Environment and Ecology 37 (3A) : 894—908, July—September 2019
Website: environmentandecology.com ISSN 0970-0420

Multitrate Phosphate Solubilizing Bacteria from *Dalbergia sissoo* Roxb. Rhizosphere in Natural Forests of Indian Central Himalayas

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Received 25 February 2019 ; Accepted 29 March 2019 ; Published on 19 April 2019

Abstract Plant growth promoting rhizobacteria (PGPR) are the rhizosphere bacteria that may be directly or indirectly utilized to augment plant growth. *Dalbergia sissoo* Roxb. (common name shisham) is a leguminous tree known internationally for its highly valued timber. The objectives of this study were to characterize bacterial diversity indigenous to *D. sissoo* rhizosphere in natural forests located at Lachhiwala, Tanakpur and Pantnagar in Uttarakhand. Rhizospheric soil from all three sites was analyzed for biotic (viz. total viable bacterial count (TVC) and soil enzyme activities) as well as abiotic (pH, organic carbon, EC and available N, P, K and micronutrients) properties. Highest bacterial count was recorded at Tanakpur followed by Lachhiwala and Pantnagar. A strong positive correlation was observed between all the enzyme activities and soil nutrients (macro and micro). Since phosphatase enzyme activity and available phosphate status in soil at all sites was higher. Therefore, phosphate solubilizing bacteria (PSB) were recovered and characterized. In addition, all isolates exhibited one or more enzymatic (amylase, lipase, pectinase, protease, urease, xylanase) and plant growth promoting (PGP) traits like the production of siderophore, HCN, IAA production, ammonia and solubilization of zinc and phosphorus. Out of all, strain L8 from Lacchiwala and P2 from Pantnagar showed a maximum number of PGP-positive traits. Amongst 18 isolates, seven were identified as *Pseudomonas* sp., four as *Streptomyces* sp., two as *Klebsiella* sp., two as *Staphylococcus* sp. and one each as *Pantoea* sp., *Kitasatospora* sp. and *Micrococcus* sp. based on morphology and 16S rDNA sequence analysis. These results enhance our knowledge of bacterial diversity in shisham rhizosphere. Moreover, Since these isolates exhibited multiple traits beneficial to the host plants, they can serve as potential bioinoculant candidates for the soil-plant system to increase their growth as well as productivity.

Keywords Natural forests, *Dalbergia sissoo* Roxb. rhizosphere, Indian central Himalaya, Phosphate solubilizing bacteria, Multiple plant growth promoting attributes.

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Introduction

Dalbergia sissoo Roxb. common name shisham is an important multipurpose leguminous tree species within the genus *Dalbergia* of family Fabacae. Primarily its timber is highly valued and important for country's economy. Shisham tree faces many biotic and abiotic stresses during growth phase leading to reduced growth and productivity (Javaid 2008). The

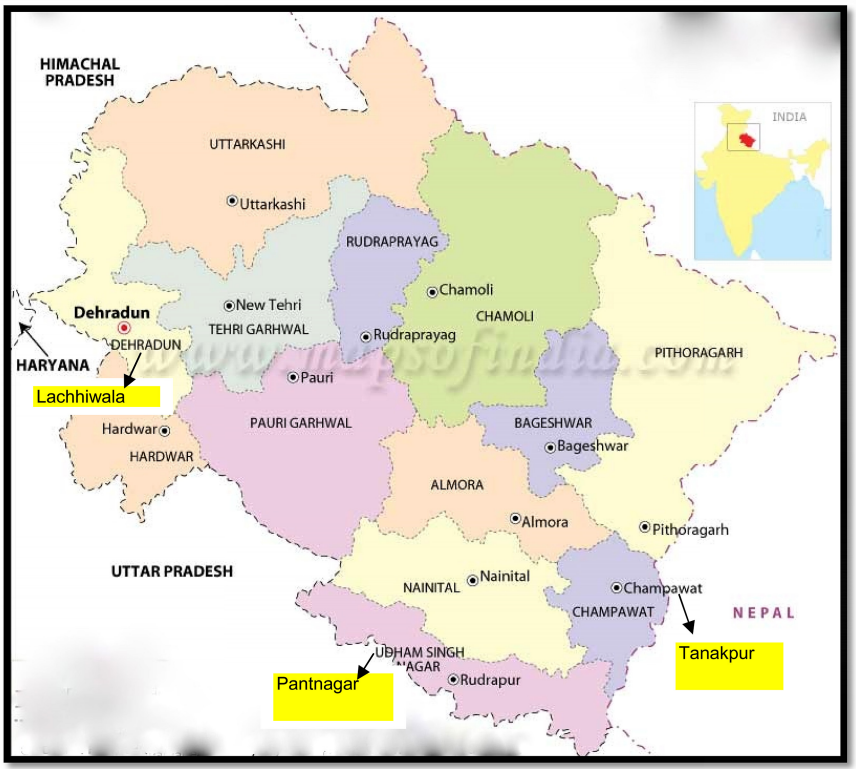

Fig. 1. Map showing the sampling site for efficient phosphate solubilizing bacteria.

plant growth and productivity is influenced by rhizospheric microbes the most. Whereas, rhizospheric bacterial population, diversity and interactions are strongly affected by plant species and soil chemical and physical properties. Since *Dalbergia sissoo*, tree has commercial value, its enhanced production can be achieved by exploring growth promotion activities of native indigenous rhizospheric microflora. For successful manipulation of microbial populations in the plant rhizosphere for the benefit of the host plant, a better understanding of soil biotic and abiotic properties is imminent. The microorganism associated with its roots may have potential for application as biofertilizer and biocontrol agent (Bhardwaj et al. 2017). Rhizospheric bacteria promote plant growth either by direct or indirect mechanisms. Direct plant growth promotion (PGP) involves production of plant hormones, accessibility of biologically fixed nitrogen, solubilizing mineral phosphates and other minerals like zinc and potassium and enhancing stress resistance through ACC (1–aminocyclopropane–1–carboxylate) deaminase enzyme production whereas, indirect mechanism includes suppression of fungal, bacterial, nematode and viral pathogens (Barea et al. 2005). These PGP mechanisms have been well documented in a large number of bacterial species within Proteobacterial and Firmicutes. Prominent examples are *Pseudomonas* and *Bacillus* (Kogel et al. 2006). The yield increase in crops like maize, tea, soybeans, alfalfa, wheat and onion simply by PGPR inoculation have been reported (Agbodjato et al. 2015). Furthermore, microbial inoculants enhanced growth of *Dalbergia sissoo* seedlings grown under stress conditions (Bisht et al. 2009a). Therefore, screening and selection of effective PGPRs and their utilization is of great importance. Phosphorus is one of the least available and the least mobile mineral nutrients in soil for plant growth. Only 0.1% of the total phosphorus from soil is available to plants (Tilak et al. 2005). Phosphate solubilizing microorganisms i.e. bacteria, fungi and actinomycetes can convert insoluble phosphates into soluble forms and make it easily available to plants. The composition and structure of phosphate-solubilizing microorgan-

