Journal of Microbiology and Antimicrobials Vol. 3(2), pp. 34-40, February 2011
Available online http://www.academicjournals.org/JMA
ISSN 2141-2308 ©2011 Academic Journals

Full Length Research Paper

Characterization of *Bacillus* sp. strains isolated from rhizosphere of soybean plants for their use as potential plant growth for promoting Rhizobacteria

Aris Tri Wahyudi[1]*, Rina Puji Astuti[1], Asri Widyawati[1], Anja Meryandini[1], and Abdjad Asih Nawangsih[2]

[1]Division of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University (IPB); Jl. Agatis-Kampus IPB Darmaga, Bogor 16680, Indonesia.
[2]Department of Plant Protection, Faculty of Agriculture, Bogor Agricultural University (IPB), Jl. Agatis-Kampus IPB Darmaga, Bogor 16680, Indonesia.

Accepted 10 February, 2011

Rhizobacteria of *Bacillus* species were isolated from the rhizosphere of soybean plant of Cirebon, Indonesia, and further examined for plant growth promoting activities. A total of 118 isolates identified as *Bacillus* sp., 90 isolates (76.3%) among them positively produced phytohormone indole acetic acid (IAA). The 12 isolates (13.3%) were selected, initially on the basis of germinating seed bioassay in which the root length, shoot length or number of lateral root of the seedling was enhanced significantly. All those 12 isolates produced siderophore and 11 isolates (91.7%) were able to solubilize phosphate. Furthermore, 3 isolates (25%) among them were able to inhibit the growth of *Fusarium oxysporum*, 9 isolates (75%) inhibited the growth of *Rhizoctonia solani*, and 1 isolate (8.3%) of *Bacillus* sp. inhibited the growth of *Sclerotium rolfsii*. DNA sequence analysis of 16S rRNA genes of those 12 isolates revealed that, all of them similar with *Bacillus* sp. cluster and was separately divided into four groups. This study has pointed out 12 isolates of *Bacillus* sp. that may be applicable as inoculants according to each supporting characters as growth promoter rhizobacteria.

Key words: Rhizobacteria, *Bacillus* sp., growth promoter, IAA, antifungal compounds, phosphate solubilization, siderophore, 16S rRNA.

INTRODUCTION

Microbial communities are abundantly present in rhizosphere or areas under the influence of the root and its close vicinity. The rhizosphere gives support to many active microbial populations capable of exerting beneficial, neutral or detrimental effects on plant growth (Whipps, 2001). Microorganisms such as bacteria inhabiting rhizosphere, which beneficially influence the growth of plants, are specifically known as plant growth promoting rhizobacteria (PGPR). PGPR can alter the stimulation of plant growth in direct or indirect mechanisms. Indirect effects are related to production of metabolites such as antibiotics, HCN, or siderophores. Direct effects are dependent on production of plant growth regulators, improvement in plant nutrients uptake, or promote induce systemic resistance (ISR) of the plant (Bloemberg and Lugtenberg, 2001).

Some genera of bacteria have been determined as PGPR including Bacillus, Pseudomonas, Azospirillum, Azotobacter, Bradyrhizobium, Rhizobium, etc. The mechanisms of PGPR-mediated enhancement of plant growth and yields of many crops are not yet fully understood. However, the possible explanations include: (i) ability to synthesize hormones like indole acetic acid (IAA) (Patten and Glick, 2002), gibberellic acid (Mahmoud et al., 1984) and cytokinins (Tien et al., 1979); (ii) the ability to produce ACC deaminase to reduce the level of ethylene in the roots of the developing plants, thereby increasing the root length and growth (Glick, 1995); (iii) asymbiotic nitrogen fixation (Kennedy et

*Corresponding author. E-mail: ariswa@ipb.ac.id. Tel/Fax: +62-251-8622833.

al., 1997); (iv) antagonism against phytopathogenic microorganisms by producing siderophores, β-1,3-glucanase, chitinases, antibiotics, fluorescent pigment and cyanide (Catellan et al., 1999; Pal et al., 2001); and (v) solubilization of mineral phosphates and mineralization of nutrients (Idriss et al., 2002).

