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CHARACTERIZATION OF RHIZOBACTERIA ISOLATES FROM SOIL AND NODULES

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ABSTRACT

The plant growth promoting rhizobacteria (PGPR) is a group of bacteria capable of colonizing plants roots, thereby developing a system and improving plants growth and yield. The objectives of the study is to characterize the PGPR activities of several bacterial isolates (*in-vitro* screening), to examine their activities in stimulating soybean growth (*in-vivo* screening), and to identify the bacterial species. These were isolated from nodules and soil samples collected from Mount Pancar in Bogor, West Java Province as well as from Bangkirai Hill and Wain River in East Kalimantan, Indonesia. The *in-vitro* PGPR activity characterization includes the N-fixing ability, ACC-deaminase, indole acetic acid (IAA) production, cellulolytic activity, P-solubilization, Phosphomonoesterase (PME-ase), and nifH-gene detection. The *in-vivo* PGPR activity with the greenhouse assay was conducted on soybean plant (*Glycine max* L.). All bacterial isolates were identified using molecular methods based on nucleotide sequence generated from 16S rRNA gene. Three isolates of soil and nodule bacteria with 7 characteristics of PGPR (N_2 fixation, ACC-deaminase, cellulolytic activity, IAA production, solubilization index, P available, and PMEase activity) were successfully identified. These isolates were B045 (*Klebsiella variicola* InaCC B827), B116 (*Klebsiella* sp. InaCC B833), and B210 (*Mangrovibacter plantisponsor* InaCC B841). The greenhouse assay showed that the plant height, plant dry weight and number of flowers in soybean seedlings significantly increased with *Bradyrhizobium* sp. strain 4167, then with *Klebsiella* sp. InaCC B833 and *Mangrovibacter plantisponsor* InaCC B841. These bacterial isolates which were characterized and screened *in-vitro* for PGPR potentials and their representative isolates which were identified by 16S rRNA sequence analysis are key factors for selecting PGPR isolates to be commercialized later as bio-stimulant.

Keywords: 16S rRNA, Bangkirai Hill, Mount Pancar, PGPR, *Rhizobacteria*, Wain River

INTRODUCTION

Bacterial characterization is one step in determining bacterial activity as plant growth promoting rhizobacteria (PGPR). This characterization process involves the activities of auxin phytohormones like indole acetic acid or IAA (Abbas *et al.* 2011), ACC-deaminase (Penrose & Glick, 2003), siderophore production (Filippi *et al.* 2011), cellulase activity in plant root invasion and colonization (Reinhold-Hurek & Hurek 1998), nitrogen fixing mechanisms (Fischer *et al.* 2007), dissolving of insoluble phosphates with acidification and phosphomonoesterase (Krey *et al.* 2013) and NifH gene detection test as the main coding gene of nitrogenase. The gene converts the atmospheric nitrogen (N_2) to ammonia which is readily used by organisms (Postgate 1998; Gilchrist & Benjamin 2017). Characterization of PGPR activities is needed for the production of bio-fertilizer, microbial rhizoremediation and effective bio-pesticides production (Adesemoye *et al.* 2008 & Bhardwaj *et al.* 2014).

The fluorescent bacteria, *Pseudomonas* which are able to fight against pathogenic bacteria and boost plant growth, were first categorized by

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Kloepper *et al.* (1980). This class of organisms is an important PGPR group as all *Rhizobacteria* having the ability to directly promote plant growth are included in this PGPR group (Kapulnik *et al.* 1981). This also covers other genera of *Rhizobacteria*, especially those which increase plant growth through different mechanisms. The *Rhizobacteria* group which forms a symbiotic interaction with legumes through nitrogen fixation and nodule formation are included in the sympathetic rhizobacteria category which consists of: *Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium* and *Actinomycetes* in symbiotic relationship with Frankia plants (Babalola & Akindolire 2011). The other nitrogen fixing, non-symbiotyic PGPR, otherwise known as diazotrophs, and living independently from plant cells are included in the free-living rhizobacteria category (Babalola & Akindolire 2011). These include *Azoarcus, Azotobacter, Acetobacter, Azospirillum, Burkholderia, Diazotrophicus, Enterobacter, Cyanobacteria, Pseudomonas* and *Gluconacetobacter (Anabaena, Nostoc), Alcaligenes, Bacillus, Klebsiella, Arthrobacter,* and *Serratia* (Saharan & Nehra 2011; Haghighi *et al.* 2011; Bhattacharyya & Jha 2012).

These aforementioned genera are the functional indigenous bacteria group which colonize plants rhizosphere or endo-rhizosphere and possess these three exceptional characteristics, i.e., biofertilization, biostimulation and biocontrol. Their production of Nitogen, Phosphorus and IAA are required for plant growth and for adaptation in stressful condition and environment (Odoh & Kenneth 2017), such as in dry and saline soil (Mapelli *et al.* 2013), abiotic stress (Lau & Lennon 2011), marginal soil (Redman *et al.* 2011) and soil packed with chemical fertilizers (Veen *et al.* 1997). These also produce environmentally friendly renewable energy (Jackson 1999) which has the capacity to sustain other agricultural practices (Noumavo *et al.* 2016) and are essential for improving the soil biological quality (Hayat *et al.* 2010) through biogeochemical cycles (Carbon, Nitrogen, Sulfur and Phosphorus), thereby making the elements available in the soil (Banig *et al.* 2008).

