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Research article

Isolation and *in vitro* screening of plant growth promoting bacteria from rhizosphere and root tissues of potato tuber (*Solanum tuberosum* L.)

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Abstract: The accumulation of chemical fertilizers that harm the environment is one of the major Indonesian agricultural problems. However, it still has less effect on potato production and yield. The discovery and use of bacteria that have the potential as plant growth-promoting agents (PGPR) is a breakthrough that can help to increase growth to increase production, especially in potato plants. In this study, several bacteria successfully isolated from the rhizosphere and root tissue of potato plants (*Solanum tuberosum* L.) were isolated from potato farms in Plaosan Village. Several in vitro screenings were carried out to assess the functional activity of plant growth promoters, including the IAA (indole-3-acetic acid) production test, siderophore production test, ACC (1-aminocyclopropane-1-carboxylate) deaminase production test and phosphate dissolution test. Based on the screening results, five isolates were considered as the best inoculants, there are R1.3, R2.2, JR2.1, E1.2 and E1.2.1. All R1.3, R2.2, E1.2 and E1.2.1 isolates were known to have the ability to produce phytohormones IAA, ACC deaminase, and siderophores. In contrast, JR2.1 isolate was not known to have the ability to fix nitrogen and produce IAA, ACC deaminase and siderophores. These isolates could be used as potential biofertilizer inoculants and provide a step towards sustainable agriculture.

Keywords: isolation; plant growth-promoting bacteria; root tissues; screening; *Solanum tuberosum*

1. Introduction

Potato (*Solanum tuberosum* L.) is one of the main food crops in the world because it contains a high amount of carbohydrates [1,2]. The nutrition of potatoes and their use in the health and culinary world causes a demand increase for potato tuber production. However, Indonesia's potato production decreased at around 1.2 million tons [3], which could be caused by soil damage due to weather changes and biotic-abiotic stresses. Moreover, the excessive use of chemical fertilizers causes the accumulation of chemical pollutants that can reduce the soil quality due to soil micronutrients and microbial deficiency [4].

Plant growth-promoting rhizobacteria (PGPR) has the potential to increase potato production by using microbes in the root area and plant root tissue that can stimulate plant growth. PGPR can form colonies and can stimulate plant growth directly or indirectly by increasing abiotic stress tolerance, inducing systemic resistance (ISR) [5], producing the indole acetic acid (IAA) hormone, providing nitrogen fixation ability, dissolving soil phosphate, forming siderophores, producing the ACC-deaminase enzyme and fighting against the pathogens [6]. Thus, in vitro PGPR testing was carried out to ensure the isolate's ability and potential to stimulate plant growth.

As a result, PGPR which has been isolated and tested in vitro can be used in conjunction with organic fertilizers and can stimulate plant growth, especially in potato tubers. Based on the research of Naqqash et al. [7], most of the PGPR bacteria found in potato root areas were *Pseudomonas*, *Bacillus* and *Azospirillum*. In this study, screening of plant growth-promoting bacteria isolated from the root system (rhizosphere and root tissue) of potato tubers was carried out to obtain pure isolates of bacteria that have the potential to be used as plant growth-promoting bacteria. In the future, this pure bacterial culture can be used either directly on plants or mixed into organic fertilizers to increase the production of potatoes or other crops in Indonesia.

2. Materials and methods

2.1. Soil samples collection

The potato plants and the soil were collected from potato plantations in Plaosan Village, Plaosan District, Magetan Regency, East Java Province (-7.684028904647954, 111.25209093554466). The samples were taken at approximately 10 cm from below the root, and then the whole plants with the soil were brought to the Microbiology Laboratory of the University of Surabaya, Indonesia for further research.

2.2. Preparation of rhizosphere soil samples and root tissue samples

The rhizosphere soil samples of approximately 1 g were taken from the soil attached to the surface of the potato's root aseptically using a sterile brush and then dissolved in 0.9% NaCl solution. Next, a serial dilution method was carried out up to the 10^{-6} dilution, 1 mL of the suspension from the 10^{-4}, 10^{-5},10^{-6} dilution was inoculated into half-strength tryptic soy agar (½TSA; Merck), soil extract agar (SEA) and Jensen (HiMedia) media, then incubated at 25 °C and 37 °C.

