubilizing bacteria indicated by discrete colony showing halo zones.

P-solubilized bacteria from qualitative assay were inoculated in 25 mL of Pikovskaya's broth and incubated for 4 days at room temperature (bacterial population is between 10^7-10^8 cfu/mL). The bacterial cultures were centrifuged at 15.000 rpm for 30 mins. 1 mL of supernatant was mixed with 10 mL of chloromolibidic acid. The volume was made up to 45 mL by adding distilled water. 0.25 mL Cholorostannous acid was added, and the volume was made up to 50 mL with distilled water. The absorbance of the developing blue colour was read at 880 nm (Ahmad et al., 2008). Statistical analysis was conducted using SPSS version 7.0 with Duncan's test at the level of $p \leq 0.05$ (1996).

IAA (Indole Acetic Acid) Production

IAA production assay was conducted using LBT medium [Luria Bertani medium amended with 5 mM L-tryptophan (1.2115 g/L LB)] overlaid with 82 mm diameter nitrocellulose membrane. Plates were overlaid immediately after inoculation and incubated until colonies reached 0.5 to 2 mm in diameter at room temperature. After incubation period, the membrane was removed from the plate and treated with Salkowski reagent (2% 0.5 M FeCl in 35% perchloric acid) (Xie et al., 1996). Membranes were saturated in a petri dish by overlaying on a reagent saturated filter paper (Whatman no. 2). The reaction was allowed to proceed until adequate color developed. IAA producing bacteria were identified by the formation pink to red halo within the membrane surrounding the colony.

Quantitative analysis was conducted using Salkowsky reagent. Isolates were grown in LBT liquid medium and incubated at room temperature for 24 h in a rotary shaker in 90 rpm and the cell density of the each bacterial isolate between 10^7-10^8 cfu/mL. After incubation period, the media were centrifuged at 5000 rpm for 25 mins. The supernatant liquid was mixed with salkowski reagent (2:1). Color appearance was measured by spectroscopy at 530 nm after 30 mins and 2 h. As standard, concentrations of IAA (0, 5, 10, 15, 20, 25, 30, 40, 60, 80, 100, 150, 200 and 300 ppm) were prepared in LB medium. This mixed medium was treated with Salkowski reagent as above and the developed color was measured. The concentration of IAA from the sample is calculated based on the curve standards with pure IAA standards (Gravel et al., 2007). Statistical analysis was conducted using SPSS version 7.0 with Duncan's test at the level of $p \leq 0.05$ (1996).

ACC-Deaminase

ACC-utilizing bacteria were determined following the procedure of Penrose et al. (2001) with modifications. Bacterial strains were inoculated in sterile DF salts medium containing 3 mM ACC as the nitrogen source and incubated on a rotary shaker at 200 rpm and 30 °C for 24 h with the cell density of the each bacterial isolate between 10^7-10^8 cfu/mL. The isolates were plated onto solid DF salts agar medium containing ACC (500 µmol/mL) and incubated for 48 h at 30 °C. Bacterial

colonies were chosen based on their growth on the DF medium.

Cellulase Activity

Cellulase activity was determined according to method described by Teather dan Wood (1982). Bacterial isolates were inoculated with a 10 mL of the following liquid basal medium: 0.5% carboxymethylcellulose (CMC), 1.5% yeast extract; 0.1% K_2HPO_4; 0.1% $NaNO_3$; 0,1% KCl; 0.05% 0.05% $MgSO_4.7H_2O$;; 1 mL distilled water. All plates were incubated at room temperature for 5 days. Congo red solution was flooded on the medium after incubation. Colonies with halo zones indicated positive result.

Microcosmos Experiment

To observe the effect of PGPR activity of selected bacteria (9 strains = can produce high IAA), pot experiment was conducted on Grobogan soybean varieties. Each strain of bacteria was inoculated into the following medium (L^{-1}): 5 g polypepton, 5 g yeast extract, 5 g glucose, 1 g $MgSO_4.7H_2O$. Incubation was conducted for three days at room temperature in rotary shaker (100 rpm).

