Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(5): 3021-3027
Received: 10-07-2018
Accepted: 12-08-2018

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Endophytic *Actinobacteria* from *Pinus roxburghii*: Isolation, diversity and antimicrobial potential against human pathogens

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Abstract
Pinus roxburghii commonly used as timber plant along with medicinal properties is used in present study as a sustainable approach for utilizing bio-resources. Plant-associated endophytic *Actinobacteria* were objected and explored for their therapeutic potential properties. The results indicated that a total of two hundred eighty-three endophytes were isolated from different tissues and belonged to predominating genus *Streptomyces* followed by *Kitastaosporia*, *Actinomadura*, *Micromonospora*, *Streptosporangium*, *Microbiospora* and *Nocardia*. During preliminary screening, 47.7% and 14.8% were active against bacterial and fungal pathogens respectively. Isolate GBTPR-137 having maximum zone of inhibition against *Micrococcus luteus* (27.3±0.5 mm) followed by *Pseudomonas aeruginosa* (18.3±0.5 mm), *Neisseria cinerea* (18.7±0.5 mm) and *Klebsiella pneumonia* (18.3±0.5 mm) While GBTPR-281 was having antibacterial as well as antifungal activity against multicellular fungi *Aspergillus fumigates* (16.7±0.5 mm), *Microsporium gypseum* (12.3±0.5 mm) and *A. flavus* (14.7±0.9 mm). The results depicted that these plants were a worthy niche for *Actinobacteria* producing important bioactive secondary metabolites.

Keywords: Endophytic *Actinobacteria*, *Pinus roxburghii*, antimicrobial activity, *Streptomyces*

Introduction
The gymnosperm forests of Garhwal Himalaya are valuable because of the resin as well as timber richness. Especially, *Pinus roxburghii* is well scattered and dominated in this region at a lower altitude between 1500-1700 meter above from sea level [1]. The resin is utilized in the formation of insecticides, disinfectants, lubricants and also be used as a folk medicine such as in gastric trouble, inflammation, asthma, liver and spleen diseases, bronchitis, piles, earache, toothache, tuberculosis, epilepsy and scabies [2, 3, 4]. Endophytic fungi (*Phialocephala* sp. and *Pestalotiopsis* sp.) [5] and endophytic *Actinobacteria* are reported from *Pinus thunbergii* but there is no report of endophytic *Actinobacteria* isolated from *P. roxburghii* [6].
Actinobacteria are well known for production of bioactive secondary metabolites with their applications in pharmaceuticals and agriculture industry [7] and have ability to resides various habitats such as soil, water (including both fresh and marine) and recently more attraction is on an endophytic *Actinobacteria*, with increase reports on their isolation from wide range of plants includes medicinal plants [8, 9] and crops [10, 11]. Typically, 70% of total antibiotics known are produced by phylum *Actinobacteria* and especially genus *Streptomyces* [12]. Although, extensive misuse of these molecules over a period of time caused in development of multidrug-resistant microorganism such as multi-drug resistant species of *Staphylococcus*, *Pseudomonas*, *Streptococcus*, *Acinetobacter*, *Enterococcus*, *Klebsiella*, and *Escherichia*. Thus, it is necessary to search for perspective unexplored niches for exploring the diversity of cultivable *Actinobacteria* for their antimicrobial potential.

Materials and methods
Sample collection: *P. roxburghii* trees in the region of district Pauri specifically en-route Kotdwar → Pauri → Devprayag were utilized for collecting samples. Total 88 plant samples including needle, root, stem, bark and female cone were collected. The plant material was safely removed and transferred to sterile poly bags. Within a week samples were taken to the laboratory for the isolation process and kept at 4ºC till further analysis.

Isolation of endophytic *Actinobacteria*
Surface sterilization: The collected samples were air dried for three days at room temperature and washed with tap water to remove the adhering soil particles from the surface. Once dried, the samples were subjected to six of steps surface sterilization procedure slightly modified

from Qin [13]. In this method, the sample was splashed with 70% ethanol for 10 minutes, followed by 10 minutes rinse in 4% sodium hypochlorite (NaOCl), a 10 minutes rinse in 2.5% sodium thiosulphate ($Na_2S_2O_3$), followed by 5 minutes rinse in 70% ethanol, three-time rinse with sterile distilled water and finally rinse for 10 minutes with 10% sodium bicarbonate ($NaHCO_3$). After surface sterilization, the samples were aseptically air dried in an oven at 70 ºC for half an hour.

