

In vitro and *in vivo* characterization of plant growth promoting rhizobacteria isolated from rice rhizospheric soil

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Abstract

The use of Plant growth-promoting rhizobacteria (PGPR) is steadily increasing in sustainable agriculture and offers an attractive way to replace chemical fertilizers, pesticides, and supplements. In this study, isolation and characterization of PGPR from the rhizospheric soil of standing rice plant was done by collecting rhizospheric soil from a rice field in Bihta region of Patna district in Bihar. Five isolates of bacteria, designated as R1, R2, R3, R4 and R5 were successfully isolated and characterized by morphological and biochemical tests. Isolates were further subjected to *in vitro* PGPR characterization tests i.e., indole acetic acid (IAA) production test, biological nitrogen fixation test and phosphate solubilization test. Subsequently, to investigate the effects of PGPR isolates on the germination and growth of Bengal gram (*Cicer arietinum* L.), an *in vivo* plant growth test was conducted where seeds were treated with PGPR isolates and results revealed that these isolates remarkably increased seed germination and overall growth and vigor of gram seeds. The present study, therefore, suggests that after further field trials and pot experiments the use of these PGPR isolates as biofertilizer will be effective.

Keywords: PGPR, rhizosphere, sustainable agriculture, *Cicer arietinum*, biofertilizer

1. Introduction

India is an agrarian country, where agriculture contributes about 17-18% of total GDP which is a major share of national income ^[1]. Today agriculture is facing precarious challenges of land degradation and susceptibility to various biotic and abiotic stresses, ultimately leading to reduced plant productivity and yield ^[2]. Conventionally agriculturists relied on application of huge amount of agrochemicals to enhance the crop produce. However their undifferentiating use adversely affects soil fertility, beneficial soil microbial community, environment, human health as well as increases the input cost for crop production especially for the marginal farmers ^[3,4]. And so, agriculturist have developed a great interest in increasing crop productivity and soil health in an eco-friendly and sustainable way.

One such effective and inexpensive approach for sustainable agriculture is the practice of using plant growth promoting rhizospheric microorganisms (PGPMs) that offers potential to meet our future agricultural needs, something that conventional agriculture is incompetent to do ^[5]. PGPMs includes bacteria, fungi, arbuscular mycorrhizal fungi (AMF) that inhabits the rhizoplane and rhizosphere and have dynamic multilateral interactions with plant roots as well as soil ^[6]. Among the many mechanisms associated with plant-rhizosphere microorganism interactions, the production of biologically active metabolites is one of the most important ways that rhizosphere microbiota influences plant growth ^[7]. PGPRs have different association with different species of host plants. The two major association are rhizospheric and endophytic. Rhizospheric relationships consist of the PGPRs that colonize the surface of the root, or superficial intercellular spaces of the host plant, often forming root nodules.

Endophytic relationships involve the PGPRs residing and growing within the host plant in the apoplastic space.

PGPR promotes plant growth in two different ways, indirectly or directly. The direct promotion of plant growth by PGPR entails either providing the plant with a compound that is synthesized by the bacterium, for example production of phytohormones and organic acids, nutrient solubilization facilitating the plant to uptake certain unavailable nutrients from the environment, nitrogen fixation ^[8, 9]. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms. This can happen by producing antagonistic substances, HCN, hydrolytic enzymes or by inducing resistance to pathogens ^[10, 9]. A particular PGPR may affect plant growth and development by using any one, or more, of these mechanisms. Bengal gram or chick pea (*Cicer arietinum* L.) belonging to Fabaceae family is one of the important pulses crop grown throughout the country. It contains about 22-26% protein, which is almost three times that of cereals and other minerals and vitamins. Besides, it is also used as nutritive fodder, especially for milch animals. Its high-yielding varieties have resulted in an increase in Bengal gram production but requires large amounts of chemical fertilizers, leading to health hazards and environmental pollution. In order to make Bengal gram cultivation sustainable and less dependent on chemical fertilizers, it is important to know how to use PGPR that can contribute to the improvement of the plant's growth ^[11]. Therefore, the present study was undertaken to screen the PGPR isolates for production of IAA, biological nitrogen fixation and phosphate solubilization and also investigated the effect of the same isolates on seed germination and growth of Bengal gram as well.

2. Materials and methods

2.1. Collection of plant roots along with the rhizospheric soil from rice field

Sampling was done from rice field on 7th August 2019 at 07:51 a.m., at 28°C (25.5809 latitude and 85.0093 longitudes) from Nasri ganj- Chandi- Bihta- Piro road, Khagaul Shivala Par, Bihar 801113, India. Shoot region of rice plant was chopped off and then was carefully uprooted and aseptically collected in sterile polythene bags.

2.2 Isolation of bacteria

The roots of rice plant were chopped down into small pieces using sterile knife.

