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Research Article

ROLE OF PHOSPHATE SOLUBILIZING BACTERIA ON GROWTH-PROMOTING ACTIVITIES IN PLANTS

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ABSTRACT
Phosphate-solubilizing microorganisms (PSMs) are a significant component of the soil microflora that facilitates the bioavailability of phosphate through the mineralization of organic phosphate, the solubilization of inorganic Phosphate minerals, and biomass storage of substantial amounts of phosphate. Phosphate-solubilizing bacteria promote plant growth by solubilizing the phosphate in soil and increasing soil fertility. The PSM bacteria occupy a significant part of the plant's growth, enabling a group of microorganisms. They produce compounds inhibiting the growth of pathogens, plant growth regulators like IAA, GA, organic acids, bacteriocins, and some antifungal substances. PSM directly or indirectly increases the nutrient absorption capacity of the plants. The present study includes three soil samples to isolate phosphate-solubilizing bacteria. Overall, nine distinct bacterial isolates were discovered and considered for future studies on phosphate solubilization. The antifungal activity of the isolates was evaluated against phytopathogenic fungi like *Fusarium, Cladosporium, Rhizomucor, Rhizopus*, and *Trichoderma* and was found to be effective against most of the tested fungi. The isolated bacterial strains were also tested for Indole acetic acid (IAA) production and produced a good amount of IAA. The strains that have a good amount of Indole acetic acid (IAA) and are analyzed by HPLC are applied to plant growth studies. The selected bacterial strains exhibited good phosphate solubilization, antifungal activity, and Indole acetic acid (IAA) production that will increase the overall growth and development of the plant. Further, these can be proposed as bioinoculants, which will help farmers obtain better crop yields.

Keywords: *Bio inoculants, Phosphate solubilization, IAA, GA*

INTRODUCTION
In recent years, scientists have focused on exploring the potential of beneficial microbes. One such aspect is using plant growth-promoting rhizobacteria (PGPR) for sustainable agriculture. These bacterial groups actively colonize plant root regions and improve plant growth and yield (Dey *et al*., 2004). The rhizosphere contains a very high microbial load, and the exudates from roots influence it (Kennedy, 2004). The Rhizosphere region predominantly harbours bacteria. Rhizobacteria are rhizosphere-competent bacteria that aggressively colonize plant roots; they can multiply and occupy all the ecological niches found on the roots at all the stages of plant growth in the presence of a competing microflora (Antoun & Kloepper, 2001). Several bacterial species are known to enhance the growth of plants by acting as PGPRs, such as *Pseudomonas*, *Agrobacterium, Bacillus, Azotobacter, Azospirillum, Xanthomonas, Klebsiella, Arthrobacter,* and *Serratia.* Plant growth can be achieved by direct or indirect methods (Glick, 1997; Aeron *et al*., 2010). Phosphate-solubilizing bacteria (PSB) are essential for improving plant growth, and hence, several research works have looked into different facets of this. PSB uses different mechanisms to solubilize the insoluble phosphate (Hussain, *et al.,* 2019). In this study, the isolated bacteria's potential to produce the hormone indole acetic acid and characterization of the bacteria were assessed.

MATERIALS AND METHODS

Isolation of microorganisms from natural sources:

Soil samples were collected from various locations in Kerala. The rhizosphere soil with roots and nodules 6- 8 inches deep was sealed into a sterile bag using a clean spatula. Soil samples were stored in an aseptic and cool environment. Serial dilution was then performed to isolate individual colonies. Suitable dilutions were plated onto nutrient agar and King's B agar plates, and incubated for 24-48 hrs at 37°C. The selected isolates were subsequently sub-cultured and used for analysis (Siddhi Gupta *et al.,* 2016).

Identification of different isolates:

The purity of the cultures obtained through many swabs was checked by colony morphology and biochemical tests (Siddhi Gupta *et al.*, 2016). Microscopic observation allowed partial identification of bacterial colonies. The bacterial cultures were studied for the colony's shape, size, color, elevation, margin, and texture. Simple tests, such as motility and Gram staining, were carried out to identify the cultures. Biochemical tests included IMViC test, catalase, oxidase, urease, nitrate reduction, gelatin hydrolysis, and starch hydrolysis tests.