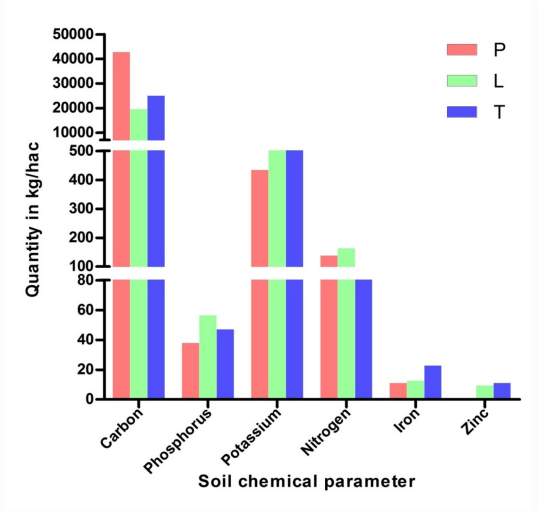

Fig. 2. Soil physico-chemical properties at three different *Dalbergia sissoo* provenances.

isms (PSM) within rhizospheric soil vary greatly and are influenced largely by the plant species and physico-chemical characteristics of soil (Khan et al. 2014). Dash et al. (2013) found that the application of phosphate solubilizing microorganism greatly affects the biomass of *Dalbergia sissoo* as compared to untreated plants. PSB can be characterized under different morphological, physiological and biochemical traits to see their diversity and adaptability for different agroecologies. This would help in selecting the most efficient strain for use as biofertilizers. This study is, therefore, aimed at isolation and characterization of efficient phosphate solubilizing bacteria from different agroecological regions of Uttarakhand and screen them for PGP capabilities.

Materials and Methods

Geographical characterization of study area

This study was carried out in natural *Dalbergia sissoo* forests located at three sites ; Pantnagar (29º3′0′′ N latitude and 79°31′0′′ E longitude), Lachhiwala (30.2099°N latitude, 78.1342° E longitude) and Tanakpur (29.0722°N latitude, 80.1066° E longitude) in Uttarakhand (Fig. 1). The three sites represent different agroecological zones and niches each diversified with distinct vegetation cover, soil and other natural resources.

Soil sampling

All the rhizospheric soil samples of *D. sissoo* forests were collected during December, 2016 from Pantnagar, Lachhiwala and Tanakpur. Five quadrads of 10 × 10 m^2 were laid down randomly at all the locations. Further, three trees of each forest location were selected within a distance of 1 to 10 m from each other. For each individual tree, the rhizosphere soil was sampled vertically along the base of the plant. Finally, samples of each tree in triplicate were mixed to generate a representative composite sample and transferred in sterilized polythene bags to rhizosphere biology laboratory at Department of Microbiology, GBPUAT Pantnagar. Soil samples were stored at –20°C for further analysis.

Soil physico-chemical characteristics

Soil samples were air-dried. Soil pH was measured with a glass electrode of micro processor based pH meter, century CP 931 (Miller and Donochue 1990) with a soil/distilled water ratio of (1/5). Soil electrical conductivity (EC) was measured in 1 : 25 solution (soil to water) at 25ºC with digital microprocessor based conductivity meter (Systronic Model 306). Total organic carbon (OC) was determined by wet oxidation method using $K_2Cr_2O_7$ and concentrated H_2SO_4 (Jones et al. 1990). Total N (TN) was estimated by the Kjeldahl digestion method (TKN). Available phosphorus (AP) content was measured by colorimetery after extraction with 0.5 mol l^{-1} $NaHCO_3$ (pH 8.5) for 30 minute (Olsen et al. 1954). Available potassium (AK) content was measured with a flame photometer after extraction with 1 $moll^{-1}$ NH_4Ac (pH 7.0) 30 minute (Yuan 1983). Minor trace elements like iron and zinc were measured using an atomic absorption spectrometry (Yao et al. 2003). Results were statistically analyzed through one-way analysis of variance (ANOVA) at $p < 0.05$ using SPSS software.

Soil enzymatic assays

The functional potential of the soil microbial communities inhabiting shisham rhizosphere was monitored

Table 1. Morphological, biochemical and PGP properties of phosphorus solubilizing bacteria from *Dalbergia sissoo* natural forests. + –Slightly positive ; Strongly positive. Stains positive for maximum number of traits are in bold.

Cultural characteristics						Biochemical characterization					
Geographical location	Isolate code	Gram reaction	Cell morphology	Amylase	Urea hydrolysis	Nitrate reduction	Lipase activity	Xylanase activity	Protease activity	Pectin hydrolysis	Catalase production
Lachhiwala	L1	Negative	Small rods	–	–	+	–	–	++	++	++
	L2	Positive	Cocci	–	–	++	–	–	++	–	++
	L3	Negative	Small rods	–	–	+	–	–	–	–	++
	L4	Negative	Small rods	–	++	–	–	–	–	–	++
	L5	Negative	Small rods	–	–	+	–	–	++	++	++
	L6	Positive	Filamentous	–	–	–	–	–	–	–	–
	L7	Positive	Filamentous	+	–	–	++	++	+	–	–
	L8	Negative	Small rods	+	–	–	+	++	+	–	++
Pantnagar	P1	Negative	Small rods	–	–	+	–	+	–	++	++
	P2	Negative	Small rods	–	+	++	++	–	++	–	++
	P3	Negative	Small rods	–	–	+	–	–	–	++	++
	P4	Negative	Small rods	–	–	+	–	+	–	++	++
Tanakpur	T1	Positive	Filamentous	–	–	–	–	++	–	++	–
	T2	Negative	Small rods	–	++	+	–	–	–	–	+
	T3	Positive	Filamentous	+	–	–	+	+	–	–	–
	T4	Positive	Cocci	–	–	–	–	+	–	–	++
	T5	Positive	Filamentous	++	–	–	+	++	+	–	+
	T6	Positive	Cocci	–	++	–	–	–	–	–	+

Table 1. Continued.

