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Isolation and characterization of plant growth promoting rhizobacteria associated with pea rhizosphere in North Himalayan region

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Abstract
PGPR are naturally occurring soil bacteria which actively colonize plant root and benefit plants by promoting growth. They help in providing atmospheric nitrogen, increase supply of other nutrients, produce plant hormones, enhance other beneficial bacteria or fungi, control bacterial and fungal diseases and help in controlling insect pests. The purpose of this study was to investigate the diversity of bacteria associated with the roots of pea and to screen them for selection of most potential PGPR for application in pea crop. The study reveals the diversity of bacterial isolates from soils under pea cultivation in district Srinagar and Baramulla of J&K. The isolates were characterized morphological and physiologically to categorize and identify them. In all 80 different bacterial isolates were recovered from 16 fields of two districts of J&K. The characteristics of the bacterial isolates were determined using the colony morphology, gram staining, biochemical properties and plant growth promoting activities. On the basis of biochemical characterization, 55 isolates belonged to genus *Bacillus*, 12 to *Pseudomonas*, 4 to *Micrococcus* and 9 to *Azotobacter*. After screening the best nine isolates PGP1, PGP10, PGP28, PGP40, PGP49, PGP52, PGP62, PGP68 and PGP72 were selected for their effect on growth and yield in field conditions using pea as test crop.

Keywords: Rhizobacteria, pea, Himalaya, diversity, rhizosphere

Introduction
Pea (*Pisum sativum*) is an important vegetable and pulse crop of India. It is relished both as fresh vegetable as well as pulse. Being a legume, it plays an important role in the eco-build up of agriculture, as it enriches the soil by fixing the atmospheric nitrogen. Pea is an important cool season grain legume, which is grown worldwide on over 6 million hectares. Important production areas of the world include France, Russia, Ukraine, Denmark and United Kingdom in Europe; China and India in Asia; Canada and USA in North America; Chile in South America; Ethiopia in Africa, and Australia (FAO, 1994) [9]. Peas require a cool, relatively humid climate and are grown at higher altitudes in tropics with temperatures ranging between 7 and 30°C (Davies *et al.*, 1985) [6]. Peas can be grown successfully during mid-summer and early fall in those areas having relatively low temperatures and a good rainfall, or where irrigation is practiced. The rhizosphere is the thin layer of soil adjacent to plant roots that is influenced by root activities (Hiltner, 1904) [11]. There are three separate, but interacting, components recognized in the rhizosphere *viz* soil, the rhizoplane and root itself. The rhizosphere is the zone of soil influenced by roots through the release of substrates that affect microbial activity. The rhizoplane is the root surface, including the strongly adhering soil particles.

Several microorganisms are able to promote plant growth either directly or indirectly through protection from diseases (Lugtenberg *et al.*, 2004) [17]. Besides root colonizing bacteria (rhizobacteria) exert beneficial effect on plant development via direct or indirect mechanisms (Nelson, 2004) [21]. The concept of plant growth promoting rhizobacteria is now well established; both for growth promotion and biocontrol. Plant growth- promoting rhizobacteria (PGPR) were first defined by Kloepper and Schroth (1978) [14] to describe soil bacteria that colonize the roots of plants following inoculation onto seed and they enhance plant growth. However, their ineffectiveness in the field has often been attributed to their inability to colonize plant roots (Benizri *et al.*, 2001; Lugtenberg *et al.*, 2001) [2, 18].

Materials and Methods

Isolation and enumeration of rhizobacteria from pea rhizosphere

Rhizobacteria were isolated from the rhizosphere soil of pea plants during the full bloom stage. To estimate the number of soil microflora, serial dilution technique using the pour plate method was used (Johnson and Curl, 1972) [13]. Each value presented in this study is an average of three individual counts. Colony forming units (CFU) were recorded after 48 hours of incubation as per below given formula

$$\text{CFU/gm} = \frac{\text{Bacterial plate count} \times \text{Dilution factor}}{\text{Dry weight of soil (gm)}}$$

Estimation of Growth Promoting activities

Siderophore production

Siderophore was detected by the formation of orange halos surrounding bacterial colonies on CAS agar plates after incubation for 48 hours at 28 ºC (Schwyan & Neilands, 1987) [25].

Phosphorous Solubilization

Phosphate Solubilization was detected by formation of transparent halos surroundings bacterial colonies on the Pikovskaya agar after 72 hour incubation at 28 ºC (Pikovskaya, 1948) [22].

