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DOI: -

To appear in : BIOTROPIA Issue

Received date : 04 April 2019
Accepted date : 25 June 2019

This manuscript has been accepted for publication in BIOTROPIA journal. It is unedited, thus, it will undergo the final copyediting and proofreading process before being published in its final form.

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CHARACTERIZATION RHIZOBACTERIA ISOLATED FROM SOIL AND NODULES

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Running title: Characterization and identification of PGPR

ABSTRACT

The plant growth promoting rhizobacteria (PGPR) is a group of bacteria capable of colonizing plants roots thereby developing the system and improving plants growth and yield. The objectives of the study is to characterize PGPR activity of several bacterial isolates (in-vitro screening), examine their activities in stimulating soybean growth (in-vivo screening), and identify the potential of the bacteria. They 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 include 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.) with a completely randomized design. All bacterial isolates were identified using molecular methods based on nucleotide sequence generated from 16S rRNA gene. Three isolate 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. The isolates were B045 (*Klebsiella variicola* InaCC B827), B116 (*Klebsiella* sp. InaCC B833), and B210 (*Mangrovibacter plantisponsor* InaCC B841). The results in greenhouse assay showed that the plant height, plant dry weight and number of flowers in soybean seedlings significantly increased with *Bradyrhizobium* sp. strain 4167, followed by *Klebsiella* sp. InaCC B833 and *Mangrovibacter plantisponsor* InaCC B841.The bacterial isolates which were characterized and screened in-vitro for PGPR potentials and representative isolates which were identified by 16S rRNA sequence analysis is a key factor for selection of PGPR isolates to be used in the future commercialization as bio-stimulant.

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

INTRODUCTION

Bacterial characterization is one of the indicators in determining bacterial activity as plant growth promoting rhizobacteria (PGPR). This characterization 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 fixation mechanisms (Fischer *et al*. 2007), dissolving of insoluble phosphates with acidification and phosphomonoesterase (Krey *et al*. 2013)

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63 and NifH gene detection test as the main coding gene of nitrogenase. The gene converts the
64 atmospheric nitrogen (N_2) to ammonia which is readily used by organisms (Postgate 1998; Gilchrist
65 & Benjamin 2017). According to Adesemoye *et al*. (2008) and Bhardwaj *et al*. (2014), the
66 characterization of PGPR activities needs to be identified for the production of bio-fertilizer,
67 microbial rhizoremediation and effective bio-pesticides production.

68 Kloepper *et al*. (1980) was the first researcher to categorize *Pseudomonas*, the fluorescent
69 bacteria, which are able to fight against pathogenic bacteria and boost plant growth. This class of
70 organisms is regarded as an important PGPR group. All *Rhizobacteria* having the ability to directly
71 promote plant growth are included in this PGPR group (Kapulnik *et al.* 1981). This also covers other
72 genera of *Rhizobacteria*, especially those which increase plant growth through different mechanisms.
73 The *Rhizobacteria* group which form a symbiotic interaction with legumes through nitrogen fixation
74 and nodule formation are included in the sympathetic rhizobacteria category which consists of:
75 *Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium,* and *Actinomycetes* in symbiotic
76 relationship with Frankia plants (Babalola & Akindolire 2011). However, other nitrogen fixing, non-
77 symbiotyic PGPR, otherwise known as diazotrophs, living independently separated from plant cells
78 are included in the free-living rhizobacteria category (Babalola & Akindolire 2011) which includes
79 *Azoarcus, Azotobacter, Acetobacter, Azospirillum, Burkholderia, Diazotrophicus, Enterobacter,*
80 *Cyanobacteria, Pseudomonas* and *Gluconacetobacter (Anabaena, Nostoc), Alcaligenes, Bacillus,*
81 *Klebsiella, Arthrobacter,* and *Serratia* (Saharan and Nehra 2011; Haghighi *et al*. 2011; Bhattacharyya
82 & Jha 2012).