Nitrogen and Phosphorus present in the rhizosphere are particularly important for plant growth in stressful conditions (Mendez *et al.* 2007) and are essential for optimal growth, metabolism and development (Meena *et al.* 2014). Plant roots could only absorb Nitrogen and Phosphorus in the form of Ammonium and inorganic Phosphorus and these elements are available for plants when both nitrogen fixing bacteria and phosphate solubilizing bacteria are in the soil (Malusá & Vassilev 2014). Therefore, indigenous bacteria were collected from marginal and upland areas like hills as well as lowlands like rivers, to serve as catalysts in the presence of elements needed for plant growth in marginal soils.

The objectives of the study were to characterize PGPR activity of several bacterial isolates (*in-vitro* screening of PGPR), to examine their activity in stimulating soybean growth (*in-vivo* screening of PGPR), and to identify the bacterial species.

MATERIALS AND METHODS

Sampling

Using the random sampling method, this research was conducted at Mount Pancar area of Bogor, West Java (6°35'0.5 S & 106°54'46.8 E; 6°35'03.9 S & 106°54'40.6 E; 6°34'30.1 S & 106°53'3.1 E; 6°34'30.1 S & 106°53'3.1 E; and 6°29'40.3 S & 106°5.0'3.1 E.), Wain River (01°05'00 S & 116°52'00 E) and Mahakam River (0°31'11 S & 117°07'09 E), and Bangkirai Hill (1°01'44.88 S & 116°52'02.2 E) East Kalimantan, Indonesia in 2012. The samples were taken from nodules of *Paraserianthes falcataria* (L.) Fosberg (1 sample), *Mimosa pudica* Linn (1 sample), *Vigna sinensis* Endl. Ex Hassk (8 samples), *Arachis hypogaea* Linn. (1 sample). The rhizosphere of *Mimosa pudica* Linn (1 sample) was obtained from the soil around the roots to a depth of 20 cm. The soil samples also taken from the soil surface layer to a depths of 20 cm from Wain and Mahakam rivers and Bangkirai Hill.

Isolation and Purification

The nodules were washed in ethanol 70%, rinsed with sterile distilled water and then cut into two parts planted in triplicate on a petri dish containing selective medium (5 g polypeptone + 5 g yeast extract + 5 g of glucose + 1 g of $MgSO_4.7H_2O$ + 20 g agar + 1 L Aquadest). Plates were incubated for 5 - 7

days at room temperature. Besides that, isolation from soil samples was carried out. As much as 1 g of soil sample was mixed with 9 mL of sterile distilled water in a test tube on a vortex until homogeneous. Then the soil extract from serial dilution (10^{-3}, 10^{-4}, 10^{-5}) were planted in triplicate on a petri dish containing selective medium (5 g polypeptone + 5 g yeast extract + 5 g of glucose + 1 g of $MgSO_4.7H_2O$ + 20 g agar + 1 L Aquadest) (Vincent 1982).

Characterization of PGPR Activity (*In-vitro* Screening)

Nitrogen Fixation Activity

The nitrogen fixing ability of the bacteria was determined using the Dobereiner's method (Dobereiner 1995). The bacteria were placed into Luria Bertani Broth media and incubated for 5 days. This was transferred into a tube containing 5 mL of semi-solid Nitrogen Free Bromthymol blue (NFB) media at pH 7.0, and incubated at 28 °C for 72 h. The nitrogen fixation activity was manifested by the formation of mist-like white rings known as pellicles, below the surface of the NFB media inside the test tube (Jha *et al.* 2014).

ACC-deaminase Production

The ACC-deaminase production was conducted by a qualitative test using the Dworkin and Foster method (1958). The Dworkin-Foster salt media enriched with 0.3033 g of 1-Aminocyclopropane 1-Carboxylic Acid (ACC) substrate and 2 g of Ammonium Sulfate were used as Nitrogen sources (Glicks *et al.* 1995). The ability of Diazotrophic bacteria to fix atmospheric N_2 (*in-vitro*) was shown by the production of ACC-deaminase (positive). In the negative control *Escherichia coli* was used (grown on DF + ACC media). The growth of the bacterial colonies on DF + ACC media after 72 h of incubation indicated the production of ACC-deaminase.

Indole Acetic Acid Production

For qualitative analysis of IAA production, the bacteria were inoculated on semi-solid King B broth media mixed with 200 µg/mL Tryptophan. Salkowski reagents were dropped on the formed colony and stored in a dark room for 30 min (Gravel *et al.* 2007). The resultant pink color indicated the production of IAA (Khan *et al.* 2014). Those bacteria capable of producing IAA were also analyzed quantitatively on Trypticase soy broth media (TSB : 20 mL) containing L-Tryptophan (200 µg/mL). The pink coloration after 72 h incubation, indicated the production of IAA on the bacterial extract (Mehboob *et al.* 2010) which was measured using spectrophotometer at λ 540 nm with interpolation on IAA calibration curve (Pattern & Glick 2002).

