ISSN: 2394-1391

Indian Journal of Biology
Volume 3 Number 2, July - December 2016
DOI: http://dx.doi.org/10.21088/ijb.2394.1391.3216.8

Original Article

Isolation and Characterization of PGPR from Wheat (*Triticum aestivum*) Rhizosphere and Their Plant Growth Promoting Traits *in Vitro*

Yogendra Singh*, Nand Lal**

Received on 13.08.2016, **Accepted on** 22.08.2016

Abstract

The PGPR have been divided into two groups based on their involvements in (i) nutrient cycling and phytostimulation, and (ii) the biocontrol of plant pathogens. A total of 72 rhizobacterial isolates belonging to genera *Bacillus, Pseudomonas* and *Rhizobium* were isolated from Wheat (*Triticum aestivum* L.) rhizospheric soils collected from various locations of Kanpur region, India. These rhizobacterial isolates were characterized biochemically and screened for their PGP (plant growth promoting) activities *in vitro.* Plant growth promoting traits screened with the test rhizobacteria included production of indole acetic acid (IAA), ammonia (NH_3), hydrogen cyanide (HCN), siderophore and catalase. All test isolates turned positive for catalase production. The rhizobacterial isolates of *Pseudomonas spp.* (100%), *Bacillus spp.* (100%) and *Rhizobium spp.* (67%) produced IAA. Production of ammonia (NH_3) was commonly detected in the rhizobacterial isolates of *Bacillus* (100%), Pseudomonas (85%) and *Rhizobium* (70%). *Bacillus Spp.* sample KNP-7 showed high level of tolerance to the multiple heavy metals tested whileas tolerance to heavy metals was observed less frequently in *Rhizobium spp*. sample KNP-36 and KNP-5. Rhizobacteria tolerant to multiple heavy metals and exhibiting a couple of PGP traits in the present study hold promise as effective PGPR with wheat and / or other compatible crops.

Keywords: Ammonia; HCN; Heavy Metal Tolerance; Indole Acetic Acid; Plant Growth-Promoting Rhizobacteria; Siderophore; *Triticum aestivum* L.

Introduction

Plant growth-promoting rhizobacteria (PGPR) form a highly diverse group of indispensable soil bacteria of plant rhizosphere that influence the plant growth through a range of mechanisms and have been classified into three broad categories i.e. bioprotectants: strains that suppress the pathogens and hence control plant diseases, biofertilizers: strains which improve the nutrient uptake of the plant and biostimulants. The rhizobacteria enhance the plant biomass and nutrient availability either by the solubilization of phosphate and other mineral complexes or by nitrogen fixation, production of siderophores for the acquisition of trace metals or by the release of phytohormones for better root growth and controlling the harmful effects of deleterious / pathogenic organisms [1-3]. Many studies have been conducted to evaluate the role of PGPR in phytoremediation efficiency action on metal contaminated soils [4]. These bacteria generate a stimulating effect on the growth of plants via giving to them a continuous supply of nutrients and hormones through their metabolic activities [5]. PGPR such as *Agrobacterium*, *Alcaligenes (Ralstonia), Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Serratia, Pseudomonas* and *Rhizobium* [6-12] are particularly interesting for metal extraction by plants since they increase both the rate of metals accumulated by plants and the plant biomass. Plant growth promoting rhizobacteria when applied to seeds or incorporated into soil reduce the toxicity of heavy metals and consequently enhance the growth and yield of plant. Further, the nodule bacteria can protect the plants against the toxic effects of nickel

Author's Affiliation: *Department of Microbiology, C.C.S. P.G. College, Heonra, Etawah- 206001, India. **Department of Life Sciences, C.S.J.M. University, Kanpur-208024, India.

Reprint's Request: Prof. Nand Lal, Head, Department of Life Sciences, C.S.J.M. University, Kanpur-208024, Uttar Pradesh India.
E-mail: nl_pr@yahoo.co.in

and zinc through adsorption or desorption mechanism [13]. In addition, the plant growth promoting rhizobacteria also synthesize plant growth promoting substances (siderophore, indole acetic acid, hydrogen cyanide and ammonia), which augment the crop productivity [14]. Thus, it may be said that there are plethora of mechanisms that may be explored for developing various PGPR strains as the successful eco-friendly tools to implement sustainable agricultural practices in all parts of the world. There is very little information regarding use of PGPR as bioinoculants / biofertilizers in wheat. Therefore, the present study was undertaken with the view to isolate and characterize PGPR strains from wheat growing fields in Kanpur agro-ecological region of Uttar Pradesh, India.