Soybean seeds were surface sterilized (0.1% HgCl for 2 mins and rinsed five times with sterilized water). For treatments, seeds were placed at a bacterial suspension of 10^7 CFU (colony forming unit)/mL for 1 h before sowing.

Treated seeds (2 seeds/pot) were sowed in each pot containing sterilized sand. The experiment was conducted using Complete Randomize Design with three replicates and two controls (K1-control without inoculation of bacteria and Nitrogen, K2-control inoculated with *Bradyrhizobium japonicum*). Pots were daily flushed using Muller solution (Saono et al., 1976). Plants growth parameters such as plant height, root length, nodulation, and dry weight were measured after 30 days. Statistical analysis was conducted using SPSS version 7.0 with Duncan's test at the level of $p \leq 0.05$ (1996).

Bacterial Identification

Bacteria were identified until genus level using Bergey's Manual of Systematic of Archaea and Bacteria (Krieg et al., 1984; Garrity, 2001). Identification of bacteria isolates were used morphological characteristic i.e. observation of colony (growth, color of aerial and substrate mycelium, and diffusible pigment), cell shape (cocci, rod, short rod), Gram of positive/negative, cell movement (motile, spore formation, single, paired or chain), and biochemical characteristics of the isolates were determined based on the guidelines of Bergey's Systematic Bacteriology.

Results

Bacterial Screening and Quantitative PGPR Activity

A total 25 strains of bacteria with PGPR activity were obtained from rhizosphere (19 strains) and nodules of soybean (6 strains) (Tab. 1). Six strains (SWNC1–SWNC6) exhibited symbiotic N-fixing activity, and 8 strains (SWRC3, SWRC7, dan SWTC2–SWTC7) exhibited non-symbiotic N-fixing activity. In P-solubilization screening, six strains (SWRC1, SWRC4, SWRC6, SWAC1-SWAC5,SWTC1) positively solublized posphate [$Ca_3(PO_4)_2$, Al_3PO_4, and $Fe_3(PO_4)_2$]. In addition, eight strains (SWRC1, SWRC4–SWRC6, SWAC1-SWAC5) showed IAA-production, ACC-deaminase, and cellulolytic activities (Tab. 1).

Table 1. PGPR strains obtained in this study

Strains	N-Fixing activity	P-solubilization			PGPR Activity				
					IAA Production with Different Sugar Sources			ACC-Deaminase	Cellulase
		Ca3(PO4)2	Al_3PO_4	Fe3(PO4)2	Fructose	Glucose	Molasses		
SWRC1 (Rhizosphere)	-	+	+	+	+	+	+	+	+
SWRC2 (Rhizosphere)	-	-	-	-	-	-	-	+	-
SWRC3 (Rhizosphere)	+	+	+	+	+	+	+	+++	+
SWRC4 (Rhizosphere)	-	+	+	+	+	+	+	+	+
SWRC5 (Rhizosphere)	-	-	-	-	-	-	-	-	-
SWAC1 (Rhizosphere)	-	+	+	+	+	+	+	++	+
SWAC2 (Rhizosphere)	-	+	+	+	+	+	+	+	+
SWAC3 (Rhizosphere)	-	+	+	+	+	+	+	++	+
SWAC4 (Rhizosphere)	-	+	+	+	+	+	+	+++	+
SWAC5 (Rhizosphere)	-	+	+	+	+	+	+	+	+

