Annals of Microbiology, **58 (4)** 561-568 (2008)

Characterisation of a psychrotolerant plant growth promoting *Pseudomonas* sp. strain PGERs17 (MTCC 9000) isolated from North Western Indian Himalayas

Pankaj K. MISHRA[1]*, Smita MISHRA[1], Govindan SELVAKUMAR[1], Shekhar C. BISHT[1], Jaideep K. BISHT[1], Samaresh KUNDU[2], Hari Shankar GUPTA[1]

[1]Vivekananda Institute of Hill Agriculture, (Indian Council of Agricultural Research), Almora-263601, Uttarakhand; [2]Indian Institute of Soil Science, (Indian Council of Agricultural Research), Bhopal, M.P., India

Received 28 March 2008 / Accepted 15 September 2008

Abstract – A psychrotolerant, Gram negative, rod shaped, plant growth promoting bacterium (PGPB) was isolated from high altitude of North Western Indian Himalayas. The identity of the bacterium was confirmed by morphological, biochemical and sequencing of the 16S rRNA gene. The sequence analysis revealed maximum similarity with *Pseudomonas vancouverensis*. It exhibited tolerance to a wide pH range (5-12; optimum 7.0) and salt concentrations up to 5% (w/v). The isolate produced 8.33 and 1.38 µg/ml of IAA at 15 °C and 4 °C respectively, on the third day after incubation. It solubilised 42.3, 66.3 and 74.1 µg/ml of tricalcium phosphate at 4, 15 and 28 °C respectively after seven days of incubation. The strain also possessed HCN and siderophore production abilities at 4 °C. It exhibited inhibitory activity against several phytopathogenic fungi in three different bioassays. The maximum relative growth inhibition was recorded against *Sclerotium rolfsii* and *Rhizoctonia solani* (100%), followed by *Pythium* sp. (73.1%) and *Fusarium oxysporum* (19.7%), in volatile compound assays. Seed bacterization with the isolate enhanced the germination of wheat seedlings grown at 18 ± 1 °C by 20.3%. Bacterized seeds also recorded 30.2 and 27.5% higher root and shoot length respectively, compared to uninoculated controls.

Key words: antagonistic activity, cold tolerant, plant growth promotion, *Pseudomonas* sp. strain PGERs17.

INTRODUCTION

Soil is a dynamic, living matrix and is a critical resource not only for agriculture production and food security but also towards maintenance of most life processes. Soil is considered a store house of microbial activity, though the space occupied by living microorganisms is estimated to be less than 5% of the total space. Therefore, major microbial activity is confined to the "hot-spot" i.e. aggregates with accumulated organic matter, rhizosphere (Tilak *et al.*, 2005). The rhizosphere, volume of soils surrounding roots are influenced chemically, physically and biologically by the plant root. This location is a highly favourable habitat for the proliferation of microorganisms and exerts a potential impact on plant health and soil fertility. Root exudates rich in amino acids, monosaccharides and organic acids, serve as the primary source of nutrients, and supports the dynamic growth and activities of various microorganisms with in the vicinity of the roots. The root colonising microorganisms could be free living, parasitic or saprophytic. An important group of these microbial communities that exerts beneficial effects on plant growth upon root colonisation were first defined by Kloepper and Schroth (1978) and termed them as plant growth promoting rhizobacteria (PGPR) (Podile and Kishore, 2006).

* Corresponding author. Phone: +91 9412589393;
Fax: +91 5962 231539; E mail: misrapank1970@yahoo.com; misrapank12@gmail.com

One of the major mechanisms of plant growth promotion is through the production of the stimulatory phytohormones, produced by PGPR within the root zone; these hormones stimulate the density and length of root hairs. The increase in root surface area improves the plant uptake potential of water and mineral nutrients from a large volume of soil. It has been observed that the plant growth promotory ability of most PGPR strains is due to the influence of phytohormones. The auxin, indole-3-acetic acid (IAA) is an important phytohormone produced by PGPR, and treatment with auxin-producing rhizobacteria has been shown to increase the plant growth (Patten and Glick, 2002). The IAA-producing capability of microorganisms is useful in their identification and provides a valuable marker when examining the physiological roles or ecological significance of IAA in the establishment and persistence of the organisms (Bric *et al.,* 1991). Apart from phytohormone production plant growth promotion is known to be mediated by a variety of mechanisms including phosphate solubilisation (Valverde *et al*., 2006), siderophore production (Katiyar and Goel, 2004), antagonism towards deleterious root microorganisms (Misaghi *et al*., 1982), deamination of the precursor molecule of the phytohormone ethylene whose accumulation in root tissue is known to be detrimental to root growth and development (Glick *et al.,* 1998), and induction of systemic resistance to plant pathogenic microorganisms.

Extreme environmental conditions are quite common on earth and the microbial diversity in such areas is of particular interest because microbes inhabiting these places are well adapted to the prevailing atmospheric and edaphic conditions. Despite this fact,

comparatively less attention has been paid to bacteria growing at low temperatures, perhaps because of the slow growth rate and the difficulty in handling these bacteria. Cold-adapted microorganisms, which include psychrophiles and psychrotrophs, are present virtually in all these areas and many of them also poses plant growth promoting traits (Premono *et al.,* 1996; Morita, 1975). One of the objectives of studying the microbial communities from the colder regions has been to select suitable bioinoculants for use in the mountains (Pandey *et al.,* 2006). Changes in the environmental conditions away from the optimum can cause the induction of many stress responses and low temperature is a major factor limiting the productivity and geographical distribution of many species, including important agricultural crops in the hilly regions of N.W. Indian Himalayas. Despite the ability of plants to adapt partially to low-temperature stress in temperate climates, plant growth and overall productivity generally decline under chilling conditions. In alpine regions of the N.W. Himalayas, winter is characterised by intermittent snow cover (November to March) and fluctuating subfreezing temperatures while summer has intense, desiccating sunshine punctuated by infrequent rains (Greenland and Losleben, 2001). Such climatic conditions warrant the use of highly adapted microbial strains that retain their biological functions, in order to harvest the beneficial effects of biofertilisation.