Cultural characteristics				PGPR properties of phosphorus solubilizing bacteria				
Geographical location	Isolate code	Gram reaction	Cell morphology	Zinc solubilization	Siderophore production	Indole acetic acid production	HCN production	Ammonia production
Lachhiwala	L1	Negative	Small rods	–	+	+	–	++
	L2	Positive	Cocci	–	–	–	–	+
	L3	Negative	Small rods	++	–	–	–	++
	L4	Negative	Small rods	+	–	++	–	++
	L5	Negative	Small rods	++	–	+	–	++
	L6	Positive	Filamentous	–	–	+	–	–
	L7	Positive	Filamentous	–	++	+	–	–
	L8	Negative	Small rods	–	++	+	–	+
Pantnagar	P1	Negative	Small rods	–	–	+	–	++
	P2	Negative	Small rods	++	–	–	+	++
	P3	Negative	Small rods	+	–	++	–	++
	P4	Negative	Small rods	+	–	+	–	++
Tanakpur	T1	Positive	Filamentous	–	++	++	–	–
	T2	Negative	Small rods	++	–	++	–	++
	T3	Positive	Filamentous	–	++	+	–	–
	T4	Positive	Cocci	–	–	++	–	+
	T5	Positive	Filamentous	–	–	+	–	+
	T6	Positive	Cocci	–	–	–	–	+

by estimating activities of five enzymes dehydrogenase, fluorescein diacetate, acid phosphatase, alkaline phosphatase and urease in DS rhizospheric soil from different provenances. The hydrolyzed product of

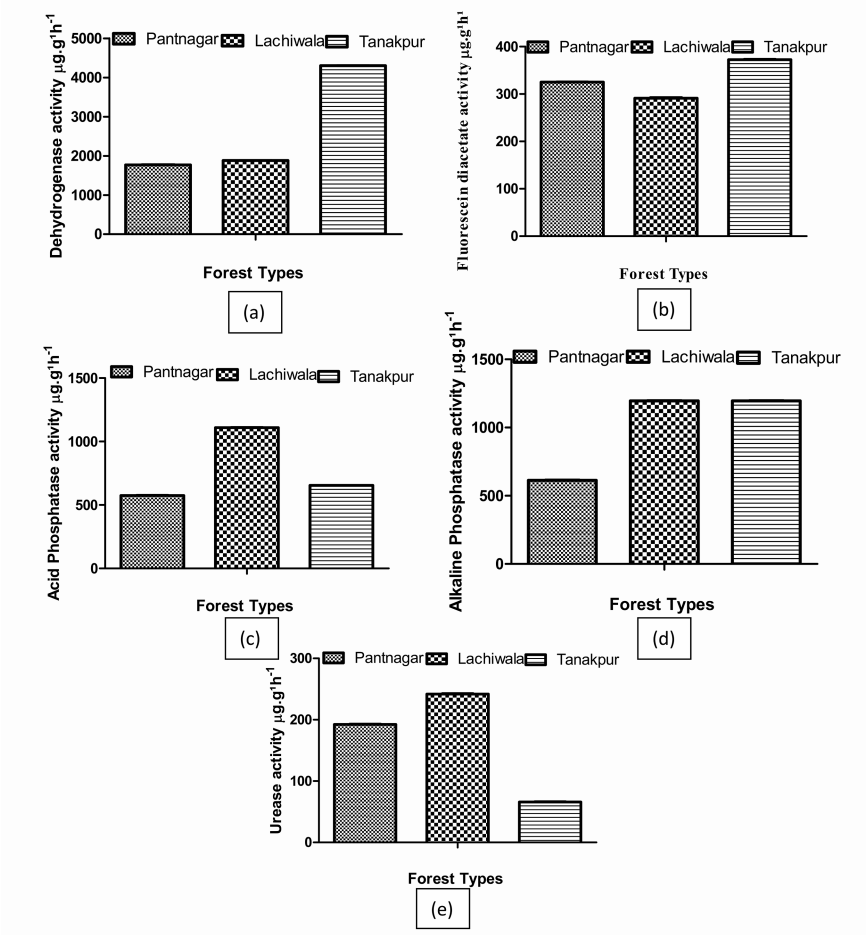

Fig. 3. Enzyme activities in rhizospheric soil of three different *Dalbergia sissoo* provenances (a) Dehydrogenase (b) Fluorescein di acetate (c) Acid phosphatase (d) Alkaline phosphatase (e) Urease. Graphs are plotted with mean values (n=3) plus standard error.

each enzyme was analyzed through spectrophotometer and compared with a standard curve. All assays were conducted in triplicates. Dehydrogenase activity was determined as reported By Thalmann (1968). FDA activity was determined according to Inbar et al. (1991). Alkaline and acid phosphomonoesterases activity was assayed according to a method of Tabatabai and Bremner (1969). Urease activity was determined as given by Kandeler and Gerber (1988). The variation in soil enzymatic activities was determined statistically by analysis of variance (one factor ANOVA).

Enumeration of bacteria in rhizospheric soil

The total aerobic bacterial count was enumerated through serial dilution pour plating on Angles's medium (Angle et al. 1991) whereas of phosphate solubilizing bacteria on Pikovskaya medium (Pikovskaya 1948). The number of bacteria per gram of soil was determined by counting and expressing as colony forming unit (CFU) after 2–3 days of incubation at 30 ±1°C. Both the media were supplemented with 100 mgL^{-1} of cycloheximide to inhibit fungal growth. Results were statistically analyzed through one-way analysis of variance (ANOVA) at $p<0.05$ using SPSS software.

