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Screening and characterization of actinomycetes isolated from soybean rhizosphere for promoting plant growth

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Abstract. *Fatmawati U, Meryandini A, Nawangsih AA, Wahyudi AT. 2019. Screening and characterization of actinomycetes isolated from soybean rhizosphere for promoting plant growth. Biodiversitas 20: 2970-2977.* Actinomycetes which colonized plant rhizosphere has a vital role in improving plant growth by producing Indole-3-Acetic Acid (IAA). The aim of this study was to evaluate the potency of actinomycetes isolated from soybean rhizosphere as promoter agents for plant growth *in vitro*. Fifty actinomycetes isolates were successfully isolated from soybean rhizosphere. Based on the colorimetric methods, 35 isolates can produce IAA in various concentration, in the range of 0.46-30.6 mg/L. Seed germination assay using Ragdoll methods revealed that 26 isolates significantly promoted germination parameters, including the hypocotyl and the radicular length, the number of the lateral roots, and dry weight of the plant. Also, 14 from 26 isolates showed phosphate solubilizing activity in different phosphate-solubilizing index ranging from 1.25-2.62. Eight isolates were able to grow in N-free medium, indicating that these isolates have the ability in fixing nitrogen. Out of 23 from 26 isolates were detected to produce siderophore. All the tested isolates show chitinase production except ASR 55. Based on the observed parameters, it showed that there are four potential isolates (ASR 46, ASR 58, ASR 75 and ASR 76) as promising plant-growth promoters, phosphate solubilizer, nitrogen fixer, siderophore and chitinase producer. Based on the result of 16S rRNA sequence analysis, four potential isolates were identified as *Streptomyces* spp. in different taxa of strains and species.

Keywords: Actinomycetes, germination, IAA production, phosphate solubilizing, soybean rhizosphere

INTRODUCTION

Soybean is one of the important food crops after rice and maize in Indonesia (Aldillah 2015). The soybean consumption in Indonesia is around 2.2 million tons per year, yet domestic soybean production only sufficient for about 40% soybean consumption. Consequently, the government has to meet the soybean needs by importing soybean from other countries. National soybean productivity is relatively low at 1.57 tons/ha compared to the genetic potential of soybean crop productivity, i.e., 3 tons/ha (Balitkabi 2016). Low productivity may be caused by limited nutrient availability in the soil and infection of some phytopathogens. Besides, the application of eco-friendly cultivation technology is still limited.

Long-term application of synthetic fertilizer can cause the alteration of soil structure and ecological damage. On the contrary, the utilization of beneficial microbes proved to increase agricultural yields. Plant rhizosphere microbes are one of the microbial groups which extensively used for agricultural purposes. Rhizosphere microbes have a vital role in improving plant growth, directly and indirectly, commonly known as Plant Growth-Promoting Rhizobacteria (PGPR). The direct mechanisms occur through producing phytohormone, improving plant nutrient uptake, and inducing plant resistance whereas indirect mechanisms occur through the production of siderophores, extracellular enzymes, antibiotics, and HCN (Palaniyandi et al. 2013).

Actinomycetes are filamentous Gram-positive bacteria that widely distributed in various environments. The most dominant actinomycetes found in the rhizosphere are the genus *Streptomyces.* Other genera such as *Nocardia, Microbispora, Micromonospora, Actinomyces, Actinoplanes*, and *Streptosporangium* are also widely isolated from soil (Franco-Correa et al. 2010). This habitat provides rich nutrition released from root exudate, which useful for the growth of actinomycetes. As rhizobacteria, actinomycetes affect plant growth, against plant pathogens and increase nutrient availability (Shimizu 2011). The existence of actinomycetes in the plant rhizosphere can promote plant growth by producing plant growth-promoting hormones such as auxins or gibberellin. Auxin is a group of indole compounds that have the ability to improve plant growth by stimulating shoot elongation, root initiation, seed germination, and plant biomass (Anwar et al. 2016). Indole-3-Acetic Acid (IAA) is a natural auxin produced from L-tryptophan metabolism (Khamna et al. 2010) in several bacteria. Rhizosphere bacteria are more efficient in the production of auxin than bacteria isolated from bulk soils (Mohite et al. 2013). Most strains of *Streptomyces* spp. which are found in the rhizosphere of various plants are capable of producing IAA. Some studies reported that actinomycetes isolated from the rhizosphere

could stimulate plant growth through IAA production. Wahyudi et al. (2019) reported that several strains of *Streptomyces* spp. isolated from maize rhizosphere were able to produce IAA ranging from 1.67 µg/mL to 26.89 µg/mL and could stimulate maize germination. Applications of IAA-producing *Streptomyces* spp. as growth-promoting bacteria are also reported in tomatoes (El-Tarabily et al. 2008), wheat (Sadeghi et al. 2012), rice (Gopalakrishnan et al. 2013), and pomegranate (Poovarasan et al. 2013). Moreover, actinomycetes can promote plant growth by other mechanisms such as phytohormone production, iron chelation, phosphate solubilization, and nitrogen fixation (Gopalakrishnan et al. 2013; Wahyudi et al. 2019).

The aims of this study are to evaluate the potency of actinomycetes isolated from soybean rhizosphere in producing IAA, and stimulating soybean germination. Potential isolates were characterized by their abilities to solubilize phosphate, fix nitrogen, produce siderophore and chitinase enzyme. Promising strains with the best activity as plant growth-promoting rhizobacteria were identified using the 16S rRNA sequence analysis. Furthermore, the findings of potential rhizobacteria in this study can be utilized for biofertilizer candidates.

