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Studies on Isolation, Characterization and *in-vitro* Screening of Plant Growth Promoting Rhizobacteria from Rhizospheric Soil of Chrysanthemum (*Dendranthema grandiflora* Tzvelev.)

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A B S T R A C T

A total of forty rhizosphere soil samples were collected from different locations of Bengaluru urban district, Karnataka and used for isolation of PGPRs by standard dilution plate method. Seventy bacterial isolates were selected, purified and were examined for morphological characters like colony morphology, cell shape, Gram reaction. Isolates were diverse in their morphology ranging from dull to milky white, rough to smooth margins, viscous, small to large, non-spreading and spreading. Among them twenty seven and seventeen isolates were identified as N_2 fixers and as Phosphate solubilizers by initial screening respectively. Further, the selected PGPR isolates were biochemically characterized and identified as *Azotobacter* spp. *Bacillus* spp. and *Pseudomonas* sp. The isolates screened qualitatively and quantitatively for plant growth promotion activities like N_2 fixation, phosphate solubalization and growth hormone production (IAA). The amount of nitrogen fixed in Waksman No.77 nitrogen free broth ranged from 4.52 to 19.33 mg per g of C utilized and highest fixed by isolate PGPR-24. Phosphate solubalization efficiency and phosphate solubilization activity in Pikovskaya's medium was ranged from 102.8 % to 168.7 % and 8.56 %to 14.32% respectively and more phosphate solubilization activity showed by isolatePGPR-9. Indole acetic activity (IAA) production ranged between 4.54 to 25.12 µg per ml.

Introduction

Plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants. They are a heterogeneous group of bacteria that can be found in the rhizosphere, on rhizoplane and in association with roots, enhancing the growth of the plant either directly and/or indirectly (Glick, 1995) through effective mobilization of major plant nutrients like Nitrogen, Phosphorus, Potash and other minor nutrients needed by the crop. These beneficial microorganisms are also known to secret plant growth promoting substance like IAA, GA, cytokinines and vitamins for the improvement of crop growth, yields and for quality produce. They not only

promote plant growth but also help in sustainable agricultural development and protecting the environment (Das *et al.,* 2013). It is well established that only 1 to 2 per cent of bacteria promote plant growth in the rhizosphere (Antoun and Kloepper, 2001).

The most predominant rhizosphere colonizing bacteria belong to genus *Bacillus*, *Pseudomonas*, *Azotobacter etc*., because of their rapid growth rate in soils having varying amount of organic matter, tolerance to different agricultural pesticides those are generally used in seed dressing (Vijaypal, 1998). The present investigation was conducted for isolation, characterization and screening of efficient strains of PGPR isolates.

Materials and Methods

The present investigation was carried out at the Department of Agricultural Microbiology, University of Agricultural Sciences, Bengaluru for screening of efficient PGPR isolates (free living N_2 fixers and phosphate solubilizing bacteria) isolated from rhizospheric soil of chrysanthemum crop grown in various parts of Anekal taluk, Bengaluru urban district, Karnataka.

Isolation and characterization

All the PGPR isolates *Azotobacter*, *Bacillus* and *Pseudomonas* were isolated from the rhizosphere soil samples of chrysanthemum collected from various parts of Anekal taluk, Bengaluru urban district, Karnataka using standard serial dilution method. The *Azotobacter* isolates were isolated by using Waksman No.77 medium, Mannitol: 20.0 g, K_2HPO_4: 0.2 g, $MgSO_4.7H_2O$: 0.2 g, NaCl: 0.2 g, K_2SO_4: 0.1 g, $CaCO_3$: 5.0 g, Agar: 15.0 g per lit of distilled water and Phosphate solubilizers were isolated by using Pikovskaya's agar medium contented Glucose: 10 g, Ca_3 $(PO_4)_2$: 5 g, $(NH_4)_2SO_4$: 0.5 g, KCl: 0.2 g, $MgSO_4$: 0.1, $MnSO_4$ and $FeSO_4$: Trace, Yeast extract: 0.5 g, NaCl: 0.1 g, Agar: 20 g for lit of distilled water (Pikovskaya, 1948). The purified isolates were transferred to the respective slants of the same medium and stored for further studies.

The PGPR isolates were characterized by their cultural conditions, morphological and biochemical characteristics (Indole production, Methyl red test, Voges-Proskauer test, Citrate utilization test, Catalase test, Oxidase test, Starch hydrolysis, Gelatine liquefaction, H_2S production, Acid and gas production, Casein hydrolysis, Urease test and Utilization of different carbon sources) using standard methods (Cappuccino and Sherman, 1992).