Effectiveness of sterility: To check the effectiveness, one ml of last wash of sterile distilled water was inoculated in 10 ml of nutrient broth and incubated for 72 hrs. After the incubation, the broth was observed for the microbial growth.

Isolation: The pretreated plant sample was processed for isolation of *Actinobacteria* following described method (Qin *et al*., 2009)

Method 1: Grind with calcium carbonate: The surface sterilized plant sample was ground in sterilized mortar and pestle with calcium carbonate ($CaCO_3$) in equal volume (1:1 w/w). The grounded sample was left for two weeks of incubation under aseptic conditions of laminar air flow. After incubation, the sample was serially diluted.

Method 2: Direct implanting of plant sample: After sterilization and drying, the samples were cut into small pieces of size 1-3 cm with the help of sterilized surgical blade. The sample was plated and distributed on to different isolating media includes starch casein agar [14], tap water yeast extract agar [15], sucrose nitrate agar (Czapek Dox agar) [16], Gause No.2 modified agar [17], yeast malt extract agar (ISP-2), Oatmeal agar (ISP-3), Arginine glycerol agar (ISP-4), glycerol asparagine agar (ISP-5) [18] and Humic acid vitamin agar[19, 20], incubated at 27°C for 21-28 days. All the isolating media was supplemented with cycloheximide and nystatin (50 µg/ml each) in order to minimize fungal contamination and nalidixic acid (25 µg/ml) to inhibit the Gram-negative as well Gram-positive bacterial contaminant. All the observed rough and tough colonies on isolating media were selected and purified on yeast extract malt extract agar (ISP-2) and preserved in 20% glycerol at -20 ºC.

Test organisms

In current study, six Gram-positive bacteria (*Staphylococcus aureus* (MTCC-96), *Micrococcus luteus* (MTCC-106), *Mycobacterium smegmatis* (MTCC-06), *Streptococcus pneumoniae* ((MTCC-1935), *Bacillus subtilis* (MTCC-441), *B. cereus* (MTCC-430)) and seven Gram-negative bacteria (*Escherichia coli* (MTCC-739), *Pseudomonas aeruginosa* (MTCC-424), *Salmonella entrica* (MTCC-733), *Neisseria cinerea* (MTCC-3583), *Proteus vulgaris* (MTCC-426), *Klebsiella pneumoniae* (MTCC-4030), *Serratia mancesens* (MTCC-97)) were used for antibacterial assay. Eight fungal pathogens including *Candida albicans* (MTCC-1637, *C. tropicalis* (MTCC-184), *C. parapsilosis* (MTCC-1744), *Microsporium canis* (MTCC-2820), *M. gypseum* (MTCC-2829), *Aspergillus flavus* (MTCC-1973), *A. fumigates* (MTCC-3070) and *Trichophytron rubrum* (MTCC-296) were assayed for antifungal activity. All the microorganisms were procured from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India.

Screening and selection of antimicrobial active strains

The cultures of *Actinobacteria* were preliminarily screened for their antibacterial activity by agar plug method [21], in which all the isolates were grown on ISP-2 agar for 7 days at 27 ºC. Six mm agar plug was prepared by using sterile cork borer and the plug was placed aseptically on Mueller-Hinton agar plates seeded with test organisms. The plates were incubated at 37 ºC and zone of inhibition against test organisms was observed after 24-48 hours of incubation as an active metabolite produced by the isolate.

Identification of endophytic *Actinobacteria*

Morphological and Chemotaxonomic characteristics

The isolates were observed for their culture characteristics (color of aerial mycelium, substrate mycelium, and pigmentation) on different ISP (International *Streptomyces* Project) media [18]. The spore patterns of the isolates were observed using coverslip technique [22] under a light microscope, grown on ISP-4 media at 27°C for one week. The diaminopimelic acid isomers of the whole cell lysate were examined through TLC [23].

Molecular profiling of promising isolate

The promising selected isolates were imperiled to 16S rDNA sequencing study for their profiling [24]. Sequence homology of the selected isolates was dignified by using online tool BLAST (Basic Local Alignment Search Tool) provided by NCBI. The obtained sequences were analyzed for chimera analysis by using online tool Decipher's [25]. Phylogenetic and molecular evolutionary analyses were directed using software MEGA version 6.0 package [26]. The obtained sequences of the isolates were aligned using Muscle program against analogous nucleotide sequences of *Actinobacteria* retrieved from GenBank. The evolutionary phylogenetic tree was concluded by neighbor-joining method. Tree topologies were assessed by 1000 bootstrap analysis [27, 28, 29].

