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Characterization of plant growth promoting rhizobacteria isolated from potato (Solanum tuberosum L.) rhizosphere

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Abstract

Plant Growth Promoting (PGP) rhizobacteria live in the rhizosphere of the plant and are frequently shown to be favourable to plant development and growth. In the current study, a sum of nine bacteria was confined from the rhizospheric soil of *Solanum tuberosum* L. gathered from three cultivated lands at Bandarawela, Sri Lanka. They were characterized for multiple PGP ability, cellulolytic enzyme synthesis, and antagonistic activity *in vitro*. Seven of nine bacterial isolates showed production of indole acetic acids in the range of 3.85 ± 0.23 to 1.02 ± 0.08 mg/L, gibberellic acid in the range of 7.32 ± 0.16 to 0.56 ± 0.02 mg/L, ammonia in the range of 0.76 ± 0.01 to 0.08 ± 0.01 µmol/mL, Phosphate solubilization in the range of 32.36 ± 0.9 to 2.14 ± 0.08 mg/L and nitrogenase activity in the range of 2.89 ± 0.06 to 0.45 ± 0.03 nmol/hr. Bacterial isolates, B4 (*Bacillus subtilis*) and B5 (*Bacillus megaterium*) showed maximum PGP ability and antagonistic activity against two phytopathogens of potato. All isolates showed cellulolytic activity except B3 and B7. These findings firmly suggest that the PGP bacteria recognized in this study can be valuable for the development of effective biofertilizers/bio-inoculants to improve the growth and productivity of *Solanum tuberosum.*

Keywords: Rhizosphere, PGP, Antagonism, Bio-inoculants

1. Introduction

Excessive use of chemical fertilizer (CF) and agrochemicals in conventional agriculture poses a major environmental threat to the functions of entire agroecosystems including reduced crop productivity by collapsing the soil microbial diversity [24]. When compared to other vegetable crops, the potato crop typically removes 1.5 times as much nitrogen (N) and 4 to 5 times as much phosphate from the soil [3]. As with most crops, potato cropping systems often employ significant quantities of CF inputs to increase yield. The use of plant growth-promoting Rhizobacteria (PGPR) for minimizing chemical inputs in agriculture is a potentially significant topic in the context of increasing global concern for food and environmental quality. The term "PGPR" refers to bacteria that colonize the rhizosphere and plant roots and promote plant development by any mechanism.

Due to its potential for sustainability, the use of PGPRs and other rhizosphere-associated beneficial microbial inoculants as bio-fertilizers has been recognized as a viable alternative. Evidence suggests that the ability to utilize the benefits of PGPRs is crucial for maintaining sustained soil fertility [26]. Direct plant growth promotion by PGPR comprises either supplying the plant with chemicals that the bacteria produce to promote plant development or assisting the plant in uptaking certain environmental nutrients [24]. Indirect plant growth promotion occurs when PGPR reduces or prevents the negative impacts of one or more phytopathogenic microorganisms and helps plants tolerate environmental challenges. Indirect plant growth promotion occurs when PGPR reduces or prevents the negative impacts of one or more phytopathogenic microorganisms and helps plants

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tolerate environmental challenges [6]. Plant growth promotion by direct mechanisms includes nitrogen fixation, phytohormones production (auxins, gibberellins, ethylene, etc.), mineral (phosphorus, zinc, and potassium) solubilization making nutrients available for the plant, and indirectly by their biological control behaviour ascribed to syntheses of antibiotic, siderophore, cyanide, hydrolytic enzymes for cell wall degradation of pathogenic fungi, ammonia and induced resistance (ISR) to repression of soil-borne pathogens [18] The goal was to find and define effective plant growth promoting (PGP) bacteria in rhizosphere soil of potato that may be employed to manage plant diseases and produce healthy crops with yield improvement.

2. Material and Methods

2.1. Sample Collection and isolation of rhizobacteria

The rhizospheric soil used for bacterial isolation was extracted from the potato roots (*Solanum tuberosum* L.) at a depth of 15-20 cm from the potato plant cultivated at the Regional Agriculture Research and Development Center, Bandarawela, Sri Lanka. Soil samples were taken from three different locations, and the bulk soil was shaken violently by hand for 10 minutes before collecting the rhizospheric soil from the roots of potato plants. Ten grams of rhizospheric soil samples were collected into sterilized black polythene bags and transported to the laboratory and stored at a temperature of 4 °C for further isolations. Root-associated soil samples were subjected to a serial dilution (10-fold) followed by the isolation of bacteria on Nutrient Agar (NA) through spread plate techniques. Isolated bacterial colonies were differentiated according to the colony morphology and the colonies were subcultured to prepare pure isolations.