83 All these aforementioned bacterial genera are the functional indigenous group which colonize
84 plants rhizosphere or endo-rhizosphere and possess these three exceptional characteristics -
85 biofertilization, biostimulation, and biocontrol. The productions of Nitogen, Phosphorus, and IAA
86 are required for plant growth and adaptation in stressful condition and environment (Odoh & Kenneth
87 2017) such as in dry and saline soil (Mapelli *et al*. 2013), abiotic stress (Lau & Lennon 2011),
88 marginal (Redman *et al.* 2011), and soil packed with chemical fertilizers (Veen *et al*. 1997). These
89 also produce environmentally friendly renewable energy (Jackson 1999) which has the capacity to
90 sustain other agricultural practices (Noumavo *et al*. 2016) and essential for improving the soil
91 biological quality (Hayat *et al.* 2010) through biogeochemical cycles (Carbon, Nitrogen, Sulfur and
92 Phosphorus) thereby making the elements available in the soil (Banig *et al*. 2008).

93 The presence of Nitrogen and Phosphorus in the rhizosphere are important for plant growth
94 in stressful conditions (Mendez *et al*. 2007) and essential for its optimal growth, metabolism, and
95 development (Meena *et al*. 2014). Plant roots could only absorb Nitrogen and Phosphorus in the form
96 of Ammonium and inorganic Phosphorus and these elements are available for plants when both
97 nitrogen fixing bacteria and phosphate solubilizing bacteria are in the soil (Malusá & Vassilev 2014).

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98 Therefore, indigenous bacteria were sought from marginal and upland areas like hills as well as
99 lowlands like rivers, in the hope that they would survive as catalysts in the presence of elements
100 needed for plant growth in marginal soils.
101 The objectives of the study were to characterize PGPR activity of several bacterial isolates
102 (in-vitro screening of PGPR), to examine their activity in stimulating soybean growth (in-vivo
103 screening of PGPR), and to identify the the potential of PGPR bacteria.
104
105 ## MATERIALS AND METHODS
106 ### Sampling
107 The random sampling method was employed in this research which was conducted at Mount
108 Pancar area of Bogor-West Java (6º 35' 10.5 S & 106º 54' 46.8 E; 6º35' 03.9 S & 106º54' 40.6 E;
109 6º34' 30.1 S & 106º53' 3.1 E; 6º 34' 30.1 S & 106º 53' 3.1 E; and 6º29' 40.3 S & 106º5.0' 3.1 E.),
110 Wain (01º 05 00 S & 116º 52 00 E) and Mahakam rivers (0º 31' 11 S & 117º 07' 09 E), and Bangkirai
111 hill (1º 01'44.88 S & 116º 52' 02.2 E, East Kalimantan, Indonesia at 2012. The samples were taken
112 from nodules of *Paraserianthes falcataria* (L.) Fosberg (1 sample) , *Mimosa pudica* Linn (1 sample),
113 *Vigna sinensis* Endl. Ex Hassk (8 samples), *Arachis hypogaea* Linn. (1 sample), the rhizosphere of
114 *Mimosa pudica* Linn (1 sample) obtained from the soil around the roots to a depths of 20 cm, and soil
115 samples also taken from soil layer to a depths of 20 cm from Wain and Mahakam rivers and Bangkirai
116 Hill.
117
118 ### Isolation and Purification
119 The nodules were washed in ethanol 70 %, rinsed with sterile distilled water and then cut into
120 two parts planted in triplicate on a Petri dish containing selective medium (5 grams polypeptone + 5
121 grams yeast extract + 5 grams of glucose + 1 gram of $MgSO_4.7H_2O$ + 20 grams agar + 1 L Aquadest).
122 Plates were incubated for 5-7 days at room temperature. Otherwise 1 gram of soil sample is mixed
123 with 9 mL of sterile distilled water in a test tube on a vortex until homogeneous. After that the soil
124 extract from serial dilution (10^{-3}, 10^{-4}, 10^{-5}) were planted in triplicate on a Petri dish containing
125 selective medium (5 grams polypeptone + 5 grams yeast extract + 5 grams of glucose + 1 gram of
126 $MgSO_4.7H_2O$ + 20 grams agar + 1 L Aquadest) (Vincent, 1982).
127
128 ### Characterization of PGPR Activity as Screening of PGPR (In-vitro)
129 #### *Analysis of Nitrogen Fixation Activity*
130 The ability of bacteria to fix nitrogen was determined using the Dobereiner's method
131 (Dobereiner, 1995). The bacteria were placed into Luria Bertani Broth media and incubated for 5
132 days. This was transferred into a tube containing 5 mL of semi-solid Nitrogen Free Bromthymol blue

