Characterization of PGPR isolated from rhizospheric soils of various plant and its effect on growth of radish (*Raphanus sativus* L.)

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Abstract. Plant growth promoting rhizobacteria (PGPR) are commonly used as biofertilizers for agricultural crops. In addition, screening and selection of effective PGPR for a particular plant is necessary to meet plants specific need and condition. Present study was aimed to obtain PGPR isolates which are effective and compatible for Radish. A total of 15 PGPR isolates had been isolated from various plant roots and in vitro screening was done for different plant growth promotion activities. The result showed that 7 isolates were able to produce IAA at the range of 10.2-125.3 µg ml⁻¹, 10 isolates were positive for phosphate solubilizing, 10 isolates had nitrogen fixing activities, 11 isolates were protease positive, 12 isolates were capable to produce ACC-deaminase enzyme, 7 isolates produced siderophore, 8 isolates produced HCN, and 9 isolates produced ammonia. The effect of 15 PGPR on Radish growth in the green house showed that all isolates had effects on increasing growth and tuber formation compared to control. Nine isolates significantly increased the wet weight shoot plants, the highest effect was reached by Kbm 10.3 isolate (168.5 g), while control only reached 81.5 g. Eight isolates had a significant effect on increasing tuber formation, the highest was Kbm 2.10 isolate (176 g), while control only reached 54.7 g. Kbm 10.3 and Kbm 2.10 isolates seemed to have the highest impact in promoting growth and tuber formation. Those two isolates were analyzed using 16s rDNA sequences, they have close evolutionary relationship with *Bacillus megaterium* (Kbm 2.10) and *Paraburkholderia tropica* (Kbm 10.3).

Keywords : Rhizosphere, PGPR, biofertilizer, Radish.

1. Introduction

The potential of plant growth promoting rhizobacteria (PGPR) for plant microbiome engineering provide great future to achieve sustainable agriculture. PGPR has been a huge part of sustainable agriculture development, but still much has to be done on both explorations as well as the implementation of PGPR. PGPR consist of a group of free-living bacteria that colonize the rhizosphere which can stimulate root growth and influence its physiology [1,2]. These bacteria have a crucial role in sustaining soil fertility and plant health. PGPR improve plant growth through direct mechanism by providing ready available nutrient for plants such as nitrogen, phosphorus and plant hormones or thought indirect mechanism by synthesizing hydrogen cyanide (HCN), antibiotics, and siderophores [4]. PGPR can also alleviate plant stress by reducing the level of ethylene via ACC deaminase enzyme which hydrolyze 1-aminocyclopropane-1-carboxylic acid (ACC). Researchers have reported that some PGPR produce ACC.
deaminase enzyme which can degrade ACC to ammonia and a-ketobutyrate, and finally decreasing ethylene concentration inside plants. [5,6,7]. Alcaligenes, Agrobacterium, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Bradyrhizobium, Burkholderia, Enterobacter, Frankia, Klebsiella, Pseudomonas, Rhizobium, and Serratia are genera that are commonly found in PGPR group with known benefits on different crop plants [8,9].

However, the diversity of PGPR is enormous, and the range of their principle of actions are very broad, and mostly are poorly understood. Studies have proven that different mechanism in plant growth promoting properties are influence by some factors include plant-specific, strain specific, and different environment condition which resulted in varying effect on plants growth and development. [10,11,12,13]. Thus, specific PGPR for targeted crop plants is important to be explored and investigated.

Research on PGPR has been extensively conducted. A number of experiments have been carried out on different crops including wheat, rice, maize, soybean and bean both in vitro and in vivo [14,15,16,17,18]. These studies have shown that although with minor inputs of agrochemicals the used of PGPR potentially to increase the growth and yield of such crops, and lowered consequences for the environment. Research reports on the application of PGPR in tuber plants are still rare, with only several research reports on potato. The data area in potato is still very limited, only on PGPR colonization, disease suppression and growth promotion [19, 20, 21]. Bacillus and Pseudomonas sp are the most commonly reported PGPR in potato and have been used to increase phosphorus uptake in plants, production of Indole acetic acid (IAA) and induce systemic resistance [22,23,24,25].