Pseudomonas, an enormously diverse genus of the gamma *Proteobacteria*, is an important member of soil microbial communities. Members of the genus have been isolated from essentially all environments studied (Palleroni, 1992), including alpine soils, where it was identified as the most prevalent cultivable genus. The genus exhibits remarkable metabolic variation (Palleroni and Doudoroff, 1972). The great metabolic flexibility of *Pseudomonas* species allows them to inhabit variable environments. One survival strategy might be the evolution of strains that are capable of utilising a large number of different carbon sources for growth. This is more relevant since the alpine environment is highly heterogeneous with pockets of specific carbon compounds, a large number of different strains that have recently gained or lost the ability to grow on particular sources of carbon may exist (Behrendt *et al.,* 2007). The utility of cold tolerant plant growth promoting strains of *Pseudomonas* has been reported earlier by Pandey *et al*., (2006), but considering the huge biodiversity of the N.W. Himalayan region, it can be safely assumed that there are many more useful bacterial strains waiting to be discovered. The present investigation deals with the isolation, characterisation, phylogenetic analysis and determination of the growth promotion and antagonistic potential of a cold tolerant, endophytic *Pseudomonas* sp. strain PGERs17 (MTCC9000), isolated from roots of garlic (*Allium sativum*).

MATERIALS AND METHODS

Isolation of cold tolerant bacterial strain from Garlic. The garlic root sample was collected from a sub alpine site in the North Western Himalayas (Heupani, Baghora, district - Pithoragarh, Uttarakhand; altitude 1900 m above mean sea level, soil temperature of the location varies from -4 to 10 °C during winters), the surrounding vegetation was dominated by pine (*Pinus* sp). Bacteria were isolated from the rhizosphere, rhizoplane and the internal tissue of the root. For isolation of rhizospheric bacteria, the soil adhering to the root was gently shaken, serially diluted and plated on Nutrient agar. Rhizoplane bacteria were isolated by thoroughly washing the roots with sterile distilled water for 2 min to remove all loosely adhering soil particles, followed by washing with sterile 0.85% (w/v) and saline triple distilled water to dislodge the adhering bacteria and plating then suspension on Nutrient agar. For the recovery of endophytic isolates from the root tissues, the roots were first blotted dry and surface sterilised with acidified $HgCl_2$ (0.1%) for 2-3 min followed by 95% ethyl alcohol for 1-2 min. The surface sterilised roots were washed with sterile distilled water four times to make it free from the sterilising agents. The roots were macerated in 0.85% saline triple distilled water in a sterile mortar and pestle. Appropriate serial dilutions of root homogenate samples were individually plated on King's B medium and half strength Nutrient agar. All the media were procured from Hi-Media Laboratories, Mumbai, India.

From the rhizosphere, rhizoplane and internal tissues of the root sample, a total of 22 bacterial colonies were recovered. These colonies were purified on the respective medium by repeated streaking. The pure cultures were maintained on Kings B and ½ strength Nutrient agar slants at 4 °C, and in 60% glycerol at -20 °C. The isolates were screened for plant growth promoting traits *viz.,* phosphate solubilisation, siderophore, HCN, ammonia and IAA production. A separate paper towel assay was carried out to determine their ability to influence the germination of wheat seeds at 18 ± 1 °C. The strain *Pseudomonas* sp PGERs17 was selected on the basis of superior PGP traits and its ability to influence germination of wheat seeds. The strain has been deposited in the Microbial Type Culture Collection (MTCC), Chandigarh, India, with the accession number MTCC 9000.

Characterisation, identification, and phylogenetic analysis. Cultures were maintained in Luria Bertani agar (Hi-Media) containing the following (g/l): tryptone, 10; yeast extract, 5; NaCl, 5 and agar, 20. The pH was adjusted to 7.0 ± 0.2 with 1 N NaOH before autoclaving (Bric *et al.,* 1991). All the experiments were conducted after raising fresh cultures.

Phenotypic genus-level characterisation of isolate was carried out by subjecting the bacterial isolate to cultural (oxygen requirement), morphological (colony morphology and pigmentation), microscopic (Gram staining and size was measured by using Image-Pro Plus version 5.1 software), biochemical (utilisation of 32 different carbon sources and enzyme activity) (Collins and Lyne, 1980; Holt *et al.,* 1994), and physiological characteristics (temperature, pH, and antibiotic sensitivity) following standard procedures (Collins and Lyne, 1980; Palleroni, 1992). All media were obtained from Hi Media.

Antibiotic sensitivity was determined on Nutrient agar using various concentrations of seven different antibiotics (rifampicin, gentamicin, streptomycin, ampicillin, penicillin, polymixin B sulphate and chloramphenicol). Antibiotic solutions were prepared in appropriate solvents (methanol, ethanol, or sterile water), and appropriate concentrations were added to Nutrient agar medium prior to gelling, and dispensed in Petri dishes. Actively growing cultures were streaked on the antibiotic supplemented plates and incubated at 28 ± 1 °C. The growth pattern of the isolate was determined at three different temperatures *viz.*, 4, 15, and 28 °C, by inoculating 5 ml of an exponentially growing culture (6.81×10^5 CFU/ml) in 100 ml of the King's B broth (initial population log 5.8 CFU/ml), and estimating the cell population at 2-h intervals. The numerical values were log transformed and plotted against time.

Based on 16S rRNA gene sequence analysis was carried out at IMTECH, Chandigarh, India using the 27F (5'-3') primer on an ABI 3031xlanalyzer. The partial gene sequence (578 bp) of isolate was aligned, using ClustalX1.8 program (Thompson *et al.,* 1997), with the sequences retrieved from the GenBank database. A graphic representation of the tree was made using Njplot software (Perri and Gouy, 1996; Saitou and Nei, 1987). The 16S

rRNA sequence of the strain has been deposited in GenBank, with the accession number EU195453.