Among PGPR cluster, *Bacillus* is one of the most potential genera due to their spore forming ability, thereby increasing the adaptation of *Bacillus* strain to commercial formulation and field application (Liu and Sinclair, 1993).

Bacillus is frequently isolated from rhizosphere, these species are also common plant endophyte. The gram-positive bacterium *Bacillus subtilis* is known to positively influence plant growth, vitality, and the ability of the plant to cope with pathogens often resulting in higher yield. *B. mucilaginous* has been observed for its capability in solubilizing potassium (Wu et al., 2005) and phosphate (Idriss et al., 2002). It has also been reported that wheat yield increased up to 30% with Azotobacter inoculation and up to 43% with *Bacillu*s inoculation, due to some exerted growth hormones such as indole acetic acid (IAA) (Kloepper et al., 1991). Other studies also revealed that inoculation of *Bacillus* with *Bradyrhizobium japonicum* enhanced the growth of soybean plant conducted to the increasing level of nodulation (Bai et al., 2003). Furthermore, Woitke et al. (2004) had proven the ability of *B. subtilis* to help hydroponically grown tomato plants to withstand salinity stress**.** The objectives of this study were to isolate and characterize *Bacillus* sp. from the rhizosphere of soybean plant, and to screen their abilities and the possession of direct and indirect plant growth promoting rhizobacteria attributes.

MATERIALS AND METHODS

Isolation of *Bacillus* from the rhizosphere

Rhizosphere soil samples were collected from soybean growing fields in Plumbon, Cirebon, West Java, Indonesia. *Bacillus* species were isolated using dilution method with Nutrient Agar medium (beef extract 3.0 g/L, pepton 5.0 g/L, agar 15 g/L). First dilution of soil was boiled at 80°C for 10 min in order to alter endospore formation of *Bacillus*. Each colony was assayed further for morphological and physiological characteristics including Gram reaction, endospore, and catalase enzyme activity. *Bacillus* species were estimated by morphologies and physiology characteristics based on Bergeys' Manual of Systematic Bacteriology (Clauss and Berkeley, 1986).

Measurement of IAA

IAA was quantified by the method of Patten and Glick (2002). *Bacillus* isolates were cultured in flasks containing 10 ml of nutrient broth supplemented with tryptophan (L-Trp) 0.2 mM and incubated at room temperature (25 to 28°C) for 48 h. The cultures were then centrifuged for 15 min at 10 000 rpm. Each 2 ml of the supernatant was mixed with 2 ml of Salkowski's reagent (150 ml H_2SO_4, 250 ml distilled water, 7.5 ml $FeCl_3.6H_2O$ 0.5 M) and incubated at room temperature for 30 min. The presence of IAA was determined by the development of pink color and the IAA concentration was measured spectroscopically at 520 nm and quantified in an IAA standard curve.

Seed germination assay

Seedling bioassay was done based on the method described by Dey et al. (2004) with slight modifications. The *Bacillus* isolates were grown in Nutrient Agar medium plate (Wahyudi et al., 2010) at room temperature for 24 h. The inoculants for treating seeds were prepared by suspending cells from agar plates in nutrient broth in order to gain approximately 10^9 cells/ml. Nine pre-germinated seeds per Petri dish with three replications for each treatment were prepared and subsequently drooped with 0.1 ml suspension of bacterial cell (approximately ~10^8 cells). Germinating parameters were measured after 7 days of incubation, including the length of the primary root and shoot, and numbers of lateral roots. The results of those measurements were analyzed statistically with one-way Analysis of Variance (ANOVA) and further analyzed with Duncan Test using SAS program.

Phosphate solubilization

Solubilization of tri-calcium phosphate was detected in Pikovskaya's Agar (Wahyudi et al., 2011). Each *Bacillus* isolate was streaked on the surface of Pikovskaya agar medium and phosphate solubilizing activity was estimated after 1 to 5 days of incubation at room temperature. Phosphate solubilization activity was determined by the development of the clear zone around bacterial colony.