Radish (Raphanus sativus L.) belongs to the Brassicaceae family, and it is a tuber plant with high economical value. Radish production in Indonesia only reached 31,861 tons, and it did not not meet domestic demand, thus the government still need to import this crop commodity. Currently, Radish productivity in several regions in Indonesia is still relatively low, one of the causes of the low production of radishes might be due to their susceptibility to environmental stresses, either in the form of waterlogging or drought which has an adverse impact on the formation of radish tubers. In present study, beneficial bacteria were isolated from rhizosphere of various plant. Furthermore, in vitro screening was done for different plant growth promotion activities to obtain the effective and compatible rhizobacteria for Radish plant.

2. Materials and Methods
2.1. In vitro screening for plant growth promoting (PGP) properties
2.1.1. Qualitative assay of phosphate solubilizing
Phosphate solubilizing ability of the isolates were tested using sterile Pikovskaya media in petri dishes. These medium were supplemented with insoluble phosphate Ca3 (PO4)2. The solubilisation zone were observed after 5 days of incubation at 30 °C. The solubilisation zone was determined by subtraction of the diameter of bacterial colony from the diameter of total zone [26, 27].

2.1.2. Qualitative assay of nitrogen fixation
First screening for N2-fixing activity of the isolates was determined on N-free semi-solid malate medium (Nfb) [28]. The inoculated plates were incubated for 2-7 days at room temperature (28°C). Positive ability of N2 fixing were indicated by a change in the colour of the media from pale green to blue.

2.1.3. Qualitative and quantitative detection of IAA hormone
For qualitative detection, 245 ml of TSA media with the addition of 5 ml of Tryptophan reagent was used to make TSA+ media. The bacterial isolates were inoculated on TSA+ media in a petri dish and then incubated for 72 hours. After incubation, the Salkowski reagent was dripped on the growing bacteria and incubated in a dark room for 24 hours. A change in colour to pink indicated positive reaction for producing the IAA hormone [29].

A spectrophotometer was used to detect IAA production quantitatively. Production of IAA was detected by using nutrient broth containing 0.1 % DL tryptophan inoculated with bacterial culture. Thus culture was incubated at hours on orbital shaker at 150 rpm. After incubation, one ml of supernatant was
mixed with 4 ml Salkowski reagent. The absorbance of the pink color was determined after 30 minutes at 535 nm by an ultraviolet or visible spectrophotometer [30].

2.1.4. Qualitative analysis of ACC deaminase
ACC deaminase activity testing was carried out on selected isolates. The media used was Dworkin Foster (DF) minimal salt media [31] enriched with 1 aminocyclopropane-1-carboxylate (ACC) or ammonium sulfate as nitrogen sources following the procedure of Glick et al. 1995 [32]. The amount of ACC or ammonium sulfate added to DF media was 2.5 mmoL of ACC or 2 g of ammonium sulfate. All media materials were sterilized by autoclave for 15 minutes at 121°C and 0.1 Mpa pressure, except for the ACC material which was not a heat-stabile substance. The ACC material was sterilized with a 0.2 µm filter membrane before it was added (mixed) to the sterilized material. The PGPR isolates were then inoculated into solid DF media, DF + ACC, and DF + ammonium sulfate with a streak technique. Isolates that could grow on DF + ACC media indicated that the isolates had ACC deaminase enzyme activity.