Quantitative estimation of phosphate solubilisation and IAA production. Initial qualitative estimation of the P-solubilising activity of the isolate was carried out on Pikovskaya agar (Pikovskaya, 1948). Quantitative estimation of tricalcium phosphate (TCP) solubilisation and IAA production were carried out at three different incubation temperatures *viz.*, 4, 15 and 28 °C. Quantitative estimation of P solubilisation was carried out as per standard methodology (Sangeeta and Shekhar, 2001), by inoculating 1 ml of bacterial suspension (2.16 x 10^7 cells/ml) in 50 ml of NBRIP broth in Erlenmeyer flasks (150 ml), and incubating the flasks for 7 days. At the end of the incubation period the cell suspension was centrifuged at 10000 rpm for 10 min. and the P content in the supernatant was spectrophotometrically estimated by the ascorbic acid method (Murphy and Riley, 1962). Estimation of indole acetic acid (IAA) was done by inoculation of 200 µl of bacterial suspension (2.16 x 10^7 cells/ml) in 10 ml Luria Bertani broth amended with L-tryptophan (5 mM) and incubating it for a period of 48 h at 4, 15 and 28 °C. The IAA content in the culture suspension was estimated by the standard procedure (Gordon and Weber, 1951). All the studies were repeated on three independent dates to confirm the results.

Qualitative measurement of siderophore and HCN production. Siderophore and HCN production by the isolate were estimated qualitatively at three different incubation temperatures *viz.*, 4, 15 and 28 °C. Siderophore production was detected by the Chrome Azurol-S (CAS) assay (Schwyn and Neilands, 1987) in 100 mm Petri dishes. HCN production was inferred by the qualitative method of Bakker and Schipper (1987). The change in the colour of the filter paper previously soaked in 2% sodium carbonate prepared in 0.05% picric acid, from yellow to dark brown was rated visually depending on the intensity of the colour change.

Assessment of in *vitro* antiphytopathogenic activity. The antiphytopathogenic ability of the strain was evaluated against four phytopathogens *viz., Sclerotium rolfsii, Fusarium oxysporum, Rhizoctonia solani* and *Pythium* using three different bioassays.

Volatile compound assay. Effects of volatile metabolites produced by strain PGERs17 on fungal growth were assessed by using the dual plate bioassay of Alstrom and Burns, (1989). A seven day old 5 mm diameter fungal disc of the respective fungus was placed in the centre of potato dextrose agar plates and incubated at 28 ± 1 °C for three days. Strain PGERs17 was spread plated on King's B agar and incubated at 28 °C for 24 h. After incubation, the dish portion of the bacterial and fungal plates, were sealed with parafilm. The paired plates were incubated at 28 ± 2 °C, with the fungal plate on the upper portion of the paired assembly, and observations were recorded after 3, 5 and 7 days after pairing. Inhibition of fungal growth was calculated using the formula: (R1-R2/R1) x 100, where R1 (a control value) represent the radial growth of the fungus in control sets without bacteria, and R2 represents the radial growth of the fungus in the sets inoculated with the bacterium. The experiment was conducted in triplicate.

Dual culture assay. For examining antagonism by the bacterium, King's B and Potato Dextrose Agar medium (1:1 v/v) were prepared according Huang and Hoes (1976). A seven day old 5 mm diameter mycelia agar disc of the respective fungus was placed at the centre of plates. A loopful actively grown culture (24 h) of strain PGERs17 was streaked towards periphery of plates 2 cm away from the tested pathogen, un-inoculated plates served as control. Plates were incubated at 28 ± 2 °C, and observations were recorded after 3, 5 and 7 days after inoculation and percent inhibition was calculated as mentioned in previous assay.

Diffusible compound assay. For examining antagonism due to diffusible compounds the assay was performed according to Schillinger and Lucke (1989). *Pseudomonas* sp. strain PGERs17 was grown for 36 h at 28 °C in King's B broth medium and centrifuged at 10000 rpm for 15 min. The supernatant containing the diffusible compound was filtered through 0.22 µm Millipore filter, to make it cell free and used as the test compound. Potato dextrose agar plates were over laid with 0.8% soft agar and a 1 cm diameter well was made with the help of cork borer in the top agar layer. The well was filled with 100 µl of filter sterilised cell free supernatant. A 5 mm disc of the respective fungus was placed in the centre of plates, 2 cm away from the well. Control plates were filled with 100 µl of sterile medium. Plates were incubated at 28 ± 2 °C, and observations were recorded on 3, 5 and 7 days after inoculation. The percent inhibition was calculated as mentioned in the previous assay.

Evaluation of plant growth promotion ability on wheat. The paper roll towel bioassay was used to determine the effect of the bacterium on the percent germination of wheat (var. VL 829). Ten surface sterilised seeds were soaked in the bacterial culture (1.1 x 10^7 CFU/ml) for 30 min and dried in the laminar flow. Ten seeds were placed at equal distance in each paper towel, rolled and placed in a 1 l beaker containing Hoagland's nutrient solution (Hoagland and Arnon, 1938), to keep the paper towel moist. Control seeds were soaked in sterile medium. The treatments were arranged in a completely randomized design with ten replications. The germination percent was recorded after seven days of incubation at 18 ± 1 °C.

A pot assay based determination of the plant growth promotion ability of the isolate was conducted using wheat seeds in non-sterile soil under glasshouse conditions. Seeds were bacterized as per standard procedure (Elliot and Lynch, 1984) with minor modifications. Surface sterilised seeds of wheat (var. VL 829) were coated with a charcoal-based inoculant of the culture (10^6 CFU/g). Control seeds were coated with sterile charcoal. Seeds were sown in cups (6.5-cm dia. and l0-cm depth) containing non sterile soil (pH 6.8, organic carbon 0.6%, available N 403 kg/ha, available P 15.2 kg/ha, available K 210 kg/ha, iron 10 mg/kg) and placed in a temperature controlled growth chamber at 18 ± 1 °C for 30 days. Thinning of seedlings was done 7 days after sowing and two seedlings per pot were maintained throughout the experimental period. The soil was moistened to 60% of its water-holding capacity. The treatments were arranged in a completely randomized design with ten replications. At the end of the experimental period, the plants were uprooted, washed under running water, and root and shoot lengths were measured.