In vitro assessment of PGP traits of rhizobacterial isolates

Screening of isolates for quantitative estimation of *in vitro* nitrogen fixation

All the free living nitrogen fixing isolates were examined for quantitative Nitrogen-fixation as per standard procedures. 100 ml of the medium (Waksman No. 77) was dispensed in 250 ml conical flasks and autoclaved. One ml of 24 hr old culture was inoculated to each flask. The flasks were incubated at 37 °C for seven days. After seven days of incubation, the culture was homogenized and 10 ml was digested with 5 ml of concentrated H_2SO_4 along with 0.2 g digestion catalyst mixture K_2SO_4: $CuSO_4$: Selenium (100:10:1). After cooling, volume was made up to 10 ml with distilled water. Later, 10 ml of aliquot was transferred to microkjeldhal distillation unit, for which 20 ml of 40 per cent NaOH was added and distilled. Ammonia evolved was trapped in 4 per cent boric acid mixed indicator (Bromocresol green 0.066 g and methyl red

0.033 g in 100 ml methanol) till the solution turned from pink to green. It was titrated against 0.05 N H_2SO_4 and total nitrogen content of the culture was determined and results were expressed as mg N fixed per g of malate.

$$\text{Nitrogen fixed (\%)} = \frac{\text{Titre value} \times 0.014 \times \text{N of } H_2SO_4 \times \text{vol. made}}{\text{Volume of sample used}} \times 100$$

Screening of PSB isolates for *in vitro* phosphate solubilization

Phosphate solubilizing ability of each PSB isolate was determined by measuring the zone of PO_4^- solubilization on the Pikovskaya's agar medium and by estimating per cent Pi released in Pikovskaya's broth medium.

Qualitative estimation [Phosphate solubilization efficiency]

For determining zone of Phosphate solubilization, about 20 ml of Pikovskaya's agar medium was added into the sterilized petriplates, after solidification 10µl broth culture was spot inoculated on the plates. The plates were incubated at 28 ± 2 °C for about 3-5 days and solubilization zone was observed around the colony. Phosphate solubilization efficiency was calculated by using the following formula.

$$\text{PSE} = \frac{\text{Solubilization diameter}}{\text{Growth diameter}} \times 100$$

Quantitative estimation of Pi released from tricalcium phosphate

The isolates showing zone of solubilization on Pikovskaya's agar were further examined for their ability to release Pi from TCP in broth medium. One ml of overnight culture was inoculated to 50 ml of Pikovskaya's broth (Pikovaskya, 1948). The inoculated flask was incubated for two weeks at 28 ± 2 °C. The amount of Pi released in the broth was estimated at 5 and 10 days of incubation from triplicate flask at each stage in comparison with a set of uninoculated controls. The broth cultures were centrifuged at 10,000 rpm for 10 minutes in a centrifuge to separate the supernatant from the cell growth and insoluble phosphate. The available Phosphorus content in the supernatant was estimated by phosphomolybdic blue color method (Jackson, 1973).

Quantitative test for IAA production by spectrophotometric method

All the PGPR isolates were further examined for the amount of IAA production. The IAA production by isolates was determined by following the method of Ivanova *et al.*, (2001) under *in vitro* condition.

Results and Discussion

Isolation and morphological characterization of PGPRs

On the basis of cultural, morphological and biochemical characteristics out of 70 bacterial isolates 44 bacterial isolates were showed plant growth promotion activity under *in vitro* condition. 27 were grouped as nitrogen fixers and 17 isolates were grouped as Phosphate solubilizers as described in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). All the N_2 fixing isolates were Gram –ve, rod, cocci, dull white or Milky white, round, non-spreading, smooth, raised, opaque, viscid colony were identified as *Azotobacter* spp. Whereas in PSB isolates some are Gram +, rod shaped cell, Large, smooth, soft, glistening, round, convex, entire, non-spreading, dense, creamy white to yellow colony were identified as *Bacillus* spp. and some are Gram -, rod shaped, Yellowish

green, round, non-spreading, glistening, convex, opaque, viscid colony were identified as *Pseudomonas* sp. (Table 1).

Biochemical characterization of PGPR isolates

Different biochemical tests were conducted to identify nitrogen fixers and phosphate solubilizers. Most of the nitrogen fixing isolates was shown positive result for indole production, methyl red test, oxidase test, gelatin liquification, ammonia production, acid production, casein hydrolysis and motility and negative for VP test and gas production. In terms of carbon source utilization most of the isolates shown good growth in medium supplemented with mannitol as carbon sources and this isolates were identified as *Azotobacter sp.* (Table 2).

PSB isolates were shown positive result for Catalase test, acid production, casein hydrolysis and motility whereas negative for Oxidase, gas production, H_2S production and most of the isolates shown variations in results (Table 3). All the PSB isolates utilize glucose and sucrose as carbon sources but fail to utilize mannitol and were identified as *Bacillus* spp. and *Pseudomonas* spp.