Test for phosphate solubility activity in bacterial isolates:

Bacterial strains were screened for their ability to solubilize inorganic phosphate by well diffusion method (Nosrati *et al*., 2014) in Sperber media (10 gglucose, 0.5 g yeast extract, 0.1 g CaCl2, 0.25 g MgSO4. 7H2O) supplemented with 2.5 g Ca3 $(PO4)_2$ (TCP) and 15 g agar (in solid medium) per liter at pH: 7.2 and Pikovskaya's media (YeastExtract 0.5 g, Dextrose 10.0 g, Calcium Phosphate 5.0 g, Ammonium Sulphate 0.5 g, Potassium Chloride 0.2 g, Magnesium Sulphate 0.1 g, Manganese Sulphate 0.0001 g, Ferrous Sulphate 0.0001 g and Agar 15.0 g) with and without calcium phosphate. Each isolated culture was spread on the plates using a sterile L-rod, and wells were made on the plates at a 3.5 cm distance apart with thehelp of a well borer. 1 ml of calcium phosphate was loaded to the wells using a sterile micropipette and incubated at 28°C for three days. After three days, the bacterial isolate that produced clear zones around the wells was evaluated as positive, leading to phosphate solubilization.

Antifungal assay:

The antifungal activity of the isolated strains was tested against several phytopathogenic fungi such as *Fusarium spp*., *Trichoderma spp*., *Mucor* spp., *Rhizomucor spp*., and *Cladosporium spp*. The bacterial culture was inoculated into the nutrient broth and kept at 37°C for 24 hours. The cultured broth was centrifuged at 3000 rpm for 30 minutes after incubation. Then, 1 ml of supernatant was mixed into the nutrient agar medium. After solidification, each fungal culture (maintained in the active sporulating stage) was inoculated onto the plate (Anjum *et al.,* 2007). The inoculated plates were maintained at 28°C for 48–72 hours.

Preparation of IAA standard curve:

The different concentrations of IAA standards were prepared in an LB culture medium accordingto the standard protocol reported by Sarker Aniruddha and Jubar Al-Rashid (2018). An initial stock concentration of 100 µg/ ml IAA was prepared by adding 1 mg IAA to 10 ml of acetone and aliquoted to 20, 40, 60, 80, and 100 µg/ ml. Then, 1 mL of each measure, including blank (pure LB broth), was taken, and to this, 2 mL of Salkowski reagent was added. After 20 minutes of incubation at room temperature, the OD values were measured at 520 nm, and the standard curvewas recorded.

Production of indole acetic acid (IAA):

For the IAA production test, bacterial isolates were cultured on nutrient broth with tryptophan (0.1 g/l) and 1.5 g of NaCl to create a stress condition. This setup was kept in an incubator shaker at 60 rpm for 24 hours at 37 °C. Centrifugation of the broth was done at 3000 rpm for 30 minutes. Then, 1 ml of each supernatant was mixed with two drops of orthophosphoric acid and 2 ml of Salkowski reagent (50 ml, 35% perchloric acid, 1 ml 0.5 FeCl3) (Sadaf Shahab *et al.*, 2009). Based on the color development, pink indicated a positive reaction to the indole acetic acid, and yellow indicated an adverse reaction (Reetha *et al*., 2014). The OD values were recorded, and IAA concentrations were determined with the help of a standard curve of commercially available IAA.

Purification of IAA:
High-performance liquid chromatography (HPLC) was performed to analyze IAA in the supernatant of the bacterial isolate. The bacterial culture was inoculated in the nutrient broth in a shaker incubator at 37°C at 60 rpm for 24-48 hours. After incubation, the culture broth was centrifuged at 3000 rpm for 30 minutes. The 20 µl of supernatant was analyzed in HPLC. The sample was loaded onto a 5 mm reversed-phase column using an HPLC syringe. HPLC was run at isocratic conditions with acetonitrile and acidic water as the mobile phase (Nighat Seema *et al.*, 2023). The eluates were detected with a differential ultraviolet detector at 254 nm and compared with standard IAA. The standard IAA was also injected into the HPLC to determine the RT (Fumio *et al*., 2005).