The endophyte samples were obtained from potato tuber roots that had been surface sterilized with the following steps: (1) soaking the roots for 90 sec in 95% ethanol and then rinsing with sterile

distilled water three times, (2) soaking the roots for 6 min in 3.1% NaOCl and then rinsing with sterile distilled water three times and (3) soaking the roots for 60 sec in 95% ethanol and then rinsing with sterile distilled water three times. Furthermore, the roots that have been surface-sterilized are pounded on a mortar aseptically. A total of 1 g of the root was mixed into 0.9% NaCl solution. Then, a serial dilution was carried out up to the 10^{-3} dilution, 1 mL of the suspension from the 10^{-1}, 10^{-2} and 10^{-3} dilution were inoculated to ½ TSA, SEA and Jensen media, and then incubated at 25 °C and 37 °C.

2.3. Bacterial isolation and purification

Isolation and purification of bacteria were initiated by inoculation of 10 µL diluted sample to ½ TSA, SEA and Jensen media separately and then incubated at 25 °C and 37 °C. Daily observations were done and the formation of colonies on the media was sub-cultured until a pure single colony was obtained. Next, the isolates obtained from isolation and purification steps were observed for their morphological appearance [8]. Last, for further analysis, the isolates were grown on trypticase soy broth (TSB; Merck) media and then stored in 30% (v/v) glycerol at -80 °C.

2.4. Morphological characterization

Individual cultures grown on TSA medium incubated at 25 °C and 37 °C were examined for the colony morphological features such as forms, color, size, margin and surface. Gram staining was performed as per standard procedures with exponentially growing cultures.

2.5. Antibiotic test

The antibiotic test was done by inoculating bacterial isolates on TSA media supplemented with three different antibiotics separately (50 g/mL kanamycin; 15 g/mL gentamicin; 5 g/mL rifampicin) and then incubated at 25 °C. After 2–3 days, the isolates growth and colony formation were observed on the antibiotic-supplemented growth media.

2.6. IAA production test

The IAA production test was carried out by inoculating one loop of isolate on TSB with and without 1 mg/mL tryptophan, incubating at 25 °C and sampling on days 1, 3, 5 and 7. Next, analysis of IAA production was performed by centrifugation (5000 rpm, 10 min). To that end, 0.5 mL of the supernatant was taken and then mixed with 1 mL of Salkowski's reagent for 30 min in the dark. Then, IAA production was measured using a spectrophotometer at a wavelength of 530 nm and the IAA concentration determination was done by comparing the results obtained with an IAA standard curve (1–10 ppm and 10–100 ppm).

2.7. Siderophore production test

The siderophore production was carried out by inoculating one loop of isolate on TSB media, which was then incubated at 25 °C for 1–2 days. Then, observations were made on the OD600. If the isolate shows an OD ranging from 0.3–0.9 and 5× concentration is carried out if the OD is above 1,

the results of this concentration will be used as a siderophore production test sample. The siderophore test was carried out by taking 10 µL of isolates from TSB inoculated into modified King's B (HiMedia) media and then 4 quadrants streaking were performed. Incubated at 25 °C for 1–2 days and observed changes in the medium from blue to orange (orange zone) as positive results.

2.8. Phosphate dissolution test

The phosphate dissolution test was carried out by inoculating one loop of isolate on TSB media, which was then incubated at 25 °C for 1–2 days. Then, observations were made on the OD600. If the isolate shows an OD ranging from 0.3–0.9 and 5× concentration is carried out if the OD is above 1, the results of this concentration will be used as a phosphate dissolution test sample. The phosphate dissolution test was carried out by taking 10 µL of isolates from TSB into half-strong Pikovskaya (HiMedia) media and then 4 quadrants streaking were performed. These samples were incubated at 25 °C for 1–2 days and then observed the presence of a clear zone around the formed bacteria colonies as positive results.

2.9. ACC deaminase production test

The ACC deaminase test was carried out by inoculating one loop of isolate on TSB media, which was then incubated at 25 °C for 1–2 days. Then, observations were made on the OD600. If the isolate shows an OD ranging from 0.3–0.9 and 5× concentration is carried out if the OD is above 1, the results of this concentration will be used as an ACC deaminase test sample. The ACC deaminase test was carried out to determine the ability of bacteria to produce the ACC deaminase enzyme. The ACC deaminase test was carried out by taking 10 µL of isolates from TSB into half-strong Pikovskaya media and then 4 quadrants streaking were performed. Incubated at 25 °C for 1–2 days and then observed the presence of a clear zone around the colonies as positive results.