Cellulolytic Activity

Using the Ponnambalam method (Ponnambalam *et al.* 2011), the cellulolytic activity of the bacteria was tested qualitatively by the presence of halo zone around the bacterial colonies.

Inorganic Phosphate Solubilization

The qualitative analysis of inorganic P solubilization was carried out on a petri dish by inoculating bacteria on Pikovskaya solid media with 0.5% $Ca_3(PO_4)_2$ as the P source which were incubated for 7 days (Chen *et al.* 2006). The formation of halo zone around the bacterial colonies indicated that P was dissolved from the Ca^{2+}. The solubilization index (SI) was then obtained using the formula: SI = Colony diameter + halo zone diameter/colony diameter (Premono & Vleck 1996). The quantitative analysis was also carried out by inoculating the bacteria on Pikovskaya liquid media and orthophosphate in a solution, and measured after incubation for 3, 6, and 9 days (Vassileva *et al.* 2000).

Phosphomonoesterase (PME-ase) Activity

The analysis of extracellular activity of PME-ase in acid was done by adding 0.5 M $CaCl_2$ and alkaline (0.5 M NaOH) to a tube inoculated with bacteria. Then, 0.115 M p-nitrophenyl phosphate disodium (pNPP) was added (Tabatabai & Bremner 1969). The absorbance of pNPP formed a yellow coloration upon incubation for 7 days. This was measured using a spectrophotometer at the wavelength of 400 nm. The PME-ase activity unit is defined as µmol/h, which is the amount of p-nitrophenol released in 1 mL extracellular enzyme solution

fractionated from 1.0 mL of culture after 7 days of incubation (Widawati & Sudiana 2016).

Bioassay in Green House (*In-vivo* Screening of PGPR)

Bioassay in the greenhouse (*in-vivo*) were prepared by using the PGPR bacteria showing good potential from the *in-vitro* test, and were applied on soybean (*Glycine max* L.) Merr. var Grobogan as the test plant. The experiment used a completely randomized design with liquid inoculant as the treatment having a single isolate (isolate codes; LIPI12-3 B136, LIPI12-3 B144, LIPI12-3 B146, LIPI12-3 B157, LIPI12-3 B183, LIPI12-3 B188, LIPI12-3 B045, LIPI12-3 B054, LIPI12-3 B100, LIPI12-3 B116, LIPI12-3 B210), *Bradyrhizobium* sp. 4167 for the purpose of comparison and the control treatments were without inoculants plus N (KNO_3 0.05%) and in other samples, minus N. Each treatment was repeated 10 times and the procedures include: 1) cleaning of Soybean seeds with ethanol 70% and soaking in sterile distilled water for one hour; 2) putting of seeds on wet filter paper in a sterile petri dish and incubating at room temperature until the seed germinate; 3) soaking of the sprouts for 1 h in 25 mL of every liquid culture of inoculant treatment (bacterial population 10^9 cfu/mL) from the tested bacteria; 4) planting of the sprouts in pots (2 sprouts per pot) filled with sterile sand covered with sand containing paraffin (to avoid contamination); 5) maintaining the humidity of the planting media at 24% by watering with Müller's solution every day through capillaries installed on the pot and covered with cotton. The application of nitrogen to control plants without the inoculants were carried out by watering Müller's solution added with KNO_3 0.05% (Saono *et al.* 1976). Plant growth measurements, like the plant height, plant dry weight and number of flowers were taken 2 months after sprout formation. The data were then analyzed with SPSS software version 16.0 and the significant differences among treatments were determined by Duncan's Multiple Range ($p \leq 0.05$).

Identification and nifH Genes Test of Bacteria

The bacteria were identified following the Otsuka (2008) method and using the 16S rRNA gene sequence with 16S-9F (5'GAGTTTGAT CCTGGCTCAG3') with 16S-1510R (5'GGCTA CCTTGTTACGA3') and 16S-785F (5'GGATT AGATACCCTGGTA3') with 16S-802R (5'CCT CTCTATGGGCAGTCGGTGAT3') primers. The phylogenetic affiliations and taxonomical hierarchy based on 16S rRNA were determined using the CLASSIFIER tool (http://www.rdp.cme.msu.edu) of RDP-II database (Cole *et al.* 2009). The *nif*H genes test of bacteria was performed using the Poly *et al.* (2010a; 2010b) method with nifHf 5'-GGCAA GGGCGGTATCGGCAAGTC-3'and nifHr 5'-CCATCGTGATCGGGTCGGGATG-3'.