MATERIALS AND METHODS

Actinomycetes isolation

Soil samples of soybean rhizosphere were collected from Cisaat Village, Subdistrict Cicurug, Sukabumi-West Java. Rhizosphere actinomycetes were isolated by serial dilution method. One gram of soil sample was preheated at 60 °C for 30 minutes and diluted in 9 ml of 0.85% NaCl solution. The soil suspension was homogenized, and 1 mL of suspension subsequently transferred to a sterile tube to obtain 10^{-5} serial dilutions. A total of 100 µL of suspension from 10^{-3} to 10^{-5} of serial dilution were spread onto the Humid Acid-Vitamin medium (HV: 1 g humic acid dissolved in 40 mL NaOH 0.4%, 5 mL of vitamin B 200x solution , 0.02 g $CaCO_3$, 0.01 g $FeSO_4$, 0.05 g $MgSO_4$, 0.5 g Na_2HPO_4, 1.7 g KCl, 20 g agar, 955 mL sterile distilled water) (Hayakawa and Nonomura 1987). Cycloheximide (50 mg/mL) and nalidixic acid (50 mg/mL) were added to the media to minimize the contamination of non targeted bacteria and fungi. The actinomycete isolates obtained from the isolation process were then purified on The International *Streptomyces* Project (ISP-2) media (4 g of yeast extract, 10 g malt extract, 4 g of dextrose, 20 g agar, 1 L of sterile distilled water) and incubated at 28°C for further analysis. The morphology of the actinomycetes colony and the type of spore chain were observed using light microscope (OLYMPUS CX21, Tokyo Japan) with 400x magnification.

Hypersensitivity assay

The hypersensitivity assay was done by inoculated actinomycetes isolates on ISP-2 broth medium for seven days at room temperature (28˚C) and agitated at 110 rpm. The actinomycetes culture were injected on the tobacco leaves through the bottom sides using a sterile syringe (Wiraswati et al. 2019). *Xanthomonas oryzae* and *Escherichia coli* BL-21 were used as a positive and negative control, respectively. The hypersensitivity was observed at 3-5 days after inoculation. Isolates that stimulate necrotic or chlorotic reactions on the leaves were considered as a pathogen for plants and were not further analyzed. The hemolytic assay was also conducted by inoculating isolates on 5% Sheep Blood Agar and incubated for 2-3 days at room temperature (28˚C). Isolates which form clear zones around the actinomycetes colony indicate hemolytic activity and considered as a pathogen for mammals.

Measurement of IAA production

IAA production was quantified by the method described by Khamna et al. (2010) and Mohite (2013) with a slight modification of incubation time. Two-discs of seven-days old actinomycete colony on ISP-2 agar were inoculated into 50 ml ISP-2 broth. supplemented with 0.1 mg L^{-1} L-Tryptophan (L-Trp). The culture was incubated for ten days at room temperature (28 ˚C) on a rotary shaker. The culture was then centrifuged for 15 min at 11.000 rpm, and 2 mL of supernatant was mixed with 2 mL of Salkowski reagent (composition: 150 mL H_2SO_4, 7.5 mL $FeCl_3.6H_2O$ 0.5 M, and 250 mL distilled water). The presence of IAA was determined by the appearance of pink color, and the concentration of IAA was measured by a spectrophotometer at 530 nm wavelength and calculated using a standard curve.

Seed germination assay

Growth-promoting activity of isolates was evaluated using ragdoll method (Shreevidya et al. 2016). Initially, each actinomycete isolate was cultured in ISP-2 broth for ten days and agitated at 110 rpm. The soybean seeds (cv. Wilis) were sterilized using 95% alcohol for 5 min, followed by 2.5% sodium hypochlorite for 30 seconds and then washed using distilled water three times. The sterilized seeds were soaked on the actinomycete culture (10^8 CFU/mL) for 60 min. Seed germination assay was performed in triplicate. Thirty-five isolates were used for seed coating treatment contains nine soybean seeds, and placed them on wet paper towel, then folded in half and rolled into a round tube. The seed soaked on the uninoculated medium was used as negative control. After five days of incubation, several germinating parameters such as the length of the primary root, shoot, the numbers of lateral roots, and dry weight were measured. The data were then statistically analyzed with one-way Analysis of Variance (ANOVA) and further analyzed with Tukey's Test using SPSS 25 program.

Phosphate solubilizing assay

Supernatant from seven-days old actinomycetes culture was used in phosphate solubilizing assay. Thirty µL of supernatants were transferred into 8 mm well on the Pikovskaya agar medium (10 g Glucose, 5 g Ca_3 $(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g $MgSO_4$. H_2O, 0.002 g $FeSO_4.7H_2O$, 15 g Agar) and incubated for seven days at

room temperature (28 °C). The formation of halo zone around the colony indicated phosphate solubilizing activity. Phosphate solubilizing index was calculated by using the following formula (Saif et al. 2014):

$$\text{Solubilization Index (SI)} = \frac{\text{Colony diameter (mm) + Halo zone diameter (mm)}}{\text{Colony diameter (mm)}}$$

Nitrogen-fixing activity

The ability of isolates in fixing nitrogen was evaluated by culturing each isolate on the N-free medium which contains (per liter): 1 g K_2HPO_4, 3 g KH_2PO_4, 0.065 g $MgSO_4$, 0.01 g $FeCl_3.6H_2O$, 0.07 g $CaCl_2.2H2O$, 5 g dextrose, 240 x 10^{-6} g $Na_2MoO_4.2H_2O$, 3 x 10^{-6} g H_3BO_4, 1.83 x 10^{-6} $MnSO_4.H_2O$, 290 x 10^{-6} g $ZnSO_4.7H_2O$, 130 x 10^{-6} g $CuSO_4.5H_2O$, 120 x 10^{-6} g $CoCl_2.6H2O$, 15 g agar. The growth of Actinomycetes was observed after seven days of incubation (Sari et al. 2014).