Indole acetic acid production

Bacterial cultures were incubated in Luria Bertani Broth at 28 ºC. The bacterial cells were removed from the culture by centrifugation at 8000 g for 10 min. A 3ml of supernatant was mixed vigorously with 2 ml of Salkowaski's reagent (2ml0.5M$FeCl_3$+98ml35%$HCLO_4$) and incubated at room temperature in dark for 30 minutes and observed for pink color formation. (Brick *et al*., 1991) [4].

Soil Analysis

The experimental soil was analyzed for various parameters in the beginning and at the end of the investigation. pH of the soil was determined in 1:2:5 soil solutions using a digital pH meter. The soil was analyzed for its available nitrogen content by Kjeldhal method (Subbaiah and Asija, 1956) [28] and organic carbon content by wet oxidation method (Jackson, 1967) [12]. The available phosphorus content was determined by Olsen's method (Muhur *et al*., 1965) [19] and the available potassium by flame photometer method (Stanford and English, 1949) [27].

Effect of PGPR on growth and yield characteristics of pea

On the basis of various plant growth promoting characteristics, nine isolates were selected and applied to the pea crop at the time of showing to evaluate their efficiency to enhance plant growth and yield characteristics under field conditions. The parameters studied were number of nodules per plant, plant height, number of pods per plant, pod length and fresh plant weight.

Results

Isolation and enumeration of rhizobacteria from pea rhizosphere

A total of 80 isolates were isolated. The most dominant isolates were identified as *Bacillus* sp (55), followed by *Pseudomonas* sp (12), *Azotobacter* sp (9) and *Micrococcus* sp (4). All the genera were tentatively identified following biochemical and morphological characterization.

Plant Growth Promoting Characteristics

Each isolate was screened for plant growth promoting traits such as siderophore production, phosphate solubulization, indole acetic acid production. Thirty isolates produced siderophores and the isolate PGP10 produced highest siderophore (18 mm) while least was observed in isolate PGP80 (2.30 mm) Table 1a. The ability to solubilize phosphate was positively exhibited by 43 isolates Table 1b. The isolate PGP72 showed maximum PSI (12.67) while minimum was noticed in isolate PGP35 (2.25). 40 isolates produce IAA that ranged from 2.10 to 25µg/ml (Table 1c).

Table 1a: Screening of PGPR, isolated from the rhizosphere of pea (*Pisum sativum*), for siderophore production

S. No.	Isolates	Qualitative siderophore estimation (zone size, mm)	S. No.	Isolates	Qualitative siderophore estimation (zone size, mm)
1	PGP1	16.00	16	PGP33	6.80
2	PGP4	4.00	17	PGP38	7.50
3	PGP5	7.52	18	PGP40	11.00
4	PGP8	8.50	19	PGP44	3.50
5	PGP9	7.00	20	PGP49	14.60
6	PGP10	18.00	21	PGP50	3.00
7	PGP12	3.15	22	PGP52	17.50
8	PGP15	6.00	23	PGP55	6.50
9	PGP19	6.50	24	PGP62	10.00
10	PGP20	4.00	25	PGP68	14.20
11	PGP22	11.00	26	PGP71	8.50
12	PGP25	3.00	27	PGP72	12.00
13	PGP28	14.50	28	PGP76	8.00
14	PGP30	8.50	29	PGP79	5.50
15	PGP32	3.50	30	PGP 80	2.30

C.D ($p \leq 0.05$): 0.345

Table 1b: Screening of PGPR, isolated from the rhizosphere of pea (*Pisum sativum*), for phosphate solubilization