2.1.5. Qualitative analysis of siderophore and HCN production
Bacterial isolates were detected siderophore production on the Chrome Azurol Sulphonate agar (CAS) plates by spot inoculated and incubated at 37°C for 2-5 days. Halo zone were observed [33]. HCN production by isolate was tested by using methodology described by Castric [34]. Bacterial strains were streaked on NA agar plates containing 4.4 g per liter of glycine. Whatmann filter paper No.1 soaked in 0.5 % picric acid solution containing sodium carbonate was place inside the lid of plate. Plate were sealed with parafilm and incubated for 72 hours at room temperatur. Development of light brown to dark-brown colour spots in filter paper indicated HCN production.

2.1.6. Qualitative analysis of Ammonia production
The medium used for the ammonia production test was pepton broth. Bacterial isolate was inoculated into 10 ml pepton broth media and incubated at room temperature and shaken for 2-4 days. Nessler’s reagent (0.5 ml) was added in each tube. Positive result of ammonia production indicated a change in color to yellow-brown [35].

2.1.7. Qualitative assay of protease production
Bacteria were inoculated on SMA agar plates containing skim milk (7 %) then incubated at 30°C for two days. Positive result of protease enzyme activity indicated by the development of clear zone around the bacterial colony [36].

2.2. The effect of PGPR inoculation on Radish growth
A study was conducted at Green House in Research Center for Biology LIPI, Cibinong, West Java. The experiment was conducted on pot using completely randomized design consisting of 16 treatments i.e. single bacterial inoculation of 15 PGPR isolates (TPK 5b2, CBT1, CBT2, CBT 4.3, Kbm 2.10, Kbm 2.15, Kbm 10.3, PK 2.4, PK 2.6, Pro 4A.A, Pro 5A Pro 7C.A, Az Th, Az Gd, Az Gu) and plant seedlings without inoculation were used as a control. The bacterial isolates were grown in 100 mL of NB for 24h at 28 C. After growth, cells were harvested, inoculum containing 10⁸ CFU mL. Radish were surface-sterilized with ethanol for 2 minute and rinsed thoroughly in sterile distilled water. The seed was sowed in germination material for 7 days then transferred to a polybag (20 x 30 cm) filled with a specific growth medium (mixture of soil and compost, with a ratio of 2: 1) in total weight of 2 kg. All treatments were arranged in 64 pots, 16 treatments with 4 replication per treatment and double seed per pot. Plants were harvested at 60 days, the parameters observed including plant weight and tuber weight. Data were statistically treated by a completely randomized design [37]. Collected data were analyzed using analysis of variance (ANOVA) followed by Duncan Multiple Range Test (DMRT) comparison at 5% level of significance using SPSS ver. 25.
2.3. Bacterial Molecular Analysis

Selected microbial isolates were further analysed based on 16s rRNA gene. Bacterial DNA was extracted using GES method [38]. DNA amplification was done using universal primers for 16s rDNA (27F and 1492R) and PCR cycle program used was as follows: (i) 2 min at 94 °C, (ii) 30 cycles of 30 s at 95 °C, 40 s at 55 °C, and 30 s at 72 °C, and (iii) 5 min at 72 °C. PCR product was sequenced and then analyzed by BLAST (Basic Local Alignment Search Tool) in https://blast.ncbi.nlm.nih.gov/Blast.cgi database. This analysis was conducted to determine the homology of bacterial genetic code (ATGC) to other known species. Multiple sequence alignment of the isolates was performed by Muscle and construction of the phylogenetic tree was done using Neighbor-Joining tree method (NJT) implemented in MEGA 6 software [39]. Strength of internal branches of the phylogenetic tree was tested with bootstrap analysis using 1000 replications.