RESULTS AND DISCUSSION

Isolation and Purification

The isolation showed various physical characteristics like shape, color, and size of different bacterial colonies. The 12 experimental isolates of indigenous bacteria consisted of 7isolates (2a, 4a, 4c, 12a, 7b, 11h, 13a) from root nodules and rhizosphere in the Pancar Mountain area and 5 isolates (7-0-1L, 18Na, 20La, 31Na, 33Na) from the soil of the Mahakam River and Bangkirai Hills in Kalimantan. The pure isolates were given the LIPI codes and numbers, and stored in a freezer maintained at -20 °C for identification, and these were LIPI-12-3 isolates, namely B045, B8054, B100, B116, B136b, B144a, B146, B184a, B157, B183a, B188a and 210 (Table 1).

Characterization of PGPR Activity (*In-vitro* Screening)

The isolates were screened for nitrogen fixation, ACC-deaminase, cellulolytic activity, IAA production, solubilization index (SI), available P and PMEase in acid and alkaline activity (Tables 2 & 3).

Table 1 Isolation results and isolates number

No	Source of sample	Isolate number	
		Private	LIPI
1	Soil (Wain River, Balikpapan, East Kalimantan, Indonesia)	18Na	LIPI12-3-B045
2	Soil (Wain River, Balikpapan, East Kalimantan, Indonesia)	20La	LIPI12-3-B054
3	Soil (Bangkirai Hill, Balikpapan, East Kalimantan, Indonesia)	31Na	LIPI12-3-B100
4	Soil (Kalimantan)	7-0-1L	LIPI12-2-B116
5	Root nodule of *Albizia falcataria* (L.) Fosberg (Pancar Mountain)	2a	LIPI12-3-B136b
6	Root nodule of *Mimosa pudica* Linn (Pancar Mountain)	4a	LIPI12-3-B144a
7	Rhizophere of *Mimosa pudica* Linn.(Pancar Mountain)	4c	LIPI12-3-B146
8	Soil (Pancar Mountain)	12a	LIPI12-3-B184a
9	Root nodule of *Vigna sinensis* Endl. Ex Hassk. (Pancar Mountain)	7b	LIPI12-3-B157
10	Rhizosphere of *Vigna sinensis* Endl. Ex Hassk. (Pancar Mountain)	11h	LIPI12-3-B183a
11	Root nodule of *Arachis hypogaea* Linn.(Pancar Mountain)	13a	LIPI12-3-B188a
12	Soil (Mahakam River, Balikpapan, East Kalimantan, Indonesia)	33Na	LIPI12-3-B210

Table 2 Screened isolates for nitrogen fixation, ACC-deaminase and cellulolytic activity

	Isolate code: LIPI12-3-	N_2 fixation	ACC-deaminase	Cellulolytic activity
1	B045	+	+	+
2	B054	+	-	+
3	B100	+	-	+
4	B116	+	+	+
5	B136b	+	-	+
6	B144a	-	-	-
7	B146	+	-	+
8	B184a	-	+	-
9	B157	-	+	-
10	B183a	+	-	+
11	B188a	-	+	-
12	B210	+	+	+

Table 3 Screened isolates for IAA, EP, available P and PMEase activity

No	Isolate code: LIPI-2-3-	IAA after 48 H (ppm)	(SI)	P available (ppm)	PMEase activity (unit)	
					Acid	Base
1	B045	84.38 ± 0.2^{bc}	40 ± 2.9^{b}	1.14 ± 0.1^{ab}	0.20 ± 0.0^{b}	0.22 ± 0.0^{b}
2	B054	76.56 ± 0.3^{a}	-	-	-	-
3	B100	96.32 ± 0.2^{d}	-	-	-	-
4	B116	104.96 ± 0.5^{e}	100 ± 1.5^{e}	1.57 ± 0.3^{cd}	0.74 ± 0.0^{d}	0.50 ± 0.0^{d}
5	B136b	88.09 ± 0.1^{c}	20 ± 1.7^{a}	0.83 ± 0.1^{a}	0.11 ± 0.0^{a}	0.11 ± 0.0^{a}
6	B144a	86.44 ± 0.2^{c}	20 ± 1.0^{a}	1.05 ± 0.0^{ab}	0.13 ± 0.0^{a}	0.10 ± 0.0^{a}
7	B146	76.46 ± 0.2^{a}	-	-	-	-
8	B184a	83.15 ± 0.1^{bc}	20 ± 1.5^{a}	1.46 ± 0.2^{bc}	0.13 ± 0.0^{a}	0.17 ± 0.0^{b}
9	B157	95.08 ± 0.0^{d}	200 ± 2.0^{f}	2.00 ± 0.0^{d}	0.86 ± 0.0^{e}	0.64 ± 0.0^{e}
10	B183a	87.26 ± 0.1^{c}	80 ± 2.1^{d}	1.87 ± 0.0^{cd}	0.83 ± 0.0^{e}	0.55 ± 0.0^{d}
11	B188a	83.15 ± 0.1^{bc}	50 ± 2.9^{c}	1.18 ± 0.0^{ab}	0.19 ± 0.0^{b}	0.20 ± 0.0^{b}
12	B210	104.13 ± 0.1^{e}	100 ± 1.0^{e}	1.51 ± 0.0^{cd}	0.52 ± 0.0^{c}	0.47 ± 0.0^{c}

Note: Means with the same superscript in the same column do not significantly differ at $p < 0.05$.