Siderophore production assay

Siderophore production capability of ten isolates was determined by inoculating the actinomycete culture onto Chrome Azurol Sulfonate (CAS) agar (Louden et al. 2011) and incubated at 28˚C for three days. The orange color formation around actinomycetes colonies indicates the production of siderophore. *Bradyrhizobium japonicum* strain BJ11 was used as a positive control.

Chitinolytic activity test

Evaluation of the chitinolytic activity from actinomycetes isolates was carried by a qualitative assay using colloidal chitin agar according to the method by Amini et al. (2016). Each actinomycetes isolate was inoculated in Colloidal Chitin Agar (CCA) contained 0.4% colloidal chitin, 0.3 g/L KH_2PO_4, 0.7 g/L K_2HPO_4, 0.5 g/L $MgSO_4.5H_2O$, 0.01 g/L $FeSO_4.7H_2O$, 0.001 g/L $ZnSO_4.7H_2O$, 0.001 g/L $MnCl_2.4H_2O$ and 20 g/L Agar, pH was adjusted to 8.0-8.5. The inoculated plates were then incubated at 28˚C for seven days. Clear zones surrounding the actinomycetes colonies indicated chitinase activity was observed after seven days of incubation. All experiments were conducted in triplicate.

Molecular identification based on 16S rRNA gene analysis

Total genome of actinomycetes were obtained by special bacterial isolation kit (Presto™ Mini gDNA Bacteria Kit from Geneaid) as following the manufacturer's instruction. The PCR was done using specific primer for actinomycetes, i.e 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 16Sact1114R (5'-GAG TTG ACC CCG GCR GT-3') as developed by Martina et al. (2008). The PCR reaction was performed in a total volume of 50 µL, containing 25 µL of DNA polymerase enzyme GoTaq Green Master Mix, 2.5 µL of each primer reverse and forward (10 pmol), 15 µL of nuclease-free water, and 5 µL of DNA template. The PCR contains pre-denaturation (95 ° C, 4 min), denaturation (95° C, 30 s), annealing (55 ° C, 30 min), elongation (72 ° C, 1 min), and post-elongation (72 ºC, 7 min) in 30 cycles. The PCR products were visualized by electrophoresis method in 1% agarose gel and migrating at 80 V for 50 minutes. The gel was stained by 1% of Ethidium Bromide (EtBr) for 15 minutes and observed in UV transilluminator. PCR products were sequenced through First Base sequencing services (Malaysia). The sequences were aligned using the Basic Local Alignment Search Tool-program Nucleotide (BlastN) from the website of the National Center for Biotechnology Information (NCBI). The phylogenetic tree was constructed by using Neighbor-Joining (NJ) method with 1000X bootstrap in MEGA 6.0 software.

RESULTS AND DISCUSSION

Actinomycetes isolated from soybean rhizosphere

Fifty isolates have been isolated from soybean rhizosphere. All isolates have a distinct morphological colony which is shown by the diversity of aerial mycelium colors and substrate mycelium, i.e. white, orange, violet, light brown and dark brown. Isolates also exhibited various types of spore chain, e.g. *Spira*, *Verticiliate*, *Retinaculiaperti*, *Rectiflexibiles*, and *Fragmented* (Figure 1).

Hypersensitivity and hemolytic reaction of actinomycetes from soybean rhizosphere

The results of hypersensitivity and hemolytic assays on 50 actinomycetes isolates resulted in 9 actinomycetes isolates stimulated necrosis reaction, one isolate formed a clear zone on blood agar medium, and one isolate shows necrosis reaction and clear zone formation. These ten isolates may indicate as pathogenic actinomycetes for plants and animals. Forty other isolates with a negative hypersensitive response and non-hemolytic characters were used for further analysis.

Actinomycetes producing IAA

Thirty-five actinomycetes isolate from soybean rhizosphere have been detected for their ability to produce IAA in various concentrations (Table 1). These actinomycetes isolates were classified into high, moderate, and low IAA producers. The results showed that 12 isolates were classified as high IAA producers (>10 mg/L), 20 isolates were moderate IAA producers (2-10 mg/L), and three isolates were low IAA producers (<2 mg/L). The highest IAA concentration was produced by ASR 67 isolate (30.6 mg/L), while the lowest IAA concentration was exhibited by ASR 57isolate (0.33 mg/L). Positive control ARK 94 isolate from soybean rhizosphere was able to produce IAA in the concentration of 7.48 mg/L, while uninoculated supernatant as negative control did not produce IAA.

Table 1. Percentage of isolates producing IAA of actinomycetes isolated from soybean rhizosphere

Group of IAA producer	Concentration (mg/L)	Number of isolates	Percentage
Negative	0.0	6	12.19%
Low	< 2	3	7.31%
Moderate	2-10	20	48.78%
High	> 10	12	31.7%