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133 (NFB) media at pH 7.0, and incubated at 28°C for 72 hours. The nitrogen fixation activity was shown
134 by the formation of mist-like white rings known as pellicles, below the surface of the NFB media in
135 the test tube (Jha *et al*. 2014).
136
137 ***Analysis of ACC-deaminase Production***
138 The production of ACC-deaminase was conducted by a qualitative test using the Dworkin and
139 Foster method (1958). In this method, Dworkin-Foster salt media enriched with 0.3033 grams of 1-
140 Aminocyclopropane 1-Carboxylic Acid (ACC) substrate and 2 grams of Ammonium Sulfate were
141 used as Nitrogen sources (Glicks *et al*. 1995). The ability of Diazotrophic bacteria to fix atmospheric
142 N2 (in-vitro) was shown by the production of ACC-deaminase (positive). In the negative control
143 Escherichia coli was used (grown on DF + ACC media). The growth of the bacterial colonies on DF
144 + ACC media after 72 hours of incubation was an indication of the production of ACC-deaminase.
145
146 ***Analysis of Indole Acetic Acid Production***
147 In the method used, bacteria was inoculated on semi-solid King B broth media mixed with
148 200 µg mL^{-1} Tryptophan for qualitative analysis of IAA production. Salkowski reagents was dropped
149 on the formed colony and stored in a dark room for 30 minutes (Gravel *et al.* 2007). The resultant
150 pink color was an indication of the production of IAA (Khan *et al*. 2014). Also, those bacteria capable
151 of producing IAA were analyzed quantitatively on Trypticase soy broth media (TSB: 20 mL)
152 containing L-Tryptophan (200 µg mL^{-1}). After 72 hours incubation, it shown a pink coloration
153 indicating the production of IAA on the bacterial extract (Mehboob *et al*. 2010) which was measured
154 using spectrophotometer at ʎ 540 nm with interpolation on IAA calibration curve (Pattern and Glick,
155 2002).
156
157 ***Analysis of Cellulolytic Activity***
158 The Cellulolytic activity of the bacteria was tested qualitatively using the Ponnambalam
159 method *et al.* (2011) with an indication of halo zone around the bacterial colonies.
160
161 ***Analysis of Inorganic Phosphate Solubilization***
162 The qualitative analysis of inorganic P solubilization was carried out by inoculating bacteria
163 on Pikovskaya solid media with 0.5% $Ca_3(PO_4)_2$ as a source of P on a Petri dish, which was incubated
164 for 7 days (Chen *et al*. 2006). The formation of halo zone around the bacterial colonies indicated that
165 P dissolved from the Ca^{2+}. Then, the solubilization index (SI) was obtained using the formula: SI =
166 Colony diameter + halo zone diameter / colony diameter (Premono and Vleck, 1996). Also,
167 quantitative analysis was carried out by inoculating bacteria on Pikovskaya liquid media and

