

Original Research Article

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Studies on *Bacillus* sp, As an Efficient Plant Growth Promoting Rhizobacteria from *Taxus wallichiana* Zucc. an Endangered Conifer of Kashmir Himalaya

Nazish Nazir^{1*}, Azra N. Kamili¹, M.Y. Zargar⁴, Imran Khan²,
Durdana Shah¹, Sumira Tyub¹ and Rubiya Dar³

¹Centre of Research for Development, ²Department of Environmental Sciences,

³Department of Biotechnology, University of Kashmir, Srinagar- 190006, J&K, India

⁴Sher-e-Kashmir University of Agricultural Sciences and Technology Kashmir,
Shalimar Campus, Srinagar- 190025 J&K, India

*Corresponding author

ABSTRACT

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The rhizospheric flora plays a vital role in the growth and establishment of the forest trees especially at early stages. *Taxus wallichiana*, being a slow growing conifer of Kashmir Himalaya, has been studied for isolation and characterization of the growth promoting bacteria from its rhizosphere. Two species of *Bacillus* namely *B. subtilis* and *B. safensis* were isolated and characterized by 16S rRNA gene technique. These bacteria have showed effective growth promoting properties like phosphate solubilization and IAA production. During this study both species were found quite effective and reliable sources for use as bio-fertilizer for improving the growth of this endangered forest tree under natural conditions.

Introduction

Forest ecosystems are highly organized systems of biotic and abiotic components and are both structurally and functionally, linked as a sensitive dynamic equilibrium (Kraigher, 1999). Soil microbial diversity plays a key role in maintaining this dynamic equilibrium along with multi functionality of forests by supporting processes such as litter decomposition and organic matter mineralization (Cardinale *et al.*, 2011) by transferring of matter and energy between above and belowground communities (Jing *et al.*, 2015). Like other forest ecosystems of world, Climate change coupled with

population pressure have deteriorated the Himalayan ecosystems too, while the degradation and habitat fragmentation are clearly evident (Shrestha *et al.*, 2012). Over the last century, Northwestern Himalayan forest coverage have showed tremendous decline because of enhanced anthropogenic interferences (Qamer *et al.*, 2016). The decrease in forest area is countered usually by afforestation programmes involving planting of the trees in deforested areas. But there are some major difficulties in successful afforestation programmes because of less percentage of adaptation and acclimatization

of planted trees. Thus some alternative strategies have to be chalked out that can ensure enhanced growth of tree species in forest ecosystems, which will provide protection and environmental safety in order to maintain long term ecological balance in forest ecosystems. The use of microbial inoculants or Plant Growth Promoting Rhizobacteria (PGPR) for the enhancement of sustainable forestry production is one such alternative which has been widely accepted and used in many parts of the world (Majeed *et al.*, 2015).

Taxus wallichiana, an evergreen medium sized conifer growing in Kashmir Himalaya (Thomas, 2011), has been over exploited owing to its durable and strong nature of wood as well as medicinal value (Rahman *et al.*, 2013). Presence of "taxol" in its shoots and leaves has shown an exciting potential as an anti-cancer drug, particularly in the treatment of ovarian cancers (Gholami *et al.*, 2014). Due to its medicinal properties and other commercial values there is increased pressure on the natural populations of this plant.

The over exploitation coupled with poor natural regeneration potential, has led to the tremendous decrease in its populations (Sharma and Uniyal, 2010). So the need of the hour is to regenerate its population in nature by planting the seedlings in the natural environment. In this regard, like other afforestation programmes use of PGPRs can be used for establishment of *Taxus walliachiana* especially during early stages. Thus, to meet the challenge, present study on the PGPRs of *T. wallichiana* was carried out. The present work emphasizes the role of predominant *Bacillus* species because of their ability of forming resistant endospores and also these have been reported to promote plant growth by indirectly affecting symbiotic N₂ fixation, nodule occupancy (Amin *et al.*,

2015). Hence the main objective of the present study was to isolate the potential *Bacillus* species from the rhizospheric soil of *T. wallichiana* for using these isolates as PGPR inoculant in order to find an alternative strategy to chemical fertilizers for its sustainable growth and development.