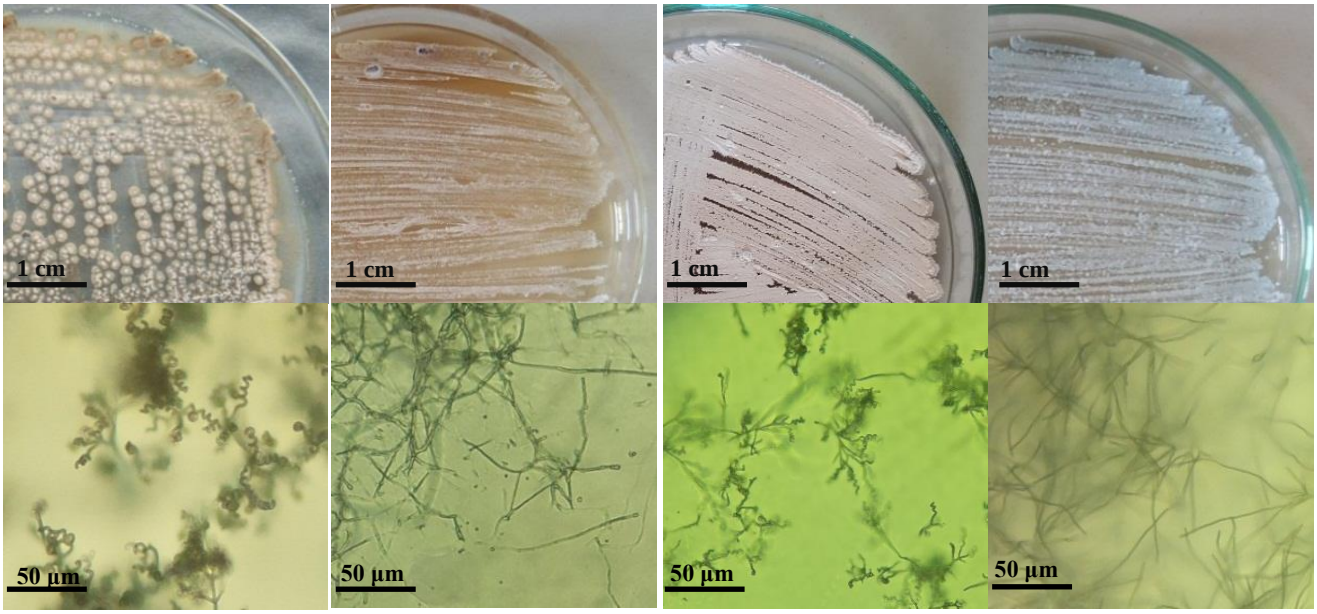

Figure 1. Morphology of actinomycetes colony (upper figure) and type of spore chain (lower figure) of actinomycetes from soybean rhizosphere. A. ASR 44 isolate, *spira*; B. ASR 67 isolate, *rectiflexibiles*, C. ASR 75 isolate, *retinaculiaperti*, D. ASR 59 isolate, *rectiflexibiles*

Plant growth-promoting activity of actinomycetes from soybean rhizosphere

Based on Ragdoll methods, among 35 isolates producing IAA, 30 isolates have significantly increased soybean sprouts growth (Table 2). Six isolates (ASR 26, ASR 43, ASR 58, ASR 61, ASR 63 and ASR 69) have the ability to improve soybean growth through hypocotyl and radicular root elongation, number of lateral roots, and dry weight, compared to the respective control. The other isolates at least showed one growth parameter activity.

Phosphate solubilizing, nitrogen-fixing, siderophore and chitinase production

Twenty-six isolates were selected for further analysis based on IAA production and plant growth-promoting activity in the soybean germination. Furthermore, 14 isolates showed phosphate solubilizing activity with different phosphate-solubilizing index ranging from 1.25 to 2.62 (Table 3). Whereas, three isolates (ASR 49, ASR 58, ASR 61) exhibited significant phosphate solubilizing activity as indicated by high phosphate solubilization index more than 2.0. Among 20 isolates evaluated for nitrogen-fixing ability showed that eight isolates were able to grow in N-free medium. Twenty-three isolates were able to produce siderophore on CAS medium by forming orange zone, and being the highest siderophore producer was ASR 41 isolate (26 mm of diameter). The results of the chitinolytic activity assay showed that all the tested isolates were able to hydrolyze colloidal chitin in the medium, except ASR 55. Isolate ASR 67 showed the highest chitinolytic index (3.09).

The identity of the potential isolates based on 16S rRNA sequences

Four isolates were identified based on 16S rRNA gene. These isolates were selected based on their activity in producing IAA, promoting soybean growth *in vitro*, solubilizing phosphate, and fixing nitrogen. The amplicon of the 16S rRNA gene resulted in the targeted DNA fragments with 1080 bp in length. Based on BlastN analysis, all isolates were identified as *Streptomyces* spp. in various taxa of strains and species (Table 4). Consistently, these results also confirmed by their position in the phylogenetic tree (Figure 2).

Discussion

Evaluation of the potential of actinomycetes isolated from soybean rhizosphere as plant growth promoters were conducted in this study. In total, 50 isolates of actinomycetes were successfully isolated from soybean rhizosphere with differences in morphology, and they used for preliminary screening. Based on the results of hypersensitivity and hemolytic assay, there were nine isolates likely pathogenic in plants as indicated by necrotic formation in tobacco leaves after injected with actinomycetes. Two isolates were pathogen to animals due to its reaction in hemolyzing the red blood cell in the blood agar medium. Other 40 isolates did not form necrotic zone nor hydrolytic zones, so that they may not be pathogenic to plants nor animal and may be used as candidates for bio-stimulant.

Based on the results on IAA production assay, there were 35 isolates out of 40 non-pathogenic isolates (87%)

were detected to have the ability to produce IAA with concentrations ranging from 0.46-30.6 mg L^{-1}. IAA production from rhizospheric actinomycetes isolates was indicated by the presence of pink color in the supernatant which was added with the Salkowsky reagent. The formation of the pink color was caused by the reaction between the oxidative IAA indole ring of tryptophan and sulfuric acid in the Salkowksi reagent (Rahman et al. 2010). The difference of IAA concentration produced by each isolate might be caused by the differences of actinomycetes species or strains with the variations in the metabolic rate of tryptophan. Besides, each isolate may require optimal conditions in producing IAA. In this study, a total of 5 isolates cannot produce IAA due to these isolates unable to express IAA encoding genes even though L-tryptophan was added as a precursor of IAA synthesis. Besides, the culture conditions might not be optimal for them for producing IAA. Therefore, to obtain maximum IAA production, it is necessary to optimize culture conditions by modifying the source of carbon and nitrogen, concentration of L-tryptophan, temperature, pH, and incubation time. This result was consistent with the research conducted by Abd-Alla et al. (2013) that *Streptomyces atrovirens* ASU14 optimally produce IAA at pH 6, temperature 30°C, with tryptophan concentration of 5 mg mL^{-1}, at nine days incubation.