Siderophore production

Siderophore production was tested qualitatively using chrome azurol S medium (CAS-medium) (Husen, 2003). Each *Bacillus* isolate was streaked on the surface of CAS agar medium and incubated at room temperature for 1 to 3 days. Siderophore production was indicated by orange halos around the colonies after the incubation, and this test was done in two replications.

Antagonism assay against phytopathogenic fungi

All *Bacillus* isolates were assayed for antifungal activities against *Fusarium oxysporum*, *Rhizoctonia solani* and *Sclerotium rolfsii* by using Potato Dextrose Agar (PDA) medium. *Bacillus* isolates were streaked on PDA medium 3 cm in distance opposite to pathogenic fungi inoculated at the center of the medium. The barrier between *Bacillus* isolate and fungi indicated antagonist interaction between them. Antagonist activity was investigated for 4 to 7 days after incubation at room temperature. The value of inhibition was measured using the formula described by Kumar et al. (2002) which is 1-(a/b) x 100% (a: distance between fungi in the center of Petri dish to *Bacillus* sp isolate, b: distance between fungi in the center of Petri dish to blank are without *Bacillus* isolate).

Sequence analysis of 16S rRNA genes

This analysis was conducted in order to investigate the species taxa of each potential isolate based on molecular assay. All potential isolates were chosen for this analysis. The isolation of genomic DNA was done with CTAB method (Wahyudi et al., 2010). Amplification of 16S-rRNA gene was carried out by PCR using specific primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and1387r (5'- GGG CGG WGT GTA CAA GGC-3') (Wahyudi et al.,

2010). Amplification will yield DNA fragment (approximately 1300 bp). The PCR condition was carried out for 30 cycles including pre-denaturation step for 2 min at 94°C, denaturation for 30 seconds at 92°C, annealing for 30 s at 55°C, polymerization for 1 min at 75°C and post PCR for 10 min at 75°C. All the PCR products were purified using QIAquick Gel Extraction Kit (Qiagen) and sequenced using ABI 310 (Perkin Elmer, USA). Similarity of each 16S-rRNA sequence was aligned against GenBank database by using BLASTN program. All the sequences were also aligned with ClustalW program for constructing a phylogenetic tree.

RESULTS

Isolation of *Bacillus* from the rhizosphere

In this study, a total 118 of *Bacillus* sp isolates have been isolated from the rhizosphere of soybean plant. All those isolates showed *Bacillus* sp characteristics either physiological or morphological criteria, including positive Gram reaction, endospore appearance, and catalase enzyme activities.

Measurement of IAA

As many as 90 isolates of *Bacillus* sp out of 118 isolates have been detected to be able to produce IAA in various concentrations. All those tested *Bacillus* sp showed IAA production in culture supplemented with tryptophan (Trp), in the range of 0.81 to 86.82 mg/L. *Bacillus* sp. designated as *Bacillus* sp. Cr-67 was able to synthesize IAA in the lowest level (0.81 mg/L) (Table 1), whereas Cr-4 was able to produce IAA in the highest level (86.82 mg/L) in the culture supplemented with L-Trp.

Seed germination assay

IAA producer isolates were determined for their plant growth promoting activity to soybean sprout. Among 90 isolates tested, 12 isolates significantly promoted soybean seedling growth. *Bacillus* sp. Cr-69 significantly promoted the length of primary root and number of lateral roots. Some other isolates showed partial sprout promoting activity. Four *Bacillus* sp isolates (Cr-33, Cr-67, Cr-68 and Cr-69) performed their ability to enhance the length of primary root, and seven isolates (Cr-66, Cr-67, Cr-68, Cr-22, Cr-28, Cr-31, and Cr-64) were determined for their ability to enhance the length of shoot and six isolates (Cr-24, Cr-44, Cr-69, Cr-71, Cr-22, and Cr-28) were observed to be able of enhancing the number of lateral root compared to the control (Table 1).