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168 orthophosphate in a solution, and measured after incubating for 3, 6, and 9 days (Vassileva *et al.*
169 2000).
170
171 ***Analysis of Phosphomonoesterase (PME-ase) Activity***
172 The analysis of extracellular activity of PME-ase in acid by adding 0.5 M $CaCl_2$ and alkaline
173 by adding 0.5 M NaOH) to a tube inoculated with bacteria. Then, 0.115 M p-nitrophenyl phosphate
174 disodium (pNPP) was added as stated by Tabatabai and Bremner (1969) method. The absorbance of
175 pNPP formed a yellow coloration upon incubation for 7 days. This was measured using a
176 spectrophotometer at the wavelength of 400 nm. The PME-ase activity unit is defined as µmol h^{-1},
177 which is the amount of p-nitrophenol released in 1 mL extracellular enzyme solution fractionated
178 from 1.0 mL of culture after 7 days of incubation (Widawati & Sudiana, 2016).
179
180 **Bioassay in Green House as Screening of PGPR (In-vivo)**
181 Bioassays in the greenhouse (in-vivo) was prepared using the PGPR bacteria showing good
182 potential from the in-vitro test, and were treated on soybean (*Glycine max* L.) Merr. var Grobogan as
183 the test plant. The experiment was a completely randomized design with liquid inoculant as the
184 treatment having a single isolate (These are the isolate codes: LIPI12-3 B136, LIPI12-3 B144,
185 LIPI12-3 B146, LIPI12-3 B157, LIPI12-3 B183, LIPI12-3 B188, LIPI12-3 B045 , LIPI12-3 B054,
186 LIPI12-3 B100, LIPI12-3 B116, LIPI12-3 B210), *Bradirhizobium* sp. 4167 for the purpose of
187 comparison and the control treatments was without inoculants plus N (KNO_3 0.05 %) and while at
188 the other times, minus N. Each treatment was repeated 10 times and the procedures include: 1).
189 Soybean seeds were cleaned with ethanol 70 % and soaked in sterile distilled water for one hour. 2).
190 Seeds were placed on wet filter paper in a sterile Petri dish, and incubated at room temperature until
191 the seed germinate. 3). The sprouts were soaked for 1 hour in 25 ml of every liquid culture of inoculant
192 treatment (bacterial population 10^9 cfu mL^{-1}) from the tested bacteria. 4). The sprouts were planted
193 in pots (2 sprouts per pot) filled with sterile sand covered with sand containing paraffin (to avoid
194 contamination). 5). The humidity of the planting media was maintained at 24% by watering with
195 Müller's solution every day through capillaries installed on the pot and covered with cotton.
196 Application of nitrogen to control plants without inoculants were carried out by watering Müller's
197 solution added with KNO_3 0.05 % (Saono *et al*. 1976). Plant growth measurements like the plant
198 height, plant dry weight and number of flowers were taken 2 months after seedling. The resultant data
199 was analyzed with SPSS software version 16.0 and the significant differences in treatment were
200 determined by Duncan's Multiple Range ($p \leq 0.05$).
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Identification and NifH Genes Test of Bacteria

The identification of bacteria was performed with reference to Otsuka (2008) method using the 16S rRNA gene sequence with 16S-9F (5'GAGTTTGATCCTGGCTCAG3') with 16S-1510R (5'GGCTACCTTGTTACGA3') and 16S-785F (5'GGATTAGATACCCTGGTA3') with 16S-802R (5'CCTCTCTATGGGCAGTCGGTGAT3') primers. The phylogenetic affiliations and taxonomical hierarchy based on 16S rRNA were determined with 95% confidence using the CLASSIFIER tool (<http://rdp.cme.msu.edu>) of RDP-II database (Cole *et al*. 2009). The NifH genes test of bacteria was performed using the Poly *et al*. (2010a, 2010b) method with nifHf 5'-GGCAAGGGCGGTATCGGCAAGTC-3'and nifHr 5'-CCATCGTGATCGGGTCGGGATG-3'.

RESULTS AND DISCUSSION

The results of isolation and purification functional characterization of PGPR activity (nitrogen fixation activity, analysis of ACC-deaminase, IAA production, cellulolytic activity, inorganic phosphate solubilization, and phosphomonoesterase activity) and bioassay in Green house results are shown in Tables 1, 2, 3, 4 and Figures 1 and 2.

Isolation and Purification

The isolation showed various physical characteristics like shape, color, and size of different bacterial colonies. There were 12 isolates of indigenus bacteria consisted of 6 isolates (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 -20ºC for identification, and these were LIPI-12-3 isolates: B045, B8054, B100, B116, B136b, B144a, B146, B184a, B157, B183a, B188a and 210 (Table 1).

Tabel 1. Isolation result and isolates number

No	Source of sample	Isolat 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 (G. Pancar)	2a	LIPI12-3-B136b
6	Root nodule of *Mimosa pudica* Linn (G. Pancar)	4a	LIPI12-3-B144a

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7	Rhizophere of *Mimosa pudica* Linn.(G. Pancar)	4c	LIPI12-3-B146
8	Soil (G. Pancar)	12a	LIPI12-3-B184a
9	Root nodule of *Vigna sinensis* Endl. Ex Hassk. (G. Pancar)	7b	LIPI12-3-B157
10	Rhizosphere of *Vigna sinensis* Endl. Ex Hassk. (G. Pancar)	11h	LIPI12-3-B183a
11	Root nodule of *Arachis hypogaea* Linn.(G. Pancar)	13a	LIPI12-3-B188a
12	Soil (Mahakam river, Balikpapan, East Kalimantan,Indonesia)	33Na	LIPI12-3-B210

229

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

231 The results of isolates screening for nitrogen fixation, ACC-deaminase, cellulolytic activity,
232 IAA production, solubilization index (SI), available P, and PMEase in acid and alkaline activity, are
233 shown in tables 2 and 3.