Table 2. Plant growth-promoting characters of actinomycetes isolated from soybean rhizosphere

No	Isolates	IAA (mg/L)	Growth Parameters			
			Shoot (cm)	Primary root (cm)	Lateral roots	Dry weight (g)
Group 1						
	Uninoculated seed		7.11 b	6.75 a	14.17 b	0.07 a
1	ASR 44	5.25	5.49 a	5.70 a	8.40 a	0.12 b
2	ASR 49	5.47	8.53 bc	11.18 c*	10.12 a	0.07 a
3	ASR 72	14.5	9.04 cd*	10.50 bc*	15.03 bc	0.07 a
4	ASR 77	8.4	9.51 cde*	9.07 b*	13.62 b	0.08 a
5	ASR 65	14.0	9.79 def*	11.51 c*	17.05 c*	0.29 e*
6	ASR 53	20.6	10.11 def*	11.28 c*	13.57 b	0.10 b*
7	ASR 69	8.27	10.54 ef*	10.33 bc*	20.94 d*	0.19 d*
8	ASR 67	30.6	11.05 f*	11.27 c*	25.08 e*	0.08 a
Group 2						
	Uninoculated seed		8.72 b	9.64 a	22 b	0.11 bc
9	ASR 40	1.54	5.83 a	11.04 ab	18.57 a	0.09 ab
10	ASR 39	0.46	8.91 b	10.41 ab	18.28 a	0.09 a
11	ASR 38	5.0	10.91 bc	14.23 c*	31.96 f*	0.12 cd
12	ASR 43	4.92	11.00 cd*	14.25 c*	26.09 de*	0.14 d*
13	ASR 70	18.93	11.18 cd*	10.59 ab	22.89 bc	0.09 ab
14	ASR 56	17.4	11.30 cd*	13.41 c*	27.12 e*	0.13 cd
15	ASR 78	10.98	11.33 cd*	13.79 c*	24.89 cd*	0.10 ab
16	ASR 46	8.13	11.75 cd*	13.63 c*	27.52 e*	0.10 ab
17	ASR 63	8.03	12.43 e*	14.22 c*	30.06 f*	0.13 cd
18	ASR 55	10.51	13.09 e*	11.67 b*	26.15 de*	0.08 a
Group 3						
	Uninoculated seed		10.35 ab	11.29 a	22.43 ab	0.11 ab
19	ASR 71	9.36	9.87 a	11.62 ab	19.88 a	0.09 a
20	ASR 76	16.46	10.25 ab	13.71 c*	29.85 c*	0.14 bc
21	ASR 59	4.24	10.71 ab	13.6 bc*	34.67 c*	0.16 c*
22	ASR 64	9.3	10.76 ab	12.78 abc	21.88 ab	0.22 d*
23	ASR 66	7.89	11.29 ab	13.20 abc	24.66 ab	0.13 bc
24	ASR 68	7.41	11.57 ab	13.16 abc	23.65 ab	0.10 ab
25	ASR 54	5.43	11.73 ab	13.69 c*	25.35 ab	0.24 d*
26	ASR 58	3.44	11.77 ab	13.37 bc*	27.78 b	0.27 d*
27	ASR 48	9.11	12.10 b	12.64 abc	26.10 ab	0.11ab
Group 4						
	Uninoculated seed		8.91 ab	11.18 b	16.21 a	0.08 a
28	ASR 41	3.19	7.83 a	8.94 a	15.66 a	0.09 abc
29	ASR 52	15.56	8.07 a	11.71 bcd	23.95 bc*	0.09 ab
30	ASR 73	6.55	8.93 ab	11.86 bcd	15.18 a	0.09 abc
31	ASR 45	15.41	10.44 bc	12.71cde*	22.41 bc*	0.11 bcd*
32	ASR 47	16.83	11.03 cd*	13.94 e*	22.47 bc*	0.08 a
33	ASR 75	9.27	11.47 cd*	13.15 de*	20.95 b*	0.12 cd*
34	ASR 61	2.63	11.75 d*	13.35 de*	22.67 bc*	0.13 de*
35	ASR 26	3.09	11.94 d*	13.44 de*	21.9 bc*	0.16 e*

Notes: Data were mean of three replicates, and each replicate consisted of 9 seeds. [a] The numbers followed different letters indicates that the data were significantly different according to Tukey's honestly significant difference test at $P \leq 0.05$. *: Isolates significantly promoted the length of the shoot, primary root, the number of the lateral roots, and dry weight of plants.