Statistical analysis. The assays were carried out in a completely randomized design. Statistical analysis was performed with SPSS software, and treatment means were compared at 5% level of significance.

RESULTS

The colonies of *Pseudomonas* sp. strain PGERs17 (MTCC 9000) were of small size (1.5-2.5 mm diameter) with entire, circular margin and produces fluorescent pigment on King's B agar at

TABLE 1 - Plant growth promotion attributes of *Pseudomonas* sp. strain PGERs17 (MTCC 9000) at different incubation temperatures

Incubation temperature (ºC)	IAA production (µg/ml)	Phosphate solubilisation (µg/ml)	HCN production	Siderophore production
4	1.4 ± 0.10	42.3 ± 0.11	+	+
15	4.2 ± 0.06	66.3 ± 0.10	+	++
28	13.2 ± 0.08	74.1 ± 0.03	++	++

Numerical values are mean ± SD of four independent observations.
+: weak, ++: moderate, +++: strong.

15 °C after 3-4 days incubation with copious production of exopolysaccharide, seen around colonies. This strain was able to grow over a wide temperature range of 4-35 °C, but maximum growth was found to occur at 28 °C. It had a pH tolerance over the range 5-10 (optimum pH 7.0) and could tolerate NaCl up to 5% (w/v). Microscopic examination revealed that the cells were highly motile, Gram negative rods [2.25 µm (L) x 0.81 µm (W), and 0.51 µm diameter]. It was positive for catalase, oxidase, methyl red tests, citrate utilisation, casein hydrolysis, ornithine and lysine decarboxylase and negative for DNAse, phosphatases (acid and alkaline), cellulase, chitinase, pectinase, ONPG, deamination, nitrate reduction, indole, Voges Proskauer tests, starch hydrolysis, urease and H_2S production. Positive carbon sources were malonate, D-arabinose, xylose, melibiose, galactose, glucose, L-arabinose and mannose utilisation. Although it was sensitive to polymyxin B sulphate (1 µg/ml), gentamycin (1 µg/ml), streptomycin (5 µg/ml) and rifampicin (20 µg/ml), it showed resistance to higher concentrations of ampicillin (500 µg/ml), chloramphenicol (200 µg/ml) and penicillin (300 µg/ml).

The different plant growth promotion traits of the isolate were determined at three different incubation temperatures (Table 1). The isolate was found to solubilise phosphorus at 4, 15, and 28 °C. P-solubilising ability of the isolate was evidently visible on plates of Pikovskaya agar, where it produced a clear halo zone and in liquid broth solubilises 42.3, 66.3 and 74.1 µg/ml at 4, 15 and 28 °C respectively after seven days of incubation. The isolate produced 1.38, 8.33 and 13.15 µg/ml of IAA after 3 days of incubation. *Pseudomonas* sp. strain PGERs17 showed the production of siderophore with the production of light orange zone on CAS agar plates and HCN (OD_{625} = 0.27, cyanogenic compound) were also observed at 15 °C. It was interesting to observe that the isolate was able to retain its functional traits even at 4 °C, which was the lower temperature extreme for its growth, while higher values for all parameters were recorded at 28 °C.

From the graphical representation of the growth pattern presented in Fig. 1 it can be inferred that the isolate attained exponential growth at both 15 and 4 °C when incubated for 24 h. To confirm the taxonomic position of the endophytic bacterium, the 578 bp 16S rRNA gene sequence was compared to different strains obtained from the NCBI database using the neighbor-joining method. The results (Fig. 2) showed that PGERs17 has the highest similarity (99.0%) with *Pseudomonas* spp. available in the public domain. The phylogenetic tree constructed using gene sequences of other species of *Pseudomonas* revealed that the isolate has separated out as a independent clade from the other two closely related 16S rRNA gene sequence clades *viz.,* the *Pseudomonas vancouverensis* ATCC 700688 clade and the clade comprising of *Pseudomonas alcaligenes* ATCC 14909, *Pseudomonas tolaasii* ATCC 33618, and *Pseudomonas agarici* ATCC 25941.