SWRC6 (Rhizosphere)	-	+	+	+	+	+	+	+	+
SWTC1 (Rhizosphere)	-	+	+	+	+	+	+	+	+
SWTC2 (Rhizosphere)	+	-	-	-	+	-	-	+	-
SWTC3 (Rhizosphere)	+	-	-	-	+	-	-	+	+
SWRC7 (Rhizosphere)	+	+	+	+	+	+	+	++	+
SWTC4 (Rhizosphere)	+	+	+	+	+	+	+	++	+
SWTC5 (Rhizosphere)	+	+	+	+	+	+	+	++	+
SWTC6 (Rhizosphere)	+	+	+	+	+	+	+	++	+
SWTC7 (Rhizosphere)	+	-	-	-	-	-	-	+	-
SWNC1 (Nodule)	+	+	+	+	+	+	+	++	+
SWNC2 (Nodule)	+	-	-	-	+	-	-	-	-
SWNC3 (Nodule)	+	-	-	-	+	-	-	-	-
SWNC4 (Nodule)	+	+	+	+	+	+	+	+++	+++
SWNC5 (Nodule)	+	+	+	+	+	+	+	+++	+
SWNC6 (Nodule)	+	-	-	-	+	-	-	+	+

Notes: - = negative. + = low activity. ++ = medium activity. +++ = high activity

In quantitative analysis, eight strains (SWAC1, SWAC3, SWAC5, SWRC6, SWTC1, SWNC1, SWNC4, and SWNC5) showed high P-solubilization and PME-ase production acitvities. Highest P-solubilization and PME-ase production acitvities produced by strain SWRC1 with 14.25 mg/L and 2.23 µg/mL p-nitrophenol/h (Unit), respectively. The activity of P-solubilization is related to pH reduction in all three different P-sources (Tab. 2).

Different sources of P turned out to have an impact on the available P dissolved by phosphate solubilizing bacteria. This can be seen from the dissolution results (Tab. 2). Sensitivity and ability of bacteria are not the same as each other in dissolving P sources, therefore to make biofertilizer based on phosphate solubilizing bacterial it is recommended to mix many phosphate solubilizing bacteria. Statistically, IAA production assay showed that all isolates capable in producing IAA hormone during incubation period. The highest IAA produced by SWAC5 strain with 5.48, 6.09, and 5.75 ppm from three different carbon sources such as fructose, glucose, and molasses, respectively (Tab. 3). These results showed that the carbon source can be influence IAA production of bacterial strains

Table 2. Quantitative analyses of P-Solubilization with $Ca_3(PO_4)_2$, Al_3PO_4, $Fe_3(PO_4)_2$ as the source of P and PME-ase production

Strain	P-Solubilization								
	$Ca_3(PO_4)_2$			Al_3PO_4			$Fe_3(PO_4)_2$		
	pH	P-Solubilize	PME-ase (unit)	pH	P-Solubilize	PME-ase	pH	P-Solubilize	PME-ase
SWRC1	4.64g	14.25p	1.47n	4.70g	12.33n	2.23o	5.10e	11.47j	2.23n
SWRC3	5.41def	5.30c	0.26c	5.61cde	2.30b	0.16c	5.63b	6.83c	0.20c
SWRC4	4.63g	2.78a	0.15b	5.85ab	0.33a	0.04a	5.85ab	5.18a	0.13a
SWAC1	4.79fg	9.10j	0.71i	4.70g	8.73j	0.63h	5.45bc	9.50i	0.93i
SWAC2	5.19ef	2.76a	0.09a	5.84ab	0.31a	0.05a	5.96a	5.17a	0.14a
SWAC3	4.85fg	7.85fg	0.58g	4.87fg	6.93ghi	0.45g	5.41cd	8.86h	0.73h
SWAC4	5.28ef	5.29c	0.58g	4.93efg	4.94d	0.27e	5.62b	6.82c	0.28de
SWAC5	5.24ef	8.50hi	0.80j	4.95ef	7.39i	0.76j	5.45bc	9.74i	0.67g
SWRC6	5.25ef	9.91k	0.63h	4.97ef	10.08k	0.68i	5.45bc	11.05j	1.05j
SWTC1	4.86fg	13.74o	1.18m	4.71g	11.47m	2.02n	5.35d	11.43j	2.01m
SWRC7	5.31def	5.89d	0.47e	5.16de	5.72e	0.20d	5.97a	7.88f	0.36f
SWTC4	5.68cde	7.59f	0.53f	5.45cde	2.29b	0.15c	5.45bc	8.41gh	0.18bc
SWTC5	5.79bcd	4.53b	0.39d	5.67bcd	4.13c	0.13bc	5.46bc	6.10b	0.19c
SWTC6	5.73bcd	6.88e	0.39d	5.71bcd	6.36f	0.40f	5.55bc	7.35de	0.33ef
SWNC1	5.98ab	12.66n	1.11l	4.71g	11.12m	1.58m	5.36d	11.31j	1.71l

