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Low Temperature Phosphate Solubilization and Plant Growth Promotion by Psychrotrophic Bacteria, Isolated from Indian Himalayan Region

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Abstract: Four hundred and fifty bacterial isolates from a culture collection of high altitude bacteria isolated from different regions of Indian Himalaya were screened for their low temperature phosphate solubilization activity at 4 and 10°C using plate based assay on Pikovskaya (PVK) agar. Five isolates (identified as psychrotrophic *Pseudomonas* species) showing solubilization efficiency >350 were selected for *in vitro* quantification studies. In PVK broth the isolates effectively solubilized phosphate at both the incubation temperature. The isolates also exhibited other plant growth promotion abilities such as production of siderophores, indole acetic acid and antagonized two test fungus viz. *Alternaria alternata* and *Fusarium oxysporum*. Under growth chamber pot assay at 10-15°C the isolates exhibited increased plant growth and mineral nutrition of wheat indicating their functionality at lower temperatures. Addition of insoluble phosphate to the inoculated soil significantly increased the growth parameters in all the cases. The present study is important with respect to enumerating microbial diversity of the colder regions as well as understanding the potential biotechnological applications of native microbes.

Key words: Plant growth promoting rhizobacteria (PGPR), low temperature phosphate solubilization, mineral nutrition

INTRODUCTION

Phosphorus (P) is an essential element for plant development and growth making up about 0.2% of plant dry weight (Schachtman *et al*., 1998). Plants acquire P from soil solution as phosphate anions. However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, depending on the particular properties of soil (Lindsay *et al*., 1962). In these forms, P is highly insoluble and unavailable to plants (Rodriguez and Fraga, 1999). Thus the release of insoluble and fixed forms is an important aspect of increasing soil P activity.

A substantial number of bacterial species, mostly associated with plant rhizosphere, may exert a beneficial effect upon plants (Glick, 1995). This group has been termed as Plant Growth Promoting Rhizobacteria (PGPR) and among them Phosphate Solubilizing Microbes (PSM) have the ability to increase the availability of P to the plants, not only by mineralizing organic P compounds but also rendering inorganic phosphates more available to them (Illmer *et al*., 1995; Richardson, 2001). PSM have been shown to improve solubilization of fixed soil P and applied phosphates resulting in higher crop yields (Goldstein, 1986; Kucey *et al*., 1989). Although PSM have been isolated from various environmental niches including highly stressed conditions and extreme environmental factors

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(Goldstein *et al*., 1999; Nautiyal *et al*., 2000; Vazquez *et al*., 2000) there are only a few reports on characterization of PSM(s) for their activity at lower temperatures (Pandey *et al*. 2002; 2006; Das *et al*., 2003).

For many years our laboratory has been conducting research on isolation and characterization of bacteria from Himalayan soils with particular reference to their low temperature adaptability and plant growth promotion abilities (Pandey *et al*., 2004; Trivedi *et al*., 2005). A culture collection of 'high altitude bacteria' isolated from different regions of Himalaya has been developed. In the present investigation efficiency of some isolates to solubilize phosphate at lower temperature (4 and 10°C) has been described. The effect of inoculation on growth and mineral nutrition (N, P and K content) of wheat plants growing at 10-15°C was also determined using pot assay in growth chamber.

MATERIALS AND METHODS

Screening for Low Temperature Phosphate-solubilizing Bacterial Isolates

Four hundred and fifty bacterial isolates from the culture collection were screened for their phosphate-solubilizing ability on Pikovskaya (PVK) agar (Pikovskaya, 1948). The halo and colony diameter were measured after 10 days of incubation at 4 and 10°C. The results are expressed as Solubilization Efficiency (SE) according to Nguyen *et al*. (1992). Five bacterial isolates which showed maximum phosphate solubilization at lower temperatures were chosen for further studies. These were further characterized for their morphological, cultural and biochemical properties using standard methods (Cappuccino and Sherman, 1996).

Quantitative Estimation of Phosphate-solubilization

Experiments were carried out in Erlenmeyer flasks (250 mL) each containing 50 mL of PVK broth, pH = 7.0, before autoclaving. Flasks were inoculated with either 1 mL of bacterial suspension grown in nutrient broth containing 10^9 colony forming units (cfu) mL^{-1}. Autoclaved, uninoculated flasks were used as controls. The flasks were incubated at 4 and 10°C as still-surface culture. Cultures were harvested by centrifugation at 7000 x g for 10 min, at 3 day interval till 30 days after incubation and the phosphate released in culture supernatant was estimated by the chlorostannus reduced molybdo-phosphoric acid blue method (Allen, 1974) and was expressed as μg mL^{-1}. pH of the medium was recorded at the same time.