Materials and Methods

Study area

Present study was undertaken in the Tangmarg forest area of Kashmir Himalaya. It is located at latitude 34°03'40.8''N and longitude 74°25'35.4''N and an elevation of 2128 m above the sea level in the District Baramulla of J&K, India. The dominant forest vegetation is mainly represented by *Pinus walliachiana* in lower altitudes (1650-2500 masl) and by *Abies pindrow* and *Picea smithiana* at higher altitudes (2300-3300 masl). The topography is mainly hilly and mountainous with stretches of plains and valleys in between. The area is characterized by temperate climatic conditions with four distinct seasons. The monthly mean temperature ranges from a minimum of -4°C to a maximum of 32.5°C accompanied by a severe cold and snow fall in winter. For the present study three sites were selected at a distance of around 1 km within sites (Figure 1).

Collection of rhizospheric soil

The rhizospheric soil samples of *Taxus wallichiana* were collected from three selected sites in the months of June-August 2014. Rhizospheric soil was shaken from the roots and collected in sterilized plastic ziplock bags and was brought to Microbiology laboratory of the Centre of Research for Development (CORD), University of Kashmir for immediate processing.

Isolation of rhizobacteria

Rhizobacteria were isolated from 1 g of soil tightly adhering to the root by serial dilution plating Luria-Bertani (LB) agar plates as described by Parray *et al.*, (2015). The plates were incubated at $28 \pm 2^\circ\text{C}$ till the appearance of bacterial colonies. Individual colonies were picked and streaked on LB plates for further purification. A total of twelve isolates were obtained in this manner and were maintained on agar slants and stored at 4°C for further processing.

Characterization of isolates

The isolates were characterized on the basis of morphology using Bergey's Manual of Systematic Bacteriology (Bergey *et al.*, 1986) and by performing Gram's staining to know whether the isolates were gram positive or gram negative. The isolates were observed under the light microscope to obtain the colony morphology and cell shape. Various carbohydrate utilizing tests like citrate, arginine, sucrose, mannitol, glucose, arabinose and trehalose for biochemical characterization were subjected to biochemical tests using strain specific biochemical kits (Hi-media), separately for Gram positive and Gram negative strains. The strains were identified as per the chart sheet of the kits to the nearest value. Pure cultures of the isolates were stored at -20°C in nutrient broth supplemented with 200 mg/g glycerol, for the screening of growth promoting activities.

Bioassays for plant growth promoting characteristics of rhizobacterial isolates

All the twelve bacterial isolates were subjected to various Plant Growth Promoting characteristics. Out of which only two isolates TR5 and TR7 showed the efficiency to produce all the essential PGP traits.

The amount of soluble phosphate of the bacterial isolates was measured by the colorimetric method as described by King (1959). Quantitative estimation of IAA production was detected as described by Brick *et al.*, (1991). HCN and Ammonia production was done using Castric's method (Castric, 1975) and as described by Cappucino and Sherman (1992) respectively. The catalase production was checked by placing a drop of H_2O_2 onto the bacterial colony on a glass slide as described by Schaad (1992). These two potential isolates (TR5 and TR7) were subjected for identification as per 16S rRNA gene sequencing.

Identification of isolates as per 16S rRNA gene partial sequencing

Total genomic DNA of two bacterial isolates was extracted using HiPurATM Bacterial Genomic DNA Purification Kit (HIMEDIA MB505-50PR). Amplification of 16S rRNA gene using universal bacterial primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1429R (5'-GGTTACCTTGTTACGACTT-3') (Sujatha *et al.*, 2012), was carried out in a 50 μl PCR reaction mixture containing miliQ (33.4 μl), dNTPs (5 μl), Taq buffer (5 μl), FP (1 μl), RP (1 μl), DNA Template (4 μl), Taq Polymerase (0.5 μl). Amplification was performed with an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 45 sec and 72°C for 45 sec and a final extension of 72°C for 10 min.