Phosphate solubilization and siderophore production

Out of 12 isolates that promoted soybean seedling significantly, 11 isolates showed phosphate solubilization activity. For siderophore production, we used the general method to investigate siderophore producing isolate. By using CAS medium, all 12 *Bacillus* sp isolates were able to produce siderophore. It confirmed by the development of orange halos surrounding those colonies (Table 2).

Antagonism assay against phytopathogenic fungi

Opposition assay was used to determine isolates that inhibit the growth of *R. solani*, *F. oxysporum* and *S. rolfsii*. One isolate showed inhibition activity against *S. rolfsii* (Cr-44), meanwhile numerous isolates were able to inhibit *F. oxysporum* and *R. solani* in various level of inhibition percentage. In this study, among 12 isolates of *Bacillus* sp that significantly promoted plant growth of soybean seedling, there were only 3 isolates (Cr24, Cr44, and Cr66) that were able to inhibit *F. oxysporum*, 9 isolates (Cr24, Cr31, Cr33, Cr44, Cr64, Cr66, Cr67, Cr68, Crb71) inhibited *R. solani*, and only 1 isolate of *Bacillus* sp (Cr44) inhibited *S. rolfsii* (Table 2).

Sequence analysis of 16S rRNA genes

Based on excellent results in previous PGPR assay, we have found 12 isolates (Cr-22, Cr-24, Cr-28, Cr-31, Cr-33, Cr-44, Cr-64, Cr-66, Cr-67, Cr-68, Cr-69 and Cr-71) that might be potential as inoculants as plant growth promoter. Each PGPR character is listed in Table 2**.** The amplification of 16S-rRNA gene of those 12 isolates yielded a specific band at approximately 1300 bp. DNA sequence analysis of 16S-rRNA genes of those isolates revealed that, all of them were similar with *Bacillus* sp cluster compared to GenBank databases (Table 2). On the phylogenetic tree construction, we used *B. subtilis* strain 168 and *B. subtilis* strain NH160 as representatives for well-known PGPR of *Bacillus* sp. According to the phylogenetic tree (Figure 1), all the plant growth promoter of *Bacillus* sp isolates were separately divided into four groups. Cr-28, Cr-67, Cr-31 and Cr-22 isolates were grouped separately and closely related to the *Bacillus pumilus* taxa. Meanwhile, Cr-44 was closely related to *B. subtilis* 168 or NH.160. Isolate Cr-24, Cr-69, Cr-68, Cr-33, and Cr-66 were clustered together and very diverse in taxa, and Cr-64 was separated with other *Bacillus* species.

DISCUSSION

Isolation of *Bacillus* species isolated from the rhizosphere area was widely studied previously. In this study, it was of interest to obtain sufficient information including the diversity and plant growth promoting activity of these *Bacillus* sp indigenously isolated from Indonesia. All *Bacillus* sp isolates collected from the rhizosphere of

Table 1. Rhizobacteria *Bacillus* sp isolates that are significantly promote the growth of soybean seedling.

No.	Isolates	IAA (mg/L)[a]	Germination assay[b]		
			Length of primary root (cm)	Length of shoot (cm)	Number of lateral root
1	Control		9.15a	9.31ab	45.70b
	Cr-24	15.16	12.21a	11.46ab	83.11a*
2	Control		10.47b	9.77a	47.63a
	Cr-33	3.25	15.58a*	10.94a	43.41a
3	Control		15.63a	11.45a	50.04b
	Cr-44	3.73	14.93a	11.20a	68.70a*
4	Control		13.94a	10.07a	61.52b
	Cr-22	3.45	16.94a	24.47b*	87.28a*
	Cr-28	12.16	11.54a	23.95b*	93.43a*
5	Control		13.31ab	10.91b	48.67ab
	Cr-31	5.45	14.73ab	13.58a*	66.95a
6	Control		10.02b	7.12b	53.19b
	Cr-69	4.32	14.51a*	9.57ab	78.81a*
	Cr-68	0.87	16.22a*	10.89a*	68.24ab
	Cr-67	0.81	15.55a*	11.50a*	65.00ab
	Cr-66	3.02	13.34ab	10.06a*	62.43ab
7	Control		13.44b	10.70c	65.14b
	Cr-64	7.56	14.68b	22.49ab*	73.53b
8	Control		13.02b	8.61b	52.24b
	Cr-71	9.63	22.25a*	14.39ab	96.86a*