Other *in vitro* Plant Growth Promotion Abilities

Detection of siderophore was estimated qualitatively on Chrome-azurol S (CAS) medium, a universal medium for siderophore production (Schwyn and Neilands, 1987). Production of hydrocyanic acid (HCN) and indole acetic acid (IAA) was determined by standard procedures as described by Bakker and Schippers. (1987) and Gupta *et al*. (2002), respectively. Antagonistic properties of the bacterial isolates were tested against *Alternaria alternata* and *Fusarium oxysporum* on Potato Carrot Agar (PCA) using a dual culture technique (Chaurasia *et al*., 2005).

Bioassay for Evaluation of Growth Promotion and Mineral Nutrition in Wheat Plants

The bioassay was conducted using wheat (*Triticum aestivum*) as test plant species. Surface-sterilized plastic pots (500 mL) were filled with steam sterilized local soil (C = 0.19%; N = 0.042%; P = 0.0908%; pH = 7.2) collected from Institute's nursery (29°38'10"N to 79°37'30"E; 1250 m above mean sea level). Pots, each containing 500 g of soil, were arranged in groups of 5 replications for each treatment. The experiment was carried out in 5 different treatments; treatment 1: uninoculated soil (control 1); treatment 2: uninoculated soil with the addition of poorly soluble phosphate (Ca_3PO_4 0.2%; control 2); treatment 3: uninoculated soil with the addition of soluble

phosphate (K_2HPO_4 0.1% and KH_2PO_4 0.1%; control 3); treatment 4: soil inoculation with either of the bacterial isolates; treatment 5: soil inoculated with either of the bacterial isolates with addition of insoluble phosphate. Soluble and poorly soluble phosphate was mixed thoroughly with soil in a plastic bag before use. Six surface sterilized (in 1% NaOCl for 3 min, then washed with H_2O) pregerminated (48 h) seeds were transplanted in each pot at 2 cm depth. The pots were placed in a plant growth chamber with mixed incandescent and fluorescent lighting (400 microeinsteins m^{-2} s^{-1}; 400-700 nm), programmed for a 16 h photoperiod, day-night cycle, with a constant temperature varying from 10-15°C (night-day) and 50-60% relative humidity. On the second week after sowing, plants were thinned down to 3 per pot and were irrigated daily as needed.

For inoculation, bacterial isolates were grown in Petri-dishes with nutrient agar for 5 days. After that, sterile water was added to the plates in order to obtain a suspension with approximately 10^{11} cfu mL^{-1}. For inoculation 1 mL of the bacterial suspension was added to each seed at the time of sowing.

At harvest (30 days), the length and dry weight of the shoot and root were determined. Plant N, P and K content was measured according to methods of the Association of Official Analytical Chemists (1990). Data were statistically analyzed using standard methods (Snedecor and Cochran, 1967).

RESULTS AND DISCUSSION

Plate based assays for screening of PSM showing activity at lower temperatures reveled that out of 450 bacteria isolates from the culture collection 69 solubilized phosphate at 4°C and 146 at 10°C. Solubilization efficiency varied from 25.8 to 375.4 and 31.7 to 456.5 at 4 and 10°C, respectively. Five isolates having solubilization efficiency of >350 were selected for further studies. The selected isolates were psychrotrophs and from preliminary characterization appear to belong to genus *Pseudomonas*. Various species of *Pseudomonas* have been isolated from higher altitudes and sites experiencing low temperatures (Lifshitz *et al*., 1986; Negi *et al*., 2005; Pandey *et al*., 2006). Several of the species of *Pseudomonas* fall in the category of promising growth-promoting rhizobacteria and a number of them have been studied for their phosphate solubilization activity (Babu-Khan *et al*., 1995; Peix *et al*., 2004; Pandey *et al*., 2006).