Isolation of phosphate solubilizers and solubilizing efficiency

Phosphate solubilizing bacteria were isolated using

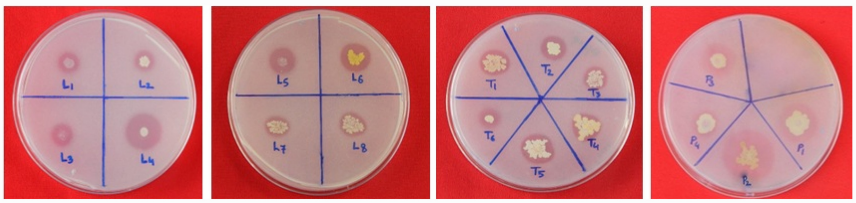

Fig. 4. Solubilization of inorganic tri-calcium phosphate *in vitro* by bacterial isolates recovered from *Dalbergia sissoo* Roxb. rhizosphere at Lachhiwala, Tanakpur and Pantnagar after 7 days of growth at 28 ± 2°C. The formation of halo zone around the colonies shows the solubilization of inorganic phosphate.

serial dilution pour plate technique on Pikovskaya's medium (PK medium) (Chen et al. 2006). Plates were incubated at 28–30°C for 3–4 d for bacterial growth. The colonies surrounded with halo zones were picked and restreaked to obtain pure cultures. All pure cultures were spot inoculated on Pikovskaya medium and incubated at 30°C for 48 h. Halos surrounding the colonies were measured and the solubilizing efficiency (SE) was calculated as follows (Pikovskaya 1948) :

$$\text{Solubilizing efficiency (SE)} = \frac{\text{Solubilization diameter}}{\text{Growth diameter}} \times 100$$

Morphological and microscopic characterization of efficient PSBs

Cell morphology and gram's reaction of all 18 isolates were studied (Prescott et al. 1999).

Biochemical characterization of PSBs

Starch hydrolysis ability of bacterial isolates was assessed by growing the bacteria on glucose yeast extract peptone agar (GYP) medium with 0.2% soluble starch pH 6.0 (Kasana et al. 2008). After incubation at 30°C for 2 days, the plates were flooded with 1% iodine in 2% potassium iodide. To assess usea hydrolysis, Log phase bacterial culture was spotted on nutrient agar plates supplemented with phenol red and 2% urea (w/v) and incubated at 30°C for 2 days. The formation of pink colored zone was indicative of positive test (Cappuccino and Sherman 1996). Nitrate reductase test was performed by inoculating 0.5 ml of log phase culture into 10 ml of the nutrient broth containing 1% KNO_3 and incubated at 28 ± 1°C for 5 days. After incubation at 28 ± 1°C for 5 days, few drops of sulphanilic acid and α - Napthylamine (5 g/1 in 5 M acetic acid) was added to the test tubes. Development of red color within minutes was considered as positive and absence of color indicates negative test. To detect Lipase production log phase bacterial cultures was inoculated on nutrient agar plates supplemented with 1% (w/v) Tween 80. The deposition of precipitate around the colony indicated positive test. Xylanolytic activity was determined by growing the bacteria in xylanase activity indicator medium containing 0.5% (w/v) xylan and 1.5% agar (w/v) (Farkas et al. 1985). After the incubation at 28±1°C for 3–4 days period, the plates were flooded with 1% iodine in 2% potassium iodide. A clear zone around the bacterial colony indicated positive test. For examining protease activity log phase bacterial isolates were spotted on Skim milk agar plates and incubated at 28°C for two days. The isolates that produced protease were identified by clear zone around bacterial colony (Shaheen et al. 2008). Pectinolytic activity was determined by growing the bacteria in Pectin Agar medium. After the incubation period, the plates were flooded with 1% iodine in 2% potassium iodide (Hankin and Anagnostakis 1975). A clear zone formation around the bacterial colony indicated pectinolytic activity. To detect the presence of catalase enzyme in different isolates, a smear of culture was made on a clean and dry glass slide. A drop of H_2O_2 was added and mixed with a smear on slide. The production of gas bubbles and effervescence constituted a positive test (Aneja 2006).

Plant growth promoting (PGP) traits of PSBs

Zinc solubilization

The log phase bacterial cultures were spot inoculated

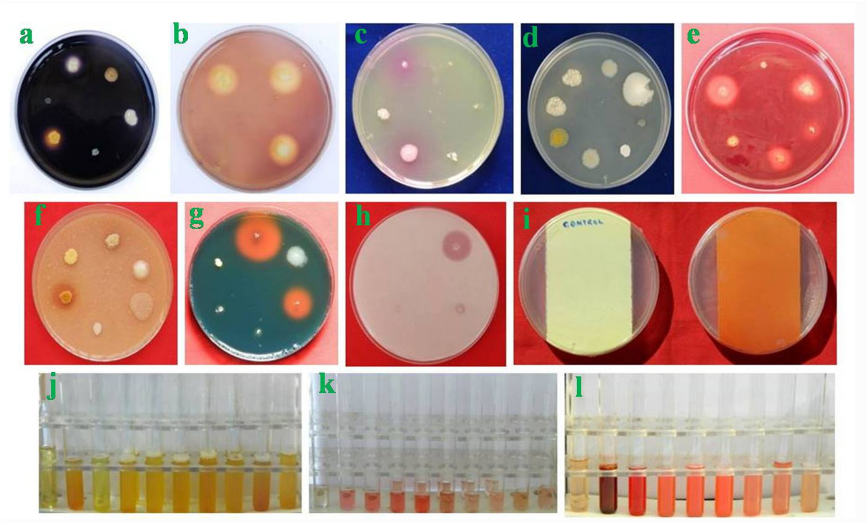

Fig. 5. Biochemical and plant growth promoting properties of isolates (a) Amylase (b) Pectinase (c) Urease (d) Lipase (e) Xylanase (f) Protease (g) Siderophore production (h) Zinc solubilization (i) HCN production (j) Ammonia production (k) IAA production (l) Nitrate reduction.

on nutrient agar medium supplemented with ZnO and $ZnCO_3$ (0.1%) . The plates were incubated at 28 ± 1°C for 5 days. The plates were observed for zone of clearance around the bacterial colonies. The zone and zone size was correlated to the zinc solubilization ability of isolates (Saravanan et al. 2004).