They were washed thoroughly with autoclaved normal saline thrice and the final rinsate was collected. The rinsate was serially diluted up to 10⁻⁶ dilution and aliquot of 0.1 ml were spread on nutrient agar (NA) plates and incubated at 37°C for 48 hrs.

Then, from spread plates, single isolated colonies were sub cultured and streaked repeatedly to obtain pure cultures. Each pure bacterial isolate were maintained and stored at 4°C for further characterization.

2.3 Characterization of bacteria

It was done on the basis of colony morphology and Gram's staining. The isolates were further subjected to different biochemical tests viz., starch hydrolysis, nitrate reduction, catalase, urease, H₂S, indole, MP-VP, citrate utilization test, etc. for preliminary identification [12].

2.4 In vitro PGPR characterization of bacterial isolates

2.4.1 Biological nitrogen fixation test

The test organisms were inoculated on Burk's medium (N-free agar medium) and incubated at 37°C for 48 hrs [13]. Appearance of growth on Burk's media indicates +ve result for nitrogen fixation.

2.4.2 Phosphate solubilization test

The test organism were inoculated on Pikovskaya's agar media and incubated at 37°C for 4-5 days). Appearance of zone of clearance (halo) around the bacterial colony indicates +ve result for phosphate solubilization [14].

2.4.3 IAA production test

Production of indole acetic acid (IAA) by bacteria was done by modified method adopted from [15].

A loopful of the test organism were inoculated in JNFB broth supplemented with tryptophan and incubated at 37°C for 48 hrs. After incubation, inoculated broth is taken and centrifuged at 8000 rpm for 15 min.

After, centrifugation 1ml of supernatant was taken in a test tube and 2ml of salkowski reagent was added to it. Appearance of pink color after 15min indicates +ve result.

2.4 In vivo characterization:

2.4.1 Effect of PGPR isolates on seed germination percentage

100 seeds of Bengal gram (*Cicer arietinum L.*) were taken and surface sterilized by soaking in 1% sodium hypochlorite solution for approximately 15 min with shaking in sterile Erlenmeyer flask. Then, the soaked gram seeds were washed thrice with sterilized D.D.W. In next step, NA broth was prepared and then inoculated with starter culture (test bacteria) and kept in shaker incubator at 170 rpm for 24 hrs. Then, broth was filtered with filter paper to make the filter paper wholly moistened with the culture. The soaked gram seeds were kept in between two moistened filter papers in a petri dish. After, seed germination of gram seeds, germination rate was calculated at 24 and 48 hrs and the length of shoot (plumule) was also measured.

2.4.2 Effect of PGPR isolates on growth of Bengal gram (*Cicer arietinum L.*) seedlings

To study the effect of PGPR isolates on the growth of Bengal gram plant, two sets of beaker were taken and were filled 3/4th with autoclaved soil. Then, one of beaker soil was left without inoculation and taken as control while others were inoculated with test cultures (10 ml). Then in all the beakers, 10 surface sterilized germinated gram seeds were sown on each beaker and kept on gnotobiotic condition for further growth and development of plant. Growth was checked after every 48 hrs and readings were noted after one week of sowing the seeds.

2.5 Statistical analysis

Graphical representation of seed germination percentage of inoculated seeds compared to control was made using Microsoft excel and bar graphs. Statistical analysis of shoot growth was done using one way ANOVA in "graph pad prism version 5.0.1" computer software. Treatments were compared via ANOVA using the least significant differences test (LSD) at 5% ($P \leq 0.05$) probability level.

3. Results and discussion

3.1 Isolation of bacteria

In the present study, five bacterial colonies were isolated from rhizospheric soil of rice plants and were designated as R1, R2, R3, R4 and R5.

3.2 Characterization of bacteria

Bacterial isolates were selected on the basis of colony morphology (shape, margin, elevation, and surface). The total of (5) isolates were obtained where isolates R1, R3 and R4 were gram positive rods while, isolates R2 and R5 were gram negative rods in long chains (Table 1). Biochemical characterization was done further done and results are shown in Table 2.

