INTRODUCTION

Plants growing at high altitudes are subjected to a variety of stressful environments namely: low temperatures coupled with frost are major constraints for crop production as plant growth and overall productivity generally decline under chilling conditions (Mishra et al., 2012). Still medicinal plants growing in such stressful conditions can grow well as their rhizosphere has diverse microbial flora which have potential to overcome all the constraints as these microbes are vital part of any ecosystem. Therefore, curiosity has been enhanced in the nature and properties of these microbes that play a major role in nutrient cycling in cold ecosystems (Häggblom and Margesin, 2005).

Psychrotolerant microbes are important in high-altitude agro-ecosystems since they survive and retain their functionality at low temperature conditions, while growing optimally at warmer temperatures (Kumar et al., 2005; Sat et al., 2013). The cold adapted microbes that possess various plant growth promotion abilities can be utilized for increased plant production in these areas. These Plant Growth Promoting Rhizobacteria (PGPR) promote plant growth by several mechanisms which include Induced Systemic Resistance (ISR), produce plant growth regulators like indole acetic acid, gibberellic acid, cytokinins and ethylene, asymbiotic nitrogen fixation, antagonism against phytopathogenic micro-organisms by production of siderophores, antibiotics and cyanide, fungal cell wall lysing enzymes which suppress the growth of fungal pathogens, solubilization of mineral phosphates and other nutrients (Modi et al., 2017).

The agri-horticultural importance of cold-tolerant microorganisms arises to protect the cropping system of temperate areas of the region and as such there is a need to...
explore cold tolerant microbes which has plant growth promoting activities and the potential to increase crop productivity in temperate areas.

Therefore, the present study was conducted in realization to explore cold adapted rarely explored bacteria from rhizosphere of *Podophyllum hexandrum* Royle which is a threatened medicinal plant found in alpine Himalayas (3000 to 4000 m.a.s.l) of Jammu and Kashmir, Himachal Pradesh, Sikkim, Uttarakhand and Arunachal Pradesh (Quazi et al., 2011).

**MATERIALS AND METHODS**

**Samples collection**

The rhizospheric soil of 5 different plants of *P. hexandrum* Royle growing in Sangla valley of Himachal Pradesh, India at an altitude of 3885 to 4205 m were collected (Figure 1). For isolation process, one composite sample was prepared by mixing 5 different soil samples in an equal proportion in a sterilized plastic bag and brought to the National Bureau of Plant Genetic Resource, Regional Station, Shimla, Himachal Pradesh, India.

**Isolation, purification and screening of bacterial culture**

Isolation of bacteria was performed by standard microbiological methods. Soil sample was diluted in a series of $10^{-2}$, $10^{-4}$, $10^{-6}$, $10^{-8}$ and $10^{-10}$ and spread on nutrient agar medium following incubation at 20°C. Bacterial colonies obtained were streaked on nutrient agar to obtain pure culture. Pure cultures were preserved on slants in refrigerator and 30% glycerol (-20°C) in deep freezer. Isolated bacteria were screened for their antagonistic potential against plant pathogens.

**Antibacterial activities of bacterial isolates against serious plant pathogens**

Antimicrobial activity was checked by Bit/disk method as highlighted.

**Indicators**

The list of indicators selected for the present study are serious plant pathogens including *Alternaria solani* MTCC 2101, *Botrytis cinera* MTCC 2350, *Rhizoctonia solani* MTCC 4633, *Collectotrichum gloesporioides* MTCC 9664, *Fusarium oxysporium* MTCC 7677, *Rosellinia* sp. MTCC 3878 and *Sclerotinia sclerotiorum* MTCC 3878. These indicators were procured from CSIR-Microbial Type Culture Collection, Institute of Microbial Culture Collection (IMTECH), Chandigarh, India.

**Antagonistic test for bacterial isolates against pathogenic fungus**

Antagonistic activity of isolated bacterial strains against plant pathogens were studied using a dual culture *in vitro* assay (Maurya et al., 2014). For testing the antagonistic activity, bit of bacterial isolate PH15 was placed on one side of the Petri dish and mycelial disc from seven days old PDA culture of selected fungus placed on the other side of the Petri dish that is, both the bits were placed on an equal
distance from the centre of the Petri plate and incubated at 27±2°C for 5 to 7 days. Petri dishes inoculated with fungal discs alone served as control. This experiment was conducted in three replicates per each bacterial species.