Response to plant growth:
Vigna radiata (Green gram) seeds were sterilized with 95% ethanol for 10 minutes and washed three times with distilled water to remove ethanol. Then, the seeds were soaked in 2.5% sodium hypochlorite (NaOCl) for 10 minutes and transferred to a beaker with distilled water. Again, the seeds were soaked for 10 minutes and then washed thoroughly (Sadaf Shahab et al., 2009). The seeds were added to culture broth for 1 hour, then planted in the pots with autoclaved soil. The length of shoots and the number of leaves were measured and recorded by a measuring scale on every alternate day (Reetha et al., 2014).

In-silico analysis of the bacterial isolate (OR 9):
The potent bacterial isolate, which showed the highest IAA production, was selected for further identification by molecular characterization using the standard protocol (Spin column) at Barcode Life Sciences Ltd., Bengaluru. The DNA was extracted by using standard protocols. The isolated DNA was further amplified and characterized using a PCR of 35 cycles (initial denaturation at 95°C for 5 mins, denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 45 sec, and final extension at 72°C for 5 mins) with primers from Bacteria- 27F and 1492R for a product length of 1500bp (Kumar *et al*., 2021).

Identification of the bacterial strain:
The data obtained from sequencing were analyzed using bioinformatics tools like BLAST. They were further investigated to identify the relationship among similar sequences and to construct a phylogenetic tree.

RESULTS AND DISCUSSION

Identification of different isolates:
Table 1 shows the results of bacterial isolates partially identified to the species level by Gram staining. Table 2 mentions the results of other preliminary tests, viz., motility test and colony characteristics. Table 3 shows the results of biochemical tests such as IMVIC, oxidase, catalase, nitrate, gelatinase, urease, and starch hydrolysis.

Table 1: Partial identification of bacterial isolates based on Gram staining.

Sl No.	Culture Number	Shape	Gram's Staining	Culture Name
1	OR 1	Rods	Gram-negative	*Pseudomonas spp*
2	OR 2	Cocci in chains	Gram-positive	*Streptococcus spp*
3	OR 3	Cocci in clusters	Gram-positive	*Staphylococcus spp*
4	OR 4	Cocci	Gram positive	*Enterococcus spp*
5	OR 5	Slender rods	Gram-positive	*Bacillus spp*
6	OR 6	Cocci in clusters	Gram-positive	*Staphylococcus spp*
7	OR 7	Rods	Gram-negative	*Enterobacter spp*
8	OR 8	Slender rods	Gram-positive	*Streptobacilli spp*
9	OR 9	Rods	Gram-negative	*Stenotropomonas spp*

Table 2: Morphological and colony characters of bacterial isolates

Sl No.	Culture Number	Shape	Size	Margin	Elevation	Opacity	Color	Motility
1	OR 1	Irregular	Small	Entire	Flat	Opaque	White	Motile
2	OR 2	Circular	Small	Entire	Flat	Opaque	White	Non-motile
3	OR 3	Circular	Small	Entire/ smooth edge	Raised	Opaque	Creamy white	Non-motile
4	OR 4	Circular/ round	Small	Entire	Raised	Opaque	White	Non-motile
5	OR 5	Circular	Large	Entire	Undulate	Opaque	White	Motile
6	OR 6	Irregular	Medium	Scalloped	Raised	Translucent	Creamy white	Non-motile
7	OR 7	Circular	Small	Entire	Raised	Opaque	Yellow	Motile
8	OR 8	Irregular	Small	Entire	Flat	Opaque	White	Non-motile
9	OR 9	Circular	Medium	Undulate	Raised	Opaque	White	Motile

Table 3: Biochemical test of the bacterial isolates

Culture Number	Indole	MR	VP	Citrate	Catalase	Oxidase	Urease	Nitrate	Gelatinase	Starch hydrolysis
OR 1	-	-	-	+	+	+	+	+	+	-
OR 2	-	+	-	+	-	-	-	-	+	+
OR 3	-	+	-	+	+	-	+	+	+	-
OR 4	+	-	+	-	-	-	-	-	-	-
OR 5	-	-	+	+	+	+	+	-	+	+
OR 6	-	+	-	+	+	-	+	+	+	-
OR 7	-	-	+	+	+	-	-	+	-	+
OR 8	-	-	-	+	-	-	-	-	+	+
OR 9	-	*	*	+	+	-	+	+	+	*