Quantitative estimation of phosphate solubilization estimated after incubation from 3 to 30 days (at 3 day intervals) at 4 and 10°C is presented in Fig. 1A and B. All the isolates efficiently release the bound P from Ca in the PVK broth at both the incubation temperatures. Maximum solubilization at 4°C for isolate PSS2 (122 μg mL^{-1}) and PSS5 (165 μg mL^{-1}) was observed after 15 days and for PSS1 (147 μg mL^{-1}), PSS3 (156 μg mL^{-1}) and PSS4 (106 μg mL^{-1}) after 18 days of incubation. At 10°C maximum solubilization for PSS1 (197 μg mL^{-1}), PSS4 (123 μg mL^{-1}) and PSS2 (169 μg mL^{-1}), PSS3 (289 μg mL^{-1}), PSS5 (310 μg mL^{-1}) was observed after incubation period of 15 and 21 days, respectively. Only a few studies have reported the phosphate solubilizing activity of PSM at lower temperature. The low temperature phosphate solubilization potential of the strains used in this study was greater then *P. corrugata* (Pandey *et al*., 2002), *P. putida* (Pandey *et al*., 2006) and cold tolerant mutants of *P. fluorescence* (Das *et al*., 2003).

In the study, a decrease in the pH values of the inoculated PVK broth was observed (Fig. 2A and B). The lowering of pH coincided with the increase in phosphate solubilization activity of the bacterial isolates. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by the PSM(s) (Kim *et al*., 1998; Vazquez *et al*., 2000). Production of organic acids results in acidification of the microbial cell and its surroundings; consequently releasing P from the mineral phosphate by proton substitution for Ca^{2+}.

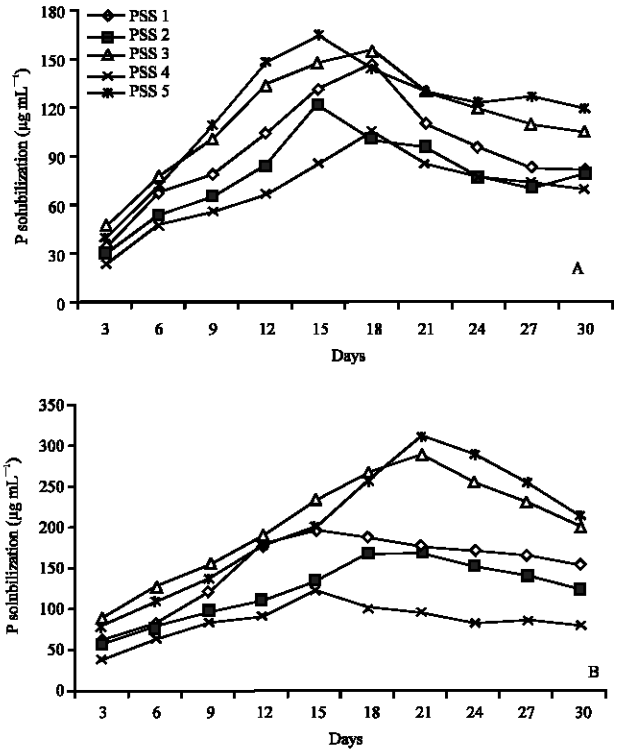

Fig. 1: Phosphate solubilization at 4°C (A) and 10°C (B) by bacterial isolates following incubation for 30 days in PVK broth

Table 1: *In vitro* plant growth promotion abilities of the bacterial isolates

	CAS blue agar				Antagonism against[c]	
Strain	Growth[a]	Halo formation[b]	HCN production	IAA production	*Fusarium oxysporum*	*Alternaria alternata*
PSS1	++	++	-	+	+++	+
PSS2	++	+++	-	+	+	++
PSS3	++	+++	-	+	+++	+++
PSS4	+	+	-	+	++	+
PSS5	++	+++	-	+	+++	+++

CAS = Chrom-blue agar; HCN = Hydrocyanic acid; IAA = Indole Acetic Acid, [a] + minimal growth, ++ normal growth [b] + small halos < 0.5 cm, ++ medium halos > 0.5 cm, +++ large halos > 1.0 cm, [c]% inhibition + < 25%, ++ > 25%, +++ > 75%

All the bacterial isolates formed orange zone around the colony on CAS agar, indicative of siderophore production (Table 1). None of the isolate produced HCN. All the isolates produced IAA in tryptone-free medium as evidenced by the production of a pink-coloured product and inhibited the growth of both the test fungus viz. *A. alternata* and *F. oxysporum* due to the production of diffusible antifungal compounds in dual culture assay. In addition to phosphate solubilization, other mechanisms based on the production of siderophores, HCN, IAA and antibiotics have been implicated in the plant growth promotion by PGPR(s) (O'Sullivan and O'Gara, 1992).