Screening of free living N_2- fixing isolates for *in vitro* nitrogen fixation

N_2 fixation is naturally the first major mechanism of action suggested for the enhancement of plant growth by *Azotobacter*. In the present investigation all the free living N_2 fixing isolates were tested for *in-vitro* nitrogen fixation, all 27 isolates fixed nitrogen but the quantity of N_2 fixation varied with individual isolate ranged from 4.52 to 19.33 mg per g of C utilized and detail was given in Table 4. Highest nitrogen fixation was observed in isolate PGPR-24 (19.33 mg per g C source) which was superior over all the isolates, followed by PGPR-45 (17.78 mg per g of C source). Kanimozhi *et al.,* (2010), the nitrogen fixation ability of the 30 isolates of *Azotobacter* from paddy rhizosphere soil of Thanjavur district by using micro kjeldhal method. Among the 30 isolates tested, 28 isolates were able to fix nitrogen. The range of nitrogen fixing ability was from 3.3 to 15.6 mg N/g malate. Similar work has done by Goswmy (1976) and Kizilkaya (2009).

Screening of PSB isolates for *in vitro* phosphate solubilization

Phosphate solubilization efficiency (PSE)

Data on qualitative analysis of the PSB isolates for P solubilization is presented in Table 5. Out of 17 isolates examined for their ability to solubilize TCP in Pikovskaya's agar medium supplemented with TCP as insoluble P source. all the isolates showed zone of solubilization, in that isolate PGPR-9 showed the highest Phosphate solubilization (168.7 %) followed by reference strain (150.00 %) whereas lowest solubilization was found in PGPR-42 (106.8 %) and also this was conformity with the findings of Sharma *et al.*, 2007 and Guar *et al*, (1973), they also reported that *Bacillus megaterium* var. *phosphaticum* recorded maximum percentage of phosphate solubilization.

Quantitative solubilization of phosphate (Per cent Pi released)

Per cent Pi released by PSB isolates at different intervals *viz*., 5^{th} and 10^{th} day of incubation (Table 5). The highest Pi released was observed by isolate PGPR-9 at 5^{th} day of incubation (7.85 %) and at 10^{th} day of incubation (14.32 %) compared to all PSB isolates and isolate PGPR-57 released considerable amount of Phosphate at 5^{th} (6.57 %) and 10^{th} (13.13 %) day of incubation respectively.

Table.1 Morphological characterization and identification of plant growth promotion activity of bacterial isolates

Sl. No	Isolate code	Morphological characters				
		Colony morphology	Cell shape	Gram reaction	Growth on N free medium	Solubilize PO_4^-
1	PGPR-1	Large, smooth, soft, glistening, round, convex, entire, non-spreading, dense, creamy white to yellow. Variations: Rough,	Rod	+ve	--	++
2	PGPR-3	Rough, opaque, dull, spreading, off white. Variations: Smooth to slimy, soft, thin, translucent, dendroid. Yellow, orange or brown.	Rod	+ve	--	+
3	PGPR-4	Milky white, round, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	+	--
4	PGPR-5	Dull white, slimy, round, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	++	--
5	PGPR-7	Yellowish green, round, non-spreading, glistening, convex, opaque, viscid colony with green pigmentation.	Rod	-ve	--	++
6	PGPR-8	Off white, irregular, nonspreading, smooth, flat, opaque, viscid colony	Rod	-ve	+	--
7	PGPR-9	Large, smooth, soft, glistening, round, convex, entire, non-spreading, dense, creamy white to yellow. Variations:	Rod	+ve	--	+++
8	PGPR-10	Milky white, round, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	+	--
9	PGPR-12	Clear white to brownish, round, non-spreading, smooth, raised, opaque, gummy colony	Rod	-ve	++	--
10	PGPR-13	Rough, opaque, dull, spreading, off white. Variations: Smooth to slimy, soft, thin, translucent, dendroid. Yellow, orange or brown.	Rod in chains	+ve	--	+
11	PGPR-17	Clear white, slimy, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	+	--
12	PGPR-18	Milky white, spherical, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	++	--
13	PGPR-19	Large, smooth, soft, glistening, round, convex, entire, non-spreading, dense, creamy white. Variations: Rough, radially ridged, thin edged	Rod in chains	+ve	--	+
14	PGPR-21	Dull white, round, non-spreading, smooth, raised, opaque, mucoid colony	Rod	-ve	++	--
15	PGPR-23	Dull white, round, non-spreading, glistening, convex, opaque, viscid colony with no pigmentation.	Rod	-ve	--	++
16	PGPR-24	Milky white, round, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	+++	--
17	PGPR-27	Rough, opaque, dull, spreading, off white. Variations: Smooth to slimy, soft, thin, translucent, dendroid. Yellow, orange or brown.	Rod	+ve	--	++
18	PGPR-28	Milky white, round, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	++	--
19	PGPR-29	Large, smooth, soft, glistening, round, convex, entire, non-spreading, dense, creamy white to yellow. Variations: Rough, concentrically or radially ridged, thin edged	Rod	+ve	--	+
20	PGPR-30	Milky white, round, non-spreading, smooth, raised,	Rod	-ve	++	--
21	PGPR-31	Dull white, spherical flat, non-spreading, smooth, raised, opaque, gummy colony	Rod	-ve	+	--
22	PGPR-33	Yellowish green, round, non-spreading, glistening, convex, opaque, viscid colony with green pigmentation.	Rod	-ve	--	++
23	PGPR-34	Milky white, round, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	++	--
24	PGPR-37	Large, smooth, soft, glistening, round, convex, entire, non-spreading, dense,	Rod	+ve	--	++