S. No.	Isolate	Colony diameter (cm)	Zone of clearance (cm)	Solubilization Index (PSI)
1.	PGP1	0.41	1.50	4.66
2.	PGP4	0.20	1.30	7.50
3.	PGP5	0.50	1.30	3.60
4.	PGP8	0.30	1.20	5.00
5.	PGP9	0.80	1.20	2.50
6.	PGP10	0.20	1.80	10.00
7.	PGP13	0.70	1.70	3.43
8.	PGP15	0.30	2.00	7.67
9.	PGP17	0.50	1.30	3.60
10.	PGP18	0.40	1.40	4.50
11.	PGP20	0.30	1.20	5.00
12.	PGP22	0.60	1.40	3.33
13.	PGP23	0.60	1.50	3.50
14.	PGP25	1.00	1.95	2.95
15.	PGP28	0.20	2.30	12.50
16.	PGP30	0.20	1.80	10.00
17.	PGP32	0.80	1.00	2.26
18.	PGP33	0.20	1.95	10.75
19.	PGP35	0.80	1.00	2.25
20.	PGP38	0.30	1.20	5.00
21.	PGP39	0.30	1.30	5.33
22.	PGP40	0.50	1.50	4.00
23.	PGP42	0.30	1.70	6.67
24.	PGP43	0.60	1.60	3.67
25.	PGP44	0.80	1.40	2.75
26.	PGP46	1.00	2.80	3.80
27.	PGP48	0.70	1.50	3.14
28.	PGP49	0.50	1.80	4.60
29.	PGP50	0.20	1.60	9.00
30.	PGP51	0.20	1.95	10.75
31.	PGP52	0.60	1.90	4.17
32.	PGP55	0.40	1.20	4.00
33.	PGP58	0.30	1.60	6.33
34.	PGP60	0.60	2.30	4.83
35.	PGP62	0.20	1.65	9.25
36.	PGP63	0.50	1.50	4.00
37.	PGP65	0.70	1.90	3.71
38.	PGP67	0.20	1.65	9.25
39.	PGP68	0.80	1.00	2.27
40.	PGP71	0.20	1.80	10.00
41.	PGP72	0.30	3.50	12.67
42.	PGP75	0.20	1.30	7.50
43.	PGP77	0.30	1.50	6.00

C.D ($p \leq 0.05$): 0.361

Table 1c: Screening of PGPR, isolated from the rhizosphere of pea (*Pisum sativum*), for Indole-3-acetic acid

S. No	Isolate	Indole-3-acetic acid (µg/ml)	S. No	Isolates	Indole-3-acetic acid (µg/ml)
1	PGP1	12.00	21	PGP49	19.00
2	PGP2	18.00	22	PGP50	7.15
3	PGP5	9.00	23	PGP52	20.50
4	PGP6	13.50	24	PGP55	7.00
5	PGP7	3.40	25	PGP56	12.50
6	PGP10	25.00	26	PGP58	5.00
7	PGP16	15.00	27	PGP60	8.50
8	PGP18	12.50	28	PGP62	17.00
9	PGP20	9.50	29	PGP64	11.00
10	PGP25	21.00	30	PGP65	14.00
11	PGP28	22.50	31	PGP68	11.00
12	PGP30	4.00	32	PGP70	6.50
13	PGP32	12.00	33	PGP72	25.00
14	PGP36	9.50	34	PGP73	14.50
15	PGP38	6.80	35	PGP74	3.80
16	PGP39	5.50	36	PGP75	11.00
17	PGP40	10.00	37	PGP76	6.00
18	PGP42	12.00	38	PGP78	9.50
19	PGP43	8.00	39	PGP79	12.00
20	PGP44	5.50	40	PGP80	2.10

C.D ($p \leq 0.05$): 0.1135

Soil analysis

Physicochemical properties of soil are presented in Table 2. The soil pH ranged from 6.32 to 6.82 with highest pH observed in control (6.82) and lowest in isolate PGP49 (6.32).The organic carbon content was found in the range of 0.83 to 0.96 per cent with maximum content noticed in isolate PGP68 (0.96 %) and minimum in control (0.83 %). The available nitrogen and phosphorus contents were highest in treatment PGP49 (291.52 kg/ha and 131.07 kg/ha) and lowest nitrogen and phosphorus contents was recorded in control (280.65 kg/ha and 107.20 kg/ha) respectively. However, highest potassium content was observed in treatment inoculated with isolate PGP62 (86.37 kg/ha) in comparison to uninoculated control (79.18 kg/ha).

Table 2: Physicochemical properties of soil

Isolate	pH	Organic carbon (%)	Available nitrogen (kg/ha)	Available phosphorus (kg/ha)	Available potassium (kg/ha)
Control	6.82	0.83	280.65	107.200	79.187
PGP68	6.38	0.96	284.78	124.812	85.412
PGP10	6.51	0.90	287.34	124.249	82.703
PGP52	6.43	0.87	289.25	124.114	81.342
PGP28	6.56	0.86	289.73	120.029	86.078
PGP72	6.48	0.95	288.50	115.294	81.755
PGP49	6.32	0.90	291.52	131.077	79.770
PGP40	6.35	0.91	290.23	122.232	84.490
PGP01	6.52	0.95	287.51	125.057	81.760
PGP62	6.38	0.88	289.51	119.200	86.379
C.D ($p \leq 0.05$)	0.100	0.860	0.924	0.104	0.124

