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Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.)

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Abstract

Plant growth promoting rhizobacteria (PGPR) are known to influence plant growth by various direct or indirect mechanisms. In search of efficient PGPR strains with multiple activities, a total of 150 bacterial isolates belonging to *Bacillus, Pseudomonas, Azotobacter and Rhizobium* were isolated from different rhizospheric soil of chick pea in the vicinity of Allahabad. These test isolates were biochemically characterized and screened *in vitro* for their plant growth promoting traits like production of indoleacetic acid (IAA), ammonia (NH_3), hydrogen cyanide (HCN), siderophore and catalase. All the isolates of *Bacillus, Pseudomonas* and *Azotobacter* produced IAA, whereas only 85.7% of *Rhizobium* was able to produce IAA. Production of ammonia was commonly detected in the isolates of *Bacillus* (95.0%) followed by *Pseudomonas* (94.2%), *Rhizobium* (74.2%) and *Azotobacter* (45.0%). All test isolates were positive for catalase but none of the isolates produced HCN. On the basis of multiple plant growth promoting activities, 20 bacterial isolates of each genus, in total 80 isolates, were evaluated for their heavy metal tolerance. Among these isolates, *Bacillus* spp. were tolerant to all the heavy metals (400 µg ml^{-1}), whereas *Pseudomonas* spp. were tolerant to Hg (100 µg ml^{-1}), Co (100 µg ml^{-1}), Cd (200 µg ml^{-1}), Cr (100 µg ml^{-1}), Cu (200 µg ml^{-1}), Pb (400 µg ml^{-1}), Zn (200 µg ml^{-1}). Tolerance to heavy metals was observed less frequently in *Azotobacter* spp. and *Rhizobium* spp. The isolates could exhibit more than two or three PGP traits, which may promote plant growth directly or indirectly or synergistically. Further, rhizobacteria tolerant to multiple heavy metals exhibited a couple of PGP activities.

Keywords: Ammonia; *C. arietinum*; HCN; Heavy metal tolerance; Indole Acetic Acid (IAA); Plant growth- promoting rhizobacteria (PGPR); Siderophore.

Introduction

Plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and/or indirectly. In last few decades a large array of bacteria including species of *Pseudomonas, Azospirillum,*

Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus and *Serratia* have reported to enhance plant growth (Kloepper et al., 1989; Okon and Labandera-Gonzalez, 1994; Glick, 1995). The direct promotion by PGPR entails either providing the plant with plant growth promoting substances that are synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment. The indirect promotion of plant growth occurs when PGPR prevent deleterious effects of one or more phytopathogenic microorganisms. The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include (i) the ability to produce or change the concentration of plant growth regulators like indoleacetic acid, gibberellic acid, cytokinins and ethylene (Arshad and Frankenberger, 1993; Glick, 1995), (ii) asymbiotic N_2 fixation (Boddey and Dobereiner, 1995), (iii) antagonism against phytopathogenic microorganisms by production of siderophores (Scher and Baker, 1982), antibiotics (Shanahan et al., 1992) and cyanide (Flaishman et al., 1996), (iv) solubilization of mineral phosphates and other nutrients (De Freitas et al., 1997; Gaur, 1990). Most popular bacteria studied and exploited as biocontrol agent includes the species of fluorescent *Pseudomonas* and *Bacillus*. Some PGPR may promote plant growth indirectly by affecting symbiotic N_2 fixation, nodulation or nodule occupancy (Fuhrmann and Wollum, 1989). However, role of cyanide production is contradictory as it may be associated with deleterious as well as beneficial rhizobacteria (Bakker and Schippers, 1987; Alstrom and Burns, 1989). In addition to these traits, plant growth promoting bacterial strains must be rhizospheric competent, able to survive and colonize in the rhizospheric soil (Cattelan et al., 1999). Unfortunately, the interaction between associative PGPR and plants can be unstable. The good results obtained *in vitro* cannot always be dependably reproduced under field conditions (Chanway and Holl, 1993; Zhender et al., 1999). The variability in the performance of PGPR may be due to various environmental factors that may affect their growth and exert their effects on plant. The environmental factors include climate, weather conditions, soil characteristics or the composition or activity of the indigenous microbial flora of the soil. To achieve the maximum growth promoting interaction between PGPR and nursery seedlings it is important to discover how the rhizobacteria exerting their effects on plant and whether the effects are altered by various environmental factors, including the presence of other microorganisms (Bent et al., 2001). Therefore, it is necessary to develop efficient strains in field conditions. One possible approach is to explore soil microbial diversity for PGPR having combination of PGP activities and well adapted to particular soil environment. So keeping in view the above constrains, the present study was designed to screen certain rhizospheric bacterial isolates belonging to the genera *Pseudomonas, Bacillus, Azotobacter,* and *Rhizobium* for their multiple plant growth promoting activities.