Results

Isolation of Endophytic *Actinobacteria*

A total of 283 endophytic Actinobacterial strains were recovered from 88 samples (including needle, root, stem, flower, and bark) of *P. roxburghii*. Of the 283 strains, highest number was recovered from the root of the plant (n=194) followed by needle (n=63) and stem (n=26). None was isolated from bark or fruiting body of the plant. Out of all isolating media, sucrose nitrate agar was found suitable for the isolation of endophytic *Actinobacteria* (Figure 1).

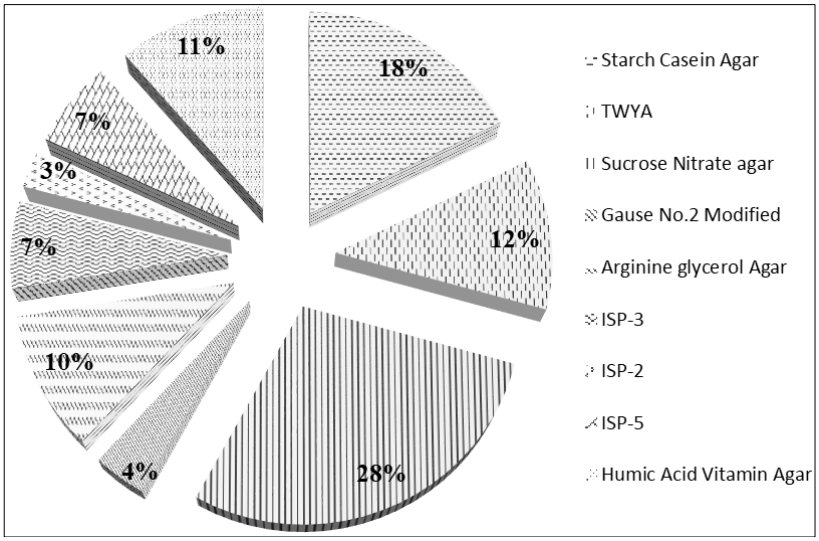

Fig 1: Isolating media for recovery of *Actinobacteria* from *Pinus roxburghii*

Preliminary Screening

Out of 283 isolates, 47.7 % (n=135) isolates displayed antibacterial activity against one or more test bacteria as summarized in Figure 2. Among these isolates, seven (GBTPR-113, GBTPR-114, GBTPR-137, GBTPR-181, GBTPR-201, GBTPR-256, and GBTPR-281) were showing broad spectrum activity against both Gram positive and Gram negative bacteria (Table 1) and belonged to genus *Streptomyces*. Maximum activity against different pathogens was exhibited by isolate GBTPR-137, displaying activity against seven pathogens with maximum zone of inhibition against *M. luteus* (27.3±0.5 mm), *P. aeruginosa* (18.3±0.5 mm), *N. cinerea* (18.7±0.5 mm), *K. pneumonia* (18.3±0.5 mm), *S. entrica*c (17.3±0.5 mm), *P. vulgaris* (17.0±0.0 mm), *E. coli* (14.7±0.5 mm) followed by isolate GBTPR-208 against *S. aureus* (20.3±0.5 mm), *B. cereus* (20.7±0.9 mm), isolate GBTPR-113 against *B. subtilis* (14.7±0.9 mm), isolate GBTPR-256 against *M. smegmatis* (20.3±0.5 mm), isolate GBTPR-167 against *S. pneumonia* (17.3±0.5 mm) and GBTPR-218 against *S. mancesens* (24.0±0.8 mm).