		creamy white. Rough, concentrically ridged, thin edged				
25	PGPR-38	Milky white, round, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	+++	--
26	PGPR-41	Clear white, round, non-spreading, raised, opaque, viscid colony with light brown pigmentation.	Rod	-ve	++	--
27	PGPR-42	Yellowish green, round, non-spreading, glistening, convex,	Rod	-ve	--	++
28	PGPR-43	Dull white, circular, non-spreading, smooth, raised, opaque,	Rod	-ve	++	--
29	PGPR-45	Milky white, circular, non-spreading, smooth, raised, opaque, viscid colony.	Rod	-ve	+	--
30	PGPR-46	Milky white, round, non-spreading, smooth, raised, opaque, viscid colony.	Rod	-ve	++	--
31	PGPR-47	Large, smooth, soft, glistening, round, convex, entire, non-spreading, dense, creamy white to yellow. Variations: Rough, concentrically or radially ridged, thin edged	Rod in chains	+ve	--	+
32	PGPR-48	Large, smooth, soft, glistening, round, convex, entire, non-spreading, dense, creamy white to yellow. Variations: Rough, concentrically or radially ridged, thin edged	Rod	+ve	--	++
33	PGPR-49	white, round, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	+	--
34	PGPR-51	Milky white, round, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	+	--
35	PGPR-53	Milky white, round, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	++	--
36	PGPR-54	Large, smooth, soft, glistening, round, convex, entire, non-spreading, dense, white to yellow. Variations: Rough,	Rod	+ve	--	+
37	PGPR-55	Rough, opaque, dull, spreading, off white. Variations: Smooth to slimy, soft, thin, translucent, dendroid. Yellow, orange or brown.	Rod	-ve	+++	--
38	PGPR-57	Yellowish green, round, non-spreading, glistening, convex, opaque, viscid colony	Rod	-ve	--	++
39	PGPR-59	light white, round, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	++	--
40	PGPR-61	Milky white, round, non-spreading, smooth, raised, opaque, viscid colony	Oval	-ve	+	--
41	PGPR-62	Large, smooth, soft, glistening, round, convex, entire, non-spreading, dense, creamy white to yellow. Variations: Rough, concentrically or radially ridged, thin edged	Rod	+ve	--	++
42	PGPR-64	white, round, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	++	--
43	PGPR-65	Milky white, round, non-spreading, smooth, raised, opaque, viscid colony	Rod	-ve	+	--
44	PGPR-66	Large, smooth, soft, glistening, round, convex, entire, non-spreading, dense, creamy white to yellow. Variations: Rough, concentrically or radially ridged, thin edged	Rod	+ve	--	+
45	PGPR-68	Milky white, round, non-spreading, smooth, raised, opaque, viscid colony	Coccid	-ve	+	--

+ : Low growth. **++:** Medium growth **+++:** Good growth
+ve: Positive -ve: Negative