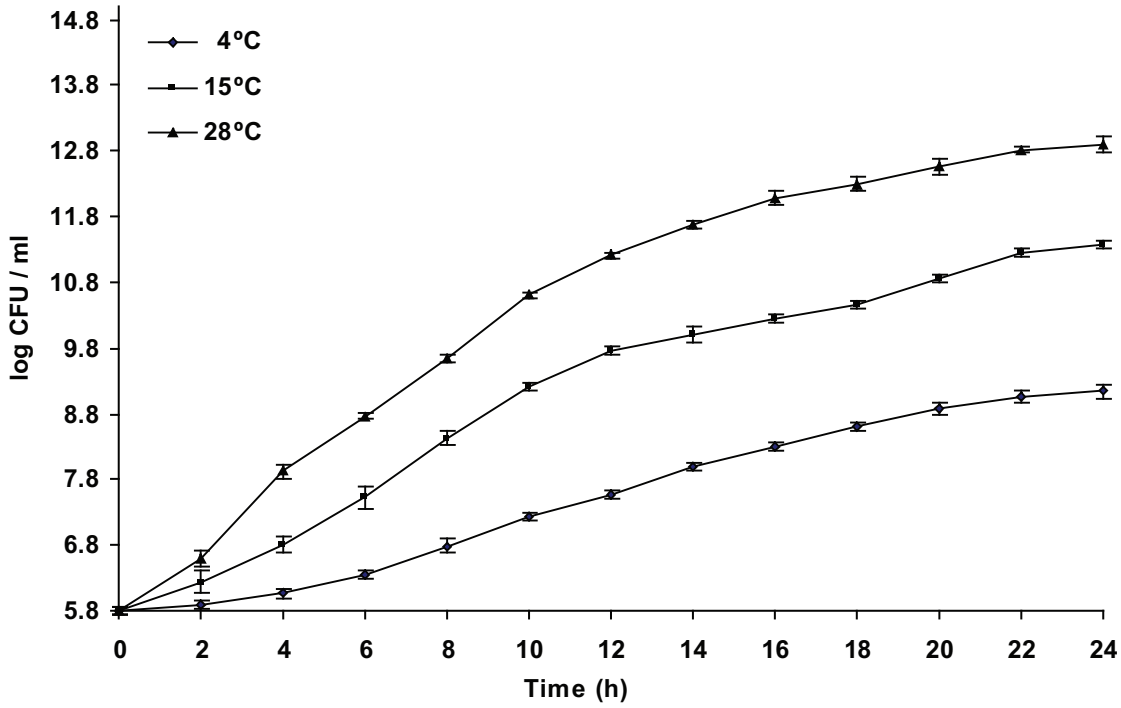

FIG. 1 - Growth curve of *Pseudomonas* sp. strain PGERs17 MTCC 9000) at three different incubation temperatures.

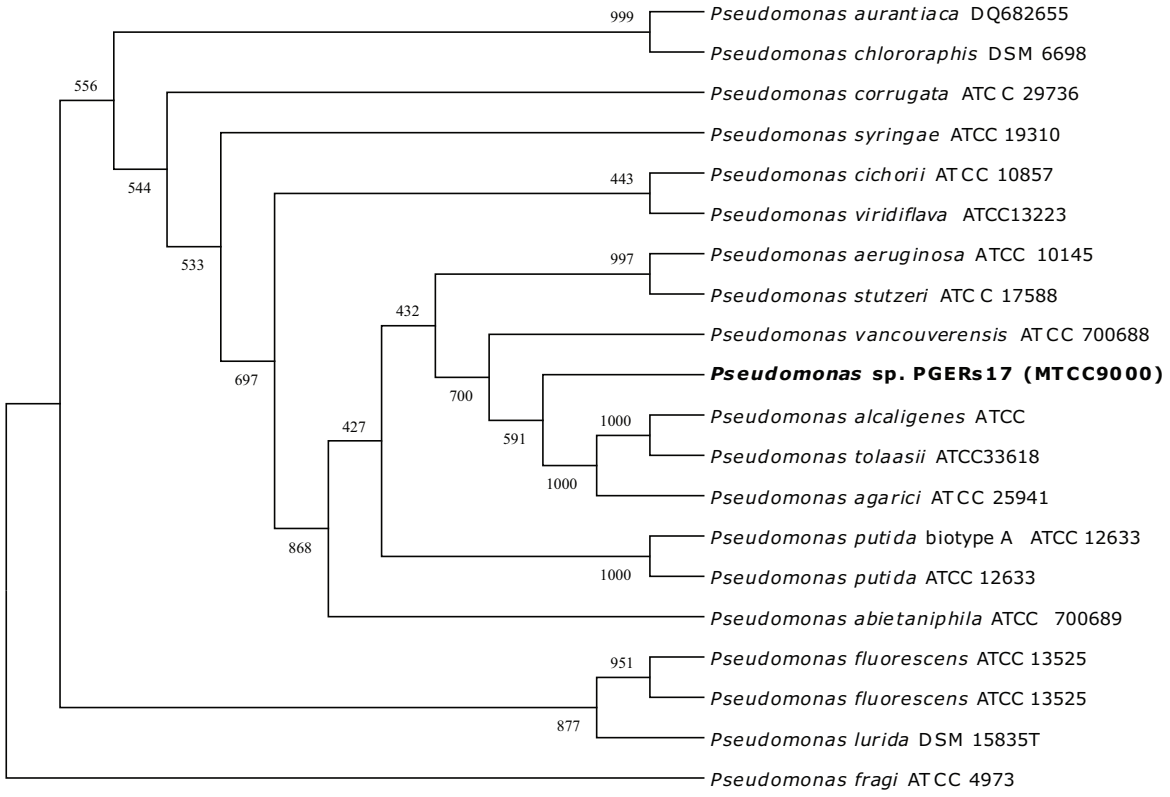

FIG. 2 - Phylogenetic tree showing the relationships between *Pseudomonas* sp. strain PGERs17 (MTCC 9000) and other Pseudomonads. The tree was constructed by using the software MEGA2.1 after aligning the sequences with ClustalX1.81 and generating evolutionary distance matrix inferred by the neighbor-joining method using Kimura parameter 2. The 16S rRNA accession numbers are given within parentheses. The bootstrap values (n = 1000) are displayed at the nodes.

Antiphytopathogenic activity was determined by volatile, dual culture and diffusible compound assay under *in vitro* condition showed the suppression of radial growth of test pathogens by the isolate after 3, 5 and 7 days (Table 2). After 7 days relative growth inhibition was observed in the range of 19.7 ± 0.05% (*Fusarium oxysporum*) followed by the 73.9 ± 0.30% (*Pythium* sp.) and maximum 100.0 ± 0.40% fungal inhibition was observed against *Sclerotium rolfsii* and *Rhizoctonia solani* under volatile compound assay. Inhibition of fungal growth ranged from 12.6 ± 0.33% *(Rhizoctonia solani)* to 27.7 ± 0.15% (*Sclerotium rolfsii)* in dual culture assay and diffusible compound assay from 3.9 ± 0.13% (*Fusarium oxysporum*) to 61.1 ± 0.24% (*Pythium* sp.).

