Growth promotion of rice by phosphate solubilizing bioinoculants in a Himalayan location

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Abstract

Three bacterial species, viz. *Bacillus megaterium*, *B. subtilis* and *Pseudomonas corrugata*, originally isolated from temperate locations in the Indian Himalayan region, were examined for their growth promotion ability using both pot and field based assays. A local landrace of rice was used as test crop. The three bacterial species exhibited *in vitro* phosphate solubilizing activity in following order: *P. corrugata* > *B. megaterium* > *B. subtilis*. The bacterial treatments (broth based in pot and charcoal based in field experiments) resulted in improved plant performance. Out of the three treatments, *B. subtilis* gave best performance resulting in 1.66 and 1.55 fold increase in grain yield of rice in pot and field trials, respectively. Inoculations also stimulated the rhizosphere associated bacterial and actinomycetes populations and suppressed the fungal flora. Colonization of roots by mycorrhizal fungi improved in all the treatments. Out of the three bacterial inoculants, *B. subtilis* was the best in affecting these changes. Bacterial treatments also resulted in higher values for phosphorus in shoots and grains in inoculated rice plants. The study indicates that the stimulation of native bacterial flora, including mycorrhizae, in and around roots is one of the important parameters playing indirect role in improving the overall plant growth. The study suggests that charcoal based *B. subtilis* cultures can be developed as an efficient bioinoculant for rice fields in the mountains.

Introduction

Occurrence, importance and use of phosphate solubilizing microorganisms in various ecological niches have been documented (Pandey and Kumar 1989; Chabot et al., 1996; Pandey and Palni, 1998a; Johri et al., 1999; Vazquez et al., 2000). While the mechanism(s) of microbial solubilization of insoluble phosphate has received some attention (Illmer et al., 1995), phosphate solubilization is considered to be an important attribute of plant growth promoting rhizobacteria (PGPRs; Kloepper et al., 1989; Tilak, 1991; Chabot, 1996; Kumar et al., 2001; Peix et al., 2001).

The beneficial effect of PGPR inoculation results from the interaction of three factors, the bacterial strain, the plant cultivar and the ecological niche. In a recent field study on maize conducted at two climatic zones-subtropical and temperate, in Mamlay watershed of Sikkim Himalaya, the bacterial inoculations resulted in statistically significant and improved plant performance at the subtropical zone; the same inoculants did not respond at the temperate site (Pandey et al., 1998). This indicated the value of isolation, identification and screening of native bacteria for selection of PGPR, suitable for use in the mountains.

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A number of landraces of rice are cultivated in the rainfed upland farming systems of Uttaranchal Himalaya (Agnihotri et al., 2000). Use of blue green algae, generally recommended in the rice fields at lowlands, is not feasible in the rainfed mountain areas. In the present study, three strains of bacteria with phosphate solubilizing activity, namely- *Bacillus megaterium*, *B. subtilis* and *Pseudomonas corrugata* have been tested as seed inoculants in rice using pot as well as field based experiments. Observations were recorded in respect of rhizosphere micrflora, mycorrhizal infection, phosphorus content, growth and yield parameters.

Materials and methods

Bacterial inoculants

The bacterial inoculants used were soil isolates, all from temperate locations: *Bacillus megaterium* from the rhizosphere soil of pine forest, *B. subtilis* from the rhizosphere of tea, and *Pseudomonas corrugata* from the maize fields (Pandey et al., 1997; Pandey and Palni, 1998b). These were maintained on Tryptone Yeast extract slants at 4 °C.

Phosphate solubilizing activity of bacterial inoculants

The spot inoculation of bacteria was carried out using petridish assay on Pikovskaya agar (Pikovskaya, 1948) at 28 °C. The halo (zone of solubilization) around the bacterial colony and colony diameter were measured following incubation for 7 days. The halo size was calculated by subtracting the colony diameter from the total (halo) diameter. Quantitative estimation of tricalcium phosphate solubilization was done at 28 °C using 100 mL of NBRIP broth in Erlenmeyer flasks (250 mL) (Nautiyal, 1999) inoculated with 1 mL (10^5 cells mL^{-1}) of bacterial suspension; uninoculated medium served as a control. The pH of the broth was adjusted to 7.0 before autoclaving. An aliquot was withdrawn from the medium aseptically from each flask after 7 days, and centrifuged at 10,000 rpm for 20 min. The supernatant was analyzed for P_2O_5 content by chlorostannous reduced molybdophosphoric acid blue colour method (Allen, 1974); the pH of the supernatant was also measured. The data are an average of three replicates.