Table.2 Biochemical characteristics of the free living nitrogen fixers

Isolate	Biochemical tests																Probable genera
	1	2	3	4	5	6	7	8	9	10	11	12a	12b	13	14a	14b	14c	
PGPR-4	+	+	-	+	+	+	-	+	+	+	+	+	-	+	3+	2+	4+	*Azotobacter* sp.
PGPR-5	+	+	-	-	+	+	+	+	-	+	+	+	-	+	3+	2+	3+	*Azotobacter* sp.
PGPR-8	+	+	-	+	+	+	+	+	+	+	+	+	-	+	4+	3+	4+	-
PGPR-10	+	+	-	+	+	+	-	+	+	+	+	+	-	+	3+	2+	3+	*Azotobacter* sp.
PGPR-12	+	+	-	-	+	+	+	+	-	+	+	+	-	+	3+	2+	4+	*Azotobacter* sp.
PGPR-17	+	+	-	-	+	+	+	+	-	+	+	+	-	+	4+	3+	4+	*Azotobacter* sp.
PGPR-18	+	+	-	+	+	+	-	+	+	+	+	+	-	+	3+	2+	3+	*Azotobacter* sp.
PGPR-21	+	+	-	+	+	+	+	+	+	+	+	+	-	+	3+	2+	3+	*Azotobacter* sp.
PGPR-24	+	+	-	+	+	+	-	+	+	+	+	+	-	+	3+	2+	3+	*Azotobacter* sp.
PGPR-28	+	+	-	-	+	+	+	+	-	+	+	+	-	+	3+	2+	4+	*Azotobacter* sp.
PGPR-30	+	+	-	+	+	+	-	+	+	+	+	+	-	+	3+	2+	3+	*Azotobacter* sp.
PGPR-31	+	+	-	-	+	+	+	+	-	+	+	+	-	+	3+	2+	4+	*Azotobacter* sp.
PGPR-34	+	+	-	+	+	+	-	+	+	+	+	+	-	+	4+	2+	4+	-
PGPR-38	+	+	-	+	+	+	-	+	+	+	+	+	-	+	3+	2+	3+	*Azotobacter* sp.
PGPR-41	+	+	-	+	+	+	-	+	+	+	+	+	-	+	3+	3+	4+	*Azotobacter* sp.
PGPR-43	+	+	-	-	+	+	+	+	-	+	+	+	-	+	4+	3+	4+	*Azotobacter* sp.
PGPR-45	+	+	-	+	+	+	+	+	+	+	+	+	-	+	3+	2+	3+	*Azotobacter* sp.
PGPR-46	+	+	-	+	+	+	-	+	+	+	+	+	-	+	3+	2+	4+	*Azotobacter* sp.
PGPR-49	+	+	-	-	+	+	+	+	-	+	+	+	-	+	4+	3+	4+	-
PGPR-51	+	+	-	-	+	+	+	+	-	+	+	+	-	+	3+	2+	4+	*Azotobacter* sp.
PGPR-53	+	+	-	+	+	+	+	+	+	+	+	+	-	+	3+	2+	3+	*Azotobacter* sp.
PGPR-55	+	+	-	+	+	+	-	+	+	+	+	+	-	+	3+	2+	3+	*Azotobacter* sp.
PGPR-59	+	+	-	+	+	+	-	+	+	+	+	+	-	+	3+	3+	3+	*Azotobacter* sp.
PGPR-61	+	+	-	-	+	+	+	+	-	+	+	+	-	+	4+	2+	4+	*Azotobacter* sp.
PGPR-64	+	+	-	+	+	+	-	+	+	+	+	+	-	+	3+	2+	3+	*Azotobacter* sp.
PGPR-65	+	+	-	-	+	+	+	+	-	+	+	+	-	+	4+	3+	4+	*Azotobacter* sp.
PGPR-68	+	+	-	+	+	+	-	+	+	+	+	+	-	+	3+	2+	3+	*Azotobacter* sp.

Table.3 Morphological and Biochemical characteristics of the Phosphate Solubilizing Bacteria (PSB)

Isolate	Spore formation	Biochemical tests																Probable genera
		1	2	3	4	5	6	7	8	9	10	11	12a	12b	13	14a	14b	14c	
PGPR-1	+	-	-	+	+	+	-	+	+	-	+	-	+	-	+	+	+	-	*Bacillus* spp.
PGPR-3	+	-	+	-	-	+	-	+	+	-	+	-	+	-	+	+	+	-	*Bacillus* spp.
PGPR-7	-	-	-	-	+	+	+	-	-	-	+	+	+	-	+	+	+	-	*Pseudomonas* sp.
PGPR-9	+	-	+	-	-	+	-	+	+	-	+	-	+	-	+	+	+	-	*Bacillus* spp.
PGPR-13	+	-	-	+	+	+	-	+	-	-	+	-	+	-	+	+	+	-	*Bacillus* spp.
PGPR-19	+	-	-	+	+	+	-	+	+	-	+	-	+	-	+	+	+	-	*Bacillus* spp.
PGPR-23	+	-	+	-	-	+	-	+	+	-	+	-	+	-	+	+	+	-	-
PGPR-27	+	-	+	-	-	+	-	+	+	-	+	-	+	-	+	+	+	-	*Bacillus* spp.
PGPR-29	+	-	-	+	+	+	-	+	+	-	+	-	+	-	+	+	+	-	*Bacillus* spp.
PGPR-33	-	-	-	-	+	+	+	-	-	-	+	+	+	-	+	+	+	-	*Pseudomonas* sp.
PGPR-37	+	-	+	-	-	+	-	+	+	-	+	-	+	-	+	+	+	-	*Bacillus* spp.
PGPR-42	-	-	-	-	+	+	+	-	-	-	+	+	+	-	+	+	+	-	*Pseudomonas* sp.
PGPR-47	+	-	-	+	+	+	-	+	+	-	+	-	+	-	+	+	+	-	*Bacillus* spp.
PGPR-48	+	-	+	-	-	+	-	+	+	-	+	-	+	-	+	+	+	-	-
PGPR-54	+	-	+	-	-	+	-	+	+	-	+	-	+	-	+	+	+	-	*Bacillus* spp.
PGPR-57	-	-	-	-	+	+	+	-	-	-	+	+	+	-	+	+	+	-	*Pseudomonas* sp.
PGPR-66	+	-	-	+	+	+	-	+	-	-	+	-	+	-	+	+	+	-	*Bacillus* spp.