2.2. Screening of isolates for PGP Traits

The isolated bacteria were characterized for PGP traits such as Phosphorus solubilization, indole acetic acid (IAA), Gibberellic Acid (GA), Nitrogen fixation, and ammonia synthesis under *in vitro* conditions.

2.2.1 Phosphate solubilization

The phosphate solubilization was measured qualitatively using Phikovkaya (PVK) agar plates incubated at 30 °C for 5 days. Phosphate solubilization resulted in the formation of a clear zone around a growing colony, as assessed by the phosphate solubilization index (PSI) calculated using the following formula:[16] PSI = colony diameter / total diameter (colony + holo zone). The phosphate solubilization ability of the bacterial isolates having the highest PSI was quantitatively estimated using PVK broth media [1]. Bacterial culture isolates were inoculated individually in PVK broth medium and cultured for 10 days at 30 °C with constant shaking. Ten milliliters of bacterial suspension were withdrawn at 2 days intervals, centrifuged (15 minutes at 10.000 g), and added 0.5 mL of Phosporos concentrated reagent (Phosporos concentrated reagent: 12 g of ammonium molybdate, 0.277 g potassium antimonyl tartrate) into 5 mL of the supernatant. After shaking the mixture and allowing it to stand for 30 minutes, the absorbance at 693 nm was measured using a UV - VIS spectrophotometer. Phosphate solubilization was estimated and represented as mg/L using a standard curve established with known amounts of PO4 (Titrisol) solution.

2.2.2 Production of IAA

The IAA production was quantified by inoculating the bacterial isolates in tryptophan-supplemented nutrition broth (NB) medium and incubating them for 48 hours at 30 °C, 150 rpm. After 48 h, 4 mL of Salkowski reagent was combined with 1 mL of the supernatant, and the absorbance of the consequent pink coloration was examined at 30 °C, after 30 min at 535 nm in UV/Visible spectrophotometer (Thermo Scientific Multiskan Go, 1510). A standard curve was plotted by 1.0 mg IAA dissolved in a few drops of methanol and made to 10 mL (0.1 mg/mL) with distilled water. The obtained results of IAA were expressed as mg/mL [7].

2.2.3 Production of GA

Gibberellic acid (GA) was quantified by using the method of [11], with minor modifications. In a brief, King's B broth medium was used to establish bacterial cultures for four days before centrifuging to separate the cells and measuring the protein content of the pellet. Ethyl acetate was used to extract the GA after adjusting the pH with 0.1 M HCl. Potassium ferrocyanide (0.2 mL) was added to 1.5 mL of extract and centrifuged at 1000 rpm for 10 min. An equivalent volume of 30% HCl was added to the supernatant, and the mixture was incubated at 20°C for 75 minutes. A blank of 5 % hydrochloric acid was used, and absorbance was measured at 254 nm using a UV-visible spectrophotometer (Thermo Scientific Multiskan Go, 1510). The concentration of GA was deduced using a standard graph and the quantity was expressed as mg/L.

2.2.4 Nitrogenase activity

Nitrogenase activity was detected by acetylene reduction/ethylene production assay as described earlier [15]. Pure bacterial colonies were inoculated into the semisolid medium of Nitrogen Free Malate vials and incubated at 28 ± 2 ºC for 48 h. Following an injection of acetylene (10% v/v) and a 16-hour incubation period at 28 °C, 100 µL of the vials' gas samples were examined on a gas chromatograph (Agilent 7890B/5977A) equipped with a Porapak Q column. The experiment was repeated twice with three replicates each and the mean was calculated.

2.2.5 Ammonia Production assay

Bacteria were tested qualitatively and quantitatively for their ability to produce am monia in peptone water following the method described by [5]. Freshly developed cultures were added to 10 mL of peptone water in each tube, and another medium was left uninoculated as a reference. The cultures were then incubated for 48 hours at 30 ºC and 150 rpm while being shaken. Nessler’s reagent (0.5 mL) was added to the supernatant of each bacterial culture and observed the color development of brown to yellow due to ammonia production. The production of ammonia was quantified spectrophotometrically at 450 nm based on a standard curve prepared using solutions of ammonium sulfate and expressed as µmol/mL.