Siderophore production

Qualitative estimation of siderophore production by the bacterial isolates was done by the method Schwyn and Neiland (1987). The Nutrient agar plates with chrome azurol'S" (CAS) dye agar plates were spot inoculated with log phase cultures of all 18 bacterial isolates and incubated at 28 ± 1°C for 5–6 days. Siderophore production was determined by the development of orange halo zone around bacterial colonies.

Indole acetic acid (IAA) production

Bacterial isolates were grown in nutrient broth supplemented with 0.01% tryptophan and incubated at 28 ± 2°C for 3 days. The broth was centrifuged at 10,000 rpm for 20 min at 4°C to collect the supernatant. The amount of IAA was qualitatively determined by adding 4 ml of Salkowasky's reagent (1 ml of 0.05 M $FeCl_3$ in 50 ml of 35% $HClO_4$) to 2 ml of culture supernatant. Uninoculated broth with Salkowski reagent served as reference. Development of red color indicates positive result (Patten and Glick 2006).

HCN production

Cyanide production was detected according to method of Bakker and Schippers (1987). The log phase culture were streaked on plates containing nutrient agar supplemented with 4.4 g of glycine per liter. Filter paper dipped in 0.5% picric acid and 2% sodium carbonate solution was placed on the lid of each petriplate. Petriplates were incubated at 28 ± 2°C for 3–7 days. The color change of filter paper from yellow to orange brown indicated cyanide production. The plates without bacterial inoculum was considered as control plates.

Ammonia production

Actively growing bacterial isolates were inoculated in 10 ml Peptone water and incubated for 72 h at 28 ± 1°C at 100 rpm on a rotatory shaker for four days. The Nessler's reagent (1 ml) was added to the bacterial culture and observed for production of ammonia (Cappuccino and Sherman 1996). Presence of yellow to brown color indicates production of ammonia.

Table 2. Antibiotic sensitivity profile of phosphorus solubilizing bacteria from *Dalbergia sissoo* natural forests. R = Resistance, S = Sensitive.

Geographical locations	ID	Ampicillin (10 mcg)	Cephalothin (30 mcg)	Chloramphenicol (30 mcg)	Clindamycin (2 mcg)	Erythromycin (15 mcg)	Gentamicin (10 mcg)	Oxacillin (1 mcg)	Vancomycin (30 mcg)
Lachhiwala	L1	R	R	R	R	R	S	R	R
	L2	S	S	S	S	S	S	S	S
	L3	R	R	R	R	R	S	R	R
	L4	R	S	R	R	R	S	R	R
	L5	R	R	R	R	R	R	R	R
	L6	S	S	S	S	S	S	S	S
	L7	S	S	S	S	S	S	S	S
	L8	S	S	S	S	S	S	R	R
Pantnagar	P1	R	R	R	R	R	S	R	R
	P2	R	R	R	R	R	S	R	R
	P3	R	R	R	R	R	S	R	R
	P4	R	R	R	R	R	S	R	R
Tanakpur	T1	S	S	S	S	S	S	S	S
	T2	R	R	R	R	R	R	R	R
	T3	S	S	S	S	S	S	S	S
	T4	S	S	S	S	S	S	S	S
	T5	S	S	S	S	S	S	S	S
	T6	S	S	S	R	R	S	S	S

Antibiotic sensitivity assay

Antibiotic sensitivity and resistance of 18 bacterial strains was assayed by disc diffusion method (Yao 2002). One ml of actively growing bacterial cultures was pour plated on nutrient agar plates. Octo disc of different antibiotic (Himedia Laboratories Pvt Ltd) ampicillin (10 µg), cephalothin (30 µg), chloramphenicol (30 µg), clindamycin (µg), erythromycin (15 µg), gentamicin (10 µg), oxacillin (1 µg) and vancomycin (30 µg) were placed on the surface of the medium and left 30 minutes at room temperature for diffusion of the antibiotics. The plates were incubated for 48 h at 30°C. Inhibition zone size was measured and bacteria were classified as sensitive or resistant.

Identification of bacterial strains

The genomic DNA was extracted by a modified method of Bazzicalupo and Fani (1996). The bacterial cultures were centrifuged at 10,000 rpm for 10 min and then 30 µl of 10% SDS and 3 µl of proteinase K were added to the pellets. The sample mixture was then incubated at 37°C for 1 h. The sample was re-centrifuged and genomic DNA was purified using phenol–chloroform–isopropanol extraction and ethanol precipitation. For amplification of 1492 bp region of the 16S rDNA gene two specific primers: Forward primer GM3f (5'TACCTTGTTGTTACGACTT3') and reverse primer GM4r (5'TACCTTGTTACGACTT3') were used (Muyzer et al. 1995). A 50 µl of reaction mixture included 5 µl template DNA and to it 45 µl of reaction mixture which consisted buffer (100 mM Tris–HCL with 15 mM $MgCl_2$), 1 µM of each dNTPs and one unit of Taq DNA polymerase. The reaction conditions included an initial denaturation of 5 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 52°C and 1 min at 72°C with the final extension of 5 min at 72°C. The amplification was carried out on Gen Amp PCR System 9700 (Applied Biosystems). Amplified DNA was electrophoresed in 1% agarose gel at 80 mA for 1 h and visualized under UV gel documentation system Gel Doc Mega (BIOSYSTEMATICA). The amplified 1492 bp 16S rDNA region of all 18 isolates was sequenced on 3730 DNA sequencer using ABI big dye terminator technology (Central Instrumental facility, Biotech Center UDSC, New Delhi) using same set of primers that were used

Table 3. Identification of the phosphate solubilizing bacteria through 16S rDNA sequence analysis.