Pot experiment

Seeds of rice (*Oryza sativa* L.; landrace: *dudil*) were obtained from a local farmer in village Katarmal, District, Almora. Five to eight seeds were sown per earthen pot (11"dia; 12"ht.), containing approximately 8 kg of soil local. Twenty pots were used for each treatment and control; a minimum of 100 plants were considered for each treatment. *B. megaterium* and *B. subtilis* cultures were grown on TY agar while pseudomonas isolation agar was used for *P. corrugata*. Following 48 h incubation at 28 °C, the bacterial growth was harvested in 100 mL of sterile distilled water obtaining an approximate population 10^5–10^6 cfu mL^{-1}. Inoculation was carried out by adding 1 mL of broth culture to the soil around each seed at the time of sowing. Seeds dipped in broth alone were used as control. The pots were kept in the Institute nursery in the open.

Field experiment

The seeds were inoculated with the bacterial inoculants using sterile charcoal as a carrier. Bacterial suspension was prepared as outlined for the pot experiment. The bacterial suspension was mixed with 200 g of seeds, 150 g of charcoal and 10 g of sticker (*gur*-raw sugar, dissolved in sterile water). The mixture was thoroughly stirred to facilitate even coating of rice seeds. Seeds treated with charcoal slurry without bacteria served as control.

Separate plots were prepared, in triplicate, for each treatment (plot size = 2.50 m × 1.25 m). The plots for different treatments were separated from each other by an adequate gap and a raised mud wall (15 cm above ground) in the middle.

The seeds were sown in the second week of May and the crop harvesting was done in the first week of October 2002, both in the pot as well as field experiments. The soil pH, before seed sowing, was found to range between 7.2 and 7.5, and the soil nutrient analyses revealed following values: C = 0.560%, N = 0.094%, P = 0.100% and K = 0.022% (Allen, 1974). The experiments were conducted in the Institute

nursery at Katarmal (29°38′10″ N to 79°37′30″ E; 1250 m above mean sea level), District-Almora in the state of Uttaranchal.

Microbial analyses

Three groups of microorganisms, viz. bacteria, actinomycetes, and fungi were enumerated to define the rhizosphere colonization, using the serial dilution technique (Johnson and Curl, 1972; soil samples analyzed in triplicate). Nutrient agar for bacteria, Actinomycetes isolation agar for actinomycetes and potato dextrose agar for fungi were used for these enumerations. Jensen's agar (Jensen, 1954), a nitrogen free medium, was also used for the enumeration of a specific group of bacteria. Following incubation at 28 °C for 1 week, the plates were observed for colonies.

Mycorrhizal colonization

Mycorrhizal colonization was determined on the basis of mycorrhizal roots per plant. The fine roots were separated, rinsed several times in tap water, cut into 1.0 cm pieces and treated with 10% KOH for 12 h at room temperature. These pieces were then bleached in alkaline hydrogen peroxide before staining in trypan blue (0.01%) (Phillips and Hayman, 1970). Microscopic observations were carried out to quantify % infection (colonization). Number of positive root pieces $\times$ 100/total number of root pieces observed gave the value for % mycorrhizal infection.

Plant growth, yield, harvest index and phosphorus analyses

Ten plants for each treatment were randomly uprooted from different pots as well as plots. Measurements were recorded for root and shoot length as well as biomass after 150 days of seed sowing. The grain yield was recorded at the time of harvesting. Harvest index was calculated using the following formula (Hall et al., 1993): Harvest index = Economic yield $\times$ 100/Biological yield. The pH of the soil after the crop harvest was also estimated. The phosphorus content of different plant parts were analyzed using the oven-dried (80 °C, for 48 h) and powdered (2 mm) samples. Triplicate samples (0.1 g) were digested on a hot plate and analyzed for phosphorous by the molybdophosphoric blue colour method (Allen, 1974).

Data were statistically analyzed as per Snedecor and Cochran (1967).