3. Results and Discussion

3.1. In vitro screening for plant growth promoting (PGP) properties

In this experiment, in vitro method was assessed to screen the potential of isolated microorganisms to promote plant growth. The assessment of growth-promoting abilities are important and is considered an effective tool in the investigation of microbes that can be used as biofertilizers before applying them to plants [40].

| Isolate code | Halo formation | Halo size (mm) | Pink colouration | Concentration (µg/ml) | NH3 (brown colouration) | N fix (blue colouration) |
|--------------|----------------|----------------|------------------|-----------------------|------------------------|------------------------|
| TPK 5b2      | -              |                | ++               | 70.6                  | -                      | -                      |
| CBT1         | +              | < 10           | +++              | 122.2                 | -                      | -                      |
| CBT2         | +              | < 10           | +++              | 125.3                 | -                      | +                      |
| CBT 4.3      | +              | < 10           | ++               | 10.2                  | +++                    | +                      |
| Kbm 2.10     | +              | < 10           | +++              | 104.8                 | +                      | ++                    |
| Kbm 2.15     | +              | < 10           | ++               | 11.2                  | ++                    | +                      |
| Kbm 10.3     | +++            | 20             | -                |                       | +                      | ++                    |
| PK 2.4       | +++            | 20             | -                |                       | +                      | +                     |
| PK 2.6       | ++             | 15             | -                |                       | +                      | ++                    |
| Pro 4A.A     | +              | < 10           | -                |                       | +++                    | -                      |
| Pro 5A       | -              | -              | -                |                       | +                      | -                     |
| Pro 7C.A     | -              | +              | +++              | -                      | -                      | -                     |
| Az Th        | +              | < 10           | -                |                       | -                      | ++                    |
| Az Gd        | -              | -              | -                |                       | -                      | ++                    |
| Az Gu        | -              | -              | -                |                       | -                      | ++                    |

- = no production; + = low production; ++ = moderate production and +++ = strong production

All of fifteen (15) bacterial isolates were tested for nitrogen fixation, phosphate solubilisation, NH₃ production and IAA production. The result showed that 10 bacterial isolates were found positive for N-fixing, 10 were positive for phosphate solubilisation, 9 for ammonia production and 7 for IAA production. (Table 1). In the present study, isolates TPK 5b2, CBT1, CBT2, CBT 4.3, Kbm 2.10, Kbm 2.15 induced the production of IAA in the presence of tryptophan. IAA production was found in the range of 10.2 to 125.3 µg ml⁻¹. Isolates CBT2, CBT1, and Kbm 2.10 were able to produce high amount of IAA i.e. 125.3, 122.2, 104.8 µg ml⁻¹ respectively. Ability to produce IAA is important trait since it was considered as the most important phytohormone. IAA have functions as signal molecule in the regulation of plant growth and development processes. Substrate availability, growth stage and culture condition were additionally influenced the production of IAA [41]. Therefore, its PGPR isolates may vary from different species and strains.
Nitrogen is crucial for plant growth, because they compulsory in the synthesis of chlorophyll, proteins, enzymes, DNA and RNA. N₂ is inaccessible to plant, N-fixing microorganisms can convert N₂ into ammonia which can be directly used by plants. To reduce dependency to synthetic nitrogen fertilizer, therefore it can be use those N fixing microbes [42]. In this study, 10 isolates had nitrogen fixing activities, 4 (Az Th, Az Gd, Az Gu, PK 2.6) of which had relatively higher activity than others. The second most important nutrient that essential for plant growth is Phosphorus. In soil, a larger portion of phosphorus are unattainable for plants because in the form of insoluble phosphates [43]. In this study, 10 isolates were positive and 3 isolates (Kbm 10.3, PK 2.4, PK 2.6) were able to solubilize phosphate at relatively higher rates (from 10 to 15 mm) than other isolates. These three isolates can change insoluble phosphates into soluble orthophosphate (H₂PO₄⁻ or HPO₄²⁻), therefore they are more suitable for their application as biofertilizers [44].

Ammonia productions are considered as direct and indirect mechanisms of growth promotion. Ammonia production can assist plants to meet its nitrogen demand and in oversupply, can lower the colonization of pathogens. Microorganisms produce ammonia by hydrolyzing urea in ammonia and carbon dioxide [45,46,47]. In this study 9 isolates produced ammonia, 3 isolates (Pro 7C.A, Pro 4A.A, Pro 5A) of which produced high ammonia.