2.3. Screening of isolates for cellulase enzymes and antagonistic activity

The reducing sugar generated during the enzymatic reaction by 3,5-dinitrosalicylic (3,5-DNS) acid was used to calculate the bacteria's cellulase activity [14]. Bacteria were inoculated into a Nutrient Broth medium supplemented with 5 g of sterilized cellulose powder and incubated at room temperature with constant shaking for five days. Then, the amount of reducing sugar produced by each bacterial isolate was using 3,5-DNS acid method. The reaction was stopped by adding 1.5 mL of DNS reagent and then heating the mixture for 5 minutes. Sugars liberated were determined by

measuring absorbance at 540 nm using UV spectroscopy and the cellulase production was estimated by using the glucose calibration curve. All the experiments were carried out in triplicates.

Isolated bacteria were screened *in vitro* for antifungal activity against two phytopathogenic fungi (*Ralstonia solanacearum* and *Alternaria solani*) for potatoes on PDA using a dual culture technique. An actively growing (5 days old) fungal culture was cut by a sterile borer and kept at the center of the PDA media plate. A loopful of 48 hours old test culture was streaked at one edge of the plate 3 cm away from the fungal plug as a straight line. Plates inoculated with equal fungus but no bacteria were used as controls, incubated at 28 ± 2 °C for five days, and the growth inhibition zone was determined. The formula: I% = [(C-T)/C] x 100 was used to calculate the percentage of fungi that were inhibited, where C represents the diameter of the fungi that developed on the control plate and B represents the diameter of the fungi that developed on the agar plates that had already been pre-inoculated with the test bacteria [12].

2.4. Molecular identification of PGP bacterial isolates

Identification of the bacterial isolates showing higher PGP activity was done through 16S rRNA sequence analysis. Using a Gene Jet DNA purification Kit and following the manufacturer's instructions, total genomic DNA from the isolates was extracted. Using a universal primer combination of 27F and 1492R, 25 µL of genomic DNA was subjected to PCR to amplify 16S rDNA [8]. The following substances were included in the PCR mixture: 4 µL of template DNA (50 ng), 1 µL of each forward 27 F and reverse 1492 R primer (10 pmol concentration), 1.5 µL of 2 mM of the dNTPs mix, 2 µL 10x PCR buffer, 0.4 µL of (3 U/mL) Taq DNA polymerase and the final volume was adjusted by adding nuclease-free water to the reaction mixture. The results of the PCR amplification were sent to Macrogen Inc. Seoul, Korea for sequencing after being amplified in a thermal cycler. Using the NCBI BLAST program, the resultant sequences were examined for similarity with the bacterial sequences in the Genebank database [13].

2.5 Statistical Analysis

The data were analyzed using a statistical program (ANOVA) in Minitab® 17.2.1, Mini Tab 2017 software. The differences in treatment means were evaluated using the least significant differences test (LSD) at a probability level of 5% ($P \leq 0.05$).

3. Results

3.1 Phosphate solubilization

A total of nine bacteria had recovered from the potato rhizosphere on NA medium and the isolates were labeled from B1 to B9. All bacterial isolates were showing a zone of solubilization on PVK medium indicating that the bacterial isolates have the capability to solubilize phosphate (Figure 1a). Out of all bacterial isolates, B2, B4, B5, and B8 isolates showed high PSI over the other isolates and B4 recorded the highest significant ($P < 0.05$) PSI (Fig. 1a). Cultures showing positive results with phosphate solubilization under plate conditions were selected for quantitative estimation of phosphate. The phosphate solubilization activity of the four best-performing bacterial isolates (B2, B4, B5, and B8) during ten days of incubation is expressed in Figure 1b. A sharp increment was observed in the Phosphate solubilization activity up to day 6 by all the bacterial isolates and the highest increment was observed by isolate B4 (*Bacillus subtilis*) at every time interval up to day 10. However, the rate of increment gradually decreased with the time from day 6 to day 10. After

day 10, it was clear that the Phosphate solubilization activity by the bacterial isolates became constant.