1.Indole production test,	**6.** Oxidase test	**11.** Ammonium production test	**14a.** Glucose
2. Methyl red test,	**7**. Starch hydrolysis test	**12a**. Acid production	**14b.** Sucrose
3.VogerProskauer test	**8**. Gelatin liquefaction test	**12b.** Gas production	**14c.** Mannitol
4. Citrate utilization test	**9**. H_2S production	**13**. Casein hydrolysis	+ : Positive
5. Catalase test	**10**. Motility test		**-** : Negative
+ : Very less growth	**2+ :** Less growth	**3+ :** Medium growth	**4+ :** Good growth

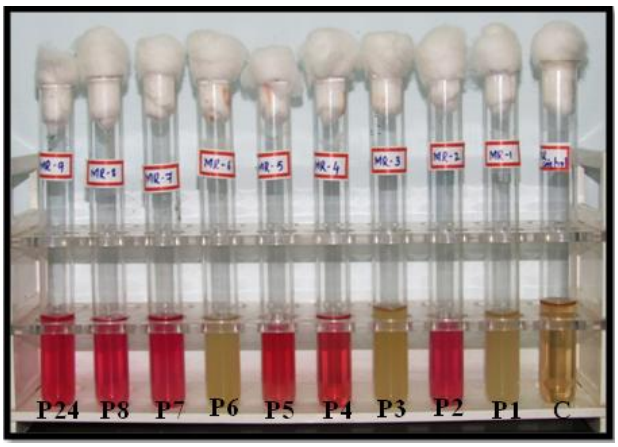
Table.4 Screening of efficient free living nitrogen fixing isolates

Sl. No	Isolate code	mg N per g of C utilized	IAA (µg per ml of medium)
1	PGPR-4	7.57	12.75
2	PGPR-5	5.32	9.46
3	PGPR-8	4.81	5.61
4	PGPR-10	7.33	11.68
5	PGPR-12	7.45	15.78
6	PGPR-17	5.13	7.78
7	PGPR-18	9.26	18.96
8	PGPR-21	4.52	6.76
9	PGPR-24	19.33	25.12
10	PGPR-28	6.24	12.73
11	PGPR-30	5.89	9.61
12	PGPR-31	8.56	13.52
13	PGPR-34	14.62	19.12
14	PGPR-38	4.71	7.45
15	PGPR-41	13.22	14.52
16	PGPR-43	15.81	17.55
17	PGPR-45	17.78	22.67
18	PGPR-46	13.59	15.32
19	PGPR-49	4.68	5.98
20	PGPR-51	5.67	9.42
21	PGPR-53	15.12	18.84
22	PGPR-55	6.23	12.45
23	PGPR-59	4.67	9.56
24	PGPR-61	15.54	12.53
25	PGPR-64	17.23	19.78
26	PGPR-65	7.48	23.54
27	PGPR-68		14.56
Reference strain (*A. chroococcum*)		18.16	24.08

Table.5 Screening of efficient phosphate solubilizing bacterial isolates

Sl. No	Isolate code	Per cent P*i* released		Phosphate Solubilization efficiency (%)	IAA Production (µg per ml of medium)
		5^{th} day	10^{th} day		
1	PGPR-1	6.12	12.86	132.6	5.89
2	PGPR-3	4.80	8.32	110.4	4.54
3	PGPR-7	5.78	10.45	114.0	10.45
4	PGPR-9	7.85	14.32	168.7	20.13
5	PGPR-13	6.23	12.32	128.0	19.95
6	PGPR-19	5.89	10.12	115.6	15.57
7	PGPR-23	4.37	8.78	108.8	17.84
8	PGPR-27	5.36	10.33	112.6	11.48
9	PGPR-29	4.39	8.59	109.6	6.32
10	PGPR-33	6.37	13.12	138.4	19.00
11	PGPR-37	4.40	8.89	110.0	9.92
12	PGPR-42	3.80	7.32	102.8	15.12
13	PGPR-47	4.52	7.45	109.0	17.67
14	PGPR-48	5.78	10.91	114.8	16.48
15	PGPR-54	5.23	9.89	106.8	18.79
16	PGPR-57	6.57	13.13	136.0	16.23
17	PGPR-62	4.35	8.56	122.8	14.99
Reference strain (*Bacillus magaterium*)		7.05	13.58	150.0	19.11
Control		0.42	0.61	0.00	0.00

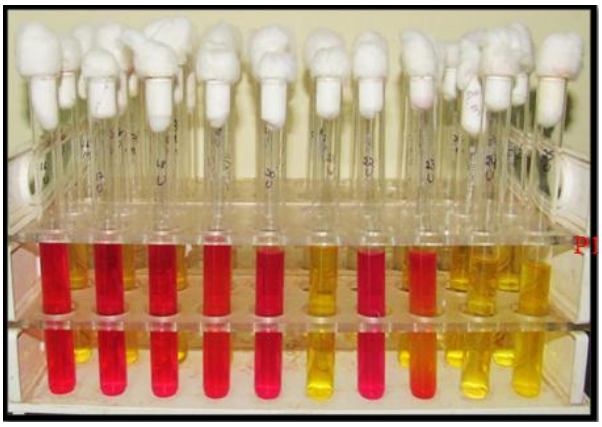
Plate.1 Different biochemical tests conducted for identification of PGPR isolates