2.10. Sequencing 16s rRNA on humanizing genomics macrogen

Sequencing was done by using 785F (5' GGA TTA GAT ACC CTG GTA-3') and 907R (5' CCG TCA ATT CCT TTR AGT TT-3') primers. PCR reactions were performed using 20 ng genomic DNA in 30 µL of the reaction mixture using EF-Taq (SolGent, Korea). For each target, 1 µL of purified genomic DNA, 1 µL of each primer (10 pmol/µL), 24 µL of distilled water and 3 µL PCR master mix were added to the PCR tubes containing a total volume of 30 µL. The PCR program used is described as follows: activation of Taq polymerase at 95 °C for 2 min; 35 cycles at 95 °C for 1 min, at 55 °C for 1 min, and at 72 °C for 1 min, and final elongation at 72 °C for 10 min. The amplification results were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reactions were performed using the PRISM BigDye Terminator v3.1 Cycle Sequencing Kit. DNA samples containing the extension product were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). Then, the mixture was incubated at 95 °C for 5 min, followed by cooling on ice for 5 min and analyzed by an ABI Prism 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA) [9].

2.11. Preparation of sequencing and identification results

The sequencing results are further processed using the SeqMan Ultra DNASTAR Lasergene 17 software to verify the obtained DNA sequence. The software will display contigs and traces to be checked and changed. Then, the results of changes based on traces will produce contig consensus sequences that will be used in sequence identification. Next, isolate identification was carried out using the NCBI Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sample nucleotide sequence is entered in the 'enter query sequence' column. Database was set by selecting rRNA/ITS databases and selecting the 16S ribosomal RNA sequences (Bacteria and Archaea) option. The top BLAST result is the result with the highest similarity, which means that the isolates obtained have similarities with the BLAST database and can be identified based on these similarities. Furthermore, additional identifying steps of the isolates using the EzBioCloud website (https://www.ezbiocloud.net/identify) are done. Then, select 16S-based ID. Choose "Identify new sequence" to enter the isolate sequence. After the sequence is entered, the results will appear in the column listed below. The results in the Top Hits column are the results with the highest similarity, which means that the isolates have similarities to the EzBioCloud database and can be identified.

3. Results

Until recently, Rhizobacteria application as a PGPR remains an active area of research for scientists all around the world. Samples found from different plants, temperatures, biotic-abiotic stress and usage of fertilizers become sources for further exploration of new and novel rhizobacteria. These rhizobacteria are associated with plant rhizosphere and plant root tissue; hence, they can stimulate plant growth directly and indirectly. In this study, different samples were obtained from the rhizosphere and root tissue of the potato plant in Indonesia for plant growth-promoting bacteria and its characteristics. Fifteen samples were obtained from the isolation process with different media incubated at 25 °C and 37 °C: 1) ½ TSA (half strength Tryptic soy agar): 4 colonies from rhizosphere and 4 colonies from root tissue; 2) SEA: 3 colonies from rhizosphere and 2 colonies from root tissue; 3) Jensen: 2 colonies from the rhizosphere; Isolates were then purified and sub-cultured on a TSA medium. Though PGPR is usually found in the rhizosphere, there is some PGPR found inside the root tissues or endophytes. Most of the isolates were obtained from the rhizosphere and some were from root tissues. There is no significant difference between total colonies obtained from incubation at 25 °C and 37 °C.

These 15 pure-colonies isolates were further analyzed by morphological identification (shape, color, size, margin, appearance, antibiotic test, Gram-staining) and in vitro screenings (IAA production, Siderophore production, Phosphate dissolution and ACC deaminase production). Five best isolates based on different morphological examinations (Table 1) and potential results of in vitro screenings (Table 2) were selected and then sequenced using the 16s rRNA Sanger sequencing.

Molecular identification for five isolates (Table 2) was performed to validate both morphological and in vitro characterization results. R1.3 was identified as *Serratia nematodiphila* (99.53% similarity). Based on morphological analysis, it was Gram-negative with coccus shape, circular, milky white, small, shiny with entire margin.