All of 15 bacterial isolates were also tested the ability to produce lytic enzyme, HCN and siderophore as a part of biocontrol properties. The result show that 11 isolates were positive for protease production, 8 isolates were positive for HCN, and 7 isolates produced siderophore (Table 2). Hydrolytic enzymes (chitinase, glucanase, lipase, and proteases) have function as agents for plant diseases prevention by instigating lysis of pathogenic microbes in plants [48]. It has been reported that PGPR which produce one or more of those lytic enzymes have exhibited biocontrol ability against a number of plant pathogenic fungi. In this experiment 11 isolates were protease positive, 6 isolates (PK 2.4, PK 2.6, Pro 4A.A, Pro 5A, Pro 7C.A, Az Th) are quite high (12-20 mm).

Another important trait of rhizobacteria that indirectly influences plants growth was HCN production. HCN is volatile product that exhibit antifungal action by playing essential role in the biological control of plant pathogens or acting as inducer of plant resistance. In our study, production of HCN were detected in Kbm 2.10, Kbm 2.15, Kbm 10.3, PK 2.4, PK 2.6, Pro 4A.A, Pro 5A, Pro 7C.A (Table 2).
Siderophore-producing bacteria can also function as biocontrol agents, they make iron available to plants, therefore these bacteria compete for this element against soil-borne pathogens [49,50]. Out of 15 bacterial isolates, seven isolates which are TPK 5b2, CBT1, CBT2, CBT 4.3, Kbm 2.15, PK 2.4 and Pro 5A showed positive activity for siderophore production and it is indicated by the development of orange halos surrounding the bacterial colonies in blue agar medium (Table 1). Three isolates, Kbm 2.15, PK 2.4 and Pro 5A had higher rates (from 12-14 mm) compared to other isolates.

The PGPR containing ACC deaminase can hinder the abiotic stress of induced ethylene production and its associated adverse effect on plants. In this study 12 isolates were capable to produce ACC-deaminase enzyme, 5 isolates (TPK 5b2, PK 2.6, Az Th, Az Gd, Az Gu) higher than the others. According to several published articles, it was suggested that plants inoculated with PGPR containing ACC deaminase could make plants more resistant to various stresses such as salinity, drought, flood and against various pathogens [51,52,53,54].

3.2. The effect of PGPR inoculation on Radish growth
The effect of 15 PGPR on Radish growth in the greenhouse showed that all isolates had some influences on increasing growth and root tuber formation compared to control. Nine isolates (TPK 5b2, CBT1, CBT2, Kbm 2.10, Kbm 2.1, Kbm 10.3, PK 2.6, Pro 4A.A) significantly increased the wet weight of shoot plants, the highest effect was reached by Kbm 10.3 isolate (168.5 g), while control only reached 81.5 g. Eight isolates (CBT1, CBT2, CBT 4.3, Kbm 2.10, Kbm 10.3, PK 2.6, PK 2.4, Pro 4A.A) had a significant effect on increasing formation of root tuber, the highest was Kbm 2.10 isolate (176 g), while control only reached 54.7 g. Six isolates (CBT1, CBT2, Kbm 2.10, Kbm 10.3, PK 2.4, Pro 4A.A) had significant effect on increasing both plant growth and root tuber formation. Two of them (Kbm 10.3 and Kbm 2.10) seemed to have the highest impact in promoting growth and root tuber formation.
3.3. Bacterial Molecular Analysis

These were six isolates capable of increasing all the plant growth variables assessed (weight of shoot plant and root tuber), two of them (Kbm 2.10 and Kbm 10.3) were analyzed using 16s rDNA sequences. The BLAST result showed that they have close evolutionary relationship with Bacillus megaterium (Kbm 2.10) and Paraburkholderia tropica (Kbm 10.3).