Results

Phosphate solubilizing activity of bacterial inoculants

All the bacterial isolates exhibited phosphate solubilizing activity and formed clear halo around the bacterial colony on Pikovskaya agar plates. Out of the three bacteria, *Pseudomonas corrugata* exhibited strongest activity, followed by *B. megaterium* and *B. subtilis*. These results were confirmed by quantitative measurements carried out with broth cultures. The bacterial inoculations also resulted in the lowering of pH of the broth indicating acid production with time (Table 1).

Pot experiment

Increase in yield and growth parameters was recorded for treated plants (Table 2). The biomass of different plant components was influenced positively, but differentially by bacterial inoculations. Out of the three bacterial species, *B. subtilis* performed best and resulted in 1.37 fold increase in the total biomass over control.

Table 1. Phosphate solubilization in Pikovskaya and broth cultures, and corresponding lowering of pH following incubation at 28 °C after 7 days

Bacterial inoculants	Halo size (mm) on Pikovskaya agar*	P solubilized in NBRIP broth**(μg/mL)	pH
Bacillus megaterium	2.3	8.0	5.08
Bacillus subtilis	1.7	5.5	5.37
Pseudomonas corrugata	9.7	11.0	4.57

* = Pikovskaya, 1948; **NBRIP (National Botanical Research Institute's phosphate growth medium = Nautiyal 1999.

Table 2. Influence of bacterial inoculation on morphological and yield attributes in rice using pot based assays

Bacterial inoculants	Length (cm)		Biomass production and yield (g dry weight)				Harvest Index	
	Root	Shoot	Root	Straw	Grain	Total		
Control	13.63 ± 2.49	122.53 ± 9.63	4.76 ± 1.07	15.40 ± 4.44	8.46 ± 3.38	28.63 ± 07.44	29.56	
B. megaterium	17.10 ± 3.16	133.20 ± 18.32	5.14 ± 1.30	16.66 ± 4.86	11.30 ± 4.75	33.11 ± 08.61	34.14	
B. subtilis	18.90 ± 4.72	137.40 ± 12.47	6.49 ± 2.42	18.73 ± 5.57	14.07 ± 6.91	39.30 ± 13.26	35.80	
P. corrugata	18.17 ± 5.23	134.21 ± 21.79	5.27 ± 2.61	16.96 ± 7.44	12.22 ± 6.51	34.43 ± 15.66	35.41	
ANOVA table								
Parameters	Length of root				Length of shoot			
Source of variation	df	MS	*F*	*P*-value	df	MS	*F*	*P*-value
Between treatments	3	58.96815	3.2414987	0.034787*	3	406.7321	1.394266	0.262422**
Within treatments	32	18.18889			32	291.7177		
Parameters	Grain yield				Total biomass			
Source of variation	df	MS	*F*	*P*-value	df	MS	*F*	*P*-value
Between treatments	3	59.66398	1.833685	0.160895**	3	230.7732	1.56824	0.216217**
Within treatments	32	32.53774			32	147.1542		

Values are a mean ± SD of ten individual plants.
*Significant at $P < 0.05\%$, ** Not significant.

The increase affected by *P. corrugata* and *B. megaterium* was 1.20 and 1.16 fold, respectively. The proportionate increase in grain yield was maximum due to bacterial inoculation in all the three treatments. The enhancement in grain yield obtained with *B. subtilis*, *P. corrugata* and *B. megaterium* was 1.66, 1.44 and 1.34 fold over control, respectively. The harvest index on per plant basis also recorded, increased irrespective of the treatments. The root length was 13.63 cm in control, 18.90 cm in *B. subtilis*, 18.17 cm in *P. corrugata* and 17.10 cm in *B. megaterium* treatments. The shoot height was also found to increase in the same order.