(indicates false positive/false negative)*

Phosphate solubility activity in bacterial isolates:
Well-defined and clear phosphate-soluble activity was observed on Pikovskaya media than on Sperber media (Fig. 1). Seven isolates (OR 1,4,5,6,7,8,9) out of nine showed higher phosphate-soluble activity, whereas OR1 and OR9 showed the highest phosphate-solubilizing activity (Table 4).

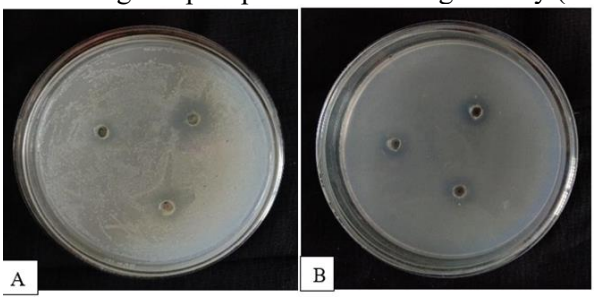

Figure 1: Phosphate solubilizing activity A. Pikovyskaya media B. Sperber media

Table 4: Phosphate-solubilizing activity of each isolate

	Zone of inhibition	
Culture no.	**Pikovskaya media**	**Sperber media**
OR 1	1.7	1.2
OR 2	-	-
OR 3	-	-
OR 4	1.2	0.9
OR 5	1.0	1.1
OR 6	1.3	1.5
OR 7	1.0	1.2
OR 8	0.8	0.8
OR 9	1.6	0.8

Antifungal assay:
The isolated organisms were tested for antifungal properties against phytopathogenic fungal species. *Fusarium, Rhizopus, Rhizomucor, Trichoderma*, and *Cladosporium*. Isolates OR-1, 4,5, 6,7, 8,9 showed potent antifungal properties against all the phytopathogens selected for the study (Table 5).

Table 5: Antifungal activity of the bacterial isolates

Culture no.	***Fusarium***	***Rhizomucor***	***Cladosporium***	***Rhizopus***	***Trichoderma***
OR 1	+	-	+	+	+
OR 2	-	-	-	-	-
OR 3	-	-	-	-	-
OR 4	+	+	+	+	+
OR 5	+	+	+	+	+
OR 6	+	+	+	-	+
OR 7	+	-	+	+	+
OR 8	+	+	+	-	-
OR 9	+	-	+	+	+

Production of indole acetic acid (IAA):

The tryptophan concentration in the growth medium considerably influences the IAA production. The productionof IAA was less on Day 1 and increased on Day 3, but later it decreased gradually. Patten and Glick (2002) have also reported an increase in IAA production up to 96 hours and attributed it to the greater availability of the precursor. Datta and Basu (2002) have studied the IAA-degrading enzymes responsible for decreased IAA production after 96-hour incubation. Since the bacterial isolate OR 9 showed a maximum concentration of IAA from day one to day 3, it was considered for HPLC analysis.

Table 6: Absorbance values of IAA production of each isolate

Sl No.	Culture Number	Day-1	Day-2	Day-3	Day 3 (IAA µg/ml)
1	OR 1	0.02	0.02	0.02	1.03
2	OR 2	0.05	0.06	0.06	6.25
3	OR 3	0.11	0.23	0.22	27.17
4	OR 4	0.08	0.25	0.24	29.76
5	OR 5	0.02	0.02	0.02	1.037
6	OR 6	0.03	0.02	0.02	1.037
7	OR 7	0.02	0.02	0.02	1.037
8	OR 8	0.01	0.01	0.16	19.31
9	OR 9	0.25	0.22	0.21	25.84

Extraction of IAA by HPLC:

High-performance liquid chromatography (HPLC) was used to analyze IAA in the supernatant of the potent bacterial isolate. In the HPLC analysis,a peak with the value of 3.081 at 15 min (Fig. 8) was observed. Calculations based on the area of the peak revealed 97.824% of IAA to be obtained from isolate OR 9. This resembled the pure IAA standard peak of HPLC analysis (Fig. 2 and Table 7).