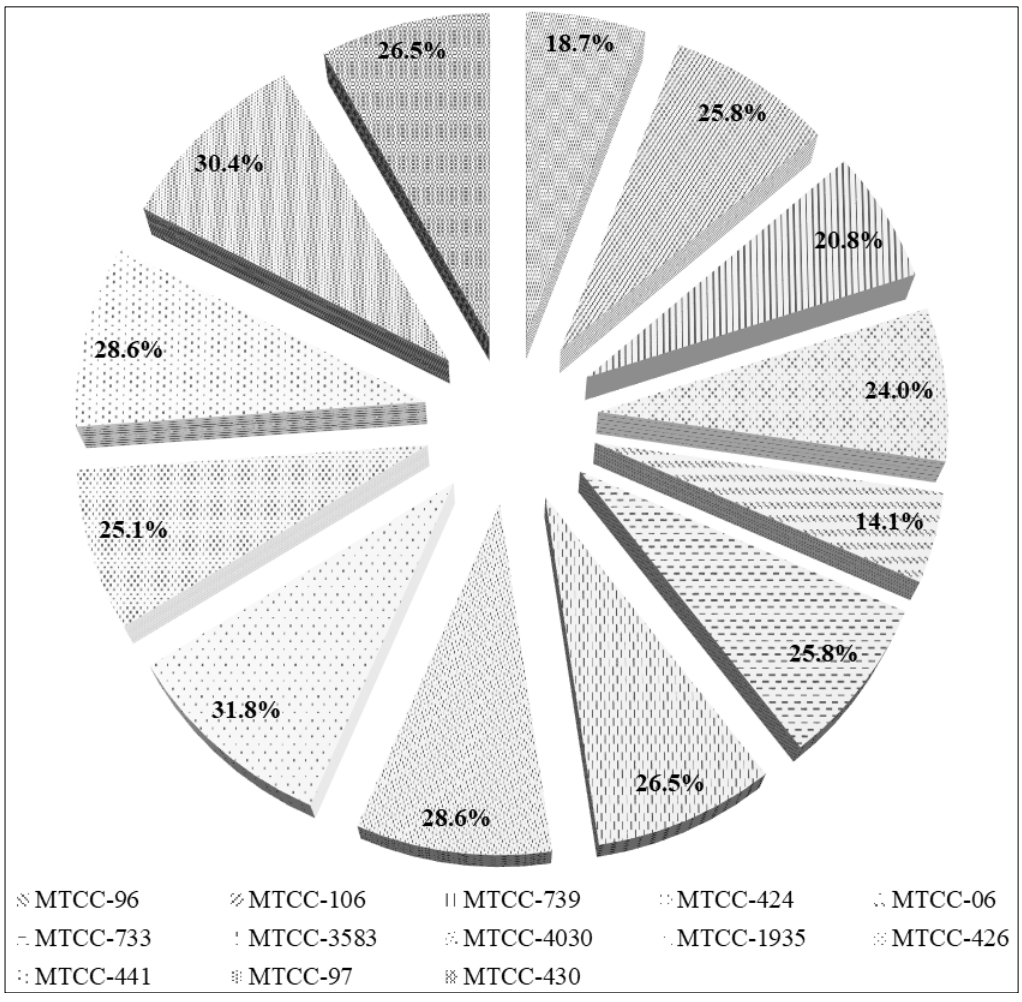

Fig 2: Active isolates against different bacterial pathogens

Only 14.8% (n = 42) of 283 isolates exhibited antifungal activity against one or more test fungal pathogens conversely 11, 13, 13, 13, 17, 16 and 15 of isolates were showing antifungal activity against *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, *Microsporium canis*, *M. gypseum*, *Aspergillus flavus* and *A. fumigates* while no activity was observed against *Trichophyton rubrum*. Isolate GBTPR-281 was active against MTCC-3070, MTCC-2829 and MTCC-1973 with a zone of inhibition of 16.7±0.5 mm, 12.3±0.5 mm and 14.7±0.9 mm respectively. Isolate GBTPR-06 against *C. albicans*, isolate GBTPR-236 against *C. tropicalis* and isolates GBTPN-45 and GBTPN-84 against *C. parapsilosis* were active with a zone of inhibition of 12.0±0.0 mm, 12.3±0.5mm and 09.3±0.5 mm respectively. A zone of 18.3±0.5 mm was observed against *M. gypseum* by GBTPR-153 and GBTPR-154 and isolate GBTPS-63, GBTPN-65 and GBTPN-81 was having a zone of 12.0 ±0.8 mm against *M. canis*.

Table 1: Comparative study of preliminary screening of isolates against different pathogens for antimicrobial activity