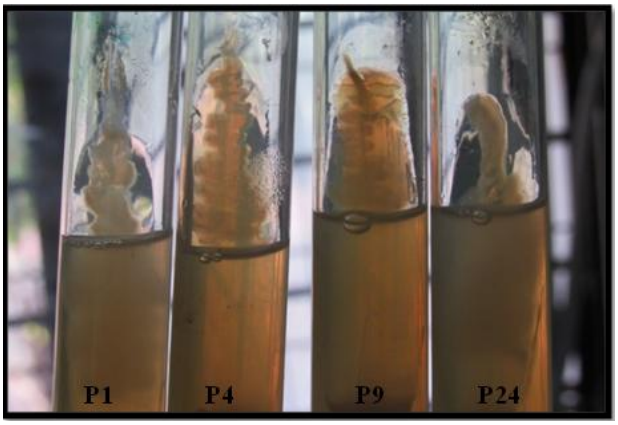
1a. Methyl red test

1b. Citrate utilization test

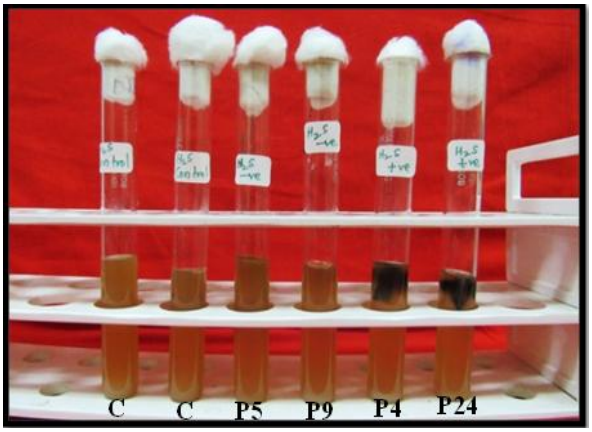
1c. Indole production test



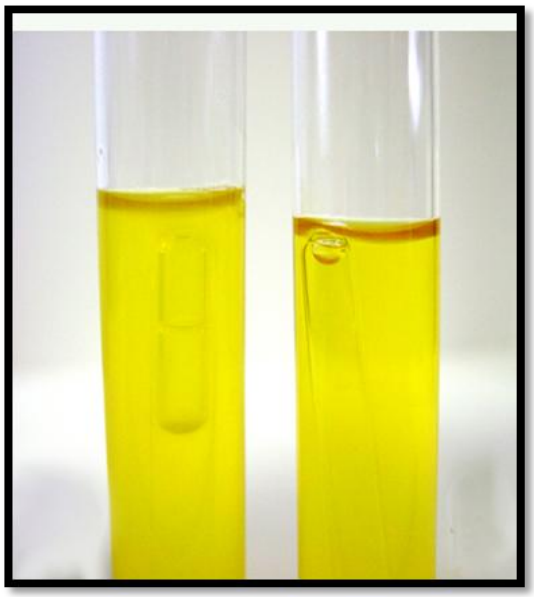
1d. Urease test



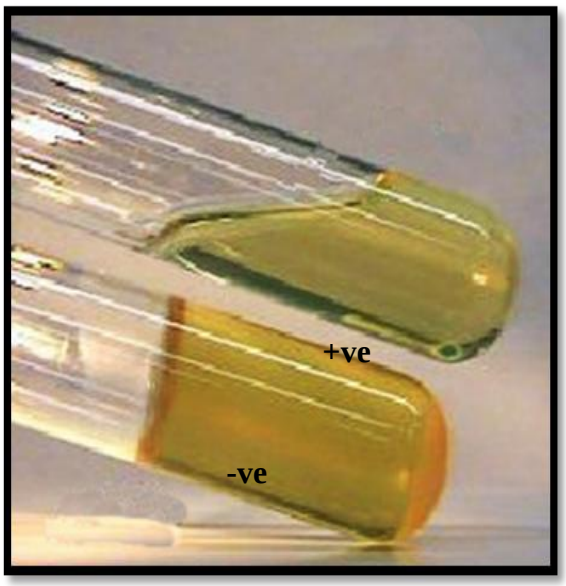
1e. Catalase test



1f. Hydrogen sulphide



1a. Gas production



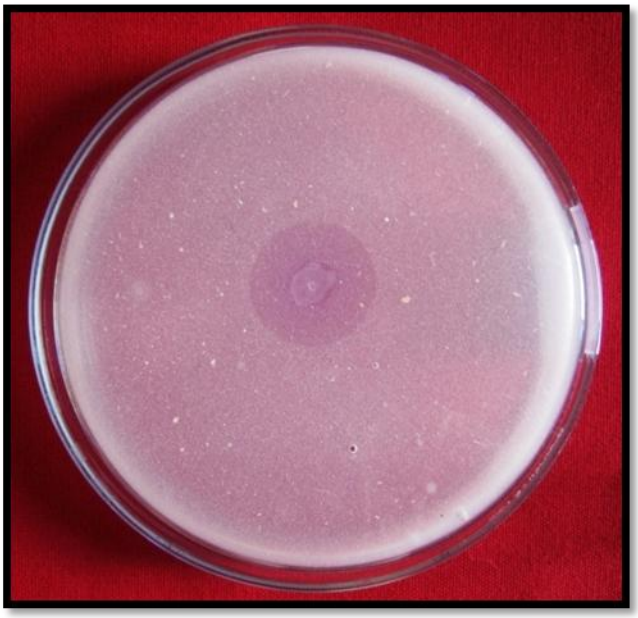
1b. Gelatin

1c. VP test +ve

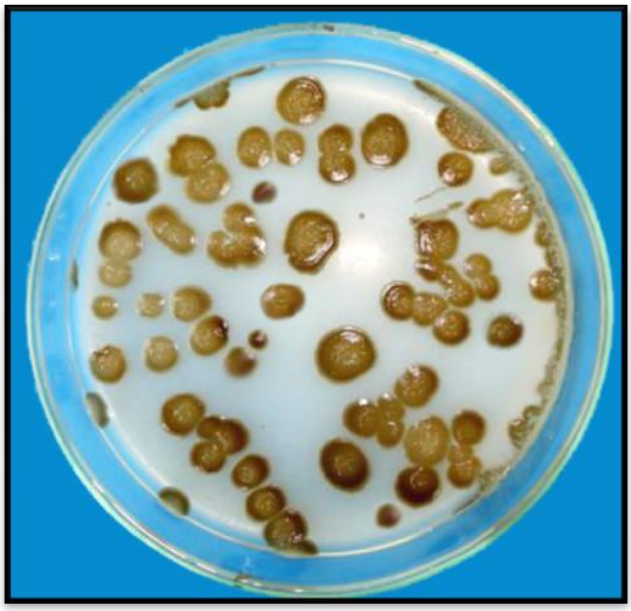
1d. Casein hydrolysis test

Plate.2 Cultures of PGPR isolates (*Azotobacter* sp. and Phosphate solubilizing bacteria)

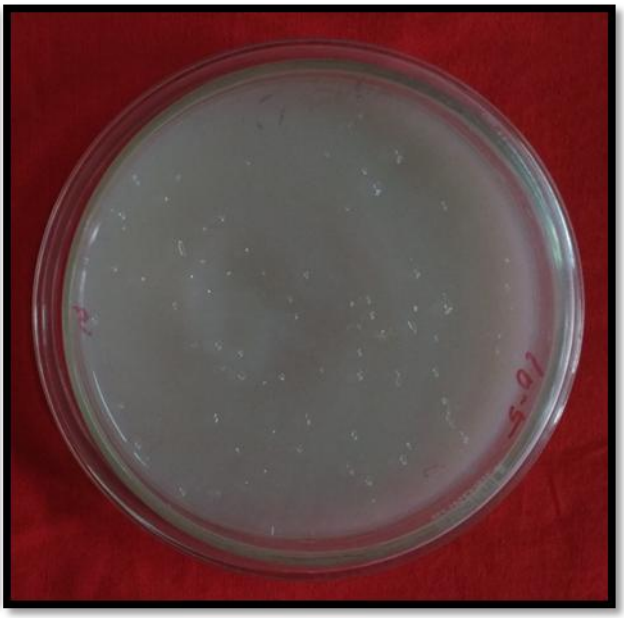
PSB on Nutrient agar media



PSB on Pikovskay's media



Pigmented *Azotobacter* sp.



Azotobacter on Whaksman No-77 media

The lowest Pi released was found in PGPR-62 (4.35 %) at 5th and (8.56 %) 10th day of incubation. Similar observation of solubilization of insoluble phosphate was made by Hu *et al.,* 2006 and Sen and Paul 1957.

Production of plant growth promoting substances by PGPR isolates

IAA production by free living N_2 fixers

Higher IAA production was recorded in PGPR-24 (25.12 µg/ml), which is superior over all other isolates. Lowest IAA production (5.61 µg/ml) was recorded in isolate PGPR-8 details were described in Table 8. The results were on par with the results of Jalan (2003) screened and twelve *Azotobacter* isolates produce the IAA only 2.09 to 3.3 µg per ml.

IAA production by the phosphate solubilizing bacteria

The higher IAA production was recorded inPGPR-9 (20.13 µg/ml) is superior over all the isolates followed by PGPR-13 (19.95 µg/ml). Lowest production of IAA was in isolate PGPR-3 (4.54 µg/ ml). Result is presented in the Table 9. Geeta (2001) studied twenty eight P-solubilizing bacterial strains for the production of PGPS. The production of IAA among the strains varied from 3.61-35.45 g/25 ml of broth. Similar results were found by Barea *et al.,* (1976) and Leinhos and Vacek (1994).

In conclusion, in the present scenario use of microorganisms in the field of agriculture to enhance growth, yield and quality of crop is the best way to reduce cost of production and maintain ecological harmony. Rhizosphere is rich source of beneficial microbes which have potential to improve plant growth.

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