Materials and methods

Isolation of Rhizobacteria

The rhizospheric soil samples (six) were collected from fields growing Chickpea (*C. arietinum* L.*)* from west of Allahabad Agricultural Institute, India. All bacterial strains were isolated on their respective media; *Rhizobium* was isolated on yeast extract mannitol agar (Vincent, 1970), *Azotobacter* on Jensen's medium (Norris and Chapman, 1968),

Pseudomonas on King's B medium (King et al., 1954) and *Bacillus* on nutrient agar. Bacterial cultures were maintained on the respective slants.

Biochemical characterization of rhizobacteria

Selected isolates of *Bacillus* (40), *Pseudomonas* (35), *Azotobacter* (40) and *Rhizobium* (35) were biochemically characterized by Gram's reaction, carbohydrate fermentation, oxidase test, O-F test, H_2S production, IMViC tests, NO_2 reduction, starch and gelatin hydrolysis as per the standard methods (Cappuccino and Sherman, 1992).

Characterization of rhizobacteria for PGP traits

Production of Indole acetic acid

Indole acetic acid (IAA) production was detected as described by Brick et al. (1991). Bacterial cultures were grown for 72 h (*Azotobacter* and *Rhizobium*) and 48 h (*Pseudomonas* and *Bacillus*) on their respective media at 36±2 °C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of pink colour indicates IAA production.

Production of ammonia

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48–72 h at 36±2 °C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman, 1992).

Production of HCN and catalase

All the isolates were screened for the production of hydrogen cyanide by adapting the method of Lorck (1948). Briefly, nutrient broth was amended with 4.4 g glycine/l and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with parafilm and incubated at 36±2 °C for 4 days. Development of orange to red colour indicated HCN production. Bacterial cultures were grown in a nutrient agar medium for 18-24 h at 36±2 °C. The cultures were mixed with appropriate amount of H_2O_2 on a glass slide to observe the evolution of oxygen.

Siderophore production

Siderophore production was detected by the universal method of Schwyn and Neilands (1987) using blue agar plates containing the dye chrom azurol S (CAS). Orange halos around the colonies on blue were indicative for siderophore production.

Physicochemical analysis

Soil samples were analyzed for physicochemical parameters like pH, conductivity, total organic carbon, total nitrogen, etc. Organic carbon was analyzed by the Walkely and Black dichromate oxidation method (Blakemore et al., 1972). Soil pH was measured in soil: water (1:2.5) slurry using a glass electrode. Nitrogen was measured by Kjeldahl method.

Heavy metal tolerance

The selected bacterial strains were tested for their resistance to heavy metals by agar dilution method (Cervantes et al., 1986). Freshly prepared agar plates were amended with various soluble heavy metal salts namely Hg, Co, Cd, Cu, Pb, Zn and Cr, at various concentrations ranging from 25 to 400 μg ml^{-1} were inoculated with overnight grown cultures. Heavy metal tolerance was determined by the appearance of bacterial growth after incubating the plates at room temperature for 24-48h.

Results

Abundance of rhizobacterial population in the rhizosphere of Chick pea is given in Figure 1. The bacterial population (cfu gm^{-1}) ranged from 0.5-2.1×10^6 of *Bacillus* spp., 1.1-2.1×10^6 of *Pseudomonas* spp., 0.3-1.7×10^6 of *Azotobacter* spp., and 0.8-2.0×10^6 of *Rhizobium* spp. The population of *Pseudomonas* dominated in the rhizosphere. On the basis of cultural, morphological and biochemical characteristics a total of 150 soil isolates were grouped into *Bacillus*, *Pseudomonas, Azotobacter,* and *Rhizobium* as described. General features of the test isolates are illustrated in Table 1.