Table 3. Phosphate solubilization, N-Fixation, siderophore and chitinase production of the 26 potential isolates

Isolate code	P-solubilizing index	Growth on N-free medium*	The diameter of siderophore production (mm)	Chitinolytic index
ASR 26	-	-	9 ± 0.0	0.5
ASR 38	-	-	7 ± 0.4	0.74
ASR 41	-	-	26 ± 0.4	1.12
ASR 43	1.25	-	-	1.12
ASR 46	1.25	+	13 ± 0.2	0.42
ASR 47	-	+	16 ± 0.4	0.27
ASR 49	2.62	-	8.5 ± 0.7	0.58
ASR 52	-	-	-	0.93
ASR 53	1.25	-	12.5 ± 1.2	0.61
ASR 54	-	+	12.5 ± 1.2	1.06
ASR 55	-	-	-	-
ASR 56	-	-	6.5 ± 0.2	0.85
ASR 58	2.50	+	24 ± 1.8	0.65
ASR 59	-	-	7 ± 0.4	2.65
ASR 61	2.12	-	14 ± 0.4	1.25
ASR 63	-	+	17 ± 1.4	0.62
ASR 64	1.25	-	16.5 ± 0.7	0.59
ASR 65	-	+	10 ± 0.4	0.95
ASR 66	1.43	-	18 ± 0.4	0.86
ASR 67	1.87	-	13.5 ± 0.2	3.09
ASR 68	-	+	9.5 ± 0.2	0.53
ASR 69	1.75	-	7.5 ± 0.2	0.79
ASR 70	-	-	17.5 ± 1.1	0.58
ASR 72	2.3	-	12.5 ± 1.1	0.42
ASR 75	1.37	+	14.5 ± 0.2	0.45
ASR 76	1.43	+	12.5 ± 0.0	0.80

Note: *: +: able to grow on N-free medium;-: not grow. All the experiment were done in three replicates

Tabel 4. The identity of four selected isolates based on 16S rRNA gene sequence analysis

Isolate code	Closest relative species	Query cover (%)	E-Value	Similarity (%)	Accession no.
ASR 46	*Streptomyces bellus* NBRC 12844	99	0.0	99	NR. 041222.1
ASR 58	*Streptomyces tendae* ATCC 19812	99	0.0	99	NR. 025871.2
ASR 75	*Streptomyces thermocarboxydus* NBRC 16323	99	0.0	99	NR. 112585.1
ASR 76	*Streptomyces ramulosus* NRRL B-2714	99	0.0	99	NR. 043503.1

IAA production by actinomycetes was influenced by the presence of L-tryptophan as a precursor of IAA synthesis which added to the culture medium. In this study, isolate ASR 67 was the highest IAA producer with a concentration of 30.6 mg/L in the medium supplemented with 0.1 mg/mL L-tryptophan. De Fretes et al. (2013) reported that *Streptomyces* sp. strain MS1 isolated from rhizosphere soils was able to produce IAA with a concentration of 120 mg/L in the 2 mg/mL L-tryptophan supplemented medium. Khamna et al. (2010) also revealed that *Streptomyces* CMU-H009 was maximums (300 mg/mL) in producing IAA when the strain was cultivated in 2 mg/mL L-tryptophan. It means that 2 mg/mL L-tryptophan was the optimum concentration added to the culture medium to produce IAA, while at the higher concentration of tryptophan utilize the adverse effect of IAA production.

IAA-producing actinomycetes were also tested for their ability to stimulate soybean growth *in vitro* using the Ragdoll method. The result showed that 26 isolates had different ability to stimulate soybean growth which characterized by several growth parameters such as hypocotyl length, radicular length, number of lateral roots, and plant dry weight. A total of 6 isolates (ASR 65, ASR 69, ASR 43, ASR 64, ASR 61 and ASR 26) out of 26 isolates were able to stimulate soybean germination significantly compared to uninoculated seeds. However, some IAA-producing isolates such as ASR 44 (5.25 mg/L), ASR 40 (1.54 mg/L), and ASR 41 (3.19 mg/L) lower soybean growth compared to uninoculated seeds. It may be due to IAA produced by these isolates failed to penetrate plant tissues so that it can not stimulate plant cell elongation. According to Patten and Glick (2002), low IAA concentrations can stimulate primary root elongation, while

high IAA concentrations stimulate ethylene production, that may result in inhibiting the growth of lateral root and apical shoots. Rapid root growth through elongation and cell division in the main root or hypocotyl, as well as cell proliferation at the lateral root provide distinct advantages for young plants in absorbing water and nutrients from the soil optimally and protecting the roots from pathogenic infections. IAA produced by actinomycetes plays a role in the stimulation of plant cell division and root elongation. Other studies also confirm that rhizosphere actinomycetes producing IAA were able to stimulate plant growth and increase plant biomass (Khamna et al. 2010; Abd-Alla et al. 2013; Shreevidya et al. 2016).

In our present study, 26 growth-promoting actinomycetes isolates were evaluated for their ability in solubilizing phosphate and fixing nitrogen. As a result, 14 isolates were capable of solubilizing phosphate in various levels as indicated by various phosphate solubilization index. ASR 49 isolate was the highest phosphate solubilizer with the index of solubilization of 2.62. Phosphate solubility was indicated by the presence of a clear zone resulting from dissolution of tricalcium phosphate by phosphate solvent enzymes produced by actinomycetes. Another mechanism of mineral phosphate solubilization by rhizosphere microbes is related to the release of low molecular weight organic acids which chelate the cations to bind phosphates through their hydroxyl and carboxyl group (Chen et al. 2006). Therefore, these isolates have the potential to mobilize insoluble inorganic phosphate for improving plant growth under low phosphate availability. Actinomycetes of the genera *Streptomyces*, *Micrococcus*, *Micromonospora* (Hamdali et al. 2008), and *Thermobifida* (Franco-Correaa et al. 2010) can promote plant growth by solubilizing phosphate. Evaluation of the actinomycetes capability to grow in N-free medium showed that eight isolates were able to grow in N-free medium which indicated that these isolates have ability in fixing nitrogen from the atmosphere. However, further tests to ensure nitrogen-fixation capacity need to done using biochemical and molecular approaches such as acetylene reduction activity, Kjeldahl analysis (Sàez-Plaza et al. 2013), N_2 isotopes dilution analysis, and amplification of nitrogenase coding gene (Gtari et al. 2011). Sari et al. (2014) and Wahyudi et al. (2019) also reported that *Streptomyces* spp were able to fix nitrogen.