Strain	Isolate	Percent similarity	NCBI Gen Bank accession no.
L1	*Pseudomonas simiae*	98.14%	MG966339
L2	*Staphylococcus petrasii*	97.98%	MG966340
L3	*Pseudomonas paralactis*	99.16%	MG966341
L4	*Klebsiella variicola*	99.51%	MG966342
L5	*Pseudomonas paralactis*	99.17%	MG966343
L6	*Streptomyces curacoi*	87.00%	MG966344
L7	*Streptomyces cellostaticus*	95.00%	MH031699
L8	*Pantoea conspicua*	96.83%	MG966345
P1	*Pseudomonas hunanensis*	98.89%	MG966346
P2	*Pseudomonas aeruginosa*	97.00%	MG966347
P3	*Pseudomonas putida*	97.00%	MG966348
P4	*Pseudomonas plecoglossicida*	98.42%	MG966349
T1	*Kitasatospora kifunensis*	93.86%	MG966350
T2	*Klebsiella singaporensis*	96.37%	MG966351
T3	*Streptomyces antibioticus*	94.22%	MG966352
T4	*Micrococcus yunnanensis*	98.00%	MG966353
T5	*Streptomyces griseoruber*	97.92%	MG966354
T6	*Staphylococcus pasteuri*	98.20%	MG966355

in 16S rRNA gene amplification. The gene sequences were further analyzed for similarity using basic local alignment search (BLASTn) algorithm at EzBioCloud's database (https://www.ezbiocloud.net/identify) (Yoon et al. 2017). The BLAST results revealed the identity of the query sequences based on their percentage query coverages and sequence identities. Sequences were submitted to NCBI GenBank data base and accession numbers were obtained.

Measuring biodiversity in natural forests at different locations

Similarity index: Percentage similarity was used to make useful comparison between different forest stands. These coefficients measure the association between samples. Such comparisons are based on the percentage composition of identified bacterial species or genera in the samples from each of the forest types (Ifo et al. 2016).

$$SI = \frac{2W}{a+b+c} \times 100$$

Where, a, b and c represents total density of forests stands.

Results and Discussion

Soil physico-chemical analysis

Soil physico-chemical analysis was performed to assess the soil nutrient status and health. Soil texture was silty loam in Lachhiwala and Tanakpur whereas silty clay loam in Pantnagar. Soil pH in Pantnagar was 6.85 which was comparatively higher than Lachhiwala and Tanakpur where it was 6.00 and 6.12 respectively. Electrical conductivity for Lachhiwala, Tanakpur and Pantnagar soil was 0.11 dsm^{-1}, 0.14 dsm^{-1} and 0.13 dsm^{-1} respectively. Total organic carbon (TC) was higher in Pantnagar (42,750 kg/hac) as compared to Lachhiwala (19,500 kg / hac) and Tanakpur (25,000 kg/hac) forests. Whereas, available phosphorus (AP) content was highest in Lacchiwala (56.48 kg/hac) followed by Tanakpur (46.87 kg/hac) and Pantnagar soil (37.86 kg/hac). Total Kjeldhal Nitrogen (TKN) in Pantnagar, Lachhiwala and Tanakpur was 137.98 kg/hac, 163.07 kg/hac and 100.35 kg/hac respectively while soil potassium was 434.11 kg/hac, 505.34 kg/hac and 520.12 kg/hac respectively. Soil iron and zinc were significantly higher in Lachhiwala and Tanakpur soil as compared to the Pantnagar (Fig. 2).

It is well known that the interplay of many factors, such as physico-chemical properties of the soil, vegetation, crop rotation and environmental

conditions, greatly influence the quantity and composition of soil microbial flora (Jha et al. 1992). Therefore analysis of physico-chemical parameters is an essential step in the present study. There are few studies that have correlated PGPR with soil type. The content of particles such as silt, clay and sand also affects the microbial load in soils (Sessitsch et al. 2001). Sessitsch et al. (2001) reported microbial population associated with sand fraction were better adapted to limited nutrient conditions and capacity to use a wider range of substrates. Da Costa et al. (2014) correlated soil quality with the isolation of PGPR, proposing that PGPR were frequently isolated from soils with low C. organic content. Similar results were obtained in this study as, most of the PGPRs were isolated from the soils at Lacchiwala and Tanakpur which had lower organic C content and a high percentage of sand (Table 1). Macronutrients N, P and K are involved in soil fertility, disease resistance and tolerance (Dordas 2008). The high soil pH may be the reason for low phosphorus availability in Pantnagar forests (Negi et al. 1999). Nitrogen and potassium content was moderate in all the three regions. Similarly, high micronutrient level is reported to play an important role in plant functioning, provide healthy environment for the growth of beneficial microbial community in rhizosphere region (Ghosh et al. 2017). Therefore, in the present study Lachhiwala and Tanakpur soil samples where micronutrient content was high could be considered better then Pantnagar soil. The ANOVA ($p < 0.05$) results revealed highly significant differences between soil nutrient values at Lachhiwala, Tanakpur and Pantnagar.

Soil enzyme activities

The difference in the soil enzyme activities in D. S. rhizosphere soils from forests at different locations were highly significant ($p < 0.05$). Rhizospheric fluorescein diacetate hydrolysis activity varied from 291.2 µg fluorescein $g^{-1}h^{-1}$ soil at Lachhiwala forest to 372.6 µg fluorescein $g^{-1}h^{-1}$ soil for Tanakpur forest (Fig. 3a). The FDA activity at Pantnagar was 325 µg fluorescein $g^{-1}h^{-1}$. Dehydrogenase enzyme levels at Tanakpur forest (4300 µg TPF $g^{-1}h^{-1}$) was almost two folds higher as than Lachhiwla t (1880 µg TPF $g^{-1}h^{-1}$) and Pantnagar forest (1770 µg TPF $g^{-1}h^{-1}$) (Fig. 3b). Lachhiwala forest reported the highest activity for acid phosphatase enzyme (1109.6 ug PNP $g^{-1}h^{-1}$) whereas the activity at Tanakpur (654.5 ug PNP $g^{-1}h^{-1}$ and Pantnagar forest soil (574.8 ug PNP $g^{-1}h^{-1}$) were almost equal but almost half than Lacchiwala (Fig. 3C). The highest alkaline phosphatase activity was found in *D. sissoo* rhizosphere at Lachhiwala forest (1196.2 ug PNP $g^{-1}h^{-1}$) followed by Pantnagar forest (613.87 µg PNP$g^{-1}h^{-1}$) and lowest in Tanakpur forest (442.8 µg PNP $g^{-1}h^{-1}$) (Fig. 3d). Similarly, highest urease activity was observed in the *D. sissoo* rhizosphere at Lachhiwala forest (241.93 µg $NH_4^+ g^{-1}h^{-1}$) followed by Pantnagar forest (192.25 µg $NH_4^+ g^{-1}h^{-1}$) and least at Tanakpur forest (65.74 µg $NH_4^+ g^{-1}h^{-1}$) (Fig. 3e).