Note: [a]Production of IAA in NB medium supplemented with L-Trp (0.2 mM); [b]Within the same column, means followed by the same letter are not different (*α:* 0.05) by Duncan test. Data are average from three replicates and each replicate consist of 9 seeds. * : Isolates which significantly enhanced length of primary root, shoot and/or number of lateral root.

soybean plant sample were confirmed further for their PGPR attributes. Some isolates showed potential activity of producing IAA, solubilizing phosphate, producing siderophore, promoting seed germination, and inhibiting the growth of plant pathogenic fungi. The ability of *Bacillus* sp isolates to use tryptophan supplemented in the cultivation medium is one of the important points to determine IAA producing activity. Tryptophan is the main precursor of IAA biosynthesis in bacteria via indole pyruvic acid (IPA) pathway (Patten and Glick, 1996). IAA is one of many secondary metabolites produced by bacteria, thereby the metabolite is observed abundantly at the stationary phase. The presence of Trp in the medium is an important factor for *Bacillus* sp isolates to produce IAA.

However, 28 isolates (23.7%) exhibited negative producing IAA ability, it might be due to loss of genetic and physiologic apparatus of IAA biosynthesis of those *Bacillus* sp isolates, whereas 90 *Bacillus* sp isolates (76.3%) were able to synthesize IAA at various concentration. The capability of Trp metabolism is different among bacterial isolates, presumably due to physiological properties. Furthermore, Patten and Glick (2002) have specified that, enzyme indolepyruvic decarboxilase (IPDC) is the principal enzyme which determines IAA biosynthesis. The activity of this enzyme is variously connected to protein structure or different level of ipdc gene expression. Several *Bacillus* sp isolates obtained from this study were also able to produce IAA constitutively, even in the culture without tryptophan supplementation. The ability of the isolates to increase plant growth in germinating seed bioassay is highly related to the IAA production, which was produced by *Bacillus* sp isolates. Varying results of germinating seed assay had also pointed out that, there was complex interaction between bacterial IAA and seedlings, therefore it caused different responses of plant growth (Table 1). Yet, there is stimulation of bacterial IAA to the development of the host plant root system (Patten and Glick, 2002). In addition, Patten and Glick (2002) also reported that, low levels of IAA can stimulate root elongation, while high levels of bacterial IAA, stimulate the formation of lateral and adventitious roots.

In this study, there were various levels of IAA concentration produced by 12 *Bacillus* sp isolates, which were shown to promote seed growth. However, IAA produced by those isolates was quite of less than 15.20 mg/L (Table 1). Therefore, it was difficult to determine the optimum IAA concentration that may promote seed growth. This may probably be due to those bacterial strains of *Bacillus* sp. The interactions between plant and bacterial IAA might be due to the fact that, IAA excreted

Table 2. Plant growth promoting attributes of the *Bacillus* isolates from the rhizosphere of soybean plants.

No.	Isolate code	Siderophore Production[a]	P-solubilizing activity[b]	Antagonism Assay[c]			Homolog with	% identity
				F. oxysporum	*S.rolfsii*	*R. solani*		
1	Cr-22	+	+	-	-	-	*Bacillus* AI 17	84
2	Cr-24	+	+	++	-	+++	*Bacillus* sp. 1Re28	85
3	Cr-28	+	+	-	-	-	*Bacillus pumilus* strain S2	91
4	Cr-31	+	+	-	-	++	*Bacillus pumilus* S6-05	98
5	Cr-33	+	+	-	-	+	*Bacillus* sp. NS-2	95
6	Cr-44	+	+	++	+	+	*Bacillus subtilis* CICC1016	92
7	Cr-64	+	+	-	-	++	*Bacillus sphaericus* NUC-5	85
8	Cr-66	+	+	++	-	+++	*Bacillus cereus* strain SS-07	94
9	Cr-67	+	-	-	-	+++	*Bacillus pumilus*	94
10	Cr-68	+	+	-	-	+++	*Bacillus cereus* KU206-3	94
11	Cr-69	+	+	-	-	-	*Bacillus cereus* isolat AD2	97
12	Cr-71	+	+	-	-	+	*Bacillus shandongensis* SD	99