The integrity of DNA was checked on 0.8% agarose gels for genomic DNA and 1.5 to 2% agarose gels for PCR amplicons. Amplified products were sequenced with respective primers using Sanger sequencing methodology at Scigenom, Cochin, India. Sequences were submitted to NCBI Gene Bank data base and accession numbers were obtained.

Results and Discussion

Twelve bacterial isolates were obtained from the rhizospheric soil of *T. wallichiana*, the bacterial isolates showed creamy, white to yellow color with variable sizes and margins on LB agar plates. The cells were mostly motile, rod shaped showing positive and negative Gram's reaction (Table 1) and were simultaneously subjected to the biochemical characterization for various carbohydrate utilizing tests, out of which two isolates TR5 and TR7 were found to be more effective in utilizing carbohydrate sources (Table 2).

All the twelve isolates were then screened for various plant growth promoting activities which included Phosphate solubilization, production of IAA, HCN, Ammonia and Catalase activity (Table 3). The isolates TR5 and TR7 showed the development of the highest phosphate solubilizing zone and quantitatively they also produced 405 µg/l and 380 µg/l inorganic phosphate respectively and produced efficient amount of IAA with the production of 290 µg/ml and 272 µg/ml respectively. The other plant growth promoting properties like HCN, Ammonia and Catalase activities were also shown by both isolates (Table 3).

Since the two bacterial isolates namely TR5 and TR7 were found to be efficient and potent for the various plant growth promoting activities so these two isolates were subjected for the identification as per 16S rRNA gene sequencing. The sequences obtained after PCR amplification using specific primers for bacteria were compared with the available data in the GenBank using the BLAST. On the basis of the percentage identity TR5 strain was identified as *Bacillus subtilis* and TR7 as *Bacillus safensis* (Table 4). It has been estimated that more than 100 million tons of NPK chemical fertilizers are used annually in

order to increase plant yield (Chandra *et al.*, 2010). The potential negative effect of chemical fertilizers on the global environment and the cost associated with production has led to research with the objective of replacing chemical fertilizers with bacterial inoculants isolated from rhizospheric soil commonly referred as Plant Growth promoting rhizobacteria. PGPRs enhance the growth of plant and reduce the damage from soil borne pathogens (Kloepper *et al.*, 1980). Inoculation with PGPR have been tried successfully in tree species like *Pinus*, *Picea*, *Tsuga*, *Pseudotsuga* and *Eucalyptus* (Kovaleva *et al.*, 2015) for increase in shoot and root length (Gujanovic *et al.*, 2012). The bacterial genera which have been frequently used in the PGPR inoculation technique are *Rhizobium* (Afzal and Bano, 2008), *Bacillus* (Orhan *et al.*, 2006), *Pseudomonas* (Naiman *et al.*, 2009), *Enterobacter* (Garcia *et al.*, 2011), *Serratia* (Nico *et al.*, 2012) and *Pantoea* (Khalimi *et al.*, 2012).

In the present study after screening of 12 bacterial isolates two isolates were found able to produce plant growth promoting activities like Phosphate solubilization, IAA, HCN, Ammonia production as well as catalase activity. These two bacterial strains after molecular characterization using specific primers for 16S rRNA gene were identified as *Bacillus subtilis* and *Bacillus safensis*. Because of the special physiological capabilities like the formation of extremely resistant spores and production of metabolites that have antagonistic effects on other microorganisms, *Bacillus* spp. are found in every environment and compete desirably with other organisms within the environment (Vargas *et al.*, 2004). Asif *et al.*, (2012) have reported that in Himalayan Cypress seedlings various species of *Bacillus* enhanced the supply of plant nutrients including essential nutrients like Nitrogen, Phosphorus, Potassium. Higher biomass production in

Scots pine and Norway spruce was found when inoculated with *Bacillus megaterium*, *B. circulans*, *B. licheniformis*, *B. pumilus*, *B. amyloliquefaciens* by Karlicic *et al.*, (2016).