Field experiment

The data showing the influence of bacterial inoculations on growth and yield of rice are presented in Table 3. The treatments resulted in improvement in biomass, in terms of root, straw and grain weight both on per plant and unit area (m^2) bases. In this experiment also, *B. subtilis* gave the best performance, with an increase of 1.40 and 1.55 fold for total biomass and grain yield, respectively, on per plant basis. For *P. corrugata* treatment the increase was 1.26 and 1.36 fold and for *B. megaterium* it was 1.17 and 1.25 fold, respectively. The harvest index per unit area also recorded an increase in all the treatments as compared to control. There was a positive increase in root length; it was 12.34, 15.60, 16.80 and 15.83 cm in control, *B. megaterium*, *B. subtilis* and *P. corrugata* treatments, respectively. The shoot height was also positively influenced by bacterial treatments in the order: *B. subtilis* > *P. corrugata* > *B. megaterium* > control.

Microbial analyses

Changes in the microbial community in the rice rhizosphere due to bacterial inoculations, under field conditions, are presented in Figure 1. The populations of bacteria (in general and those grown on Jensen's medium) and actinomycetes were found to be stimulated due to inoculations. The maximum stimulation was found in case of *B. subtilis* treatment, where the counts increased by 1.5–2.4 fold for bacteria, 1.5–2.9 fold for actinomycetes and 1.7–3.5 fold for the bacteria on Jensen's medium. In case of *B. megaterium* treatment the counts were found to increase between 1.3–2.1, 1.6–2.4 and 1.7–3.3 fold, and in *P. corrugata* treatment the increase was 1.3–2.1, 1.7–2.2 and 1.1–2.2 fold for bacteria, actinomycetes and for bacteria on Jensen's medium, respectively. The counts were higher in all the three treatments as compared to control, during the entire period of plant growth. The microbial populations were highest during the middle of the growth period, after which a decline was recorded. Contrary to this, the fungal population in the rhizosphere was not stimulated and the counts remained lower than the counts recorded on zero day of sampling. Also during the entire period the plant growth, the fungal counts in all the bacterial treatments remained lower as compared to control. Results were similar for the rhizosphere soil samples collected from the pot experiment (data not presented).

Mycorrhizal colonization

The per cent roots colonized by mycorrhizae increased up to 90 days following seed sowing, after which the per cent colonization remained more or less constant (Figure 2). The maximum root colonization was found in case of *B. subtilis* treatment (88.4%), followed by *B. megaterium* (80.6%), *P. corrugata* (78.4%), and untreated control (76.4%), 90 days after sowing.

Phosphorus content of plant parts

The bacterial inoculation positively influenced the phosphorus (P) content of various plant components; *P. corrugata* treatment was most effective in this respect. The treatments were found to enhance enhance the P content of shoots and grains. The P content of roots was not enhanced (Table 4).

Discussion

The observations recorded on growth and yield related parameters demonstrate the beneficial effects of bacterial inoculation on rice; the best response was obtained with *B. subtilis* treatment. The other effects recorded were in terms of

Table 3. Influence of bacterial inoculation on morphological and yield attributes in rice following a field experiment

Bacterial inoculants	Length (cm)		Biomass production and yield (g dry weight)							
			Per plant basis				Per unit area (m^2) basis			
	Root	Shoot	Root	Straw	Grain yield	Total	Crop residue	Grain yield	Total	Harvest index
Control	12.34 ± 2.66	119.50 ± 11.24	2.85 ± 1.06	12.50 ± 3.40	6.85 ± 3.18	22.21	460.98	205.89	666.87	30.87
B. megaterium	15.60 ± 3.03	127.70 ± 17.54	3.58 ± 1.32	13.74 ± 4.33	8.60 ± 4.23	25.92	520.00	258.10	778.10	33.17
B. subtilis	16.80 ± 4.37	131.17 ± 11.05	4.72 ± 2.17	15.59 ± 5.14	10.76 ± 6.24	31.07	609.43	323.04	932.47	34.64
P. corrugata	15.83 ± 4.51	129.40 ± 21.26	3.53 ± 1.71	15.02 ± 7.47	9.36 ± 7.35	27.91	560.96	281.05	842.01	33.37
ANOVA table										
Parameters	Length of root				Length of shoot					
Source of variation	df	MS	*F*	*P*-value	df	MS	*F*	*P*-value		
Between treatments	3	33.91213	2.304433	0.095595**	3	190.5463	0.79243	0.507124**		
Within treatments	32	14.71604			32	240.4583				
Parameters	Grain Yield				Total biomass					
Source of variation	df	MS	*F*	*P*-value	df	MS	*F*	*P*-value		
Between treatments	3	15002.85	0.604915	0.616622**	3	65861.9	0.653472	0.586658**		
Within treatments	32	24801.57			32	100787.7				

Values are a mean ± SD of ten replicates.
**Not significant.