The *in-vitro* screening produced eight isolates (B045, B054, B100, B8116, B136b, B146, B183b and B210) which were positive for nitrogen fixing activity as indicated by the formation of pellicle-like white ring under the surface of NFb media inside the test tube after 5 days of incubation. Six isolates (B045, B116, B184a, B157, B188a and B210) were positive in the production of ACC deaminase, indicated by the formation of colonies in DF + ACC media after 48 hours of incubation. Eight isolates (B045, B054, B100, B116, B136b, B146, B183a and B210) were positive in the cellulolytic activity as indicated by the formation of halo zone around the colonies on CMC media. Another, nine isolates (B045, B116, B136b, B144a, B184a, B157, B183a, B188a and B210) exhibited their ability to solubilize phosphate in the Pikovskaya

solid media as indicated by the formation of some halo zones around the colonies, as well as in Pikovskaya liquid media after 5 days incubation (Table 3). All the twelve isolates produced the growth hormone IAA as indicated by the formation of a reddish pink color after the bacterial colonies were given Salkowski reagent and incubated for 3 hours in a dark room (Gordon & Weber, 1951). In conclusion, the presence of this IAA primarily indicated that these bacteria are potential PGPR. This confirms the study of Abbas *et al.* (2011), as well as Mano and Nemoto (2012), that the main character of the PGPR group is its ability to produce IAA, since it is the first identified plant growth promoting hormone. The quantitative analysis also showed that the IAA yield ranging from 76.56 - 104.96 ppm (with isolates B116 and B210 having the highest IAA) which were achieved after 48 hours of incubation. This IAA yield was far higher than those of Kishi *et al.* (2012) (58.34 ppm), Widawati (6.08 ppm), Widawati and Sudiana (2016) (9.56 ppm), as well as Suliasih and Widawati (2017) (19.98 ppm). This fluctuating yield may be influenced by the condition of bacterial culture in the synthetic media. The condition of the culture extract, the substrate growth stage, the presence of L-tryptophan (200 µg/mL) which is an essential amino acid synthesized in plant chloroplasts and used as a precursor by bacteria to produce IAA, are all fundamental to the amount of IAA produced by bacteria in the synthetic media (Ahmad *et al.* 2008).

The PGPR *in-vitro* screening produced three isolates (B045, B116, and B210) with 7 characteristics, namely N_2 fixation, ACC-deaminase, cellulolytic activity, IAA production, solubilization index, P available, and PMEase activity (Tables 2 & 3; Figs. 1 & 2). These results were superior to that of Kusumawati *et al.* (2017) having only 1 isolate with 4 PGPR characteristics, namely N_2 fixation, cellulolytic activity, IAA production and solubilization index.

Bioassay in Green House (*In-vivo* Screening of PGPR)

The isolates were tested *in-vivo* and were found positive for their effectiveness as PGPR (Table 4). All the isolates tested on the soybean plants showed positive effects on plant height, plant dry weight and number of flowers compared to control (-N). These results were inferior compared to control (+N) and plant growth was perceived to be smaller. This was caused by the N from chemical fertilizers which was easily absorbed by the plant roots compared to the N produced by nitrogen fixing bacteria wherein more time was needed for the roots to absorb. Chemical fertilizers showed an immediate impact on the plant growth, however, the environment got contaminated in the process. Although the biological organic fertilizers, i.e., fixation of N with the help of bacteria, showed slow plant growth, these are environmentally friendly and produced plants that are safe for human consumption. The PGPR contained in bio-stimulant Mix (14 isolates) increased the soil fertility by raising the population of bio-fertilizer bacteria, boosting root nodule formation, increasing soil pH, and by positively improving soybean growth and production, even in a marginal soil (Widawati *et al.* 2015).

The PGPR screening (*in-vivo*) on soybean has obtained the best performing isolates which were LIPI2-3-B116 and LIPI2-3-B210 (single isolate). The isolate had similar efficacy as that of the inorganic fertilizer "NPK" and comparative isolate (isolate number 4167= *Bradyrhizobium* sp.) (Table 4). These were the PGPR isolates with the highest IAA yield, P available, and highest PMEase (Widawati, 2015). Similar results were also observed in the study on *Bradyrhizobium japonicum*, which produced IAA, available P, and highest PME-ase, also had the highest positive effect on the plant growth (Marinković *et al.* 2016).

NifH Genes Detection and Identification

The identification and detection of *nifH* gene on the eight isolates from root nodules and rhizosphere in the Pancar Mountain region, as well as the four isolates from the soil in the Mahakam river and Bangkirai Hills, Kalimantan, resulted in two *Rhizobium radiobacter* strains, two *Burkholderia anthina* strains, *Ochrobactrum pseudogrignonense, Pseudomonas beteli, Klebsiella* sp., *Klebsiella variicola, Enterobacter* sp., *Pantoea cypripedii, Tolumonas osonensis* (presumably a new species), and *Mangrovibacter plantisponsor* (Table 5).