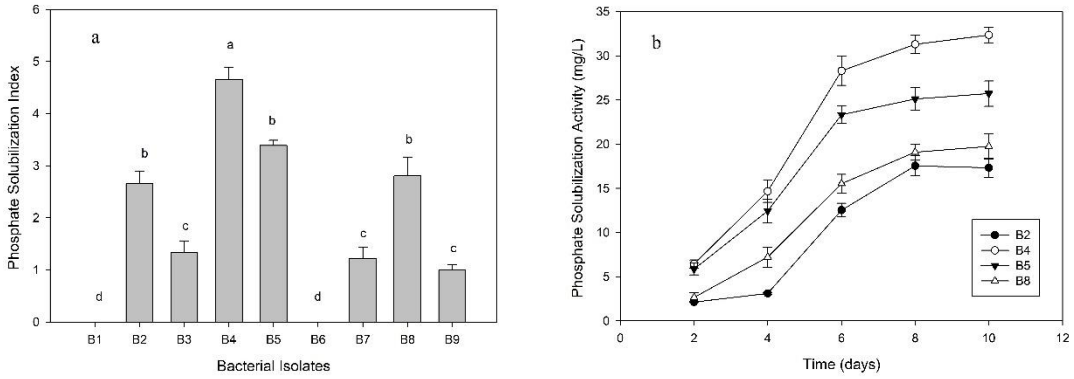

Figure 1- Phosphate solubilization activity of the isolated bacteria a) PSI b) Phosphate solubilization activity with time. At the 5% probability level, there is no significant difference between columns with the same letter. vertical bars show standard deviations.

3.2 Plant Growth Promoting (PGP) Traits

All the isolates were screened for diverse PGP traits *in-vitro viz.*, IAA production, GA production, Nitrogenase activity, and Ammonia production, results of which are given below (Table 1). Varied levels of PGP traits were found with the test bacteria. Out of nine bacterial isolates, bacterial isolate B5 showed maximum production of IAA (3.85 ± 0.23 mg/L) followed by B4 (3.76 ± 0.15 mg/L), B8 (2.45 ± 0.1mg/L) and B2 (1.64 ± 0.04 mg/L). A similar pattern was observed in the production of GA from which the maximum GA production was recorded by B5 (*Bacillus megaterium*) isolate. However, significant differences were not observed between B4 and B5 in the production of both plant growth hormones (IAA and GA). Phytohormone production was not detected from the isolates B6 and B7. Nitrogenase activity from these isolates ranged from 2.89 ± 0.06 to 0.45 ± 0.03 nmol/hr. The highest nitrogenase activity was shown by bacterial isolate B4 (*B. subtilis*) with the activity of 2.63 ± 0.01 nmol/hr followed by B5 (2.63 ± 0.01nmol/hr), B2 (1.95 ± 0.04 nmol/hr), B1 (1.79 ± 0.01 nmol/hr), B3 (1.78 ± 0.02 nmol/hr), B8 (1.44 ± 0.01 nmol/hr) and B6 (0.45 ± 0.03 nmol/hr). However, any significant difference was not observed between B4 and B5 in nitrogenase activity. A similar pattern was observed in ammonia production except in isolates B1 and B8, from which B4 (0.76 ± 0.01 µmol/mL) showed the highest value. Nitrogen fixation ability was not observed from the isolates B7 and B9.

Table 1- Plant Growth Promoting (PGP) Traits of the isolated bacterial strains

Bacterial Isolate	IAA (mg/L)	GA (mg/L)	Nitrogenase Activity (nmol/hr)	Ammonia Production (µmol/mL)
B1	1.02 ± 0.08^{d}	3.34 ± 0.03^{c}	1.79 ± 0.01^{bc}	0.21 ± 0.01^{d}
B2	1.64 ± 0.04^{c}	3.42 ± 0.06^{c}	1.95 ± 0.04^{bc}	0.49 ± 0.01^{b}
B3	0.86 ± 0.03^{d}	1.12 ± 0.08^{de}	1.78 ± 0.02^{bc}	0.18 ± 0.02^{d}
B4	3.76 ± 0.15^{a}	6.97 ± 0.14^{ab}	2.89 ± 0.06^{a}	0.76 ± 0.01^{a}
B5	3.85 ± 0.23^{a}	7.32 ± 0.16^{a}	2.63 ± 0.01^{a}	0.68 ± 0.04^{a}
B6	0 ± 0^{e}	0 ± 0^{f}	0.45 ± 0.03^{d}	0.08 ± 0.01^{e}
B7	0 ± 0^{e}	0 ± 0^{f}	0 ± 0^{e}	0 ± 0^{f}
B8	2.45 ± 0.1^{b}	6.23 ± 0.11^{b}	1.44 ± 0.01^{c}	0.31 ± 0.01^{c}
B9	1.38 ± 0.06^{c}	0.56 ± 0.02^{e}	0 ± 0^{f}	0 ± 0^{f}
	(P = 0.00, F = 422.05)	(P = 0.00, F = 3187.43)	(P = 0.00, F= 3038.48)	(P = 0.00, F = 429.52)

Mean ± SD. B1-B9 are bacterial isolates. Means in the same column followed by the same letter are not significantly different at a 5 % probability level.