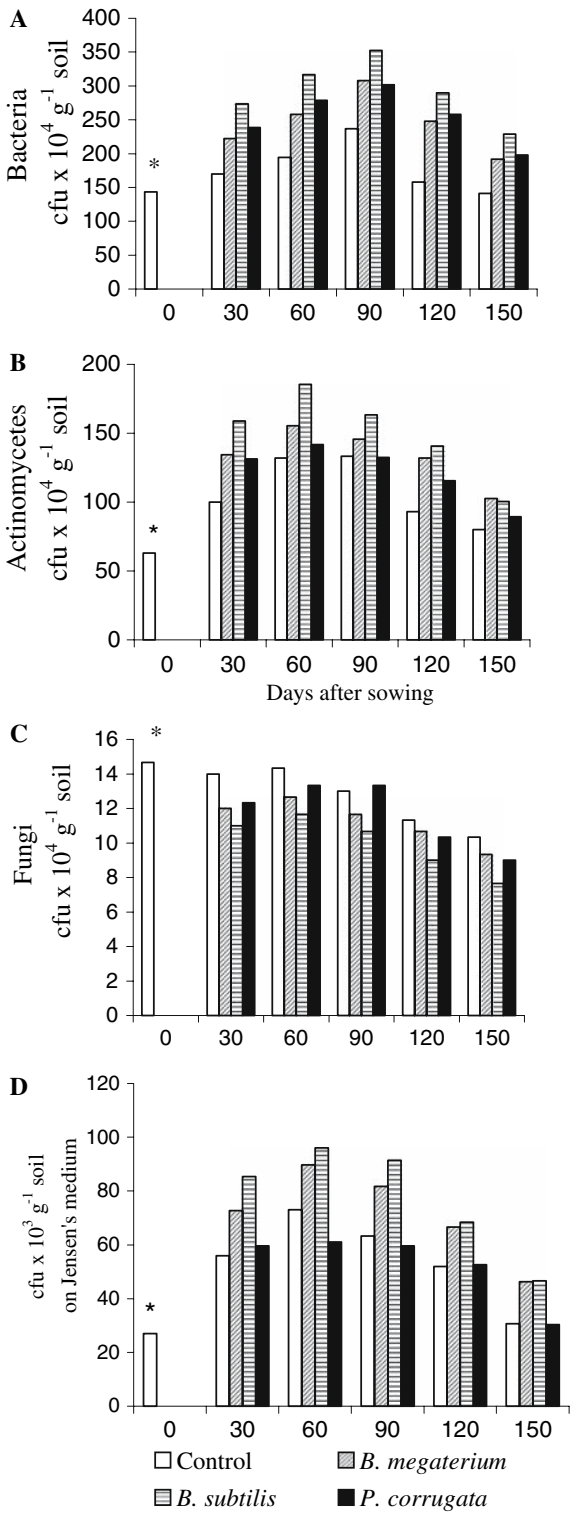

Figure 1. Influence of bacterial inoculation on the microbial communities in the rhizosphere of rice. The LSD values for various microbial communities are: 9.01, 7.78, 10.61, 5.43, 6.21 for bacteria; 8.23, 8.32 7.07 8.41, 7.31 for actinomycetes; 1.24, 2.03, 1.52, 1.70, 1.31 for fungi; and 6.37, 5.74, 4.64, 5.02, 5.42 for the cfu(s) recorded on N-free medium at 30, 60, 90, 120 and 150days, respectively after seed sowing. Cfu = colony forming units. * Bars indicate counts at the time of sowing.

stimulation of rhizosphere associated native bacterial and actinomycetes populations, increase in mycorrhizal colonization of roots and suppression of fungal population in the rhizosphere. Improved phosphorus content of plants was also related to the bacterial inoculations. A number of physiological properties like N-fixation, P-solubilization, production of antifungal and plant growth promoting substances are given importance while selecting effective strains of bioinoculants. Besides these, original habitat of the isolates and their ability to positively influence the native microflora are other parameters of importance. In previous pot as well as field based studies, the beneficial effects of bacterial inoculations have been correlated with the stimulation of native microbial communities in the rhizosphere (Pandey et al., 1998; Pandey et al., 1999). Similar observations have been recorded in this study. Besides the stimulation of general bacterial and actinomycetes flora, root colonization of ectomycorrhizal fungi was also found to be stimulated in all the treatments. The role of ectomycorhizal fungi in improving the phosphorus nutrition of plants is well documented (Lapeyrie