Table 4 Stimulating bacterial activity in soybean growth (*in-vivo* screening of PGPR)

No	Isolate code	High (cm)	Dry weight per plant (g)	Number of flowers
1	LIPI2-3-B045	41.16 ± 0.1^{cf}	4.84 ± 0.4^{ab}	15.5 ± 2.9^{de}
2	LIPI2-3-B054	37.24 ± 2.0^{ed}	3.87 ± 0.0^{ab}	15.16 ± 0.1^{de}
3	LIPI2-3-B100	31.33 ± 0.1^{b}	3.05 ± 0.1^{ab}	10.5 ± 0.3^{cd}
4	LIPI2-3-B116	44.83 ± 0.1^{fg}	5.55 ± 0.1^{ab}	23.16 ± 0.1^{f}
5	LIPI2-3-B136b	42.00 ± 1.5^{df}	4.20 ± 0.1^{ab}	16.66 ± 0.1e
6	LIPI2-3-B144a	38.66 ± 0.1^{de}	4.10 ± 0.5^{ab}	15.5 ± 0.3^{de}
7	LIPI2-3-B146	38.33 ± 0.1^{de}	3.66 ± 0.4^{ab}	10.5 ± 0.2^{cd}
8	LIPI2-3-B184a	32.69 ± 0.3^{bc}	3.33 ± 0.1^{ab}	3.16 ± 0.6^{ab}
9	LIPI2-3-B157	37.25 ± 0.1^{ed}	3.54 ± 0.3^{ab}	15 ± 2.9^{de}
10	LIPI2-3-B183a	32.70 ± 0.5^{bc}	3.06 ± 0.0^{ab}	6.66 ± 0.0^{bc}
11	LIPI2-3-B188a	37.66 ± 0.0^{ed}	3.55 ± 0.1^{ab}	15 ± 3.0^{de}
12	LIPI2-3-B210	44.33 ± 0.2^{fg}	4.90 ± 0.4^{ab}	20.33 ± 0.1^{ef}
13	4167 (comparative control)	46.00 ± 3.1^{fg}	5.58 ± 0.1^{ab}	25.00 ± 2.9^{f}
14	Control +N	47.16 ± 0.1^{g}	5.99 ± 0.5^{b}	22.50 ± 0.6^{f}
15	Control - N	17.88 ± 0.5^{a}	2.57 ± 0.3^{a}	0.33 ± 0.2^{a}

Note: Means with the same superscript do not significantly differ at $p < 0.05$.

Table 5 Identification of *nifH* genes and InaCC list code

Code	Scientific name by Eztaxon search	Pairwise similarity (%)	Taxonomic group	Nifh genes	InaCC number
LIPI2-3-B045	*Klebsiella variicola*	98.16	Gammaproteobacteria	Detected 1 *nifH* genes	InaCC B827
LIPI2-3-B054	*Pantoea cypripedii*	99.63	Gammaproteobacteria	Detected 1 *nifH* genes	InaCC B828
LIPI2-3-B100	*Teiumonas asonensis*	99.932	Gammaproteobacteria	Detected 1 *nifH* genes	InaCC B831
LIPI2-3-B116	*Klebsiella* sp.	100	Gammaproteobacteria	Detected 1 *nifH* genes	InaCC B833
LIPI2-3-B136b	*Rhizobium radiobacter*	99.65	Alphaproteobacteria	Undetected	InaCC B834
LIPI2-3-B144a	*Rhizobium radiobacter*	99.72	Alphaproteobacteria	Undetected	InaCC B835
LIPI2-3-B146	*Ochrobactrum pseudogrignonense*	100	Alphaproteobacteria	Undetected	InaCC B836
LIPI2-3-B184a	*Enterobacter* sp.	98.539	Gammaproteobacteria	Undetected	InaCC B837
LIPI2-3-B157	*Burkholderia anthina*	99.84	Betaproteobacteria	Undetected	InaCC B838
LIPI2-3-B183a	*Pseudomonas beteli.*	99.719	Gammaproteobacteria	Undetected	InaCC B839
LIPI2-3-B188a	*Bukholderia anthina*	99.863	Betaproteobacteria	Undetected	InaCC B840
LIPI2-3-B210	*Mangrovibacter plantisponsor*	99.927	Gammaproteobacteria	Detected 2 *nifH* genes	InaCC B841

The isolates LIPI2-3-B136b, LIPI2-3-B144a, LIPI2-3-B157 and LIPI2-3-B188a from the same material (root nodules) comprised two different genera (Table 3). The LIPI2-3-B136b and LIPI2-3-B144a isolates were identified as the genus *Rhizobium*, from the family "Alphaproteobacteria" and are known as root nodule bacteria. The LIPI2-3-B157 and LIPI2-3-B188a were identified as the *Burkholderia* of the "Betaproteobacteria" family. This was possible because the *Burkholderia* sp. also have the ability to form nodules. This confirmed the results of Moulin *et al.* (2001), that the strains of *Burkholderia* sp. (STM678 and STM815) showed nodulation ability.