Materials and Methods

Collection of Sample

The rhizospheric soil samples were collected from sewage irrigated fields growing *Triticum aestivum* L. from rural areas of Kanpur region, India. The fields are being irrigated with domestic sewage last 7 to 8 years. Randomly chosen plants from different locations were uprooted carefully and the excess of soil was removed by gentle shaking and the soil adhering to roots formed composite samples. The collected samples were placed in plastic bags and kept at 4°C in the laboratory until processed further.

Isolation of Rhizobacteria

Soil samples were serially diluted up to 10^{-5} to 10^{-7} in sterile phosphate-buffered saline (Hi-Media, pH-7.2) and plated on the appropriate culture medium for isolating different rhizobacteria. All bacterial strains were isolated on their respective media; *Rhizobium* was isolated on yeast extract mannitol agar [15]. *Pseudomonas* and *Bacillus* were isolated on King's B agar selective medium [16] and nutrient agar, respectively. Isolated colonies of rhizobacterial strains were randomly selected and further purified by streaking. Pure isolates were maintained as glycerol stocks at -80°C for further use.

Identification and Biochemical Characterization of Rhizobacteria

Isolated rhizobacterial strains were characterized and tentatively identified on the basis of their morphological, biochemical and / or physiological characteristics according to Bergey's manual of determinative bacteriology. Selected isolates of *Bacillus* (55), *Pseudomonas* (45) and *Rhizobium* (45) were biochemically characterized for Gram's reaction, carbohydrate fermentation, oxidase test, O-F test, H_2S production, IMVIC tests, NO_2 reduction, and starch and gelatin hydrolysis as per the standard methods [17].

Characterization of Rhizobacteria for PGP Traits

Production of Indole Acetic Acid

Indole acetic acid (IAA) production was detected as described by Brick *et al.* [18]. *Pseudomonas, Bacillus* and *Rhizobium* cultures were grown separately on their respective media with 100 and 200 μg / ml of L-tryptophan at 30°C for 48 hours. Fully grown cultures were centrifuged at 8000 rpm for 10 min. The supernatant (2 ml) was mixed with two drops of ortho-phosphoric acid and 4 ml of the Salkowski reagent (concentrated H_2SO_4:150 ml, 0.5M $FeCl_3 \cdot 6H_2O$:7.5 ml, distilled water: 250 ml). 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 hours 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 [17].

Siderophore Production

Siderophore production was detected by the method of Schwyn and Neilands [19] using blue agar plates containing the dye chrom azurol S (CAS). Orange halos around the colonies on blue were indicative for siderophore production.

Phosphate Solubilization Activity

All isolates were first screened on Pikovskaya's agar plates for solubilization of insoluble inorganic phosphate as described by Gaur [20]. Bacterial cultures were inoculated on centre of agar plate through inoculation loop under aseptic condition. Inoculated plates were incubated for 3 days at 30°C. Presence of clear zone (halozone) around the colony was recorded on Pikovskaya's agar plates. Formation of halozone showed positive phosphate solubilization ability.

Catalase Production

Bacterial cultures were grown in nutrient agar medium for 18-24 hours. The cultures were mixed with appropriate amount of H_2O_2 on a glass slide to observe the evolution of oxygen.

HCN Production

Hydrogen cyanide (HCN) production from glycine was tested growing the bacteria in 10% trypic soy agar (TSA) supplemented with glycine (4.4 g l^{-1}) and cyanogenesis was revealed using picric acid and Na_2CO_3 (0.5 and 2%, respectively) using the method of Donate-Correa *et al.* [21]. Impregnated filter paper fixed to the underside of the Petridis lids. Results were read after five days of culture at 28°C. A change in filter paper colour from yellow to orange-brown indicated production of HCN as indicated below:

Yellow (1) - limited cyanide production, orange (2) - moderate cyanide production, light brown (3) - relatively high cyanide production and brown (4) - high cyanide production.

Heavy Metal Tolerance

The selected bacterial strains were tested for their resistance to heavy metals by agar dilution method. Freshly prepared agar plates were amended with various soluble heavy metal salts namely Cr, Pb, Hg, Cd, Zn, and Cu, at various concentrations ranging from 25 to 200 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 24-48 hours.

Results and Discussion

In the present study, the rhizobacterial isolates were identified based on morphological and biochemical characteristics and were tested for their beneficial traits like ability to production of indole acetic acid ammonia and production of other plant growth promoting substances. Efficient rhizobacterial isolates selected based on the above characters were examined for their *in vitro* screening methods.