Furthermore, based on this study, 23 isolates were also detected to produce siderophore. Siderophore is a non-protein compound that could increase the availability of Fe in soil and could inhibit the fungal growth by competing Fe intake (Barucha et al. 2013). The study by Gopalakrishnan et al. (2011) showed that rhizosphere actinomycetes were able to synthesize siderophores and responsible for inhibiting the growth of pathogenic fungi (Gopalakrishnan et al. 2011). Almost all isolates produced chitinase based on the result of *in vitro* chitinolytic activity assay. Chitinase is a cell wall degrading enzyme that has been investigated for biocontrol of phytopathogenic fungi (Yandegeri et al. 2015). Actinomycetes isolates that have the ability in phosphate solubilizing, nitrogen-fixing, producing siderophore and having chitinolytic activity are potential to enhance soil nutrient availability and to protect plant from pathogen.

Based on the overall parameters observed, it can be concluded that there were four isolates that very promising as a plant growth promoter and selected for molecular identification. The result of 16S rRNA partial gene analysis showed that all isolates belong to *Streptomyces* group in various taxa of strains and species. *Streptomyces* group are commonly known as the most promising actinomycetes group as IAA producers (Khamna et al. 2010) and plant growth promoters (Wahyudi et al. 2019). Based on these results, four isolates (ASR 46, ASR 58, ASR 75 and ASR 76) have the potential to be developed further for plant growth-promoting rhizobacteria.

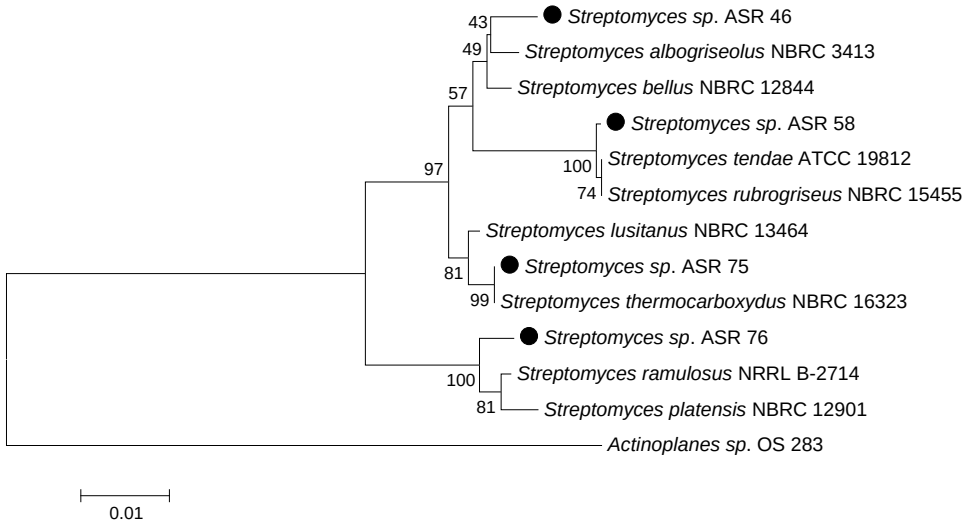

Figure 2. Phylogenetic-tree of actinomycetes isolated from soybean rhizosphere compared to their closest relative species constructing with the neighbor-joining method with 1000x bootstrap value. The black circle are the isolates in this study

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REFERENCES

Abd-Alla MH, El-Sayed EA, Rasmey AHM. 2013. Indole-3-Acetic Acid (IAA) production by Streptomyces atrovirens isolated from rhizospheric soil in Egypt. J Biol Earth Sci 3 (2): 182-193

Aldillah R. 2015. Soybean Production and Consumption Prediction in Indonesia. J Ekonomi Kuantitatif Terapan 8 (1): 9-23. [Indonesian]

Amini J, Agapoor Z, Ashengroph M. 2016. Evaluation of Streptomyces spp. against *Fusarium oxysporum* f. sp. Ciceris for the management of chickpea wilt. J Plant Prot Res 56 (3): 257-264.

Anwar S, Ali B, Sajid I. 2016. Screening of rhizospheric actinomycetes for various in-vitro and in-vivo plant growth promoting (PGP) traits and for agroactive compound. Fontiers Microbiol 7: 1334.

Balitkabi. 2016. Balitbangtan Gelar Teknologi Budidaya Kedelai Grobogan 3 ton per hektar. Balitkabi, Malang. [Indonesian].

Chen YP, Rekha PD, Arun AB, Shen FT, Lai WA, Young CC. 2006. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl Soil Ecol 34: 33-41.

Barucha UD, Patel KC, Trivedi UB. 2013. Antifungal activity of chatecholate type siderophore produces by *Bacillus* sp. Intl J Res Pharm Sci 4 (4): 528.

De Fretes CE, Sembiring L, Purwestri YA. 2013. Characterization of *Streptomyces* spp. producing indole-3-acetic acid as biostimulant agents. Indon J Biotechnol 18 (2): 83-91.

El-Tarabily KA. 2008. Promotion of tomato (*Lycopersicon esculentum* Mill.) plant growth by rhizosphere competent 1-amino cyclo propane-1-carboxylic acid deaminase-producing Streptomycete actinomycetes. Plant Soil 308: 161-74.