Hence, it is generally accepted that legumes are not only nodulated by members of the *Rhizobiaceae* in the *Alphaproteobacteria*, but also by members of the *Betaproteobacteria* (usually called 'legume-nodulating *β-proteobacteria*' or 'β-

rhizobia"). The PCR result on several isolates (*Pantoea cypripedii, Tolumonas osonensis* and *Mangrovibacter plantisponsor*) detected the nifH gene and the presence of this gene indicated that the isolates have nitrogen fixation ability (Ash *et al.* 1993). *Pantoea cypripedii* isolates (LIPI2-3-B054), *Tolumonas osonensis* (LIPI2-3-B100), and *Mangrovibacter plantisponsor* (LIPI2-3-B210), showed positive nitrogenase activity (Table 2). Similar results were found in *cyanobacteria* (Ben-Porath & Zehr 1994) and *Paenibacillus azotofixans* (Rosado *et al.* 1998).

These results were also confirmed by the phylogenetic trees. The isolates showed the best ability both *in-vitro* and *in-vivo*, and had a significant impact on the soybean plants (Figs. 1 & 2).

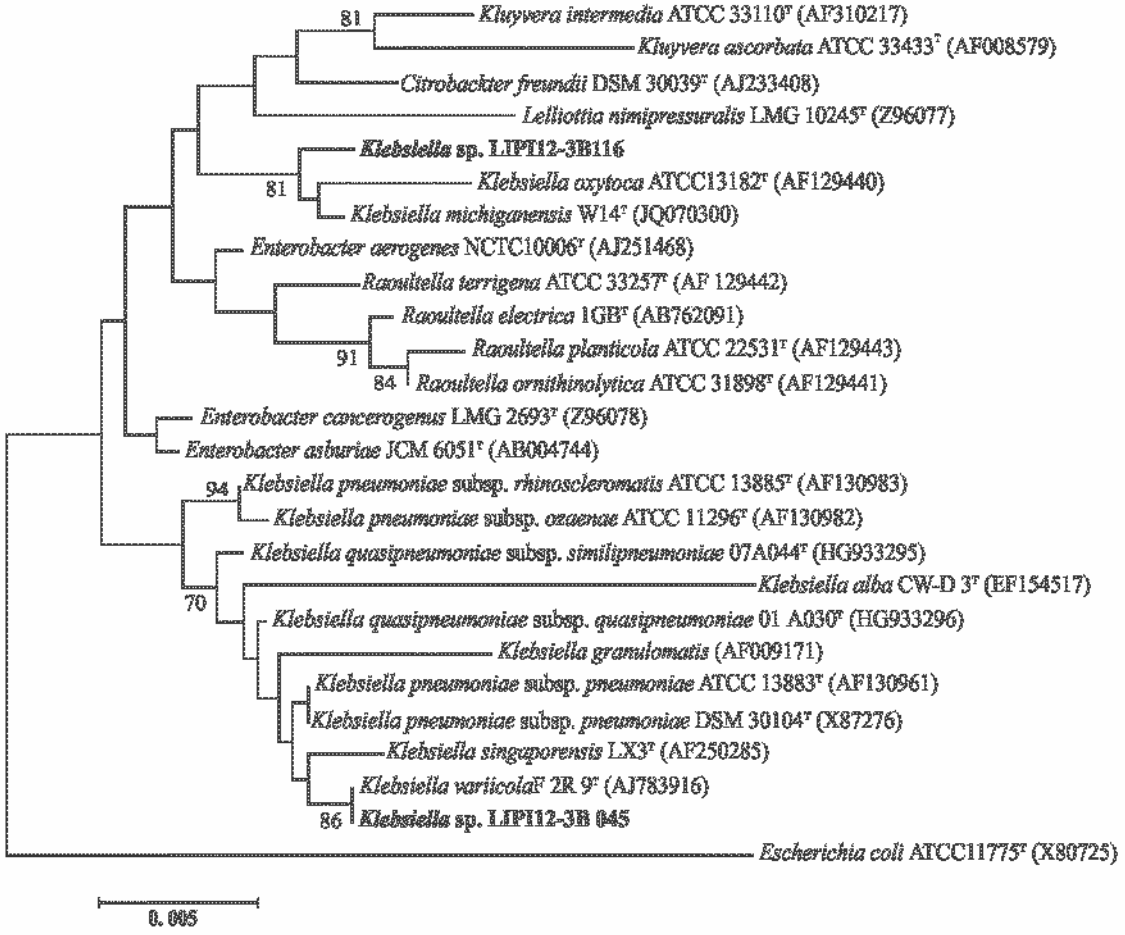

Figure 1 *Klebsiella* sp. LIPI12-3B045 performed by MIEGA6

Notes: Neighbor-joining tree based on rRNA gene sequences showing the phylogenetic positions of LIPI112-3B045 and related species in the family Enterobacteriaceae; *Escherichia coli* ATCC 11775T (X80725) was used as a group; Bar, 0.005 K_{mc}. Bootstrap values greater than 70 in 100 replicates are shown.