Table 1: Morphological characterization of bacterial isolates

S. No.	Isolate	Grams stain	Shape	Color of Colony	Elevation	Surface	Margin	Form	Density
1	R1	+ve	Long rods	Whitish	Convex	Mucoid	Entire	Irregular	Opaque
2	R2	-ve	Short rods	Off white	Raised	Mucoid	Undulate	Round	Opaque
3	R3	+ve	Short rods	Creamy White	Flat	Dry	Entire	Irregular	Opaque
4	R4	+ve	Coccus	Creamy White	Slightly raised	Mucoid	Entire	Irregular	Opaque
5	R5	-ve	Long rods	Off white	Convex	Mucoid	Entire	Irregular	Translucent

"-" indicates negative response and "+" indicates positive response

Table 2: Biochemical characterization of bacterial isolates

S. No.	Tests	Isolates				
		R1	R2	R3	R4	R5
1	Starch hydrolysis	+ve	-ve	+ve	+ve	-ve
2	Nitrate reduction	+ve	+ve	-ve	+ve	+ve
3	Catalase	+ve	+ve	+ve	+ve	+ve
4	Urease	-ve	-ve	-ve	-ve	-ve
5	H ₂ S production	-ve	-ve	-ve	-ve	-ve
6	Indole production	+ve	-ve	-ve	+ve	+ve
7	MR test	-ve	-ve	-ve	-ve	-ve
8	VP test	+ve	+ve	+ve	+ve	+ve
9	Citrate utilization	+ve	+ve	+ve	+ve	+ve

"-" indicates negative response and "+" indicates positive response

3.3 In vitro PGPR characterization of bacterial isolates

These isolates were further subjected to *in vitro* PGPR activity tests like IAA production test, phosphate solubilization test and nitrogen fixation test. Only one isolates i.e., isolate R5 showed positive result for phosphate solubilization. For IAA production test, out of 5 isolates, 3 (R1, R2, R5) gave positive results for IAA production. The other two isolates i.e., R3 and R4 showed moderate to low results, which was considered as negative result. Isolates were also subjected to biological nitrogen fixation test using Burk's media. All the 5 isolates, i.e., R1, R2, R3, R4 and R5 grew on this media showing positive results for nitrogen fixation (Table 3, Fig. 1).

Table 3: Characterization of PGPR traits

S. No.	Test	Isolates				
		R1	R2	R3	R4	R5
1	Nitrogen fixation	+ve	+ve	+ve	+ve	+ve
2	IAA production	+ve	+ve	-ve	-ve	+ve
3	Phosphate solubilization	-ve	-ve	-ve	-ve	+ve

(+ve) indicates positive test, (-ve) indicates negative test

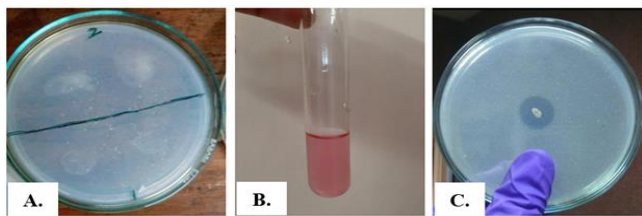


Fig 1: Positive test for PGP activities of the isolates. A. Nitrogen fixation B. IAA production C. Phosphate solubilization

3.4 In vivo characterization

Effect of isolates on seed germination and plumule length of *Cicer arietinum* were studied by germinating the seeds in presence of PGP isolates (treated seeds) and in their absence (untreated/control) for 2 days. It was observed that there was an increase in seed germination rate as well as plumule length of seedling in PGP treated seeds with respect to untreated/control seeds (Table 4). Out of 5 isolates, R2 and R5 showed a pronounced increase in seed germination rate as compared to control. While isolates R2 and R4 showed pronounced enhancement in plumule length.

Table 4: The effect of PGPR on seed germination and growth of Gram seeds

S. No.	Isolates	Seed Germination (%)		Plumule Length on Day 2 (cm)
		Day 1	Day 2	
1	Control	55	70	2.4 cm
2	R1	60	69	2.2 cm
3	R2	70	82	2.9 cm
4	R3	62	78	2.5 cm
5	R4	69	74	3.2 cm
6	R5	75	90	2.1 cm

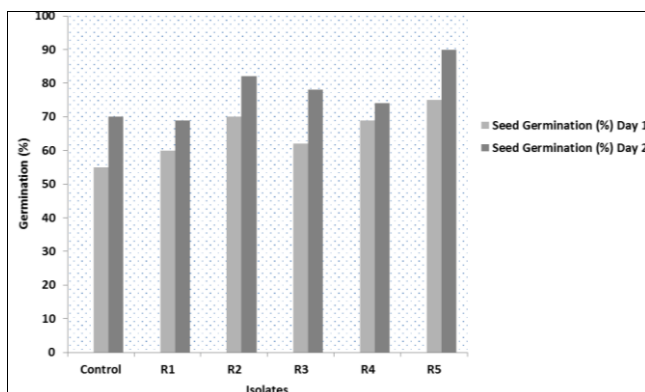


Fig 2: Graph showing % germination of Bengal gram seeds

Further a comparative study was also done to check the effect of PGPR isolate on the growth of Bengal gram seedlings.

It was observed that after 7-8 days of sowing the gram seeds, there was significant increase in both the root and shoot growth which were inoculated with test bacteria as compared to control (without inoculation). R5 showed best significant increase in shoot length as compared to other isolates (Table 5, Fig. 3 and Fig. 4).