Table 1. Results of isolation and purification of isolate

Colony	Morphology observations	Gram staining	Antibiotic test		
			Kanamycin	Gentamicin	Rifampicin
R1.3	Circular, Milky white, Small, Entire, Shiny	Negative, Coccus	R	R	R
R2.2	Circular, Milky white, Small-Medium, Entire, Shiny	Positive, Bacil	R	R	R
JR2.1	Circular, White, Small, Entire, Shiny	Negative, Bacil	S	S	S
E1.2	Circular, Transparent white, Big, Not Entire, Shiny	Negative, Bacil	R	S	S
E1.2.1	Circular, Milky-yellow, Small-Medium, Entire, Shiny	Negative, Coccus	R	R	R

Table 2. Results of identification and *in vitro* screenings.

Lab no	Accession Number	Similarity with closest type strain	Identity (%)	*Plant Growth Promoting Activities*				
				IAA production		Siderophore production	ACC deaminase production	Phosphate dissolution
				TSB	TSB+Trp			
R1.3	NR_044385.1[a] JPUX01000001[b]	*Serratia nematodiphila DZ0503SBS1*	99.53	0.08*	24.796**	++	+++	-
R2.2	NR_117706.1[a] HF586506[b]	*Achromobacter insuavis strain LMG 26845*	99.79	0	27.019**	++	+++	-
JR2.1	NR_024691.1[a] BCVD01000224[b]	*Bacillus flexus strain IFO15715*	99.73	3.057**	25.969**	++	+++	-
E1.2	NR_117706.1[a] HF586506[b]	*Achromobacter insuavis strain LMG 26845*	99.86	2.169*	37.389**	++	+++	-
E1.2.1	NR_113706.1[a] AB100738[b]	*Sphingobacterium multivorum strain NBRC 14947*	98.97	1.191**	25.846**	+	++	-

Note: [a] Accession number from NCBI Blast Database, [b] Accession number from EzBioCloud Database.

4. Discussion

R2.2 and E1.2 isolates were both identified as *Achromobacter insuavis* (99.79% and 99.86% similarity). Based on morphological analysis, both isolates were bacilli shape cells, and their colonies were circular and shiny. In contrast, its colony color, size, margin and its Gram-staining differ significantly. The result was relatively consistent with research by Zhang et al. [10], except for the bacilli shape and red colony color. Colony morphology can be slightly different due to different growth mediums [11]. From in vitro screenings, isolate R1.3 was found to produce IAA, Siderophore and ACC deaminase, but unfortunately, it could not dissolve phosphate. Based on research by Basharat et al. [12], it is concluded that *Serratia nematodiphila* acquired biocontrol property towards pathogen

fungi towards plants. According to a past research report by Chester and Cooper [13], the color of colonies can vary from white to translucent white, has various sizes and some Gram-positive were found, except for the colonies margin that was supposed to be entire. Based on in vitro screenings, isolate R2.2 and E1.2 were found to produce IAA, Siderophore and ACC deaminase, but unfortunately, it has no phosphate solubilization ability. However, there was no other past evidence and report about *Achromobacter insuavis* application as PGPR.

JR2.1 isolate was identified as *Bacillus flexus* (99.73% similarity). Based on morphological analysis, it was a Gram-negative with bacilli shape, circular, white, small, shiny and entire margin. There was still no specific report about colony characteristics of *Bacillus flexus*. However, *Bacillus* spp. was found to be a Gram-negative bacterium with bacilli shape, circular, white to slightly yellow, and entire margin. Based on in vitro screenings, isolate JR2.1 was found to produce IAA, Siderophore and ACC deaminase, but unfortunately, it could not dissolve phosphate. There is also no evidence and report about *Bacillus flexus* as PGPR. Nevertheless, it is known that *Bacillus flexus* potentially have alkaliphiles and salt-tolerant traits that can be valuable in extreme environments [14].