SWNC4	6.09a	12.10m	1.09l	4.79fg	11.40m	1.28l	5.36cd	11.27j	1.13k
SWNC5	6.06a	10.84l	1.02k	4.69g	10.55kl	1.09k	5.37cd	11.23j	1.11k

Note: The number followed by the same letter are not significantly different at (p<0.05) level of Duncan's test**.**

Table 3. Quantitative analysis of IAA production

Strain	IAA Production based on different Carbon sources as a sole carbon		
	Fructose (ppm)	Glucose (ppm)	Molasses (ppm)
SWRC1	3.67j	3.520h	4.11fg
SWRC3	0.38b	1.420b	1.44ab
SWRC4	083a	1.213a	0.75a
SWAC1	2.14h	4.497j	3.45defg
SWAC2	0.87d	2.740f	1.09ab
SWAC3	2.42i	3.300g	3.81efg
SWAC4	1.69f	2.383e	2.29bcd
SWAC5	5.48m	6.090m	5.75hi
SWRC6	5.45m	5.477l	4.11fg
SWTC1	2.52i	4.170i	3.21cdef
SWRC7	2.13h	1.580bc	2.63bcde
SWTC4	1.07e	2.333e	1.97abc
SWTC5	1.88g	1.830d	1.92abc
SWTC6	0.62c	1.776cd	1.56ab
SWNC1	3.96k	2.720f	3.76defg
SWNC4	3.83k	2.780f	4.83ghi
SWNC5	4.33l	4.807k	4.58fgh

Note: The number followed by the same letter are not significantly different at (p<0.05) level of Duncan's test**.**

Table 4. Effect of selected PGPR to soybean (*Glycine max* L.)

Treatment	Nodule Number	Nodule Wet Weight (g)	Nodule Dry Weight (g)	Number of Flower	Plant Dry Weight (g)	Plant Height (cm)
K 1	0a	0a	0a	1a ± 0.00	2.50a ± 0.006	17.88a ± 0.06
K 2	32f ± 1.15	0.63e ± 0.017	0.09c ± 0.006	23g ± 0.33	5.99g ± 0.00	47.16b ± 0.57
SWRC1	0a	0a	0a	20f ± 0.58	3.87c ± 0.069	37.25b ± 0.58
SWAC1	9b ± 0.33	0.19b ± 0.006	0.04b ± 0.012	16d ± 0.58	4.20d ± 0.115	42.00b ± 0.58
SWAC3	9b ± 0.88	0.18b ± 0.012	0.04b ± 0.012	16d ± 0.58	4.84e ± 0.029	41.16b ± 0.58
SWAC5	0a	0a	0a	1a ± 0.00	3.33b ± 0.191	37.66b ± 0.00
SWRC6	0a	0a	0a	3b ± 0.33	3.26b ± 0.035	38.03bc ± 13
SWTC1	0a	0a	0a	4c ± 0.58	3.54bc ± 0.577	15.33a ± 11.67
SWNC1	30e ± 0.33	0.61d ± 0.006	0.09c ± 0.006	23g ± 0.00	5.57f ± 0.035	46.00bf ± 2.31
SWNC4	24d ± 0.33	0.56c ± 0.006	0.08c ± 0.006	4c ± 0.58	5.55f ± 0.087	44.83b ± 0.02
SWNC5	17c ± 0.58	0.55c ± 0.012	0.08c ± 0.012	18e ± 0.67	4.90e ± 0.058	38.33b ± 0.19

Note: The number followed by the same letter are not significantly different at (p<0.05) level of Duncan's test. K1 = untreated seeds and medium lacking Nitrogen; K2 = seeds treated using Bradyrhizobium japonicum; ± SD is score from three replicates.