Soil nutrients and soil enzyme activities are closely related. Soil organic carbon (SOC), nitrogen, phosphorus, potassium and other elements significantly affect the activities of the soil enzymes (Burke et al. 2011). In the present study Fluorescein diacetate (FDA) and Dehydrogenase activity correlated with culturable microbial population or respiratory metabolism (Wolinska and Stepniewska 2012). The dehydrogenase and FDA activities were higher in DS rhizosphere from Tanakpur where the aerobic bacterial population was also highest. Soil phosphatase activity is pH sensitive, (Nannipieri et al. 2017). The acid and alkaline phosphatase and urease activities were higher in D. S. rhizosphere from Lachhiwala. The reasons for higher acid and alkaline phosphatase activity could be soil pH (Renella et al. 2006). Urease is an important enzyme involved in soil nitrogen (N) cycling. It catalyzes the hydrolysis of organic N to inorganic forms, the former using urea-type substrates and the latter ammonia or ammonium ion substrates (Cookson 1999). Gianfreda et al. (2005) found a significant positive correlation of urease and phosphatase with available nitrogen and phosphate. Thus BNF by *sissoo* trees positively contributed towards urease activities (Bisht et al. 2009b). Higher nutrient availability could promote rhizosphere colonization of beneficial microbes which further affects the rhizosphere microbiome.

Culturable bacterial population
in rhizosphere soil

Total population as enumerated on Angle's medium

in *Dalbergia sissoo* rhizosphere at Tanakpur, Lachhiwala and Pantnagar was 2.76 $\times 10^4$, 1.87 $\times 10^4$ and 1.96 $\times 10^4$ cfu g^{-1} of soil. Whereas, P solubilizing bacterial count on Pickovskaya medium was 1.20 $\times 10^4$ cfu g^{-1}, 1.55 $\times 10^4$ cfu g^{-1} and 1.06 $\times 10^4$ cfu g^{-1} respectively.

The present study revealed that PSB population level varied in rhizosphere soil from different forests. This is mainly due to the soil abiotic factors. In an earlier study Kucey (1983) detected PSB in almost all soils tested, although PSB population varies with soil organic carbon content, climatic factors, cropping history (Griffiths et al. 1999).

PGPR isolation and morphological characteristics of the strains

Eighteen PSBs were selected from all three forest sites based on their P solubilization efficiency. The gram's reaction and cell morphology for all bacterial isolates have been summarized in Table 1.

Qualitative estimation of P solubilization potential of bacterial isolates

All eighteen bacterial isolates exhibited P solubilizing potential (Fig. 4). Phosphate solubilizing index (PSI) that represents the amounts of hydrolyzed Pi from tricalcium phosphate substrate were assessed. The isolates from Lachhiwala forests depicted higher phosphorus solubilizing index as compared to Tanakpur and Pantnagar forests. Highest PSI was detected in isolate L4 (4.75) and lowest in isolate T4 (1.16).

The ability of PGPR strains to solubilize insoluble P and convert it to plant available form is an important characteristic under conditions where P is a limiting factor for crop production (Saravanan et al. 2007). Therefore, the widespread and commercial application of PSM in plant growth promotion can help reduce the spiralling cost of chemical phosphate fertilizers and maintain soil fertility simultaneously.

Biochemical and functional characteristics of PSBs recovered from *Dalbergia sissoo* rhizosphere

All PSBs were positive for more than one enzyme activities like amylase, urease, nitrate reductase, lipase, xylanase, protease, pectinase and catalase in *in vitro* assay (Fig. 5, Table 1). Among the 18 isolates, four each were positive for amylase (L7, L8, T3 and T5) and urease (L4, P2, T2 and T6), five ; L7, L8, P2, T3 and T5 for lipase activity, eight ; L7, L8, P1, P4, T1, T3, T4 and T5 for xylanase, seven ; L1, L2, L5, L7, L8, P2 and T5 for protease production, six ; L1, L5, P1, P3, P4 and T1 for pectinase enzyme production. All the isolates except L4, T1, T3, T4, T5 and T6 exhibited nitrate reductase activity and except L6, T1 and T3 for catalase production. Only Seven isolates exhibited zinc solubilization and eight isolates siderophore production. Fourteen isolates were positive for IAA. Except four isolates L6, L7, T1 and T3 all were positive for ammonia production. All the isolates except P2 were negative for HCN production. Hence, all bacterial isolates exhibited one or more PGP traits in addition to inorganic P solubilization (Fig. 5, Table 1).

A considerable worldwide research has focused on the exploration of varied agro-ecological niches for the existence of native beneficial micro-organisms. The PGPR can competitively colonize plant roots and stimulate plant growth through various direct and indirect mechanisms. The direct mechanism include production of hormones, enzymes, metabolites and acquisition of macro and micro nutrients from soil while indirect mechanisms include inhibition of growth of phytopathogens through production of antibiotics or induced systemic resistance in plants (Meena et al. 2017). Microorganisms with amylase, pectinase, xylanase, lipase and protease activity are not only helpful in organic matter decomposition and plant growth promotion, but also are important in the disease suppression (Kavamura et al. 2013). The enzyme urease is involved in regulation of N supply to plant. It catalyzes the hydrolysis of urea to CO_2 and NH_3 with concomitant rise in soil pH ; whereas, nitrate reductase (NR) catalyzes the reduction of NO_2^- to N_2O under anaerobic conditions. Nitrate reductase is an adaptive enzyme synthesized only