Franco-Correa MF, Quintana A, Duque C, Suarez C, Rodríguez M X, Barea JM. 2010. Evaluation of actinomycete strains for key traits related with plant growth promotion and mycorrhiza helping activities. Appl Soil Ecol 45: 209-217.

Gopalakrishnan S, Vadlamudi S, Vidya MS, Rathore A. 2013. Plant growth-promoting activities of *Streptomyces* spp. in sorghum and rice. SpringerPlus 2 (574): 1-8.

Gopalakrishnan S, Pande S, Sharma M, Humayun P, Kiran BK, Sandeep D. 2011. Evaluation of actinomycete isolates obtained from herbal vermicompost for biological control of *Fusarium* wilt of chickpea. Crop Prot 30:1070-8.

Gtari M, Gtari FG, Nouioui I, Beauchemin N, Tisa LS. 2012. Phylogenetic perspectives of nitrogen-fixing actinobacteria. Arch Microbiol 194 (1): 3-11.

Hamdali H, Bouizgarne B, Hafidi M, Lebrihi A, Virolle MJ, Ouhdouch Y. 2008. Screening for rock phosphate solubilizing Actinomycetes from Moroccan phosphate mines. Appl Soil Ecol 38:12-19.

Hayakawa M, Nonomura H. 1987. Humic Acid-Vitamin Agar, a new medium for the selective isolation of soil actinomycetes. J Ferment Technol 65 (5): 501-509.

Khamna S, Yokota A, Peberdy JF, Lumyong S. 2010. Indole-3-acetic acid production by *Streptomyces* sp. isolated from some Thai medicinal plant rhizosphere soils. Eur Asian J Biol Sci 4: 23-32.

Louden BC, Haarmann D, Lynne AM. 2011. Use of Blue Agar CAS Assay for Siderophore Detection. J Microbiol Biol Educ 12 (1): 51-53.

Martina K, Jan K, Tamas F, Ladislav C, Marek O, Genevieve LG, Yvan ML, MASReta SM. 2008. Development of a 16S rRNA gene-based prototype microarray for the detection of selected actinomycetes genus. Antonio Van Leeuwenhoek 94:439-453.

Mohite B. 2013. Isolation and Characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. J Soil Sci Plant Nutr 13 (3): 638-649.

Palaniyandi SA, Yang SH, Zhang L, Suh JW. 2016. Effects of actinobacteria on disease suppression and growth promotion. Appl Microbiol Biotechnol 97 (22): 9621-36.

Patten CL, Glick BR. 2002. Role of Pseudomonas putida indole acetic acid in development of the host plant root system. Appl Environ Microbiol 68 (8): 3795-3801.

Poovarasan S, Mohandas S, Paneerselvam P, Sharita B, Ajay KM. 2013. Mycorrhizae colonizing actinomycetes promote plant growth and control bacterial blight disease of pomegranate (*Punica granatum* L.cv Bhagwa). Crop Prot 53: 175-181.

Rahman A, Sitepu IR, Tang SY, Hashidoko Y. 2010. Salkowski's reagent test as a primary screening index for functionalities of rhizobacteria isolates from wild dipterocarp saplings growing naturally on medium-strongly acidic tropical peat soil. Biosci Biotechnol Biochem 74 (11): 2202-2208.

Sàez-Plaza P, Navaz JM, Wybraniec S, Michalowski T, Asuero AG. 2013. An overview of the Kjeldahl method of nitrogen determination. Part II. Sample Preparation, Working Scale, Instrumental Finish, and Quality Control. Crit Rev Anal Chem 43: 224-272.

Sadeghi A, Karimi E, Dahazi PA, Javid MG, Dalvand Y, Askari H. 2012. Plant growth promoting activity of an auxin and siderophore producing isolate of Streptomyces under saline soil condition. World J Microbiol Biotechnol 28:1503-9.

Saif S, Khan MS, Zaidi A, Ahmad E. 2014. Role of phosphate-solubilizing actinomycetes in plant growth promotion: current perspective. In: Khan MS, Zaidi A, Musarrat J (eds) Phosphate Solubilizing Microorganisms Principle and Application of Microphos Technology. Springer International Publishing, Switzerland.

Sari WE, Solihin DD, Lestari Y. 2014. Identification of endophytic actinomycetes from Indonesian rice plant based on 16S rRNA and nifH genes analyses. Adv Environ Biol 8 (7): 2357-2365.

Shimizu M. 2011. Endophytic actinomycetes: biocontrol agents and growth promoters. Maheshwari DK [editorial]. Bacteria in Agrobiology: Plant Growth Responses. Springer Berlin Heidelberg 201-220.

Shreevidya M, Gopalakrishnan S, Kudapa H, VASRhney NK. 2016. Exploring plant growth-promotion actinomycetes from vermicompost and rhizosphere soil for yield enhancement in chickpea. Braz J Microbiol 47: 85-95.

Wahyudi AT, Priyanto JA, Fijrina HN, Mariastuti HD, Nawangsih AA. 2019. *Streptomyces* spp. from rhizosphere soil of maize with potential as plant growth promoter. Biodiversitas 20 (9): 2547-2553.

Wiraswati SM, Rusmana I, Nawangsih AA, Wahyudi AT. 2019. Antifungal activity of bacteria producing bioactive compound isolated from rice phyllosphere against *Pyricularia oryzae*. J Plant Prot Res 59 (1): 86-94.

Yandegeri MS, Malviya N, Solanki MK, Shrivastava P, Sivakumar N. 2015. Chitinolytic Streptomyces vinaceusdrappus S5MW2 isolated from Chilika lake, India enhances plant growth and biocontrol efficacy through chitin supplementation against *Rhizoctonia solani*. World J Microbiol Biotechnol 31 (8): 1217-1225.