In the present investigation 40 isolates of *Azotobacter* spp., *Bacillus* spp. each and 35 isolates belonging to *Rhizobium* spp., and *Pseudomonas* spp., were screened for *in vitro* PGP activities. Screening results of PGP traits are depicted in Table 2. IAA production was shown in all the isolates of *Bacillus, Pseudomonas* and *Azotobacter* (100%) followed by *Rhizobium* (85.7%). Ammonia production was detected in 95% of isolates of *Bacillus* followed by *Pseudomonas* (94.2%), *Rhizobium* (74.2%) and *Azotobacter* (45.0%). Production of siderophore was detected less frequently than other PGP characteristics. None of the isolates of *Azotobacter* and *Rhizobium* spp. produced siderophore. The isolates of *Pseudomonas* spp. were strong siderophore producers (74.2%) while few isolates of *Bacillus* were able to produce siderophore (12.5%). Production of catalase was exhibited by all the isolates of rhizobacteria. However, production of HCN was not detected in rhizobacterial isolates under study (data not shown). Catalase activity was detected in all the bacterial strains that may be potentially very advantageous.

The levels of total organic carbon (0.73-1.29 %) pH (6.8-7.7), conductivity (0.27 - 0.33 μm cm^{-1}), nitrate (45.00-50.65 kg ha^{-1}) and ammonical (14.62-18.83 kg ha^{-1}) nitrogen in rhizosphere were at normal levels (Table 3). Among all the rhizobacteria studied, majority of the isolates of *Bacillus* spp. were tolerant to all the heavy metals (200 μg ml^{-1}). However, the isolates were susceptible to higher levels of Hg, Co. (Table 4). Majority of the *Pseudomonas* spp. were tolerant to Hg (100 μg ml^{-1}), Co (100 μg ml^{-1}), Cd (200 μg ml^{-1}), Cu (200 μg ml^{-1}), Pb (400 μg ml^{-1}), Zn(200 μg ml^{-1}) Cr (100 μg ml^{-1}). However, only few

isolates were able to tolerate higher metal concentration. Tolerance to heavy metals was observed less frequently in *Azotobacter* spp. None of these isolates were tolerant to Co, Cd, Cu (200 µg ml^{-1}), Zn and Pb (400 µg ml^{-1}). *Rhizobium* spp. was fully susceptible to 400 µg ml^{-1} of metals like Cu, Zn and Cr.

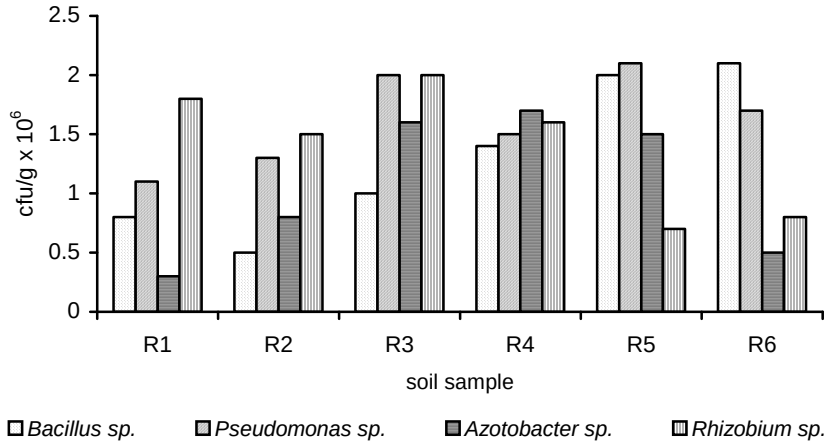

Figure 1. Microbiological analysis of soil samples.

Table 1. Morphological and cultural characteristics of rhizobacteria.