Isolation and Identification of Rhizobacteria

On the basis of cultural, morphological and biochemical characteristics (Table 1), a total of *Bacillus* (55), *Pseudomonas* (45) and *Rhizobium* (45) were identified from domestic sewage irrigated rhizospheric soil samples as described in Bergey's Manual of Determinative Bacteriology. *Bacillus* represents the predominant bacterial genera of the tested wheat rhizosphere. This observation is in conformity with Rawat *et al.* [22] and may be attributed to the ability of *Bacillus* to form endospores and produce antimicrobial substances that inhibit other competitors in the rhizosphere. Among the 145 isolates, 72 isolates (*Bacillus*-35, *Pseudomonas*-20 and *Rhizobium*-17) were selected for further studies based on the efficiency of multiple plant growth promoting activities *in vitro*.

Plant Growth Promoting Characteristics of Test Isolates

Screening results for PGP traits of selected isolates are presented in Table 2. IAA production was shown in most of the isolates of *Bacillus* (100%), *Pseudomonas* (100%) and *Rhizobium* (67%) thus showing positive PGP activities in relation to IAA. IAA is the most important auxin (phytohormone) produced by plants and many soil bacteria. It has a crucial role to play in a variety of plant activities, including embryo development; root initiation and development; apical dominance; leaf formation and fruit development. IAA is derived mainly from tryptophan through multiple enzymatic pathways by many different genera of PGPR like *Rhizobium*, *Bacillus*, *Pseudomonas*, *Azotobacter*, *Enterobacter*, *Bradyrhizobium*, *Xanthomonas* and *Alcaligenes* [23]. All rhizobacterial test isolates turned positive for catalase production. Catalase activity in the bacterial strains may be potentially very advantageous and bacterial strains showing catalase activity must be highly resistant to environmental, mechanical and chemical stress. Selected rhizobacterial isolates did not exhibit significant phosphate solubilisation activity in the present study. Siderophore production was detected in *Pseudomonas* (70%), *Bacillus* (33%) and *Rhizobium* (0%). Siderophore ability was detected significantly higher among isolates of *Pseudomonas* spp. Siderophores are low-molecular-weight molecules that are secreted by many microorganisms and act as solubilising agent for iron from minerals under iron shortage condition. In addition, siderophores form stable complexes with heavy metals including U, Np, Al, Cu, Cd, In, Ga, Zn and Pb and increases the soluble metal concentration [24], thus, it helps to alleviate the stresses imposed on plants by heavy metals in soil. Siddiqui *et al.* [25] reported that AY197010 isolate of *Pseudomonas* and AY197006 and AY197009 isolates of *Flavobacterium* could manufacture siderophore. HCN production was detected higher in *Pseudomonas spp.* as compared to the *Bacillus spp.* and *Rhizobium spp.* isolates. Higher HCN production by *Pseudomonas fluorescens*, *P.*

aeruginosa and *Chromobacterium violaceum* has also been reported by other researchers [11, 26]. Ammonia production was detected in *Bacillus* (100%), *Pseudomonas* (85%) and *Rhizobium* (70%) of test isolates which is an important attribute of PGPR that influences plant growth indirectly [27]. Hydrogen cyanide was detected in *Bacillus* (70%), *Pseudomonas* (100%) and *Rhizobium* (50%) among the test isolates. HCN production by soil bacteria is reported to play a role in disease suppression, as in the case of tobacco

Table 1: Morphological, cultural and biochemical characteristics of rhizobacteria associated with rhizosphere of *T. aestivum* L

Morphological and Biochemical Characterization	*Bacillus* (35)	*Pseudomonas* (20)	*Rhizobium* (17)
Gram's reaction	G +ve	G -ve	G -ve
Shape	Rods	rods	rods
Pigments	-	+	+/-
Dextrose	+	+	-
Sucrose	+	+	-
Mannitol	+	-	+
Oxidase	-	+	+
OF test	-	+	-
H_2S production	-	+	+
Indole	-	-	+
Methyl red	-	-	+
Vogues Proskauer	+	-	+
Citrate utilization	+	+	-
Starch hydrolysis	+	+	+
Gelatin hydrolysis	+	-	-

Table 2: Plant Growth Promoting Characteristics of rhizobacteria associated with rhizosphere of *T. aestivum* L.

Organism	Sample No.	IAA	Catalase	HCN	Ammonia	Siderophore
Bacillus spp.	KNP-4	+	+	+	+	+
Bacillus spp.	KNP-7	+	+	-	+	-
Bacillus spp.	KNP-13	+	+	+	+	-
Pseudomonas spp.	KNP-19	+	+	+	+	+
Pseudomonas spp.	KNP-11	+	+	+	+	+
Pseudomonas spp.	KNP-27	+	+	+	+/-	+/-
Rhizobium spp.	KNP-36	-	+	-	+	-
Rhizobium spp.	KNP-5	+	+	-	+/-	-
Rhizobium spp.	KNP-22	+	+	+	+	-

Table 3: Heavy metal tolerance of selected of rhizobacterial isolates associated with rhizosphere of *T. aestivum* L.