According to these results, a significant increase of most of the growth parameters measured in terms of length and dry weight of root and shoot was observed when the soil was inoculated with the bacterial strains compared to the soil without inoculum (Table 2). Addition of insoluble

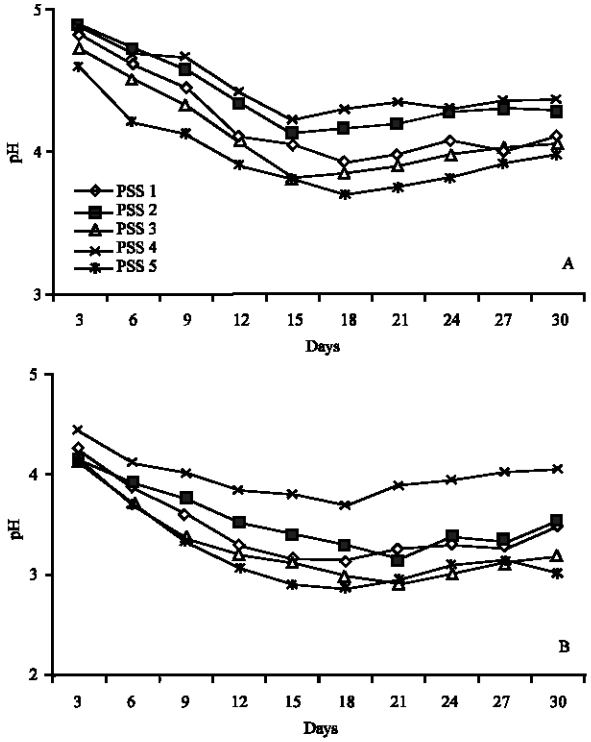

Fig. 2: Change in pH of culture broth at 4°C (A) and 10°C (B) by bacterial isolates following incubation for 30 days

Table 2: Effect of inoculation with bacterial isolates on growth parameters and nutrient content of wheat

	Length (cm)		Dry weight (mg/plant)				
Treatment	Root	Shoot	Root	Shoot	Total N (mg/plant)	Total P (mg/plant)	Total K (mg/plant)
Control1	08.49	38.62	09.33	60.0	1.12	2.48	09.42
Control2	08.98	39.24	10.43	62.12	0.99	2.53	09.97
Control3	11.67	45.20	14.95	64.55	1.10	4.98	11.72
PSS1	12.96	47.39	16.64	78.00	1.56	2.82	12.02
PSS2	12.54	49.43	15.59	82.36	1.64	2.81	13.35
PSS3	12.18	47.66	15.27	81.92	1.61	3.72	13.44
PSS4	11.21	46.65	15.44	73.74	1.53	2.66	12.74
PSS5	12.27	50.19	15.77	82.84	1.54	2.78	12.02
PSS1+ $Ca_3(PO_4)_2$	13.91	56.01	18.30	80.75	1.68	3.85	13.21
PSS2 + $Ca_3(PO_4)_2$	13.89	54.57	18.00	84.48	1.70	3.92	13.88
PSS3 + $Ca_3(PO_4)_2$	14.12	57.78	20.92	89.67	1.77	4.34	13.78
PSS4 + $Ca_3(PO_4)_2$	13.42	55.56	18.42	88.70	1.74	4.36	13.43
PSS5 + $Ca_3(PO_4)_2$	13.75	59.70	23.03	91.25	1.69	4.03	14.00
CD at 0.05%	1.44	4.91	2.77	5.86	0.21	1.15	1.90

phosphate to the inoculated soil significantly increased the growth parameters in all the cases. Although the P content was higher in soils with soluble phosphate, the P content of plants growing in soil inoculated with the bacterial isolates (with or without insoluble phosphate) increased significantly as compared to uninoculated soil (with no amendment or addition of insoluble phosphate). Inoculation also resulted in higher N and K content. The use of PSM in augmenting the overall plant growth and mineral nutrition has been reported by various workers (Jisha and Alagawadi, 1996;

Chabot *et al*., 1996; Peix *et al*., 2001). No correlation was found between the phosphate solubilization *in vitro* and growth promotion effect in plant based assays. It is postulated that inoculation of soil with PSM can increase crop yield by other mechanism, such as the growth factor production (Tinker, 1984).

CONCLUSIONS

The results suggest that the bacteria isolated from the Himalayan soils have been able to evolve with the ability to solubilize phosphate at lower temperatures. The selected bacterial isolates showing low temperature adaptation and possessing various plant growth promotion abilities are suitable for the development of carrier based, easy to use inoculants for improved plant performance in colder regions. The isolates may also serve as suitable model to study the physiological, biochemical and molecular mechanism(s) of phosphate solubilization under stressed conditions. Further studies are required to investigate their beneficial activities in actual field conditions.

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