Morphological and biochemical characterization	*Bacillus* (40)*	*Pseudomonas* (35)*	*Azotobacter* (40)*	*Rhizobium* (35)*
Grams reaction	G +ve	G -ve	G -ve	G -ve
Shape	rods	rods	rods	rods
Pigments	-	+	+/-	+/-
Lactose	+	-	+	w
Dextrose	+	+	+	-
Sucrose	+	+	+	-
Mannitol	+	-	+	+
Oxidase	-	+	+	+
OF test	-	+	+	-
H_2S production	-	+	-	+
Indole	-	-	+	+
Methyl red	-	-	+	+
Vogues Proskauer	+	-	+	+
Citrate utilization	+	+	+	-
Nitrate reduction	+	+	+	+
Starch hydrolysis	+	+	+	+
Gelatin hydrolysis	+	-	-	-

Table 2. Plant growth promoting characteristics of rhizobacterial isolates.

Bacteria	No. of isolates	PGP characteristics (%)			
		IAA production	Ammonia production	Siderophore production	Catalase production
Bacillus spp.	40	100	95.0	12.5	100
Pseudomonas spp.	35	100	94.2	74.2s	100
Azotobacter spp.	40	100	45.0	0	100
Rhizobium spp.	35	85.7	74.2	0	100

Table 3. Physiochemical parameters of the rhizosphere soil of Chickpea.

Samples	Physiochemical parameters				
	pH	Electrical conductivity ($\mu m\ cm^{-1}$)	Organic carbon (%)	Nitrogen availability	
				Nitrate kg ha^{-1}	Ammonical kg ha^{-1}
R1	7.5	0.27	0.73	50.62	18.83
R2	7.7	0.29	1.29	45.00	14.62
R3	7.6	0.30	1.20	50.65	14.62
R4	6.8	0.33	1.06	50.65	14.62
R5	7.2	0.28	0.83	50.62	16.87
R6	7.6	0.31	0.74	50.65	18.00

Discussion

Plant rhizosphere is known to be preferred ecological niche for soil microorganisms due to rich nutrient availability. Reports are available on *Azotobacter* spp. isolated from different sources showed IAA production (Gonzalez-Lopez et al., 1986; Jagnow, 1987; Nieto and Frankenberger, 1989). In the present study IAA production in *Azotobacter* isolates are in agreement with earlier reports. The ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant. Growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere and other PGP activities (Arshad and Frankenberger, 1993; Glick, 1995). Production of IAA by *Bacillus*, *Pseudomonas* and *Azotobacter* is a general characteristic of our test isolates. Higher level of IAA production by *Pseudomonas* was recorded by other workers (Xie et al., 1996). Another important trait of PGPR, that may indirectly influence the plant growth, is the production of ammonia. Mostly all the isolates were able to produce ammonia. However, ammonia production was observed less frequently in *Azotobacter* isolates. *Pseudomonas* and *Bacillus* spp. were siderophore producers. Siderophores chelates iron and other metals contribute to disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitats (Hofte et al., 1992; Loper and Henkels, 1997). Siderophores may directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria. Antibiotic and siderophores may further function as stress factors or singles including local and systematic host resistance.

Table 4. Heavy metal tolerance among PGPR isolated from rhizosphere soil of Chickpea.

PGPR	No. of Colonies tested	Heavy metals (μg ml^{-1})																			
		Hg		Co		Cd			Cu			Pb			Zn			Cr		
		100	200	100	200	100	200	400	100	200	400	100	200	400	100	200	400	100	200	400
Bacillus sp.	20	20 (100)	11 (55)	0 (0)	0 (0)	20 (100)	14 (70)	1 (5)	20 (100)	20 (100)	4 (20)	20 (100)	20 (100)	10 (50)	20 (100)	20 (100)	4 (20)	20 (100)	20 (100)	8 (40)
Rhizobium sp.	20	20 (100)	9 (45)	20 (100)	8 (40)	20 (100)	16 (80)	1 (5)	20 (100)	9 (45)	0 (0)	20 (100)	20 (100)	5 (25)	20 (100)	7 (35)	0 (0)	20 (100)	3 (15)	0 (0)
Pseudomonas sp.	20	20 (100)	2 (10)	20 (100)	3 (15)	20 (100)	17 (85)	1 (5)	20 (100)	9 (45)	2 (10)	20 (100)	20 (100)	4 (20)	20 (100)	19 (95)	2 (10)	20 (100)	9 (45)	0 (0)
Azotobacter sp.	20	6 (30)	1 (5)	6 (30)	0 (0)	5 (25)	0 (0)	0 (0)	3 (15)	0 (0)	0 (0)	12 (60)	1 (5)	0 (0)	13 (65)	1 (5)	0 (0)	15 (75)	3 (15)	1 (5)