TABLE 2 - *In vitro* antiphytopathogenic activity of *Pseudomonas* sp. strain PGERs17 (MTCC 9000) against select phytopathogenic fungi at different periods of incubation

Phytopathogen	Days	Relative growth inhibition (%)		
		Bioassay used		
		Volatile compound	Dual culture	Diffusible compound
Fusarium oxysporum	3	35.1 ± 0.30	6.6 ± 0.06	32.6 ± 0.08
	5	20.0 ± 0.10	7.2 ± 0.19	7.4 ± 0.10
	7	19.7 ± 0.05	13.5 ± 0.06	3.9 ± 0.13
Rhizoctonia solani	3	100.0 ± 0.20	49.4 ± 0.29	NI
	5	100.0 ± 0.50	47.8 ± 0.66	NI
	7	100.0 ± 0.40	12.6 ± 0.33	NI
Pythium sp.	3	71.9 ± 0.06	51.5 ± 0.13	70.9 ± 0.14
	5	72.7 ± 0.05	31.0 ± 0.66	82.1 ± 0.15
	7	73.9 ± 0.30	-	61.1 ± 0.24
Sclerotium rolfsii	3	100.0 ± 0.10	7.7 ± 0.06	1.2 ± 0.05
	5	100.0 ± 0.06	25.5 ± 0.13	56.0 ± 0.08
	7	100.0 ± 0.02	27.7 ± 0.15	58.1 ± 0.08

Values are mean of three replicates.
Numerical values are mean ± SD of five independent experiments.
NI, -: no fungal growth inhibition observed.

TABLE 3 - Effect of *Pseudomonas* sp. strain PGERs17 on root, shoot lengths and percentage germination on wheat (var. VL 829) under greenhouse condition at 18 ± 1 °C after 30 days

Treatments	Shoot length	Root length	Germination (%)
Control	21.30	17.03	71.30
Pseudomonas sp. PGERs17	27.2	22.73	87.50
LSD	5.32	0.96	6.38
SEm±	2.17	0.39	2.61

Values are mean of ten replicates; $P = 0.05$.
LSD: Least Significant Difference.
SEm: Standard Error of mean.

The plant growth promotion potential of *Pseudomonas* sp. strain PGERs17 (MTCC 9000) was determined by a pot assay under non-sterile soil conditions at sub-optimal temperatures. It was observed that the bacterized wheat seedlings recorded 20.3% higher seed germination, 30.2 and 27.5% higher root and shoot lengths respectively, as compared to uninoculated control (Table 3). A corresponding increase in the root and shoot biomass was also observed in bacterized seedlings. Seed bacterization resulted in greater enhancement of the root growth, as compared to the shoot growth.

DISCUSSION

The N.W. Indian Himalayan region is a hot spot of biodiversity and several new cold-tolerant bacterial species have been isolated from this region (Shivaji *et al.,* 2005). The bacterium described in the present study is an addition to the beneficial biodiversity inventory of the region. Since the isolate was obtained from the internal tissue of surface sterilised garlic root tissue, it would be appropriate to call it an endophyte. The biochemical characteristics of the bacterium are in accordance with the descriptions of *Pseudomonas* spp. by Holt *et al*. (1994). The phylogenetic tree constructed based on the 16S rRNA gene sequence, revealed its close similarity with *Pseudomonas vancouverensis,* a Gram negative bacterium that grows on pulp effluent with resin acids recovered from forest soil of Vancouver, Canada (Mohn *et al.,* 1999). Considering the fact that this strain was isolated at 4 °C, and expressed plant growth promoting traits at temperatures ranging from 4 to 35 °C, we would call it a psychrotrophic (cold-tolerant) plant growth-promoting bacterium, rather than a psychrophilic (cold loving) one.

Bacterial plant growth promotion is a well established and complex phenomenon, and is often achieved by the activities of multiple plant growth promoting traits exhibited by the associated bacterium (Lifshitz *et al*., 1987). Among the PGP, traits IAA production by the bacterium has a cascading effect on the plant development due to its ability to influence root growth, which in turn affects the nutrient uptake and ultimately the plant productivity. IAA production by cold tolerant bacteria has received little interest in the past. But the available studies suggest that IAA production is higher in the mesophilic range compared to the cold temperatures (Basu and Ghosh, 1998). A similar trend was observed by Selvakumar *et al*. (2008a), who observed higher levels of IAA production by the bacterium *Pantoea dispersa* at 30 °C, compared to 4 °C. Solubilisation of insoluble phosphorous compounds in the rhizosphere by microorganisms is another important means of achieving plant growth promotion (Gull *et al.,* 2004). We strongly suspect that the P solubilisation ability of the bacterium could have played an important role in the observed plant growth promotion.

The availability of iron for microbial assimilation in environments such as the rhizosphere is extremely limiting. Since it is the fourth most abundant element in the earth's crust it is largely required by all living organisms for direct microbial assimilation. In aqueous solution, iron can exist in either the ferrous (Fe^{2+}) or (Fe^{3+}) form, the latter being the less soluble. However, in highly oxidised and aerated soils, the predominant form of iron is the ferric form, which is soluble in water (pH 7.4) at about 10^{-18} M (Neilands *et al.,* 1987). This is too low to support the growth of microorganisms, which generally need concentrations approaching 10^{-6} M for normal growth. Consequently, to survive in such environments, organisms secrete iron-binding ligands (siderophores), which can bind the ferric iron and make it available to the host microorganisms. These compounds have been defined as "low molecular mass", virtually ferric specific ligands, the biosynthesis of which is carefully regulated by iron and the function of which is to supply iron to the cell (Neilands, 1986). The role of such iron chelating siderophores in plant growth promotion is well established (Katiyar and Goel, 2004). While siderophores are mainly implicated in the biological control of plant pathogenic fungi, their role in iron nutrition of plants still remains unclear. Since our isolate possessed both antagonistic and plant growth promotion activity, the role of siderophores produced by the bacterium is a researchable issue.