Note: [a] : + : able to produce siderophores; [b] : + : able to solubilize P in Pikovskaya medium; - : not able to solubilize P; [c] : + : low inhibition percentage (<30%); ++ : moderate inhibition percentage (30%≤x≤40%); +++ : strong inhibition percentage (> 40%); -: no inhibition activity

by a bacterium may promote primary root, shoot growth, and increase lateral root directly by stimulating plant cell elongation or cell division, or indirectly by influencing bacterial Amino Cyclopropane-1-Carboxylate (ACC) deaminase activity (Patten and Glick, 2002). ACC deaminase produced by many plant growth promoting bacteria including *Bacillus* genera is involved in the promoting root elongation in seedlings. ACC deaminase hydrolyzes plant ACC, the immediate precursor of the phytohormone ethylene, and thereby prevents the production of plant growth-inhibiting levels of ethylene (Patten and Glick, 2002).

The ability of several isolates to solubilize tri-calcium phosphate *in vitro* shows the possible application of the isolates in crop fields. Rodriguez and Fraga (1999) demonstrated that, Pseudomonas and other phosphate solubilizing bacteria (PSB) like *Bacillus* sp were capable of increasing the availability of phosphorus in soil. Specifically, all isolates showed their potential to be developed as inoculants for alkaline soil, based on the ability to solubilize phosphate bounded by calcium which mostly exists in alkaline soils, whereas in the acidic soil, phosphate was mostly fixed by Fe or Al (Glodstein, 1995). Our study revealed that, all *Bacillus* sp isolates that significantly promoted soybean seedling, were able to solubilize phosphate, except one isolate (Cr67) which was unable to solubilize phosphate (Table 2). Siderophore is one of the biocontrol mechanisms belonging to PGPR groups, including *Bacillus* sp. under iron limiting condition. PGPR produces a range of siderophore which have a very high affinity for iron. Therefore, the low availability of iron in the environment would suppress the growth of pathogenic organisms including plant pathogenic fungi (Whipps, 2000). In addition to siderophore, there are other mechanisms of biocontrol including antibiotics compounds, elicitation of induced systemic resistance (ISR) of plant, and lytic enzyme secretion (Haas and Defago, 2005). This study has demonstrated that, the 12 *Bacillus* sp isolates classified as plant growth promoter, and produced siderophores. Based on the siderophore produced by those isolates, it has been determined that, all the isolates which produced the bioactive compound siderophore were able to inhibit the phytopathogenic fungi (Table 2). The results of this study suggest that, siderophore produced by those bacteria functions as suppressor to the growth of phytopathogenic fungi such as *F. oxysporum*, *R. solani*, or *S. rolfsii*. 16S rRNA sequence analysis showed that, all isolates were diverse in terms of species taxa. Interestingly, based on 16S rRNA sequence, all isolates of plant growth promoter of *Bacillus* sp were divided into four groups. Group 1 (Cr-28, Cr-67, Cr-31, and Cr-22) was closely