Number in parenthesis shows percentage of isolates

It has been assumed that inoculation with bacteria like *Bacillus, Pseudomonas*, *Rhizobium,* and *Azotobacter* may enhance the plant growth as a result of their ability to fix nitrogen. All the bacterial isolates in the present study were able to produce catalase. Bacterial strains showing catalase activity must be highly resistant to environmental, mechanical, and chemical stress. Some of the above-tested isolates could exhibit more than two or three PGP traits, which may promote plant growth directly or indirectly or synergistically. Similar to our findings of multiple PGP activities among PGPR have been reported by some other workers while such findings on indigenous isolates of India are less commonly explored (Gupta et al., 1998).

The organic content in soil samples was considered as one of the key determinants driving the microbial community structure (Zhou et al., 2002). Recent study by Sharma et al. (2005) addressing the effect of legume rhizodeposition on bacterial communities, showed a distinct plant-dependent rhizosphere effect on the distribution of different bacterial groups present in legume rhizosphere. However, reports on plant-dependent rhizosphere effect on microbial community functions are limited. Plant roots influence soil borne microbial communities via several mechanisms, including excretion of organic compounds, competition for nutrients, and providing a solid surface for attachment. The nature of this influence is highly variable and depends upon both the amount and composition of organic materials released by the plants (Griffiths et al., 1999). Any microbial utilization in agriculture requires an evaluation of the environmental risks associated with the introduction of indigenous or non-indigenous microorganisms into the plant rhizosphere (Jackman et al., 1992) as well as an assessment of the most suitable conditions for effective and successful establishment of the PGPR inoculation in the rhizosphere of the host plant (De Leij et al., 1994; 1995). Furthermore, it is known that some PGPR strains are able to express multiple beneficial functions (Kloepper and Schrot, 1978).

Microorganisms have developed the mechanisms to cope with a variety of toxic metals for their survival in the environment enriched with such metals. We observed few rhizobacteria tolerant to multiple heavy metals and exhibiting a couple of PGP activities. It was also apparent that more cultures of PGPR isolated from chickpea rhizosphere were tolerant to elevated levels heavy metals. Burd et al. (1998) found that by decreasing heavy metal toxicity, PGPR increases plant growth. The selection of microorganisms both metal tolerant and efficient in producing PGP compounds can be useful to speed up the re-colonization of the plant rhizosphere in polluted soils (Carlot et al., 2002). Heavy metals, at higher concentration, are toxic to cells and may cause cell death by interacting with nucleic acids and enzymes active site (Ohsumi et al., 1988; Hazel and Williams, 1990; Cervantes and Gutierrez-Corana, 1994). *Azotobacter* spp, when inoculated into heavy metal contaminated soil, inhibited N_2-fixation (Briely and Thornton, 1983). Rother and co-workers (1983) reported a reduction in nodule and plant size and in nitrogenase activity of clover at sites heavily contaminated with Cd and Pb. Chromium-resistant pseudomonads,

isolated from paint industry effluents, were able to stimulate seed germination and growth of *Triticum aestivum* in the presence of potassium dichromate (Hasnain and Sabri, 1996).

In addition to these traits, plant growth promoting bacterial strains must be rhizospheric competent, able to survive and colonize in the rhizospheric soil. Unfortunately, the interaction between associative PGPR and plants can be unstable. The good results obtained *in vitro* cannot always be dependably reproduced under field conditions. It is expected that inoculation with rhizobacteria containing PGP characteristics consequently promote root and shoot growth as well as nodulation. Further evaluation of the isolates exhibiting multiple plant growth promoting (PGP) traits on soil–plant system is needed to uncover their efficacy as effective PGPR.

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