In the present study bacterial species isolated from the rhizospheric soil of *Taxus wallichiana*, the TR5 and TR7 showed more potential to be used as PGPR's inoculum. Asif *et al.*, (2013) reported *Bacillus subtilis* with other bacterial strains producing efficient amount of phosphate helps to increase the nutrient uptake and availability to increase the growth of Blue Pine (*Pinus wallichiana*) seedlings under the nursery conditions. *Bacillus licheniformis*, *Aeromonas*

hydrophila, *Pseudomonas putida* and *Burkholderia cepacia* rich in inorganic phosphate were observed to enhance the growth of *Robinia pseudoacacia* L. and *Pinus sylvistris* L. seedlings (Vera *et al.*, 2015).

Indole acetic acid (IAA) is a common natural auxin and is a product of L tryptophan metabolism in microorganisms. *Bacillus subtilis* and *Bacillus safensis* tested in the present study were positive for IAA production which is an important PGPR and alters root architecture and upgrade plant development (Spaepen and Vanderleyden 2011).

Table.1 Morphological characteristics of rhizobacterial species isolated from *Taxus wallichiana*

Isolate	Colony size	Colony color	Cell motility	Cell Shape	Gram Reaction
TR1	Large round	Creamy	Slowly motile	Medium rods	-
TR2	Small round	White	Slowly motile	Oval	+
TR3	Large round	Yellow	Motile	Medium rods	-
TR4	Medium round	Creamy	Slowly motile	Small rods	-
TR5	Medium wavy	Off- white	Highly motile	Small rods	+
TR6	Small round	Yellow	Motile	Oval	-
TR7	Medium round	Creamy	Highly motile	Medium rods	+
TR8	Large round	White	Motile	Small rods	+
TR9	Large round	Off-white	Slowly Motile	Oval	-
TR10	Small wavy	Creamy	Highly motile	Thin rods	-
TR11	Large round	Yellow	Motile	Thin rods	+
TR12	Small round	Off-White	Motile	Thin rods	-

Table.2 Biochemical characterization of rhizobacterial isolates

Isolate	Citrate	Arginine	Sucrose	Mannitol	Glucose	Arabinose	Trehalose
TR1	-	-	-	+	+	-	-
TR2	+	-	-	-	+	+	-
TR3	-	-	-	+	-	-	+
TR4	+	-	-	-	-	-	-
TR5	+	+	+	+	+	+	+
TR6	+	+	-	-	-	-	+
TR7	+	+	+	+	+	+	+
TR8	-	-	-	-	-	+	+
TR9	+	+	-	-	+	-	-
TR10	-	-	-	-	-	+	-
TR11	+	+	-	-	-	+	-
TR12	-	-	-	+	-	-	+

+ shows presence; - shows absence

Table.3 Plant growth promoting trait analysis of Rhizobacterial strains

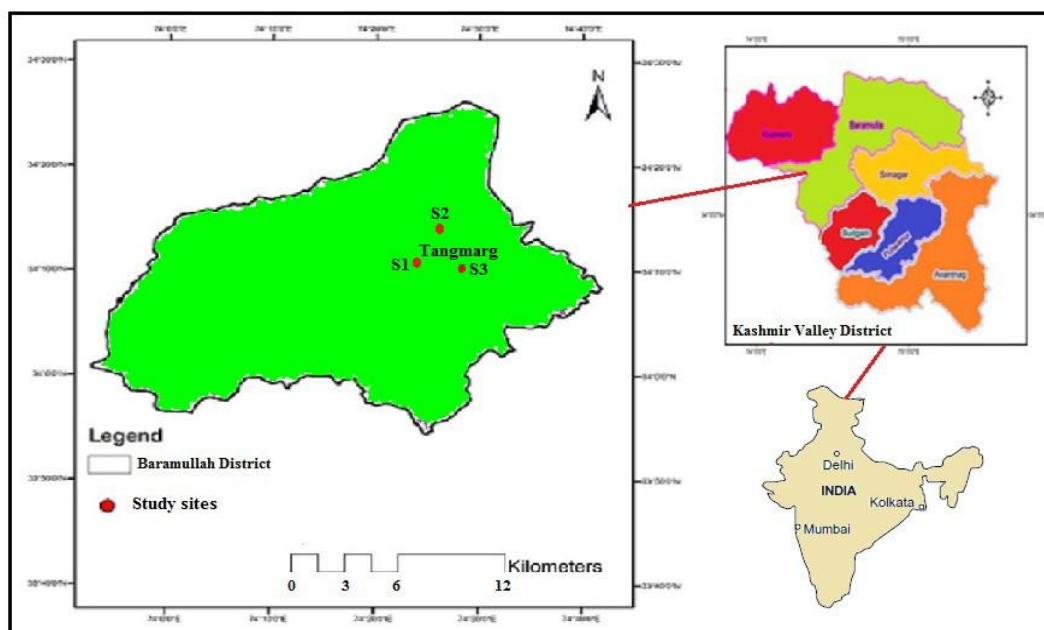
Isolate	PGP trait analysis				
	Phosphate solubilization (µg/l)	IAA production (µg/ml)	HCN production	Ammonia production	Catalase activity
TR1	—	—	—	—	+
TR2	205	—	—	+	+
TR3	220	—	—	—	—
TR4	240	—	—	—	+
TR5	405	290	+	+	+
TR6	—	—	+	—	—
TR7	380	272	+	+	+
TR8	340	—	—	—	—
TR9	320	—	+	—	+
TR10	—	—	—	—	—
TR11	—	—	—	—	—
TR12	195	—	—	—	+