Percentage inhibition was calculated using the formula:

\[
\text{Percent inhibition (I)} = \frac{C - T}{C} \times 100
\]

Where: C represent mycelia growth of pathogen in control and T representing mycelia growth of pathogen in dual culture plate.

On the basis of antagonistic potential of bacterial isolate PH15 it was selected for the present study and identified using 16SrRNA gene technique.

**Identification of screened bacterial isolate by 16SrRNA gene technique**

**Isolation of genomic DNA**

Potential screened isolate PH15 was subjected to molecular identification by 16S rRNA gene sequencing. Genomic DNA of AH1 was isolated using standard protocol of DNA prepkit (ZymoBIOMICS™ Make), DNA was quantified using nanodrop spectrophotometer (Thermo Fisher Scientific make).

**Amplification and sequencing of genomic DNA**

PCR amplification of 16S rRNA region was carried out with the following concentration of reagents Taq buffer (10 X)-5.0 μl; dNTP 2 mM - 2.5 μl; primer (F)-1.0 μl; primer (R)-1.0 μl; Taq polymerase-0.2 μl; glycerol- 0.5 μl; water-12.8 μl; DNA-1 μl; MgCl-1 μl. The procedure consisted of 35 cycles of 92°C for 1 min, 55°C for 1 min and 72°C for 1 min. Samples were amplified by universal primers of 16S rRNA (8F 5’AGAGTTTGATCCTGGCTCAG3) and (1492 R 5’GGTTACCTTGTTACGACTT3). The amplified PCR product was cleaned up using PCR clean up kit (Real Genomics Hi Yield TM Make). Eluted PCR product was submitted to Xcelar, Ahmdabad, India for sequencing process. Sequences were edited with the Sequence Navigator program (Applied Biosystems) and aligned using ClustalW (version 1.8; Infobiogene). For further analysis, sequence was compared with 16S DNA sequences from appropriate reference strains registered in the GenBank database. After comparison it was submitted in blank to obtain accession number and phylogenetic tree inferred using neighbor joining method (Saitou and Nei, 1987).

**Plant growth promoting traits of isolated bacterial strains**

**Siderophore production**

Siderophore was detected by chrome azurol S (CAS) plate assays (Schwyan and Neiland, 1987; Loudan et al., 2011). CAS plates were prepared as follows:

**Solution A:** Sterilized blue agar was prepared by mixing chrome azurol (CAS) 60.5 mg/50 ml distilled water with 10 ml iron solution and 1 mM FeCl3.6H2O in 10 mM HCl. This solution was added to hexadecyltriethyl ammonium bromide (HDTMA) (72.9 mg/40 ml distilled water).

**Solution B:** 750 ml nutrient agar was mixed with 1, 4 piperoxazineethane sulphonic acid (30.24 g). pH was adjusted to 6.8 with NaOH (6.0 g) and autoclaved. To prepare CAS agar plates solutions A and B were mixed and poured in Petri dishes. Bit of 72 h old isolate PH15 was placed on CAS agar plate incubated at 20°C for 72 h. Siderophore production was observed by formation of yellow or orange halo around the bit.

**Phosphate solubilization**

Bacterial isolates PH15 was screened for its tri-calcium phosphate (TCP) solubilizing activity on Pikovoskaya’s agar plate (Pikovoskaya, 1948). A bit of 72 h old PH15 was placed on Pikovoskaya’s agar plate (Hi Media make) followed by incubation at 20°C for 5 days. Phosphate solubilization was observed with a zone formation around bit. % Solubilization efficiency (SE) was calculated as:

\[
\% \text{Solubilization efficiency (SE)} = \frac{Z-C}{100*100}
\]

**HCN production**

Isolate PH15 was streaked on nutrient agar (Hi Media Make) amended with glycine (4 g/L). Whatman filter paper No. 1 was soaked in 2% sodium carbonate and thereafter in 0.5% picric acid inside the top of the plate. The plate was sealed with parafilm and incubated at 20°C for 5 days. Production of HCN was observed in terms of change of colour of paper from deep yellow to orange brown to dark brown (++ to +++ to ++++). Bakker and Schipper, (1987). Control was kept un-inoculated for comparison of results.

**Ammonia production**

Bacterial isolate V. arenosi PH15 was inoculated in 5 ml peptone water in test tubes followed by incubation at 20°C for 5 days. 1 ml Nessler’s reagent’s was added to each test tube to check for the presence of ammonia (Lata, 2003). Ammonia production was expressed in terms of change of colour of culture from faint yellow to brown (++ to ++++).