234

235 Table 2 Screening 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	+	+	+

236

237 Table 3. Screening isolates for IAA, EP, P available, and PMEase activity

No	Isolate code: LIPI-2-3-	IAA after 48 H (ppm)	(SI)	P available (ppm)	PMEase activity (unit)	
					Acid	basa
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}

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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}

238 Note: The number followed by the same letter are not significantly different at *($p<0.05$) level of*
239 *Duncan's test.*
240

241 The in-vitro screening resulted in eight isolates (B045, B054, B100, B8116, B136b, B146,
242 B183b, and B210) which were positive for nitrogen fixation activity, indicated by the formation of
243 pellicle-like white ring under the surface of NFb media in the test tube after 5 days incubation, as
244 shown in Figure 1. Then, six isolates (B045, B116, B184a, B157, B188a, and B210) were positive
245 for the production of ACC deaminase, indicated by the formation of colonies in DF + ACC media
246 after 48 hours of incubation, as also shown in Figure 1. Another eight isolates (B045, B054, B100,
247 B116, B136b, B146, B183a, and B210) were positive for cellulolytic activity, indicated by the
248 formation of halo zone around the colonies on CMC media, as shown in Figure 1. Also, nine isolates
249 (B045, B116, B136b, B144a, B184a, B157, B183a, B188a, and B210) exhibited the ability as
250 phosphate solubilizing bacteria in the Pikovskaya solid media, indicated by the formation of some
251 halo zones around the colonies, as shown in Figure 1, as well as in Pikovskaya liquid media after 5
252 days incubation as shown in Table 3. All the twelve isolates produced the growth hormone IAA
253 indicated by the formation of a reddish pink color after the bacterial colonies were given Salkowski
254 reagent and incubated for 3 hours in a dark room, as shown in Figure 2. Therefore, the presence of
255 this IAA is the major indication that these bacteria have the potential as PGPR. This is in line with
256 statements from Abbas *et al*. (2011) and Mano & Nemoto (2012), that the main indication of the
257 PGPR group is the isolates ability to produce IAA, since it was the first identified plant hormone that
258 promotes growth in plants. Also, the quantitative analysis of IAA resulted in its maximum amount
259 ranging from 76.56 - 104.96 ppm. This was achieved after 48 hours of incubation and the highest
260 IAA yield of 104.96 ppm was produced by isolates B116 and B210. The IAA yield in this study was
261 higher than that of Kishi *et al.* (2012) (58.34 ppm), Widawati (6.08 ppm), Widawati and Sudiana
262 (2016) (9.56 ppm), and Suliasih and Widawati (2017) (19.98 ppm). A fluctuating yield may be
263 influenced by the condition of bacterial culture in the synthetic media. These results were in
264 accordance to the study conducted by Ahmad *et al.* (2008), that the condition of the culture extract,
265 the substrate growth stage, the presence of L-tryptophan (200 µg mL^{-1}) which is an essential amino
266 acid synthesized in plant chloroplasts and used as a precursor by bacteria to produce IAA, are all
267 fundamental to the amount of IAA produced by bacteria in the synthetic media.

268 The general results of PGPR in-vitro screening produced three isolates with 7 characteristics
269 of PGPR tested (N_2 fixation, ACC-deaminase, cellulolytic activity, IAA production , solubilization
270 index, P available, and PMEase activity), the isolates were B045, B116, and B210 as shown in tables
271 2 and 3, as well as in figures 1 and 2. This result - 3 isolates with 7 PGPR characters - was superior

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272 to the result of the research conducted by Kusumawati *et al.* (2017) having only 1 isolate with 4
273 characteristics of PGPR (N_2 fixation, cellulolytic activity, IAA production, and solubilization index).
274

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

276 The results of the effectiveness test of PGPR on the soybean plants are presented in Table 4,
277 and it is obvious that all the isolates tested in-vitro proved to be effective as PGPR.