Bacterial antagonism towards phytopathogenic fungi is known to be mediated by a variety of compounds of microbial origin, *viz.,* bacteriocins, enzymes, toxic substances, volatiles, and others. The effect of volatile compounds has received only limited attention in comparison to the diffusible compounds. In the present study, volatile compounds produced by the bacterium showed 100% inhibition of the test pathogens, which was much superior to the antagonism levels noticed in the diffusible and dual culture assays. The growth inhibition noticed in the volatile compound assay may be attributed to cyanogenesis from glycine, resulting in the production of HCN, which is volatile in nature and plays a key role in the inhibition of phytopathogenic fungi namely *Sclerotium rolfsii, Rhizoctonia solani* and *Pythium* sp. under *in vitro* conditions (Bakker and Schipper, 1987).

The plant growth promotion ability of the isolate was demonstrated through a pot culture assay. Its use as inoculant resulted in a significant increment in roots and shoot biomass of 30 day old wheat seedlings. These results are in line with earlier findings of Pandey *et al.* (1999), where plant growth promotion was found to correlate with the colonisation of introduced bacteria. It would be difficult to attribute to attribute the growth promotion effect to a single trait, since the bacterium possessed multiple traits. Such a study would require the construction of knock out

mutants that are impaired in a particular trait. Never the less the plant growth promotion ability of the bacterium has been proved beyond doubt. The intrinsic resistance exhibited by the bacterium against multiple antibiotics can be used as a marker evaluating the survival of inoculated bacteria in pot/field trials (Kluepfel, 1993; Trivedi *et al.,* 2005).

The selection of native functional plant growth promoting microorganisms, is a mandatory step for reducing the use of energy intensive chemical fertilisers. The strain reported in this study seems to be an ideal candidate for promotion as a bio-inoculant, due to its cold tolerance and multiple abilities of plant growth promotion traits. Earlier attempts in this regard have examined psychrotrophic bacteria such as *P. corrugta, Pantoea dispersa* and *Serratia marcescens* that were isolated from different location in the N.W. Indian Himalayas (Pandey and Palni, 1998; Pandey *et al.,* 1999, 2006; Trivedi *et al.,* 2005; Selvakumar *et al.,* 2008a, 2008b). *Pseudomonas* sp. strain PGERs 17 (MTCC 9000) is another important isolate that could be developed as a suitable inoculant for winter season crops grown in the alpine and sub-alpine regions.

Acknowledgements

This research work was supported by Indian Council of Agricultural Research (I.C.A.R.), New Delhi, under the project "Application of Microorganisms in Agriculture and Allied Sectors (AMAAS)".

REFERENCES

Alstrom S., Burns R.G. (1989). Cyanide production by rhizobacteria as a possible mechanism of plant growth inhibition. Biol. Fert. Soils, 7: 232-238.

Bakker A.W., Schippers B. (1987). Microbial cyanide production in the Rhizosphere in relation to potato yield reduction and *Pseudomonas* spp. mediated plant growth stimulation. Soil Biol. Biochem., 19: 451-457.

Basu P.S., Ghosh A.C. (1998). Indole acetic acid and its metabolism in root nodules of a monocotyledonous tree *Roystonea regia*. Curr. Microbiol., 37: 137-140.

Behrendt U., Ulrich A., Schumann P., Meyer J. M., Sproer C. (2007). *Pseudomonas lurida* sp. nov., a fluorescent species associated with the phyllosphere of grasses. Int. J. Syst. Evol. Microbiol., 57: 979-985.

Bric J.M., Bostock R.M., Silverstone S.R. (1991). Rapid in situ assay for indole acetic acid production by bacteria immobilized on a nitrocellulose membrane. Appl. Microbiol., 57: 535-538.

Collins C.H., Lyne P.M. (1980). Microbiological Methods. Butterworth and Co. (Publishers) Ltd, London.

Elliot L.F., Lynch J.M. (1984). *Pseudomonas* as a factor in the growth of winter wheat (*Triticum aestivum* L.). Soil Biol. Biochem., 16: 69-71.

Glick B.R., Penrose D.M., Jiping L. (1998). A model for the lowering plant ethylene concentrations by plant growth promoting bacteria. J. Theor. Biol., 190: 63-68.

Gordon S.A., Weber R.P. (1951). Colometric estimation of Indole Acetic Acid. Plant Physiol., 26: 192-195.

Greenland D., Losleben M. (2001). Climate. In: Bowman W.D., Seastedt T.R., Eds, Structure and Function of an Alpine Ecosystem Niwot Ridge, Colorado, Oxford University Press, New York, N.Y., pp. 15-31.

Gull F.Y., Hafeez I., Saleem M., Malik K.A. (2004). Phosphorus uptake and growth promotion of chickpea by co-inoculation of mineral phosphate solubilizing bacteria and a mixed rhizobial culture. Aust. J. Exp. Agric., 44: 623-628.

Hoagland D.R., Arnon D.I. (1938). The water culture method for growing plants without soil; Circ. Calif. Agric. Exp. Stn. 347 32.

Holt J.G., Kreig N.R., Sneath P.H.A., Stanley J.T., Williams S.T. (1994). Bergey's Manual of Determinative Bacteriology, 9th edn., The Williams & Wilkins Co, Baltimore, MD.

Huang H.C., Hoes J.A. (1976). Penetration and infection in *Sclerotium* by *C. minitans*. Can. J. Botany, 54: 406-410.

Katiyar V., Goel R. (2004). Siderophore mediated plant growth promotion at low temperature by mutant of fluorescent pseudomonad. Plant Growth Regul., 42: 239-244.

Kloepper J.W., Schroth M.N. (1978). Plant growth-promoting rhizobacteria on radishes. In: Proceedings of the 4th International Conference on Plant Pathogenic Bacteria. Gilbert-Clarey, Tours, France, pp. 879-882.

Kluepfel D.A. (1993). The behaviour and tracking of bacteria in the rhizosphere. Annu. Rev. Phytopathol., 31: 441-472.