Effect of PGPR for growth and yield parameters of pea

The data presented in Table 3 shows that the application of isolates increased the number of nodules, plant height, number of pods, fresh plant weight and pod length in all selected isolates as compared to control where no culture was applied. The isolate PGP49 showed increased number of nodules (60.44) as compared to control (40.70). Maximum plant height was recorded with respect to isolate PGP1 (47.53 cm) while minimum was recorded in control (44.12 cm). The maximum number of pods was observed in isolate PGP68 (32.66) while it was minimum in control (21.33). Highest plant fresh weight was also recorded with respect to isolate PGP1 (427 g) than control (229 g). Highest pod length was recorded in isolate PGP62 (8.52 cm) and lowest in control (6.55 cm).

Table 3: Effect of selected isolates of PGPR for growth and yield characteristics on pea rhizosphere

Isolate	Number of nodules per plant	Plant height (cm)	Number of pods per plant	Plant fresh weight (g)	Pod length (cm)
Control	40.70	44.127	21.334	229	6.559
PGP68	54.20	46.712	32.667	397	8.342
PGP10	58.78	46.305	29.667	377	7.657
PGP52	58.69	44.727	30.000	282	8.270
PGP28	49.76	45.217	31.500	312	7.289
PGP72	52.85	45.679	29.667	364	7.375
PGP49	60.44	45.087	28.334	362	8.308
PGP40	57.90	45.112	29.167	360	6.785
PGP01	47.92	47.535	29.667	427	7.570
PGP62	54.94	44.130	29.000	357	8.522
C.D ($p \leq 0.05$)	1.072	0.588	1.146	0.121	0.253

Discussion

Rhizosphere is a rich habitat for microorganisms and should be explored for obtaining potential PGPR, which can be useful in developing bioinoculants for enhancement of growth and yield of crop plants. The beneficial effect of plant growth promoting rhizobacteria, particularly those belonging to the genus *Bacillus* or *Pseudomonas*, in enhancing growth and overall plant establishment is well established. This has been attributed to various mechanisms, such as providing fixed nitrogen to the host plant, production of phytohormones, solubilization of insoluble phosphate, production of metabolites, including antibiotics and siderophores (Compant *et al.*, 2005) [5].

The results of the present studies, based on the rhizobacteria isolation on the rich media and classification of the isolates on the morphological and biochemical features, can be criticized. In our study, four different genera were identified *viz. Bacillus*, *Pseudomonas*, *Micrococcus*, and *Azotobacter*.

In our study, *Bacillus* (55) was dominant group. *Bacillus* species are also a major component of the microbial flora, which live in close association with various types of agricultural crops. Many authors cite *Pseudomonas* as the dominant genus in the rhizosphere, probably because under favorable environmental conditions, its growth rate is higher than that of *Bacillus* (Bowen & Foster, 1978) [3]. Predominance of *Bacilllus* sp. is due to its ability to efficiently use the nutrients provided by the plant through exudates. In additions, *Bacillus* has the ability to inhibit the growth of other strains. Many strains of *Bacillus* have been reported to produce substances that act as growth inhibitors for other microorganisms (Lilinares *et al.*, 1994) [16]. However, previous study shows *Bacillus* as the dominant genus in the rhizosphere of *Elaeagnus angusti folia* L. (Ramos, 1998) [23].

The results show 37.5% isolates have ability to produce siderophore on CAS agar medium, 53% isolates solubilize phosphate and 50% produce IAA. These results indicate that the tested isolates could exhibit two or three plant growth promoting (PGPR) traits, which may promote plant growth directly, indirectly, or synergistically. In corroboration to our

findings multiple PGP activities among PGPR have been reported by some other investigators (Gupta *et al*., 1998; Dey *et al*., 2004) [10, 7]. Several studies have demonstrated that production of siderophore by PGPR was most effective in controlling the plant root pathogens (Mullen, 1998; *Diaz et al.*, 2002; and *D*ey *et al*., 2004) [20, 8, 7]. The potential to produce siderophores by microorganisms in improving iron availability to plants was also reported by some workers (Bar-Ness *et al*., 1992; Rroco *et al*., 2003; Sharma *et al*., 2003) [1, 24, 26]. Phosphate solubilization is considered to be the most important attribute of plant growth promoting rhizobacteria (Kloepper *et al*., 1989) [15]. Bacterial IAA stimulates the development of the host plant root system.

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