Pot Experiment

Inoculation assay of selected PGPR to the soybean showed that nine strains (SWRC1, SWAC1, SWAC3, SWAC5, SWRC6, SWTC1, SWNC1, SWNC4, SWNC5) and control K2 (seeds treated using *Bradyrhizobium japonicum*) showed positive plant growth supporting activity in all parameters (nodule number, nodule wet weight, nodule dry weight, number of flower, plant dry weight, and plant height) (Tab. 2). Among them, SWNC1 showed highest PGPR activity to the plant (nodule number = 30, nodule wet weight = 0.61 g, nodule dry weight = 0.09 g, number of flower = 23, plant dry weight = 5.57 g, and plant height = 46 cm). High plant growth promoting activity also showed by K2 (nodule number = 32, nodule wet weight = 0.63 g, nodule dry weight = 0.09 g, number of flower = 23, plant dry weight = 5.99 g, and plant height = 47.16 cm) (Tab. 2).

Bacterial Identification

Identification of PGPR based on their morphology, physiology activities, and phenotype characters is presented in Table 5a, 5b, 5c, 5d, 5e, and 5f. The result of data analysis showed that the selected PGPR belong to two phyla, namely, firmicutes and Proteobacteria; three classes (Bacilli, α-Proteobacteria, γ-Proteobacteria), and six genera (*Bacillus* sp. = SWTC1; *Bradyrhizobium* sp. = SWNC1; *Rhizobium* sp. = SWAC1, SWAC3, SWAC4, SWNC5; *Azospirillum* sp. = SWRC6; *Azotobacter* sp. = SWAC5; and *Pseudomonas* sp. = SWRC1).

Table 5a. Identification of PGPR promoting bacteria

Domain	Phylum	Class	Species
Bacteria	Firmicutes	Bacilli	*Bacillus* sp. (SWTC1)
	Proteobacteria	α-Proteobacteria	*Bradyrhizobium* sp. (SWNC1)
			Rhizobium sp. (SWAC1, SWAC3, SWAC4, SWNC5)
			Azospirillum sp. (SWRC6)
		γ-Proteobacteria	*Azotobacter* sp. (SWAC5)
			Pseudomonas sp (SWRC1)

Table 5b. Phenotype characteristics of the genus Bacillus

Phenotype characteristics of the genus *Bacillus*	
Colony form	
Gram	
Spore	
Predominant type of flagellation	
Aerobic	

Table 5c. Phenotype characteristics of the genus Bradyrhizobium

Phenotype characteristics of the genus *Bradyrhizobium*	
Colony form	Short rods / rods, slow growing
Gram	-
Spore	Nonsporeforming
Predominant type of flagellation	One polar / subpolar flagellum
Aerobic	+

Table 5d. Phenotype characteristics of the genus Rhizobium

Phenotype characteristics of the genus *Rhizobium*	
Colony form	Rods 0.5-1.0 (µm), gram -, Motyle, Aerobic, mucous,
Peritrichouse flagella / one subpolar flagellum	1-6
Reproduce by budding at one pole of the cell	-
Bioti	+
Thiamine	+
Growth with 1% and 2 % NaCl	-
Optimal growth temperature (°C)	28
Citrate	-
Gluconate	+

Table 5e. Phenotype characteristics specie of the genus Azospirillum

Phenotype characteristics specie of the genus *Azospirillum*	
Vibroid	+
Helical	-
Cel diameter (µm)	0.6-1.7
Colony form	Plump, slightly-curved and straight rods
Gram (cultured in MPPS)	-
Motility	+
Predominant type of flagellation	Monopolar single flagellum, lateral flagellata
Nitrogenase activity	+
Nitrogen fixed only under microaerophilic conditions	+
Cell width (µm)	1.0-1.7
Enlarged, pleomorphic develop in alkaline media	+
Biotin requirement	+
Growth with 3 % NaCl	Differs among strains
Pectin hydrolysis	-
Optimal growth temperature (°C)	37
Glucose	+
Fructose	+
Mannitol	+
Glycerol	+
Sucrose	-
Colony type on BMS agar medium	Pink, raised. curled
Colony type on Congo red medium	Scarlet
Succinate, malate, lactate, fumarate	+