		Heavy Metal Tolerance (µg ml^{-1})					
Organism	Sample No.	CR	PB	HG	CD	ZN	CU
Bacillus spp.	KNP-4	200	100	50	100	100	100
Bacillus spp.	KNP-7	200	200	100	100	200	100
Bacillus spp.	KNP-13	200	200	50	100	100	100
Pseudomonas spp.	KNP-19	200	100	100	100	50	100
Pseudomonas spp.	KNP-11	150	100	100	100	100	100
Pseudomonas spp.	KNP-27	200	200	100	100	200	100
Rhizobium spp.	KNP-36	100	100	100	100	50	100
Rhizobium spp.	KNP-5	100	100	50	50	100	50
Rhizobium spp.	KNP-22	200	100	200	100	100	200

where *Pseudomonas fluorescens* helped suppression of black root rot disease [28].

Heavy Metal Tolerance of Test Isolates associated with Rhizosphere of T. aestivum.

Thirty-five (35) *Bacillus*, 17 *Rhizobium* and 20 *Pseudomonas* rhizobacterial isolates was checked against different heavy metals Cr, Pb, Hg, Cd, Zn and Cu and data on few selected isolates is presented in Table 3. This study observed rhizobacteria particularly *Bacillus* and *Pseudomonas* isolates tolerant to multiple heavy metals and exhibiting a couple of PGP activities (Table 2 and 3). The metal-microbe interaction in natural environment is influenced by pH and organic matter content. In the present study, selected strains showed heavy metal tolerance up to 200 µg / ml. *Bacillus sp.* (KNP-7) tolerated Pb and Cr (200 µg / ml), Hg (50 µg / ml) and tolerance exhibited

towards Zn, Cu and Cd was 100 µg / ml, respectively. *Pseudomonas sp.* (KNP-27) proved more heavy metal tolerant as compared to other isolates, tolerating up to 200 µg / ml of Cr, Zn and Pb, and 100 µg / ml of Hg, Cd and Cu. A varying level of resistance to heavy metals among the PGPR (*Bacillus* and *Pseudomonas*) have also been reported [29] as seen in present findings. *Bacillus* and *Pseudomonas* induced larger inhibition zones showing their high heavy metal tolerance activity and exhibiting high metal tolerance activities against heavy metals compared to the other *Rhizobium* isolates. Tolerance to heavy metals was observed less frequently in *Rhizobium spp.* isolates. The present study shows the significance of rhizobacteria under *in vitro* conditions for multiple PGPR traits and their evaluation under controlled conditions for selection of effective PGPR isolates of *Bacillus*, *Pseudomonas* and *Rhizobium.* Their multiple plant growth promoting activities are highly effective in improving the plant growth parameters. Several studies have also established a correlation between bacterial antibiotic resistance and metal tolerance [27, 30].