Last, E1.2.1 isolates were identified as *Sphingobacterium multivorum* (98.97% similarity). Based on its morphological analysis, it was a Gram-negative with coccus shape, circular, milky-yellow, small to medium, shiny, and entire margin. The result was relatively consistent with research by Barahona and Slim [15], except for the bacilli shape. From in vitro screenings, isolate E1.2.1 was found to produce IAA, Siderophore and ACC deaminase, but it also could not dissolve phosphate. Based on research by Barahona and Slim [15], these bacteria can be found in soil, on plants, in foodstuffs and water sources; thus, these bacteria also have the potential to contaminate laboratory culture media and blood culture systems. At the same time, there is still no evidence or report about *Sphingobacterium multivorum* as PGPR.

The most common PGPR characteristic is its ability to produce IAA. The biosynthesis of IAA consists of tryptophan-dependent and independent pathways [16]. Most bacteria are tryptophan-dependent; hence, the addition of tryptophan will increase the IAA synthesis [7]. In this study, IAA production was evaluated in both tryptophan-supplemented medium and without supplementation and then observed by the spectrophotometry assay [17]. Aligned with previous studies, IAA production with tryptophan addition was significantly higher than without supplementation on all five isolates. Based on past research on plant inoculation of IAA producing PGPR, it has a positive effect on root elongation and lateral root development which is known to increase nutrients and water uptake efficiency [17,18]. Another method to detect IAA production is using high-performance liquid chromatography (HPLC), Naqqash et al. [7] described using the HPLC method after spectrophotometry method as a comparison, which is useful for screening large bacterial population.

Another important enzyme synthesized by PGPR is ACC-deaminase, an important enzyme that affects ethylene production and auxin regulation [17,19]. In this study, all the isolates were able to form colonies, which indicates that the isolates were able to degrade ACC, an ethylene precursor, in the medium to ammonia and α-ketobutyrate [17]. It is in line with an earlier study by Belimov et al. [19] that found inoculation of PGPR with ACC-deaminase enzyme increases plant resistance to stress by lowering ethylene production, which caused retardation root development, promote aging, inhibition of seed germination, senescence and abscission [19,20]. ACC deaminase activity can also be assessed spectrophotometrically with Bradford methodology by detecting α-ketobutyrate, this quantification method is used after qualitative assessment of ACC deaminase production [17].

In this study, all isolates produced an orange zone around the colony which is a positive sign of

PGPR siderophore production ability. Siderophore production bacteria can transform the essential insoluble iron (Fe^{3+}) to its soluble form [17,21], which is essential for the plants' enzyme synthesis, metabolism, cellular development and defense mechanism [22,23]. In Fe-deficient conditions, these bacteria have an important role to acquire soluble iron and are also able to compete with pathogenic microbes for iron by forming colonization around the root, protecting plants from pathogens [21].

Phosphate is also an essential nutrient for plants and is required for several characteristics of plant growth. Though essential, phosphate is usually found in an insoluble form. Phosphate solubilizing bacteria play an important role in the solubilization and mineralization of phosphate from inorganic into a more soluble form that is easily absorbed by plants [23,24]. It has been reported that phosphate solubilizing bacteria also enhance the formation of plat growth hormones and availability of trace metals (Zinc, magnesium, iron) and stimulate nitrogen fixation [17]. However, all isolates in the current study cannot dissolve phosphate using the agar plate method, which is indicated by its inability to form a clear zone around the colony. There is no previous report on this characteristic for the same species.

There is an additional test that can be conducted along with all the four above, such as Acetylene reduction assay (ARA) to determine nitrogen fixation bacteria by nitrogenase activity [7,18]. In addition, isolates can also be further characterized by its ability to utilize amino acids as 'sole' carbon and nitrogen source [19]. The wide range of positive effects of PGPR, including increased levels of phytohormones (IAA), increase stress resistance (ACC deaminase) and improved nutrients uptake (Siderophore production and phosphate solubilization), are influenced by plants species, plants growth, cultivation practices, soil type, climate and the bacteria itself [24,25]. Our results strongly indicate that these five isolates have the potential to be used as plant growth-promoting rhizobacteria (PGPR), which could help maintain a healthy environment and ultimately can be used as biofertilizers to enhance the production of potato or perhaps other food crops in Indonesia.

5. Conclusions

Our results strongly indicate that these five isolates have the potential to be used as plant growth-promoting rhizobacteria (PGPR), which could help maintain a healthy environment and ultimately can be used as biofertilizers to enhance the production of potato or perhaps other food crops in Indonesia.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

No conflict of interest declared.

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