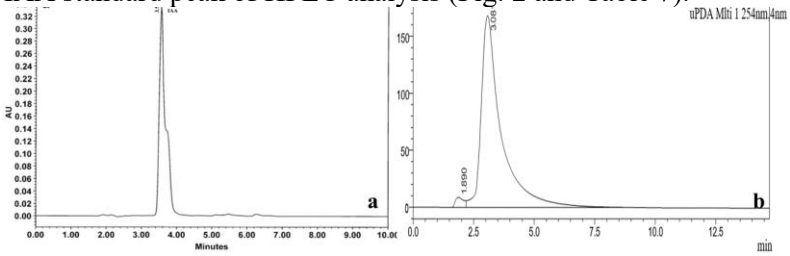

Fig 2: HPLC analysis of standard and bacterial isolate IAA. The peaks obtained during the HPLC analysis of standard IAA and bacterial isolate (OR 9) purified IAA are shown in Figures A and B, respectively.

Response to plant growth:

The plant's shoot length was found to be maximum in the soil supplemented with the isolate OR 9 (Fig 3). The maximum shoot and root length indicated the plant's increased absorption ability for water, minerals, and nutrients deep inside the soil (Table 7).

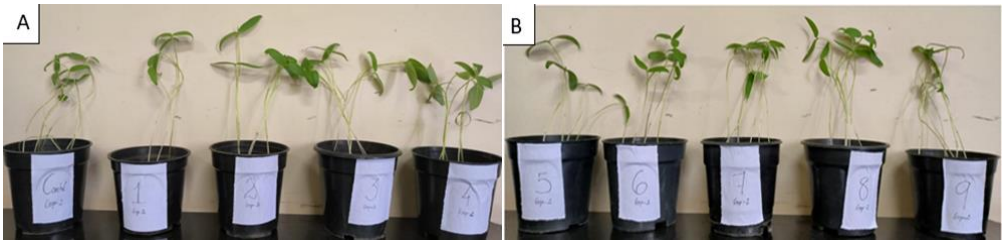

Fig 3: The *Vigna radiata* plants with various isolates. The plants (cultivated with isolates OR 1 to 9) were observed on the 15th day of the growth period.

Table 7: Table of shoot length of plants cultivated with bacterial isolates.

		Control	1	2	3	4	5	6	7	8	9
DAY 1	Plant 1	0	0	0	0	0	0	0	0	0	0
	Plant 2	0	0	0	0	0	0	0	0	0	0
	Plant 3	0	0	0	0	0	0	0	0	0	0
DAY 3	Plant 1	1	1	2	5	2	0	5	8	6	9
	Plant 2	0	0	2	4	0	0	7	4	5	8
	Plant 3	0	0	1	5	0	0	6	4	6	9
DAY 5	Plant 1	9.8	13	13	15	12.3	9.4	13	8	12.3	14
	Plant 2	10	11	12	14	9	6	10	14.3	11	11.6
	Plant 3	9.5	7.6	11.4	12.5	7	5	9	12	12.8	12.4
DAY 7	Plant 1	10.5	14	10	13.3	15	12.6	14	15	15	16
	Plant 2	11	10.5	14.9	12.2	12.1	9.2	13	12	13	13.5
	Plant 3	12	12	14	13	11.4	9	13	13	12	1
DAY 9	Plant 1	16	15	12	13	15.5	14.8	16.4	16	16	17
	Plant 2	12	12	15.2	13.7	17.2	11.8	14.5	13	14	15.5
	Plant 3	13	13.5	14.7	14	12.5	10	15	14.5	15	15.7
DAY 11	Plant 1	17	16.1	17	15	17	15.9	17.2	17	17	18
	Plant 2	15	13.8	16.4	15	16.3	13	16	17.2	15.5	17.5
	Plant 3	14	15.3	15.6	16.1	13.9	11.2	15.3	16	16.3	17.5
DAY 15	Plant 1	19	18.2	21.5	22.3	22.3	18.5	21.3	22.2	21.7	25
	Plant 2	17.2	19.5	18	19	18.5	15	19	21.3	23	25.5
	Plant 3	17.8	17.2	18.2	19.5	17.5	14	17.8	18.5	23	26