+ shows presence; - shows absence; Data are the mean values of three replicates each and all experiments were repeated twice for confirmation of results.

Table.4 BLAST search results of isolated bacterial species

Isolate	Name	% Similarity	Gene bank acct. No.	Fragment size (bp)
TR5	<i>B. subtilis</i>	99%	NR_118486.1	1206
TR7	<i>B. safensis</i>	85%	NR_113945.1	1208

Fig.1 Map of the study area showing study sites



Earlier Khin *et al.*, (2012) have reported IAA production from the *Bacillus* spp. and subsequent role in development of maize seedlings. Similarly, Rodge *et al.*, (2016) have studied the bacterial isolates including *Bacillus* spp. for the production of IAA from the roots of *Ficus religiosa*. Effects of IAA producing *Bacillus subtilis* were also observed by Reetha *et al.*, (2014) to enhance the germination of onion seeds.

A few bacterial species are known to produce and excrete hydrogen cyanide (HCN), a potent inhibitor of cytochrome *c* oxidase and several other metallo enzymes (Blumer and Haas, 2000). Hydrogen cyanide (HCN) suppresses the growth of fungal pathogens and promotes a successful competition with pathogens for nutrients or specific niches on the root surface (Cartieaux *et al.*, 2003). Rayavarapu and Padmavathi, (2016) have used the *Bacillus* spp. as biofertilizer for increasing the crop yield instead of using chemical fertilizers. Earlier, Agarwal and Agarwal, (2013) reported that *Bacillus* species produces HCN from rhizospheric soils and hence indirectly influences plant growth promotion. The *Bacillus* spp. Isolated in present study also showed the HCN production so can also be used as biofertilizer.

Ammonia production by the plant growth promoting bacteria helps to influence plant growth indirectly (Geetha *et al.*, 2014). In the present study both *B. subtilis* and *B. safensis* isolated from rhizospheric soil of *T. wallichiana* showed ammonia production and similar results were reported by Kumar *et al.*, (2012) from French bean soils. Evolution of gas bubbles from the H₂O₂ solution showed positive test for the presence of catalase enzyme by the organisms. Our results are in line with Kannahi and Kowsalya, (2013) pertaining to catalase activity in *Bacillus* spp. However, the presence of catalase activity in *Bacillus* spp. has been reported by some other workers as well (Singh *et al.*, 2015).

Current study is first of its kind in the Kashmir Himalayan region regarding the insight into the bacterial community present in rhizospheric region of this endangered forest tree. P-solubilizing and IAA are considered important PGP traits, hence in the present study we found *B. subtilis* and *B. safensis* efficient P-solubilizing and IAA producing bacteria among the natural population. These isolates offer potential field application as PGP agents in *Taxus wallichiana* an endangered conifer of Kashmir Himalaya. Further studies should be carried out in order to get a detailed functional characterization of these *Bacillus* species for the practical application in natural environs.

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