Table 5: Effect of PGPR on plant growth observations at interval of 2 days

Isolates	Average shoot length (cm) N*=10			
	Day 2	Day 4	Day 6	Day 8
Control	2.30	5.20	9.10	11.9
R1	3.40	5.25	10.22	14.11
R2	4.56	9.78	12.00	17.75
R3	2.28	5.04	11.01	14.99
R4	2.37	6.01	13.25	15.93
R5	5.00	8.32	14.01	19.93

N*-number of seeds sown



Fig 3: Uprooted Bengal gram seedling: Without inoculation (left) and seedling inoculated with R5 isolate (right)

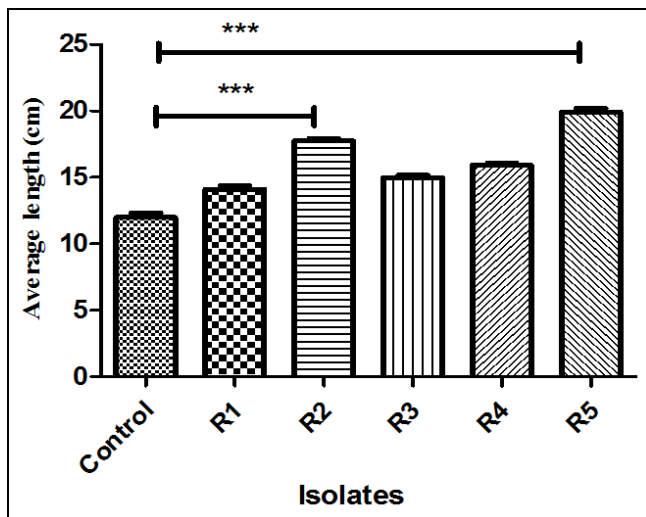


Fig 4: ANOVA graph of average shoot length on Day 8 *. “***” shows significant difference

Discussion

Nitrogen fixation is one of the important feature of PGPR because plant directly cannot use atmospheric nitrogen for their growth unless it fixed to freely available form like ammonia and nitrates by atmosphere and bacteria [16, 17]. These microbes possessing the skill of nitrogen fixation makes them potent candidate of PGPRs. Inaccessibility of soluble phosphate and widespread use of chemical phosphates as fertilizer have compelled the researchers to search for phosphate solubilizing microorganism which can solubilize the inexplicable phosphates, a major macronutrients for plant growth. So, phosphate solubilization is one of the important plant growth promoting traits [18]. Ability to synthesize exogenous phytohormone such as IAA is most frequently found property of rhizospheric bacteria. Higher levels of IAA production by *Pseudomonas* was recorded by other workers. The ability of the bacteria to produce IAA in the rhizosphere depends on the precursor available and the ability of the plant to uptake microbial IAA [19].

The study showed that seed treatment of Bengal gram with selected isolates benefitted the plant during the early growth stages by increasing seed germination, and root lengths of seedlings, as well as shoot length of the plant. Increases in early seedling growth due to seed treatment with bacterial strains have also been reported in other grains such as wheat [20] and rice [21]. The percentage germination of gram seeds increased to 7.5 - 12.7%, which compared with the percentage germination of untreated seeds. Similar results have been reported in earlier studies on pearl millet in which seed germination was enhanced by up to 8% due to seed treatment with two *Bacilli* strains [22]. Increased vigour improves a seedlings ability to withstand infections by pathogens and survive under harsh environmental conditions [23]. Seed treatments which increase germination and vigour could be important for carryover seeds that have been stored for a long time, since seed ageing are one of the major aspects influencing seed quality [24].

Significant increase in shoot length by these these reveals that these isolates have plant growth promoting activity and can be used as biofertilizer inoculant, which might be beneficial for crop cultivation as they enhanced growth of bengal gram (seeds) and induced IAA production and phosphorus solubilization and biological nitrogen fixation.

4. Conclusion

Thus, on the basis of above research work and biochemical tests result, we can conclude that the isolates R1, R3 and R4 may be of *Bacillus sp.*; isolates R2 and R5 may be of *Pseudomonas sp.* And further species specification can be done by 16sRNA. Furthermore, these isolates remarkably increased seed germination rate, plant root and shoot growth of bengal gram. These aspects can be used as the growth promoter of crops and can be used as superior bio fertilizer. Thus, Plant growth promoting rhizobacteria (PGPR) shows an important role in the sustainable agriculture industry. As the increasing demand for crop production with a significant reduction of synthetic chemical fertilizers and pesticides use is a big challenge nowadays. The use of PGPR has been proven to be an environmentally sound way of increasing crop yields by facilitating plant growth through either a direct or indirect mechanism.

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