in the presence of NO_3^- ions. Hence, its activity is commonly used as an indicator for ability of plants to utilize NO_3^- from the soil (Barford and Lajtha 1992). In present study, all 18 bacterial isolates were efficient phosphate solubilizers and seven of them were positive for Zn solubilization. Along with phosphate most natural soils are also deficient in zinc. Mechanism of zinc solubilization by bacteria is also similar to phosphate solubilization (Pietr et al. 1990). Siderophores may enhance plant growth by acquisition of metal cations including Fe and Cu (Gururani et al. 2013). Most of the phosphate fertilizers applied to soil from complexes with soil cations such as Fe, Ca and Al. Siderophore positive bacterial strains scavenge Fe^{3+} from these complex compounds and thus indirectly release P in soil (Sharma et al. 2013). Moreover, they deprive phytopathogen from iron and hence lead to disease suppression. Phytohormone producing bacteria enhance plant root system thus facilitating in higher nutrient uptake (Ribeiro and Cardoso 2012). Although HCN plays an important role in disease suppression (Ramette et al. 2003). In the current study only one isolate was positive for HCN production. Production of ammonia helps in plant growth directly by supplying nitrogen to plants (Marques et al. 2010) and indirectly by suppressing plant pathogens (Minaxi et al. 2012).

Intrinsic antibiotic resistant (IAR) pattern of shisham associated rhizobacteria

Out of 18 isolates majority exhibited resistance towards ampicillin (10 µg), cephalothin (30 µg), chloramphenicol (30 µg), clindamycin (2 µg), erythromycin (15 µg), oxacillin (1 µg) and vancomycin (30 µg) except gentamicin (10 µg) and two L5 and T2 were resistant to all eight antibiotics. The conclusion is that bacterial strains L2, L6, L7, T1, T3, T4 and T5 were sensitive to all antibiotics (Table 2). Considering that antibiotic use in agriculture has enhanced, antibiotic resistant bacteria have added ecological advantage to survive in an environment affected with antibiotic stress.

16S rDNA sequence based identification of PSB isolates from DS rhizosphere

Amongst 18 bacterial isolates exhibiting multiple PGP-traits, seven were identified within genus *Pseudomonas*. Out of these seven isolates, 3 were from Lachhiwala (L1, L3 and L5) whereas four were from Pantnagar (P1, P2, P3 and P4). Out of remaining eleven, four isolates were identified as *Streptomyces* sp. (L6, L7, T3 and T5), two each as *Klebsiella* sp. (L4 and T2) and *Staphylococcus* sp. (L2 and T6) and one each as *Pantoea* sp. (L8), *Kitasatospora* sp. (T1) and *Micrococcus* sp. (T4). The 16S rDNA sequences of all 18 isolates have been deposited in NCBI Gen Bank under accession number MG966339-MG966355 (Table 3). Previously also, these genera have been found to be associated with rhizosphere of leguminous plants (Kandjimi et al. 2015, Rfaki et al. 2014, Saidi et al. 2013). The two isolates showing maximum positive traits *in vitro* were L8 and P2. Strain L8 has been identified as *Pantoea conspicua* and P2 as *Pseudomonas aeruginosa*.

Application of P solubilizing *Pantoea* sp. and *Pseudomonas* sp. have resulted in beneficial effects on plant growth parameters of medicinal plant *Coleus forskohlii* (Malleswari et al. 2014). In the present study, majority of the phosphate solubilizers identified in phyla Proteobacteria and Actinobacteria. Efficient P solubilizers are distributed among the phylum Proteobacteria and Actinomycetes (Franco-Correa et al. 2010). Moreover genera such as: *Pantoea*, *Pseudomonas* and *Streptomyces* (Sturz et al. 2000), *Klebsiella* and *Micrococcus* (Felici et al. 2008, Swain and Ray 2009), *Kitasatospora* (Shrivastava et al. 2008) and *Staphylococcus* (Tariq et al. 2010) have previously been shown to exhibit PGP traits. Considering these above-mentioned facts, the PSB bestowed with multiple plant growth promoting traits have greater potential to be used in the future for enhancing the productivity and growth of plants (Zaidi et al. 2017).

Similarity index

The values of coefficient of similarity based on genera identified from different forest types varied from 0% to 57.14%. Between the forest types studied the lowest Jaccard index value was obtained between T–P (0%) and the highest value was noted between L–T (57.14%) ; 50% similarity was observed between L–P. In present study, three forest soil samples were compared and several factors could explain the

issue of heterogeneity of forests and variations of biodiversity i.e. topography (Basnet 1992) or edaphic factors (Gartlan 1986). Lower similarity index value of Lachhiwala and Tanakpur with Pantnagar enables us to conclude that there is obviously a difference in point of microbial composition, thus confirming the data presented above.

Conclusion

The present study showed that PSB recovered from shisham rhizosphere were genetically diverse and exhibited multiple plant growth promoting traits such as zinc solubilization, IAA production, ammonia production and siderophore production. They were distributed amongst seven genera *Pseudomonas*, *Klebsiella*, *Streptomyces*, *Pantoea*, *Kitasatospora*, *Micrococcus* and *Staphylococcus*. From the above results obtained in this study, it can be clearly concluded that two rhizobacterial isolates such as L8 identified as *Pantoea conspicua* and P2 identified as *Pseudomonas aeruginosa* can be used as bioinoculants for cultivation of agricultural crops in a sustainable way. Also by analyzing PGPR diversity, enzymatic activities and physico-chemical properties of three different regions it is possible to determine the changes between soil of different provenances. The identification of bacteria in the soils showed that there were differences between them and that each forest had some exclusive bacterial species. Such investigation is necessary as it advocates the changes brought about by the agricultural manipulation of the soils studied. Simultaneous screening of PGPR from field is a good tool to select effective PGPR for bio fertilizer development technology. It can be used as bioinoculants either alone or in combination to enhance the growth and productivity of agricultural crops under local agro-climatic conditions in a sustainable way. Thus, this study can be assumed as the first reported analysis of culturable PSBs associated with DS rhizosphere. Moreover, further studies targeting microbiome analysis of DS rhizosphere are needed to reveal actual diversity present.

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