278 All the isolates tested on the soybean plants showed positive effects for plant height, plant dry
279 weight, and number of flowers compared to control (-N). The results were inferior compared to
280 control (+ N) and plant growth was perceived to be smaller. This was as a result of the N from
281 chemical fertilizers which was easily absorbed by the plant roots compared to the N produced by
282 nitrogen fixing bacteria. More time was needed for the roots to absorb it. Thus, chemical fertilizers
283 showed an immediate impact on the plant growth although the environment got contaminated in the
284 process. However, the biological organic fertilizers - fixation of N with the help of bacteria - showed
285 slow plant growth, environmentally friendly and produced plants safe for human consumption.
286 According to Widawati *et al.* (2015), PGPR contained in bio-stimulant Mix (14 isolates) increased
287 soil fertility by raising the population of bio-fertilizer bacteria, boosting root nodule formation,
288 increasing soil pH, and positively improving soybean growth and production, even in a marginal soil.
289

290 Tabel 4. Examine bacteria activity in stimulating soybean growth (in-vivo screening of PGPR)

No	Isolate code	High (cm)	Dry weight per plant (g)	Number of flower
1	LIPI2-3-B045	41.16±0.1[df]	4.84±0.4[ab]	15.5±2.9[de]
2	LIPI2-3-B054	37.24±2.0[cd]	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[cd]	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[cd]	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	Kontrol +N	47.16±0.1[g]	5.99±0.5[b]	22.50±0.6[f]
15	Kontrol - N	17.88±0.5[a]	2.57±0.3[a]	0.33±0.2[a]

291 Note: The number followed by the same letter are not significantly different at *($p<0.05$) level of*
292 ***Duncan's test.***
293

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The PGPR screening (in-vivo) on soybean obtained isolates with the best performance, which were LIPI2-3-B116 and LIPI2-3-B210 (single isolate). The isolate had similar efficacy like the chemical fertilizer "NPK" and comparative isolate (isolate number 4167= *Bradyrhizobium* sp.) as shown in Table 4. These isolates were PGPR with the highest IAA yield, P available, and highest PMEase. Similar results were also observed in a study conducted by Widawati *et al.* (2015) which showed that the isolates, *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 isolated from the soil in the Mahakam river and Bangkirai Hills, Kalimantan, resulted in two *Rhizobium radiobacter* strains, two *Bukholderia anthina* strains, *Ochrobactrum pseudogrignonense* , *Pseudomonas beteli, Klebsiella sp., Klebsiella variicola, Enterobacter sp., Pantoea cypripedii, Tolumonas osonensis (*presumably a new species*),* and *Mangrovibacter plantisponsor* as shown in Table 5.

Tabel 5. Identification of Bacterial , strain list detected *nifH* genes test, and list code of InaCC

Code	Scientific name by Eztaxon search	Pairwise similarity (%)	Taxonomic group	Nifh genes	InaCC number
LIPI2-3-B045	*Klebsiella variicola*	98.16	Gammaproteobacteria	Detected nifh genes 1	InaCC B827
LIPI2-3-B054	*Pantoea cypripedii*	99.63	Gammaproteobacteria	Detected nifh genes 1	InaCC B828
LIPI2-3-B100	*Tolumonas osonensis*	99.932	Gammaproteobacteria	Detected nifh genes 1	InaCC B831
LIPI2-3-B116	*Klebsiella* sp.	100	Gammaproteobacteria	Detected nifh genes 1	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

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LIPI2-3-B210	*Mangrovibacter plantisponsor*	99.927	Gammaproteobacteria	Detected nifh genes	2	InaCC B841

311

312 The identification in Table 3 showed that LIPI2-3-B136b, LIPI2-3-B144a, LIPI2-3-B157, and
313 LIPI2-3-B188a isolated from the same material (root nodules) comprised of two different genera.
314 LIPI2-3-B136b and LIPI2-3-B144a isolates were identified as the genus *Rhizobium,* from the family
315 "Alphaproteobacteria" and are known as root nodule bacteria. But LIPI2-3-B157 and LIPI2-3-B188a
316 were identified as the *Burkholderia* of the family "Betaproteobacteria". This was possible since
317 *Rhizobium* and *Bradyrhizobium* are not the only noodles-forming bacteria, the *Burkholderia* sp. also
318 have that ability. This is in line with the results of Moulin *et al.* (2001), that *Burkholderia* sp. STM678
319 and STM815 strains showed nodulation ability.