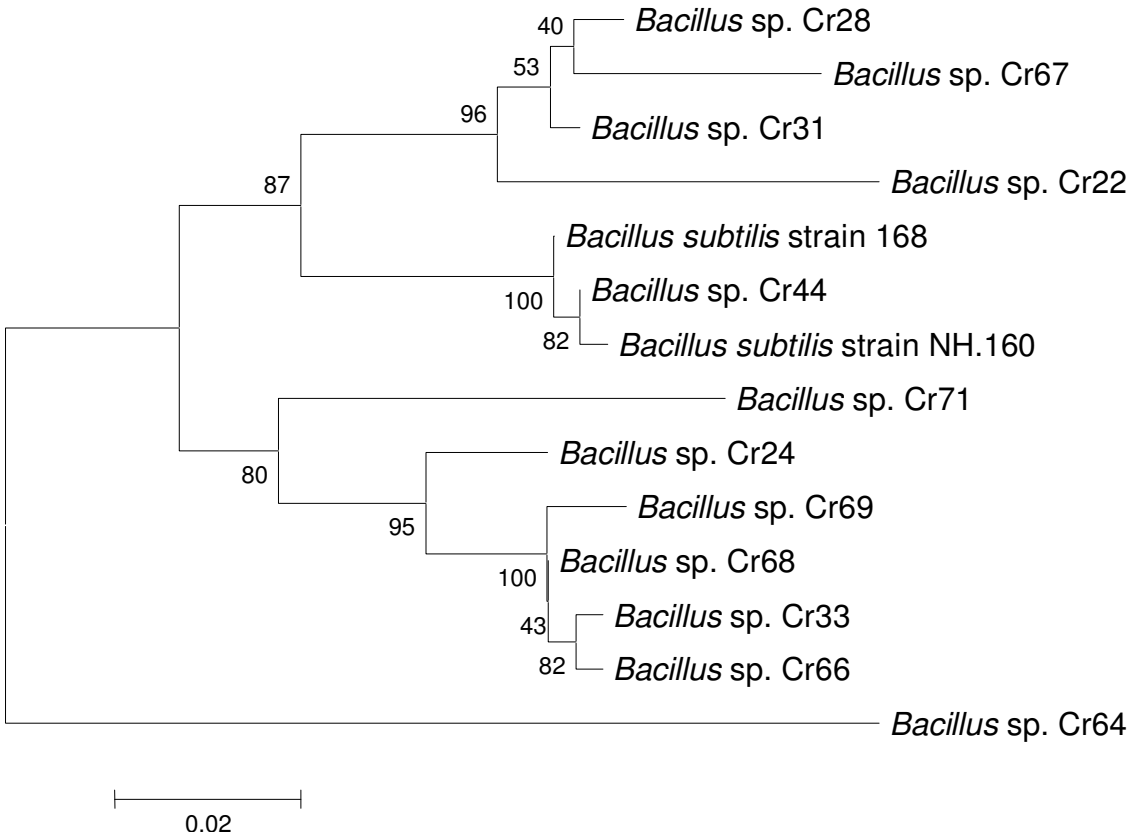

Figure 1. Dendrogram phylogenetic of *Bacillus* sp. isolates based on 16S rRNA sequence. All 12 isolates were divided into four groups. Group 1 (Cr-28, Cr-67, Cr-31, and Cr-22) were closely related to each other with *B. pumilus*, Group 2 (Cr-44) was closely related to *B. subtilis*, Group 3 was pretty diverse in taxa (Cr-71, Cr-24, Cr-69, Cr-68, Cr-33, and Cr-66) and Group 4 only contain of Cr-64, which identified as *B. sphaericus.*

related to each other with *B. pumilus*, Group 2 (Cr-44) was closely related to *B. subtilis*, Group 3 was also diverse in taxa (Cr-71, Cr-24, Cr-69, Cr-68, Cr-33, and Cr-66), and Group 4 only contained Cr-64, which was identified as *Bacillus sphaericus.* The PGPR characters of each isolated *Bacillus* sp was investigated in this study and according to the *in vitro* assay, it has been determined that the12 may have a role as plant growth promoter. All isolates were identified as *Bacillus* sp according to the homology analysis of 16S rRNA gene sequence with GenBank database. This research has also revealed the diversity of *Bacillus* sp in the rhizosphere of soybean plant and its potential to develop as inoculants under field conditions.

ACKNOWLEDGEMENT

This research was funded by Incentive Program for Basics Research (“Program Insentif Riset Dasar”) from the Ministry Department of Reseach and Technology (KNRT) of Indonesia to ATW. The authors thank and appreciate all the supports given to carry out this research.

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