Gluconate	+
Citrate	+
A-Ketoglutarate	+
Maltose	-
Lactose	-

Table 5f. Phenotype characteristics of the genus Azotobacter

Phenotype characteristics of the *Azotobacter*	
Colony form	Straight rods with rounded ends to more ellip-soidal or
Cell diameter (µm)	2
length	4
Motile	+
Number of flagella	Peritrichous flagella
Gram	-
Aerobic	+
Water soluble	+
Water soluble pigments: Yellow green fluorescent, Green, Brown	-
Temperature growth	9 °C - 37 °C
Denitrification	+
Produce of Urease	+
Produce of peroxidase	-
Produce of oxidase	+
Fructose, glucose, acetate, pyruvate, fumarate, malate, succinate,	+
Sucrose	+

Table 5g. Phenotype characteristics specie of the genus Pseudomonas

Phenotype characteristics specie of the genus *Pseudomonas*	
Colony form	Straight / slightly curved rods
Cel diameter (µm)	0.5-1.0 x 1.5-5.0
Motyle	+
Number of flagella	by one / several polar fagella
Gram	-
Aerobic	+
Nonfluorescent pigment, colour: Green, ornge, yellow,Blue	-
Denitrification	+
Grwth	4°C -40°C
D-Fructosa	+
D-Glucose	+

Discussion

PGPR Activity

Plant-microbes interaction studies have attracted more attention from agricultural researcher worldwide (Philippot et al., 2013). In this study, we obtained 25 bacterial strains with PGPR activity from rhizosphere (19 strains) and nodules of soybean (6 strains). The obtained PGPR isolates in this experiment were more than the experiment conducted by Widawati and Made (2016) which obtained 17 isolates from rhizosphere of mung bean in CSC (Cibinong), but lower than the experiment conducted by Kusumawati et al. (2017) which obtained 48 isolates from rhizosphere of *shorgum bicolor* in CSC (Cibinong). In the previous study of PGPR in Cibinong Science Center area, 17 strains PGPR were obtained from rhizosphere of rice and 8 strains from mung bean (Widawati and Sudiana, 2016). This showed that rhizosphere bacterial community is higher than another bacterial community associated with plant. White et al. (2015) noted that microflora in the rhizosphere is more diverse and high in population than other parts of plant. Community of bacteria in rhizosphere is affected by plant species and organic compounds produced by the plants in the rhizosphere area (White, 2015). Roots exudates production by plant is affected not only by plant species, but also environmental factors (temperature, CO_2 concentration, light, soil chemical, and soil microorganisms (Mimmo et al., 2011). Effect of the environmentals factor to bacterial diversity, bacterial population, adaptation of bacteria to plants and the environment to absorb nutrients (Kohler et al., 2011; Pérez-Monta˜no et al., 2014); besides that it also affects the variation of the composition of the root exudate (Mimmo et al., 2011), where the exudate is a C source that is easily accessible to bacteria in the rhizosphere (Hinsinger et al., 2009).

In this study, 14 strains exhibited N-fixing activity, 17 strains P-solubilization activity, and 17 strains showed IAA-production, 22 strains showed ACC-deaminase production, and 19 strains cellulolytic activity. The results of the analysis of PGPR activities and production from the experiment were better than the experimental results of Kusumawati et al. (2017) which produced a total of 8

strains exhibited N-fixing activity, 15 strains P-solubilization activity, and 4 strains showed IAA-production, and 4 strains cellulolytic activity.