References

1. Verma SC, Ladha JK, Tripathi AK. Evaluation of plant growth promoting and colonization ability of endophytic diazotrophs from deep water rice. J Biotechnol 2001; 91: 127-141.
2. Zhuang X, Chen J, Shim H, Bai Z. New advances in plant growth promoting rhizobacteria for bioremediation. Environ Int 2007; 33: 406-413.
3. Rau N, Mishra V, Sharma M, Das M, Ahaluwalia K, Sharma RS. Evaluation of functional diversity in rhizobacterial taxa of a wild grass (*Saccharum ravennae*) colonizing abandoned fly ash dumps in Delhi urban ecosystem. Soil Biol Biochem 2009; 41: 813-821.
4. Khan AG. Role of soil microbes in the rhizosphere of plants growing on trace metal contaminated soils in phytoremediation. J Trace Elem Med Biol 2005; 18: 355–364.
5. Wu SC, Cao ZH, Li ZG, Cheung KC, Wong MH. Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. Geoderma 2005; 125: 155–166.
6. Cervantes-Vega C, Chavez J, Cardova NA, de la Mora P, Amador Velasco J. Resistance to Metal by *Pseudomonas aeruginosa* clinical isolates. Microbios 1986; 48: 159-163.
7. O'Sullivan DJ, O'Gara F. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol Rev 1992; 56: 662-676.
8. Carlot M, Giacomini A, Casella S. Aspects of plant microbe interactions in heavy metal polluted soil. Acta Biotechnol 2002; 22: 13-20.
9. Glick RB. Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotechnol Adv 2003; 21: 383-393.
10. Singh Y, Ramteke PW, Shukla PK. Characterization of *Rhizobium* isolates of pigeon pea rhizosphere from Allahabad soils and their potential PGPR characteristics. Int J Res Pure Appl Microbiol 2013; 3: 4-7.
11. Singh Y, Ramteke PW, Shukla PK. Isolation and characterization of heavy metal resistant *Pseudomonas spp.* and their plant growth promoting activities. Adv Appl Sci Res 2013; 4: 269-272.
12. Singh Y, Ramteke PW, Shukla PK, Khan K. Isolation and characterization of multi heavy metal resistant *Bacillus* spp. and their plant growth promoting activities. Int J Rec Sci Res 2013; 4: 148-150.
13. Glick BR, Penrose DM, Li J. A model for the lowering of plant ethylene concentrations by plant growth promoting bacteria. J Theor Biol 1998; 190: 63–68.
14. Rajkumar M, Freitas H. Effects of inoculation of plant growth promoting bacteria on Ni uptake by Indian mustard. Biores Technol 2008; 99: 3491–3498.
15. Vincent JM. A manual for the practical study of the root nodule bacteria. Blackwell Scientific publications, Oxford and Edinburgh, 1970. pp: 1-3.
16. King EO, Ward MK, Randey DE. Two simple media for the demonstration of pyocyanin and fluorescein. J Lab Clin Med 1954; 44: 301-307.
17. Cappuccino JC, Sherman N. In: Microbiology: A Laboratory Manual, New York; 1992. pp. 125–179.
18. Brick JM, Bostock RM, Silverstone SE. Rapid in situ assay for indole acetic acid production by bacteria immobilized on nitrocellulose membrane. Appl Environ Microbiol 1991; 57: 535–538.
19. Schwyn B, Neilands JB. Universal chemical assay for detection and determination of siderophores. Anal Biochem 1987; 160: 47-56.
20. Gaur AC. Physiological functions of phosphate solubilising microorganisms. In: Gaur AC editor. Phosphate Solubilizing Microorganisms as Biofertilizers. Omega Scientific Publishers, New Delhi, 1990. pp. 16-72.
21. Donate-Correa J, Leon-Barrios M, Perez-Galdona R. Screening for plant growth-promoting rhizobacteria in *Chamaecytisus proliferus* (tagasaste), a forage tree-shrub legume endemic to the Canary Island. Plant Soil 2004; 266: 261-272.
22. Rawat S, Izhari A, Khan A. (2011). Bacterial diversity in wheat rhizosphere and their characterization. Adv Appl Sci Res 2011; 2: 351-356.
23. Ahmad F, Ahmad I, Khan M. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol Res 2008; 163: 173-181.

24. Rajkumar M, Ae N, Prasad MNV, Freitas H. Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. Trends Biotechnol 2010; 28: 142-149.

25. Siddiqui IA, Shaukat SS, Khan GH, Ali NI. Suppression of *Meloidogyne javanica* by *Pseudomonas aeruginosa* IE-6S+ in tomato: the influence of NaCl, oxygen and iron level. Soil Biol Biochem 2003; 35: 1625-1634.

26. Ngoma L, Esau B, Babalola OO. Isolation and characterization of beneficial indigenous endophytic bacteria for plant growth promoting activity in Molelwane Farm, Mafikeng, South Africa. Afric J Biotechnol 2013; 12: 4105-4114.

27. Belimov AA, Hontzeas N, Safronova VI, Demchinskaya S, Piluzza G, Bulitta S, Glick BR. Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* (L.) Czern.). Soil Biol Biochem 2005; 37: 241-250.

28. Haas D, Keel C, Laville J, Maurhofer M, Oberhansli T, Schnider U, Voisard C, Wuthrich B, Defago G. Secondary metabolites of *Pseudomonas fluorescens* strain CHAO involved in the suppression of root diseases. In: Hennecke H, Verma DPS editors. Advances in Molecular Genetics of Plant-Microbe Interactions, Vol. 11. Kluwer Academic Publishers, Dordrecht, 1991; p. 450–456.

29. Niranjan-Raj S, Shetty NP, Shetty HS. Seed-bio-priming with *Pseudomonas fluorescens* isolates enhances growth of pearl millet plants and induce resistance against downy mildew. Int J Pest Manage 2004; 50: 41-48.

30. Wani PA, Khan MS, Said A. Effect of metal tolerant plant growth promoting *Bradyrhizobium* sp. (*Vigna*) on growth, symbiosis, seed yield and metal uptake by green gram plants. Chemosphere 2007; 70: 36-45.