It has been reported that the root system in A. thaliana WT plants inoculated by Bacillus megaterium was affected, including a suppression in primary root growth followed by an increase in lateral root number, lateral root growth and root hair length, suggested the response was due to phytohormones, [55]. The Paraburkholderia was previously bacteria of the genus Burkholderia that were split into the environmental genera (Paraburkholderia) and pathogenic genera (Burkholderia) [56]. Paraburkholderia were identified as PGPR in several C4 plants, such as sugarcane, maize, rice, and numerous grasses [57].

PGPR shows direct effect on the growth of host plant via direct or indirect mechanism. Through mechanism by which PGPR promote plant growth are not yet completely clear. Phosphate solubilisation, production of phytohormones, promotion of the mineral nutrient uptake and suppression of soil borne pathogens are believed to be involved in promote plant growth. In the present study, PGPR were isolated from various rhizosphere plant samples and screened for different plant growth promoting traits. The result showed that all of 15 isolates exhibited multiple PGPR properties (Table 1, 2). These findings are important for future studies. In the plant inoculation assays, all bacterial strains improved plant growth and root tuber of Radish as compared to the uninoculated control. Inoculation with Bacillus megaterium (Kbm 2.10) and Paraburkholderia tropica (Kbm 10.3) exhibited maximum beneficial effects on plant growth, and root tuber formation which we tentatively link to the ability of strain to produce IAA, solubilize P as well as fix nitrogen. Kbm 2.10 isolate has a fairly high IAA productions (104.8 µg ml⁻¹) and has activities of P solubilizing and N fixing, and also produce Siderophore, HCN and Ammonium.

Bacterial secretion of phytohormone such as IAA can impact root architecture by stimulating overproduction of root hairs and lateral roots and subsequently increase nutrient and water uptake, thus contributing to growth. Kbm 10.3 is also potential isolate, it had a fairly high P-solubilizing activity (20 mm), N fixing, produce HCN and Ammonia. It has been reported that rhizobacteria which have phosphate solubilizing activity as well as nitrogen fixation, will be the most important plant growth promotion factors [58]. Both isolates also produced ammonia that indirectly influences the plant growth.
It is suggested that the Radish growth was caused by a sum of factors, and not only by individual values obtained in vitro but there is also compatibility between microbes and plant.

![Phylogenetic tree](image)

**Figure 3.** Neighbor joining tree showing the phylogenetic relationship of the PGPR strains based on the sequences of the 16S rRNA gene (1100bp). Closely related sequences were obtained from NCBI Gen Bank. Numbers at the branching points are bootstrap values >90% (percentages of 1000 resamplings).

Sometimes, the in vitro test results are not directly proportional to the bioassay test results. Bacteria that have the highest plant growth-promoting character sometimes do not show the highest plant growth. To obtain potential microorganisms to use in agriculture, the most studied mechanisms in vitro are auxin production, nitrogen fixation and phosphate solubilizing. However, evaluation of indirect plant growth mechanisms, such as production of siderophores, production of hydrogen cyanide (HCN), ammonia and hydrolytic enzyme should be done. Microorganisms used in plant tests should ideally showed different growth promotion factors, in both direct and indirect mechanism.

### 4. Conclusion
In the present study, 15 isolates exhibited multiple PGPR properties. PGPR inoculation significantly increased the growth of Radish plants under greenhouse condition. Six isolates (CBT1, CBT2, Kbm 2.10, Kbm 10.3, PK 2.4, Pro 4A-A) showed significant effect on growth and root tuber formation of Radish compared to control. Two of the isolates (Kbm 10.3 and Kbm 2.10) seemed to have the highest impact in promoting growth and root tuber formation. Those two isolates were analyzed using 16s rDNA sequences, they have close evolutionary relationship with Bacillus megaterium (Kbm 2.10) and Paraburkholderia tropica (Kbm 10.3). These two isolates may be exploited as microbial inoculants for Radish crop as they enhanced plant growth via diverse mechanisms and offered an attractive strategy to replace synthetic fertilizers.

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