3.3 Cellulase and antagonistic activity of the isolated bacterial strains

Cellulolytic activity is expressed as the amount of reducing sugar formation (Fig. 2a) and the antagonistic activity of the bacterial isolates against phytopathogenic fungi is expressed as the percentage of inhibition (Fig. 2b). Out of nine bacterial isolates, bacterial isolate B2 (0.46 ± 0.05 nmol/L), showed significantly the highest ($P < 0.05$) sugar formation over the other isolates indicating the highest cellulolytic activity. Moderate cellulolytic activities were shown by B5, B4, B8, and B1 in descending order. Results of dual culture assay revealed that out of nine isolates, seven bacterial isolates (B1-B6 and B8) reduced the mycelial growth of *R. solanacearum* and six bacterial isolates (B2-B6 and B8) reduced the mycelial growth of *A. solani* on PDA media. Among them, the highest inhibition was observed by bacterial isolate B4 (76 %) for *R. solanacearum* and B5 (83 %) for *A. solani.*

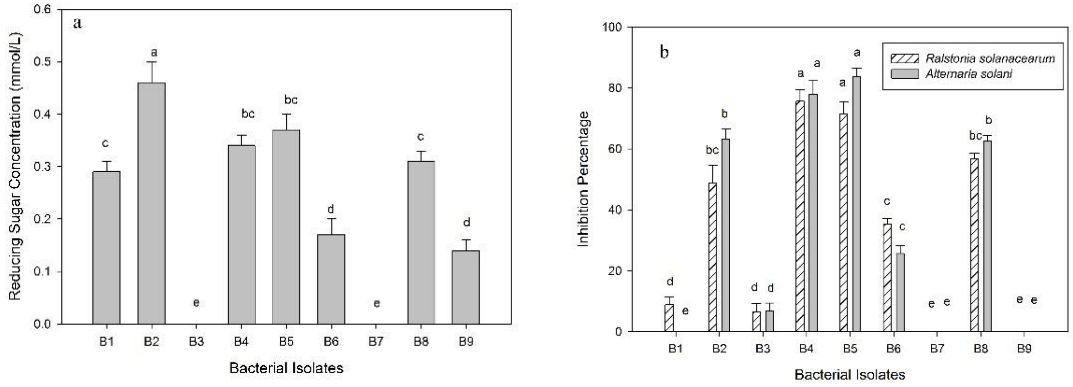

Figure 2- a) Cellulolytic activity b) antagonistic activity of the bacterial isolates. Columns with the same letter are not significantly different at a 5 % probability level. vertical bars show standard deviations.

3.4 Molecular identification of PGP bacterial isolates

According to sequence analysis through GenBank search showed that the isolates had high sequence similarity to the species B2- *Pseudomonas aeruginosa* (CP050330.1), B3- *Bacillus cereus* (AP023014.1), B4- *Bacillus subtilis* (CP046448.1), B5- *Bacillus megaterium* (CP001984.1), and B8- *Pseudomonas putida* (CP096581.1) (Table 2).

Table 2- Molecular identification of PGP bacterial isolates

Sample identity	Length of the fragment (bp)	Closest Relative	Similarity (%)	Accession Number
B2	582	*Pseudomonas aeruginosa*	100	CP050330.1
B3	590	*Bacillus cereus*	100	AP023014.1
B4	655	*Bacillus subtilis*	100	CP046448.1
B5	686	*Bacillus megaterium*	100	CP001984.1
B8	745	*Pseudomonas putida*	100	CP096581.1