Statistical analysis:
All the experiments were performed in triplicate, and a correlation coefficient was calculated for each isolate (OR 1 to OR 9) with the number of days required for an initial increase in shoot length. There is a positive correlation coefficient value of 1 for isolates (OR 1, 3, 4, 6, 7, 8, and 9). A positive correlation coefficient value of 0.99 was observed for isolates OR 2 and OR 5.

In-silico analysis of the bacterial isolate (OR 9):
After the 16s rRNA sequencing, BLAST analysis using the NCBI-GenBank database revealed a % sequence identity of 86.08% with *Stenotrophomonas spp.* Alignment of the sequence with similar species and generating a phylogenetic tree is shown in Fig. 4.

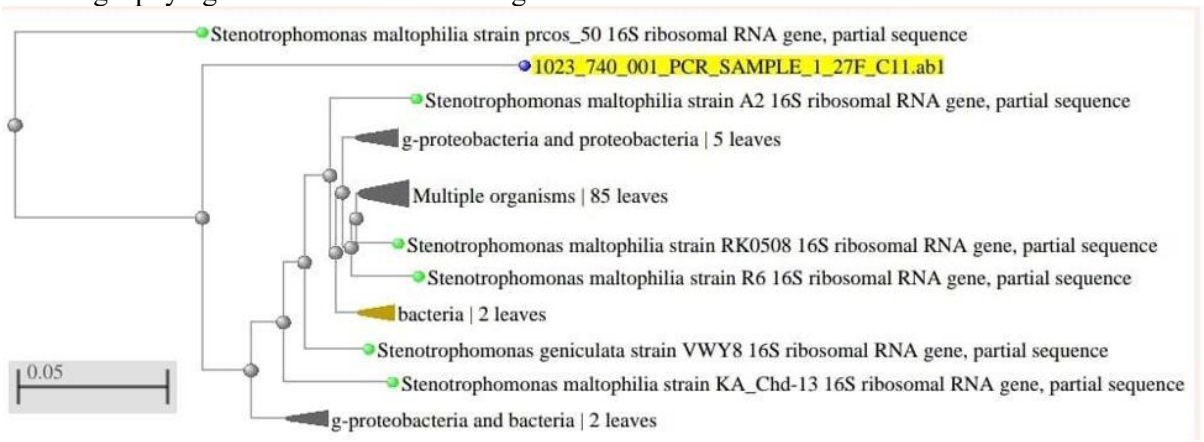

Fig 4: Phylogenetic tree depicting the lineage of the bacterial isolate OR 9

Discussion
Based on this study, it can be inferred that PGPR is a promising source of biologically active compounds. They play an essential role in maintaining the nutrient levels in soil, in the growth and development of the plant, in disease control, and in overcoming drought conditions (Lindsay *et al.,* 1989; Li HP *et al.,* 1989). All isolated rhizobacteria produced different amounts of IAA; the ninth isolate produced the highest amount of IAA on all three days. Scientists combined *Pseudomonas, Azotobacter, and Bacillus* inoculants with Mussoorie Rock Phosphate (MRP) and increased the phosphorus availability in soil. Further, they have indicated that the plant growth-promoting ability of these bacteria is due to the production of Indole-3-acetic acid (IAA) (Patten & Glick, 2002; Timofeeva *et al*., 2022). Among the nine, seven isolates exhibited phosphate-solubilizing activity, improving phosphate levels in the soil. According to Kumar and Dangar 2013, several species are effective phosphate solubilizers correlated with rice plants in nitrogen-fixing nitrogen. Pot studies performed by us have also shown an increase in the growth and development of plants daily when observed for fifteen days. Hence, PGPR can be used as bio-inoculants to replace fertilizers, leading to eco–friendly agricultural practices.

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Conflict of Interest
The authors declare no conflicts of interest related to this work.

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