**Protease production**

Skimmed milk agar plates were prepared by adding
sterilized skim milk to nutrient agar. A bit of 72 h old V. arenosi PH15 was cut with the help of sterilized borer and placed on skim agar plate followed by incubation at 20°C for 72 h. Production of proteolytic activity expressed in terms of mm diameter of clear zone was produced around the well (Aneja, 2000).

RESULTS AND DISCUSSION

Isolation of bacterial isolate

Psychrotrophic bacterial isolate PH15 was isolated from the composite soil sample of the rhizospheres of P. hexandra Royle growing in Sangla valley, Himachal Pradesh, India which lies in northwestern Himalaya. P. hexandra is a threatened high value Ayurvedic medicinal herb found in temperate forests and alpine meadows usually in humus rich soils. The plant contains podophyllin, which has an antibiotic effect (it interferes with cell division and can thus prevent the growth of cells). It is therefore, a possible treatment for cancer and has been used especially in the treatment of ovarian cancer. In total fifteen number of bacterial isolates were isolated by standard microbiological techniques on nutrient agar medium followed by incubation at 20°C.

Screening and identification of bacterial isolate PH15

Screening of potential bacterial isolate out of 10 isolated bacterial isolates was done on the basis of its antagonistic behavior against serious plant pathogens. Antagonism was checked by Bit/disk method. Bacterial isolate PH15 was found to inhibit three deadly plant pathogens out of 6 selected (Table 1). The most promising isolate PH15 was selected for further study as it exhibited maximum antagonistic activities against A. solani MTCC 2101 (87%), B. cinerea MTCC2350 (62.5%) and F. oxysporum MTCC 7677 (87.5 %). Although the isolate did not show antagonism against other selected indicators namely: C. gloesporioides MTCC 9664, Rosellinia sp. MTCC 3878, S. sclerotiorum MTCC 3878 but the antibiotic potential of isolate was quite good against three deadly plant pathogens which are the major cause of plant diseases. The isolate PH15 was identified by 16SrRNA gene technique as V. arenosi (Figure 2a and b) and has been submitted to Genbank databases. It is registered under the accession number MG742327. Phylogenetic tree for the organism was inferred using Neighbour joining method (NJ). The principle of this method is to find pairs of operational taxonomic units (OTUs) that minimize the total branch length at each stage of clustering of OTUs starting with a star like tree. The merit of NJ method is fast. Therefore, it is practical to analyze a large database (100 or 1000 taxa) (Horikke, 2016). This is the first report of V. arenosi as a plant growth promoting rhizobacteria.

Table 1: Antagonistic activity of isolate PH 15 in terms of inhibition.

<table>
<thead>
<tr>
<th>Name of pathogen</th>
<th>Inhibition percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria solani (MTCC 2101)</td>
<td>87</td>
</tr>
<tr>
<td>Botrytis cinerea (MTCC 2350)</td>
<td>62.5</td>
</tr>
<tr>
<td>Fusarium oxysporum (MTCC 7677)</td>
<td>87.5</td>
</tr>
</tbody>
</table>

Plant growth promoting traits of Virdibacillus arenosi PH15

Antagonistic activity

As earlier mentioned V. arenosi PH15 inhibited three deadly plant pathogens out of six tested (Figure 3). The antagonistic potential of this strain could be useful as biological control of plant disease which may be an alternative approach to the use of hazardous chemical fungicides. Many microbial antagonists have been reported so far to possess antagonistic activities against plant pathogens. Maurya and Tomer (2014) reported that the P. fluorescens inhibited growth of tested fungi significantly. Inhibition percentage against Fusarium moniliforme, R. solani and A. alternata was 65.45, 68.23 and 48.13%, respectively. Similarly, Mardanova et al. (2017) reported highest antagonistic activity of Bacillus subtilis against Fusarium sp.