320 Since then, it is generally accepted that legumes could not only be nodulated by members of
321 the *Rhizobiaceae* in the *Alphaproteobacteria* but also by members of the *Betaproteobacteria* (usually
322 called ‘legume-nodulating β-*proteobacteria*’ or ‘β-rhizobia‘). The identification of PCR result on
323 several isolates (*Pantoea cypripedii, Tolumonas osonensis*, and *Mangrovibacter plantisponsor*)
324 detected the nifH gene and the presence of this gene indicated that the isolates have nitrogen fixation
325 ability (Ash *et al.* 1993). *Pantoea cypripedii* isolates (LIPI2-3-B054), *Tolumonas osonensis* (LIPI2-
326 3-B100), and *Mangrovibacter plantisponsor* (LIPI2-3-B210), showed positive nitrogenase activity as
327 shown in Table 2. Similar results were found in *cyanobacteria* (Ben-Porath & Zehr 1994) and
328 *Paenibacillus azotofixanz* Rosado *et al*. 1998).

329 The identified results can also be confirmed from the phylogenetic trees. Thus, the results of
330 this study from these trees showed isolates with the best ability to activities test, both in-vitro and in-
331 vivo, and had a significant impact on the soybean plants as shown in figures 1 and 2.

332

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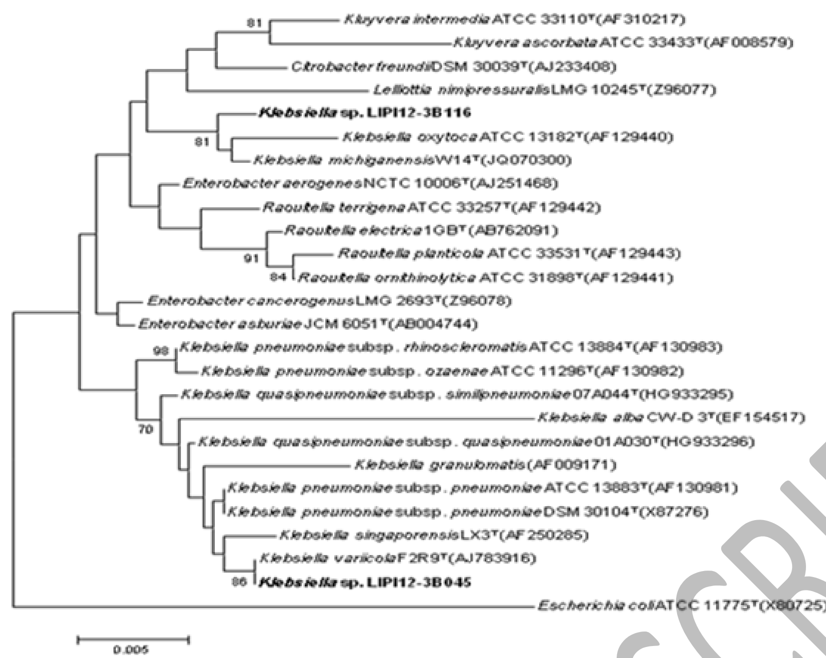
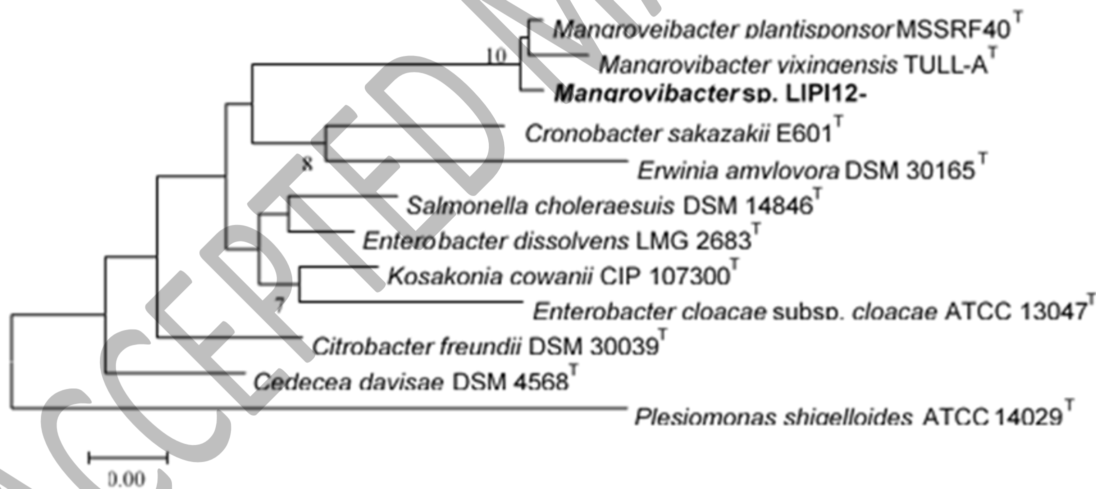