Strains that have the ability of N-fixing activity, P-solubilization activity, IAA-production, ACC-deaminase production, and cellulolytic activity are included in the PGPR category and are biostimulants for plants to absorb N and P bound in the soil (Ruzzi and Aroca, 2015). Nitrogen and phosphorus are the most important nutrient for plant growth compared to the other essential nutrients. The use of N-fixing bacteria with ability in solubilizing organic and inorganic phosphates is common practice for the sustainable agriculture because it can supply sufficient N_2 and assist the hydrolysis of a wide range of P compounds in soil, leading to increased crop production.

Effect of Selected PGPR to Soybean (Glycine max L.) Growth

Nine selected PGPR showed plant growth promoting activity (Tab. 4). Significant improvement based on several parameters such as nodule number, nodule wet weight, nodule dry weight, number of flower, plant dry weight, and plant height was distinctly observed between treated and untreated plants. It was found that highest PGPR activity was found in soybean plant treated by SWNC1 inoculum and *B. japonicum* (K2) (Tab. 4). Identification based on morphology and biochemical characters showed that SWNC1 strain belongs to genus *Bradyrhizobium* (Tab. 4). *Bradyrhizobium* has been known for their effectiveness in promoting maize and soybean growth (Botha et al., 2004). In soybean, *Bradyrhizobium* spp. form symbiotic effectiveness for nodulation. Symbiosis effectiveness between *Bradyrhizobium* spp. and soybean is determined by types of cultivars (Payakapong et al., 2004).

Prévost et al. (2012) noted that majority of *Bradyrhizobium* strains from capable in producing siderophores, but less number of *Bradyrhizobium* strains capable in producing IAA hormone, the main auxin in plants of which very important for plant physiological processes such as cell enlargement and division, and tissue differentiation (Maor et al., 2004). The relationship between IAA production and nodule formation in legume crops was previously elucidated by Pii et al. (2007). Nodule development of legumes requires local accumulation of auxin, especially in *Medicago* species. In addition IAA effects on soybean growth is affected by and nitric oxide (NO). Involvement of NO in auxin-induced adventitious root and lateral root formation providing evidence that this compound is possibly related to auxin signalling pathway. In our previous study, *B. japonicum* inoculation to soybean yield higher soybean growth caompared to IAA or P-solubilized bacteria inoculation (Widawati et al., 2015). Application of symbiotic N-fixing bacteria to soybean often exhibited different effectiveness in nodule formation. It is probably affected by sources of symbiotic N-fixing bacteria and soybean cultivars (Prévost et al., 2012).

Soybean plant has been known as very demanding plant on nitrogen (N). Therefore, this plant requires biological fixation of N_2 (BNF) as efficient as possible to obtain high yields. N-fixing symbiotic bacteria such as *Rhizobium* and *Bradyrhizobium* have been reported increasing N uptake of various soybean cultivars, especially during the stage of flowering (Paradiso et al., 2015). The symbiotic bacteria increase usable N supply (i.e., ureides) and stimulating plant photosynthesis (Imsande and Schmidt, 1998).

This study also showed that application of non-symbiotic PGPR (*Pseudomonas* sp., *Azotobacter* sp., *Azospirillum* sp., and *Bacillus* sp.) to soybean less effective than symbiotic PGPR (*Bradyrhizobium* sp.) (Table 4). Ineffectivity of non-symbiotic PGPR inoculant to soybean growth possibly due to their presence in the root did not affect physiological quality of the soybean. Another factor for not maximizing soybean growth is root nodule bacteria. Root nodules bacteria that are symbiotic with soybean plants as their host are specific bacteria, *Bradyrhizobium* sp. Rhizosphere bacteria such as *Pseudomonas* sp., *Azotobacter* sp., *Azospirillum* sp., and Bacillus sp. (Glick, 2012) is unable to infect soybean roots and make nodules. Thus influencing physiological of soybean is very important in improvement of agronomical characters of soybean such as days to flowering, days to maturing, plant height, number of branches per plant, number of reproductive nodes per plant, number of grains per plant, yield per plant, etc.

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