Siderophore production

Siderophore production by V. arenosi PH15 was confirmed by incubating it on CAS agar plates. A yellow to orange colored zone of 5 mm was developed on CAS agar plate indicating siderophore production by PH15 (Figure 3). The color changed from blue to orange resulting from siderophoral removal of Fe from the dye. Similar finding was reported in the literature that siderophores are organic compounds whose main function is to chelate the ferric iron (Fe (III)) from the environment (Ali et al., 2011). Microbial siderophores also provide plants with Fe, enhancing their growth when Fe is limiting (Frail et al., 2014). Rhizobial strains able to produce siderophores have been reported to be potential biofertilizers, improving the production of carrots, lettuce, peppers and tomatoes (Frail et al., 2012; Felix et al., 2013).
Figure 2: A) Genomic DNA and PCR product of bacterial isolate PH 15 and B) Phylogenetic relationship between PH15 and representative species based on neighbour joining tree and pairwise alignment.

Figure 3: Plant growth promoting traits of *Virdibacillus arenosi* PH15. A) Antagonistic activity, B) Phosphate solubilization, C) Proteolytic activity and D) HCN production.
Phosphate solubilization

V. *arenosi* PH15 was found to be a phosphate solubilizer as it forms a yellow zone around the bit after incubation on Pikovskaya’s agar medium (Figure 3). Phosphate solubilization was measured in terms of % solubilization efficiency which was found to be 40%. The zone formation could be due to the activity of phosphatase enzyme in bacterial isolates (Tripti et al., 2012). Phosphorus is the second most important nutrient for plants, after nitrogen. This element is fairly insoluble in soils. It exists in soil as mineral salts or incorporated into organic compounds. Although these phosphorus compounds are abundant in agricultural soils, the majority of them occur in an insoluble form (Otenio et al., 2015). Different bacterial species, particularly rhizosphere colonizing bacteria, have the ability to liberate organic phosphates or to solubilize insoluble inorganic phosphate compounds such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite and rock phosphate. These bacteria make available the soluble phosphates to the plants and in return gain root borne carbon compounds, mainly sugars and organic acids, necessary for bacterial growth (Khan et al., 2014).

**HCN production**

In the present study, *V. arenosi* PH15 was depicted to produce strong HCN. Production of HCN was observed in terms of change of colour of Whatman filter paper No. 1 from deep yellow to orange brown to dark brown (Figure 3). It is reported in the literature by many authors that HCN is the common secondary metabolite produced by rhizospheric *Pseudomonas* (Schipper, 1990). Here, it is interesting to note that HCN production by bacterial or fungal isolate provides a beneficial effect in terms of biocontrol activity. HCN is an example of a metabolite that can differentially affect plant growth depending on the producer strain, the amount of HCN accumulating in microsites in the rhizosphere and the crop species grown (Weller, 2007).

**Ammonia production**

Ammonia was suspected to be responsible for this alkalization. In a time course experiment, bacterial ammonia and amine emissions were determined using Nessler’s reaction. *V. arenosi* PH15 was found to emit ammonia at substantial levels. Ammonia production is an important characteristic of PGPR, which indirectly influences plants growth. The high ammonia release generated a pH shift in the plant medium. These results substantiated the observation that bacteria growing on peptone-rich media released ammonia in concentrations that were sufficient to alkalize the MS medium which in turn retarded plant growth (Weise et al., 2013). Likewise, all *Bacillus* and *Pseudomonas* isolated from chickpea rhizosphere in India have been found to produce ammonia (Yadav et al., 2013).

**Protease production**

Isolate *V. arenosi* PH15 was observed to be positive for protease production expressed in terms of clear zone (5 mm) around the bit placed on skimmed milk agar plates (Figure 3). Protease producing micro-organisms has their role in eradication of some fungal and bacterial pathogens as this enzyme hydrolyzes proteins. This comes under the category of biocontrol agent.

**Conclusion**

In the current study, bacterial isolate *V. arenosi* PH15 isolated from rarely explored rhizosphere of *P. hexandrum* - a medicinal plant grown in District Kinnaur Himachal Pradesh, India. This strain was chosen on the basis of *in vitro* antagonistic assay and was identified by 16SrRNA gene technique. This isolate exhibited diverse plant growth promoting traits namely: siderophore production, phosphate solubilization, HCN production, ammonia and protease production. Thus, the study divulges the *V. arenosi* PH15 as a potential plant growth promoting rhizobacteria in the context of cold agro-ecosystems, where introduced mesophilic inoculants would fail. Hence, it is concluded that first time reported *V. arenosi* PH15 as a PGPR can be set up as an inoculant to attain the preferred results of bacterization. However, different field trials and the interaction of this PGPR with other native soil microflora have to be assessed in future.

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**REFERENCES**


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