333

334 Figure 1 *Klebsiella* sp. LIPI12-3B045 performed by MIEGA6. Neighbour-joining tree based on
335 rRNA gene sequences showing the phylogenetic positions of LIPI112-3B045 and related
336 species in the family *Enterobacteriaceae*. *Escherichia coli* ATCC 11775^T (X80725) was
337 used as a group. Bar, 0.005 K_{nuc}. Bootstrap values greater than 70 in 100 replicates are
338 shown.

339

340



341

342 Figure 2 *Mangrovibacter* sp. LIPI2-3B210 performed by MIEGA6. Neighbour-joining tree based on
343 16S rRNA gene sequences showing the phylogenetic position of LIPI12-3B210 and related
344 species in the family Enterobacteriaceae. Plesiomonas shigelloides ATCC 14029T
345 (X74688) was used as an outgroup. Bar, 0.005 Knuc. Bootstrap values greater than 70 in
346 100 replicates are shown.

347

348 The phylogenetic tree of Figure 1 showed that LIPI12-3B116 isolate was supported by
349 bootstraps value 81% at branch points with *Klebsiella oxytoca* ATCC 13182^T estimated the degree
350 of confidence with sequence of *Klebsiella oxytoca* ATCC 13182^T with bootstraps value greater than
351 70%. It showed that LIPI12-3B116 isolate is similar to *Klebsiella oxytoca* ATCC 13182^T sequences

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from the database. The same result shown in the Figure 1 for LIPI12-3B045 isolate was supported by bootstraps value 86% with *Klebsiella variicola* F2R9^{T}. The LIPI12-3B045 isolate has similarity with *Klebsiella variicola* F2R9^{T} sequences from the database with a degree confidence of 86%. The phylogenetic tree of Figure 2 showed that LIPI12-3B210 isolate was supported by bootstraps value 100% at branch points with species of *Mangrovibacter yixingennis* TULL-A^{T}. Since the degree of trust seen from the bootstraps value of more than 70 is 100, then the isolate LIPI12-3B210 is similar with *Mangrovibacter yixingennis* TULL-A^{T}. However, in the phylogenetic tree, the bootstraps value that appears there are 100 and still branching again, and seen the closest position with isolate LIPI12-3B210 more similar to *Mangrovibacter yixingennis* TULL-A^{T} compared *Mangroveibacter plantisponsor* MSSRF40^{T}.

CONCLUSION

Three isolates of soil and nodule bacteria with 7 characteristics of PGPR (N2 fixation, ACC-deaminase, cellulolytic activity, IAA production, solubilization index, P available, and PMEase activity) were successfully identified in this study. These isolates were B045 (*Klebsiella variicola* InaCC B827), B116 (*Klebsiella* sp. InaCCB833), and B210 (*Mangrovibacter plantisponsor* InaCC B841). The results in greenhouse assay showed that the plant height, plant dry weight and number of flowers in soybean seedlings significantly increased with *Bradyrhizobium* sp. strain 4167, followed by *Klebsiella* sp. InaCC B833 and *Mangrovibacter plantisponsor* InaCC B841. The bacterial isolates which were characterized and screened in-vitro for PGPR potentials and representative isolates which were identified by 16S rRNA sequence analysis is a key factor for selection of PGPR isolates to be used in the future commercialization as bio-stimulant.

ACKNOWLEDGEMENTS

This work was funded by JICA-JST SATREP 2011-2016. We thank Dr Shigeto Otsuka from the University of Tokyo for guidance throughout this research. We are grateful to Rinatu Siwi, Dinihari Indah Kusumawati, Ety Suryati, Gunawan for their assistance in laboratory works and Greenhouse. The authors also thank to Annisaa Justicia Mutiara Rany, Ria Anggun Tripambudi, Nurul Hasanah for technical help especially in data collection.

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