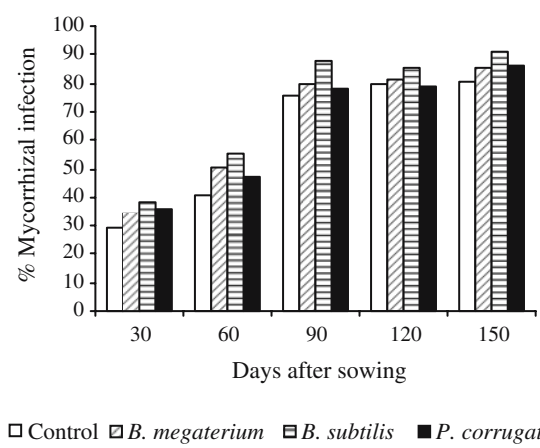

Figure 2. % Mycorrhizal colonization in the roots of rice. The LSD values are 4.88, 3.40, 2.06, 1.81, 3.14 at 30, 60, 90, 120 and 150 days respectively after sowing.

Table 4. Phosphorus content (%) of different parts of rice plant following bacterial inoculation

Bacterial inoculants	Root	Shoot	Grain
Control	0.0221	0.039	0.0307
Bacillus megaterium	0.0207	0.041	0.032
B. subtilis	0.0193	0.050	0.0343
P. corrugata	0.0221	0.059	0.0357

et al., 1991). This group of fungi is well known for a number of other properties associated with plant growth promotion, e.g., improved water status and nutrient uptake, and the protection of root system against phytopathogens (Marschner and Dell, 1994). While the inoculants used in this study possessed phosphate solubilizing property, the overall positive influence obtained may have resulted from a combined effect exerted by the stimulated microbial communities, including mycorrhizae. *B. subtilis*, the weakest phosphate solubilizer among the three bioinoculants, was most effective in stimulating the general microflora, mycorrhizal colonization and the suppression of fungal flora in the rhizosphere. In fact, improvement in the mycorrhizal colonization would seem to be an important attribute of the use of native strains in inoculation trials. The pH of the soil (7.2–7.5) recorded at the time of sowing was found to decline (up to 6.8–6.9) in various treatments after harvest. The decline in the soil pH may be an outcome of the microbial activity in the rhizosphere. Suppression of the general fungal flora in the rhizosphere of treated plants is indicative of antifungal property of the inoculants. (Pandey et al., 1997; Pandey and Palni, 1998b).

Results of the present study represent a step forward of a systematic programme initiated for the isolation of native bacteria, screening for plant growth promoting rhizobacteria, and subsequent selection of suitable inoculants for use in the colder regions of mountains. The programme began with the isolation of a large number of bacteria from the soil samples collected from various temperate/alpine (up to 3600 m above mean sea level) locations. The initial *in vitro* experiments revealed the dominance of species of *Bacillus* and *Pseudomonas* in these soils (Pandey and Palni, 1998a, b). The isolates were screened for various beneficial properties, e.g., ability to solubilize tricalcium phosphate, production of antifungal compounds, intrinsic antibiotic resistance, nitrogen fixing ability, etc., with emphasis on their ability to tolerate lower temperatures (Pandey et al., 1997; Pandey and Palni, 1998a, b; Pandey et al., 2002). Based on the results of above cited studies, the efficient bacterial isolates were tested as inoculants using bioassays and pot assays (Pandey et al., 1999, 2000, 2001). The programme has now progressed to the stage of testing the potential inoculants in field trials using local hill crops. The growth promotion of rice observed in this investigation seems to result from a combined effect of various mechanisms, involved directly or indirectly. Regardless of the mechanism(s) involved, the study suggests the suitability of native bacterial species to be developed as carrier based bioinoculants for use in the colder regions of mountains.

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