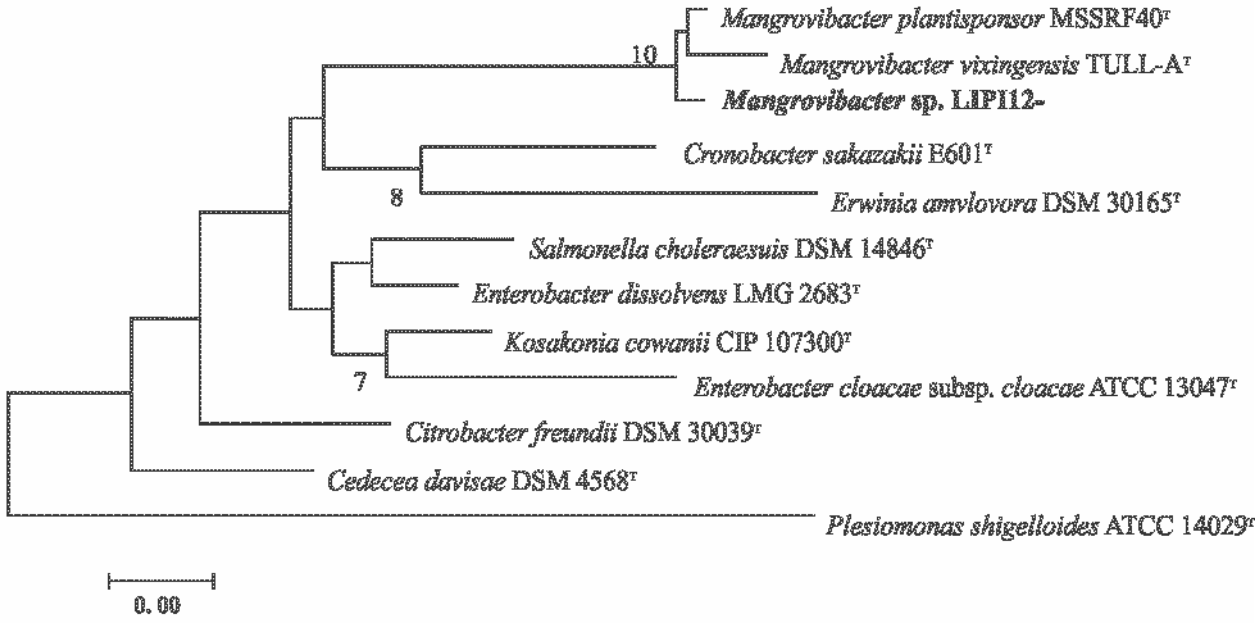

Figure 2 *Mangrovibacter* sp. LIPI2-3B210 performed by MIEGA6

Notes: Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of LIPI12-3B210 and related species in the family Enterobacteriaceae; *Plesiomonas shigelloides* ATCC 14029T (X74688) was used as an outgroup; Bar, 0.005 Knuc. Bootstrap values greater than 70 in 100 replicates are shown.

The phylogenetic tree showed that LIPI12-3B116 isolate was supported by bootstraps value 81% at branch points with *Klebsiella oxytoca* ATCC 13182^T that estimated the degree of confidence with the sequence of *Klebsiella oxytoca* ATCC 13182^T with bootstraps value greater than 70% (Fig. 1). The LIPI12-3B116 isolate is similar to *Klebsiella oxytoca* ATCC 13182^T sequences as shown in the database. The LIPI12-3B045 isolate was also supported by bootstraps value 86% with *Klebsiella variicola* F2R9^T (Fig. 1). The LIPI12-3B045 isolate has similarity with *Klebsiella variicola* F2R9^T sequences from the database with a confidence degree of 86%. The phylogenetic tree showed that LIPI12-3B210 isolate was supported by bootstraps value 100% at branch points with species of *Mangrovibacter yixingennis* TULL-A^T (Fig. 2). As seen in the 100 degrees of trust from the bootstraps value of more than 70, the isolate LIPI12-3B210 is similar to *Mangrovibacter yixingennis* TULL-A^T. However, the bootstraps value that appears in the phylogenetic tree, which is 100 and still branching, is showing that isolate LIPI12-3B210 is closer to *Mangrovibacter yixingennis* TULL-A^T than to *Mangroveibacter plantisponsor* MSSRF40^T.

CONCLUSION

Three isolates of soil and nodule bacteria were identified as B045 (*Klebsiella variicola* InaCC B827), B116 (*Klebsiella* sp. InaCCB833) and B210 (*Mangrovibacter plantisponsor* InaCC B841). The seven PGPR characteristics they exhibited include N2 fixation, ACC-deaminase, cellulolytic activity, IAA production, solubilization index, P available and PMEase activity. The greenhouse assay showed that the plant height, plant dry weight and number of flowers of the soybean seedlings significantly increased with *Bradyrhizobium* sp. strain 4167, then with *Klebsiella* sp. InaCC B833 and with *Mangrovibacter plantisponsor* InaCC B841. The bacterial isolates which were characterized and screened *in-vitro* for PGPR potentials and their representative isolates which were identified by 16S rRNA sequence analysis, are key factors in selecting PGPR isolates to be commercialized later as the future bio-stimulant.

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