Lifshitz R., Kloepper J.W., Kozlowski M., Simonson C., Carlson J., Tipping E.N., Zaleska I. (1987). Growth promotion of canola (rape-seed) seedlings by a strain of *Peudomonas putida* under gnotobiotic conditions. Can. J. Microbiol., 8: 102-106.

Misaghi I.J., Stowell L.J., Grogan R.G., Spearman L.C. (1982). Fungistatic activity of water-soluble fluorescent pigments of fluorescent pseudomonads. Phytopathology, 72: 33-36.

Mohn W.W., Wilson A.E., Bicho P., Moore E.R.B. (1999). Physiological and phylogenetic diversity of bacteria growing on resin acids. Syst. Appl. Microbiol., 22: 68-78.

Morita R.Y. (1975). Psychrophilic bacteria. Bact. Rev., 39: 144-167.

Murphy J.P., Riley J.P. (1962). A modified single solution method for the determination of the phosphate in natural waters. Anal. Chem. Acta, 27: 31-36.

Neilands J.B. (1986). Microbial iron compounds. Annu. Rev. Biochem., 50: 715-731.

Neilands J.B., Konopka K., Schwyn B., Coy M., Francis R.T., Paw B.H., Bagg A. (1987). Comparative biochemistry of microbial iron assimilation, In: Winkelmann G., Van der Helm D., Neilands J.B., Eds, Iron Transport in Microbes, Plants and Animals, Verlagsgescellschaft mbh, Weinheim, pp. 3-33.

Palleroni N.J., Doudoroff M. (1972). Some properties and taxonomic subdivisions of genus *Pseudomonas.* Annu. Rev. Phytopathol., 10: 73-100.

Palleroni N.J. (1992). Introduction to the family Pseudomonadaceae. In: Balows A., Truper H., Dworkin M., Harder W., Schleifer K.H., Eds, The Prokaryotes, 2nd edn., vol. 1, Springer-Verlag, New York, N.Y., pp. 3071-3079.

Pandey A., Palni L.M.S. (1998). Isolation of *Pseudomonas corrugata* from Sikkim Himalaya. World J. Microbiol. Biotechnol., 14: 411-413.

Pandey A., Durgapal A., Joshi M., Palni L.M.S. (1999). Influence of *Pseudomonas corrugata* inoculation on root colonization and growth promotion of two important hill crops. Microbiol. Res., 154: 259-266.

Pandey A., Trivedi P., Kumar B., Palni L.M.S. (2006). Characterization of a phosphate solubilizing and antagonistic strain of

Pseudomonas putida (B0) isolated from a sub-alpine location in the Indian Central Himalaya. Curr. Microbiol., 53: 102-107.

Patten C.L., Glick B.R. (2002). Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. Appl. Environ. Microbiol., 68: 3795-3801.

Perrire G., Gouy M. (1996). WWW-query: an on-line retrieval system for biological sequence banks. Biochimie, 78: 364-369.

Pikovskaya R.I. (1948). Mobilization of the phosphorous in soil in connection with the vital activity of some microbial sp. Mikrobiologiya, 17: 362-370.

Podile A.P., Kishore K.G. (2006). Plant growth promoting Rhizobacteria. In: Gnanamanickam S.S., Ed., Plant Associated Bacteria, Springer, Netherlands, pp. 195-230.

Premono M.E., Moawad A.M., Vlek P.L.G. (1996). Effect of phosphate-solubilizing *Pseudomonas* Psychrophilic bacteria. Bact. Rev., 39: 144-167.

Saitou N., Nei M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol., 4: 406-425.

Sangeeta M., Shekhar N.C. (2001). An efficient method for qualitative screening of phosphate-solubilizing bacteria. Curr. Microbiol., 43: 51-56.

Schillinger U., Lucke F.K. (1989). Antibacterial activity of *Lactobacillus* stain isolated from meat. Appl. Environ. Microbiol., 55: 1901-1906.

Schwyn B., Neilands J. (1987). Universal assay for detection and determination of siderophores. Anal. Biochem., 160: 47-56.

Selvakumar G., Kundu S., Joshi P., Nazim S., Gupta A.D., Mishra P.K., Gupta H.S. (2008a). Characterization of a cold-tolerant plant growth-promoting bacterium *Pantoea dispersa* 1A isolated from a sub-alpine soil in the North Western Indian Himalayas. World J. Microbiol. Biotechnol., 24: 955-960.

Selvakumar G., Mohan M., Kundu S., Gupta A.D., Joshi P., Nazim S., Gupta H.S. (2008b). Cold tolerance and plant growth promotion potential of *Serratia marcescens* strain SRM (MTCC 8708) isolated from flowers of summer squash (*Cucurbita pepo*). Lett. Appl. Microbiol., 46: 171-175.

Shivaji S., Chaturvedi P., Reddy G.S.N., Suresh K. (2005). *Pedobacter himalayensis* sp. nov., from Hamta glacier located in the Himalayan mountain range in India. Int. J. Syst. Evol. Microbiol., 55: 1083-1088.

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F., Higgins D.G. (1997). The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools; Nucleic Acids Res., 25: 4876-4882.

Tilak K.V.B.R., Ranganayaki N., Pal K.K., De R., Saxena A.K., Nautiyal C. Shekhar M.S., Tripathi A.K., Johri B.N. (2005). Diversity of plant growth and soil supporting bacteria. Curr. Sci., 89: 136-150.

Trivedi P., Pandey A., Palni L.M.S., Bag N., Tamang M.B. (2005). Colonization of rhizosphere of tea by growth promoting bacteria. Int. J. Tea Sci., 4: 19-25.

Valverde A., Burgos A., Fiscella T., Rivas R., Velazquez E., Rodrıguez-Barrueco C., Cervantes E., Chamber M., Igual J.M. (2006). Differential effects of coinoculations with *Pseudomonas jessenii* PS06 (a phosphate-solubilizing bacterium) and *Mesorhizobium ciceri* C-2/2 strains on the growth and seed yield of chickpea under greenhouse and field conditions. Plant Soil, 287: 43-50.