4. Discussion

The employment of beneficial microorganisms, which may either directly or indirectly increase plant development, help in nutrient uptake, and protect crops from a range of biotic and abiotic challenges, is an important component of sustainable agricultural practices. The development of effective microbial formulations could aid to boost crop yield and also reduce environmental risks associated with the indiscriminate use of industrial chemicals such as fertilizers and pesticides. In the present research, eight bacteria had been isolated from the rhizosphere soil of potato and all of the isolated bacteria have been screened for a couple of plant growth tendencies *in vitro*. Results found out bacterial isolates B4 and B5 confirmed maximum production of a growth-stimulating hormone (IAA and GA), phosphate solubilization, ammonia production, and nitrogenase activity (Figure 1 and Table 1). 16S rRNA gene sequences of the potent bacterial isolates (B4 and B5) had been determined as most similar to *B. subtilis* and *B. megaterium.* Further, *P. aeruginosa* (B2) and *P. putida* (B8) isolated from potato rhizosphere showed high performances in all tested parameters. Many researchers have isolated and identified numerous species of *Bacillus* including *B. subtilis* and *B. megaterium* [10, 21] from the rhizosphere of potatoes.

Many rhizobacteria reported being supplied with numerous plant hormones including IAA [25]. In the existing research, out of nine bacterial isolates, seven isolates have been capable to produce IAA in a range from 3.85 ± 0.23 to 1.02 ± 0.08 mg/L and the highest was recorded by *B. megaterium* (Table 1). IAA is a very significant phytohormone that acts as a signal molecule in the control of plant growth. According to [19], 80 % of PGP bacteria found in the rhizosphere of different crops can synthesize and excrete IAA in significant amounts. It significantly affects cell growth, division, and differentiation as well as tuber and seed germination, etc. A large fraction of phosphorous exists in soil as insoluble inorganic forms and is consequently unavailable to plants. In this context, phosphate solubilizing bacteria (PSB) play a key role in solubilizing phosphate [25]. It has been noted that rhizosphere soil frequently has larger concentrations of phosphate-solubilizing bacteria than nonrhizospheric soil [19]. Different bacterial genera together with *Bacillus sp, Pseudomonas sp, Rhizobium* sp., etc., are determined to be potential phosphate-solubilizing microorganisms [21]. The findings of the current study agree with the earlier research by [17], which described the solubilization of inorganic phosphate by *Bacillus* sp. and *Pseudomonas* sp., both of which were isolated from the rhizosphere of wheat. The promotion of plant growth may also involve nitrogen fixation. According to a study, *B. subtilis* application

improved potato tuber growth [21]. Except for B7 and B9, all of the chosen isolates used in this work exhibited acetylene reduction activity, a commonly used indicator for nitrogenase activity and N2 -fixing ability [2]. Numerous crops' roots and diazotrophic N-fixing rhizobacteria have been shown to transfer nitrogen (N) [23].

In the present study, seven of nine bacterial isolates were able to produce cellulase enzyme under *in vitro* conditions (Fig. 2a). Cellulolytic enzymes synthesized by rhizospheric bacteria cause lysis of fungal cell walls and defend the host plants from various fungal diseases [9]. An environmentally benign alternative to chemically manufactured fungicides, which are one of the main causes of environmental contamination, is the employment of microorganisms that produce cellulolytic enzymes. The results are in line with the previous finding of many researchers who reported the production of cellulolytic enzymes by various species of *Pseudomonas* and *Bacillus* [22]. In the present investigation, bacterial isolates B4 and B5 showed antagonistic activity against *R. solanacearum* and *A. solani* (Fig. 2b). *R. solanacearum* and *A. solani* are soil-borne fungal pathogens that are reported to cause wilt disease [9] and early blight in potato plants respectively [4]. The use of PGP bacteria can serve as cheap and environmentally safe alternatives to synthetic products [22]. Several studies reported that to control numerous soil-borne diseases *Pseudomonas* sp and *Bacillus* sp. are the most effective microbes and they can be used as potential biocontrol agents [20]. The results of this study are in line with earlier work of [21], who reported antagonistic activity of various species of *Pseudomonas* sp. and *Bacillus* sp. against *R. solanacearum* and *A. solani*. to enrich the growth and yield of the potato crop.

5. Conclusion

The plant-beneficial PGP's rhizobacterial bio-inoculum would reduce reliance on toxic agrochemicals which harm ecosystems globally. This study was successful in identifying two effective PGP isolates *B. megaterium* and *B. subtilis* from the rhizosphere of potato, which can be utilized to manage some pathogenic diseases of potato. The isolated PGPR can be further studied for their potential to be developed as bioinoculants for plant growth promotion in *Solanum tuberosum*.

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7. References

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