Test Organism	Zone of inhibition (mm) of isolates						
	GBTPR-113	**GBTPR-114**	**GBTPR -137**	**GBTPR-181**	**GBTPR-201**	**GBTPR-256**	**GBTPR-281**
Gram-positive bacteria							
Staphylococcus aureus (MTCC-96)	13.3±0.5	13.7±0.9	14.0±0.8	08.7±0.5	11.3±0.5	10.7±0.5	11.7±0.5
Micrococcus luteus (MTCC-106)	24.7±0.5	10.3±0.5	27.3±0.5	10.3±0.5	24.3±0.5	16.3±0.5	11.3±0.5
Mycobacterium smegmatis (MTCC-06)	16.3±0.5	11.7±0.9	17.0±0.8	10.7±0.5	13.3±0.5	20.3±0.5	19.3±0.5
Streptococcus pneumonia (MTCC-1935)	16.7±0.5	12.7±0.5	16.3±0.5	07.7±0.5	12.0±0.0	12.0±0.0	11.7±0.5
Bacillus subtilis (MTCC-441)	14.7±0.9	11.3±0.5	12.0±0.0	07.3±0.5	13.3±0.5	07.7±0.5	09.3±0.5
B. cereus (MTCC-430)	13.0±0.8	11.7±0.5	18.3±0.5	07.7±0.5	12.7±0.5	09.7±0.5	13.0±0.0
Gram-negative bacteria							
Escherichia coli (MTCC-739)	12.3±0.5	10.3±1.2	14.7±0.5	10.3±0.5	10.7±0.5	08.7±0.5	07.7±0.5
Pseudomonas aeruginosa (MTCC-424)	16.3±0.5	14.3±0.5	18.3±0.5	09.7±0.5	12.3±0.5	10.7±0.5	14.3±0.5
Salmonella entrica (MTCC-733)	16.0±0.8	14.7±0.5	17.3±0.5	07.7±0.5	16.3±0.5	12.3±0.5	12.3±0.5
Neisseria cinerea (MTCC-3583)	16.0±0.0	17.3±0.5	18.7±0.5	09.7±0.5	12.0±0.0	13.3±0.5	14.7±0.5
Proteus vulgaris (MTCC-426)	14.3±0.5	12.3±0.5	17.0±0.0	07.7±0.9	13.7±0.5	10.7±0.5	12.7±0.5
Klebsiella pneumonia (MTCC-4030)	14.3±0.5	12.0±0.0	18.3±0.5	08.7±0.5	16.3±0.5	07.7±0.5	12.7±0.9
Serratia mancesens (MTCC-97)	14.0±0.8	11.3±0.5	13.0±0.0	17.3±0.5	12.0±0.0	14.3±0.5	08.7±0.5

Value are means ±Standard deviation

Identification and molecular profiling

Based on the obtained preliminary screening results, six strains (GBTPR-113, GBTPR-114, GBTPR-137, GBTPR-181, GBTPR-201 and GBTPR-281) were selected for their molecular profiling. Taxonomic characterization of isolates was done by using Genbank and constructed a phylogenetic tree using Mega 6. Based on the 16s rDNA gene sequencing, it was found that the selected promising isolates abundantly belonged to the genera *Streptomyces* and the sequences were submitted to the NCBI (National Centre for Biotechnology Information) Gene Bank database with the accession number (Table 2). The obtained data of sequence was compared with relative sequence available on 16S rDNA of Archea and bacteria Genbank database using BLAST tool available on NCBI. The sequences have the highest similarity of 99-100% with the sequence of *Streptomyces* sp. (Table 2). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree using neighbor-joining method (Figure 3). The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site along with 1000 times of bootstrapping. The phylogenetic tree also confirmed the above results and the promising isolate was shorted with their closest type strain recovered from Gen Bank of NCBI.

Table 2: Based on nearly complete sequence of the 16SrRNA gene with the nearest type of strains from the NCBI database

S. No.	Isolate	Accession No.	Nearest type strain	Similarity
1.	GBTPR-113	MF138867	*Streptomyces pratensis* ch24	99 %
2.	GBTPR-114	MF138873	*Streptomyces sampsonii* NRRLD12325	100 %
3.	GBTPR-137	MF138868	*Streptomyces pratensis* ch24	99 %
4.	GBTPR-181	MF138874	*Streptomyces spectabilis* NBRC 15441	99 %
5.	GBTPR-201	MF138876	*Streptomyces coelicolar* DSM 40233	99 %
6.	GBTPR-281	MF138875	*Streptomyces capillispiralli* NBRC 14222	99 %

According to morphological and culture characteristics, the isolates were representing the genera *Streptomyces*, *Kitastaosporia*, *Actinomadura*, *Micromonospora*, *Streptosporangium*, *Microbiospora*, and *Nocardia*. The maximum isolates recovered and belonged to genus *Streptomyces* (81.9%) followed by *Actinomadura* (6.4%), *Microbiospora* (3.2%), *Micromonospora* (2.5%), *Kitastaosporia* (2.1%), *Streptosporangium* (2.1%) and *Nocardia* (1.8%).

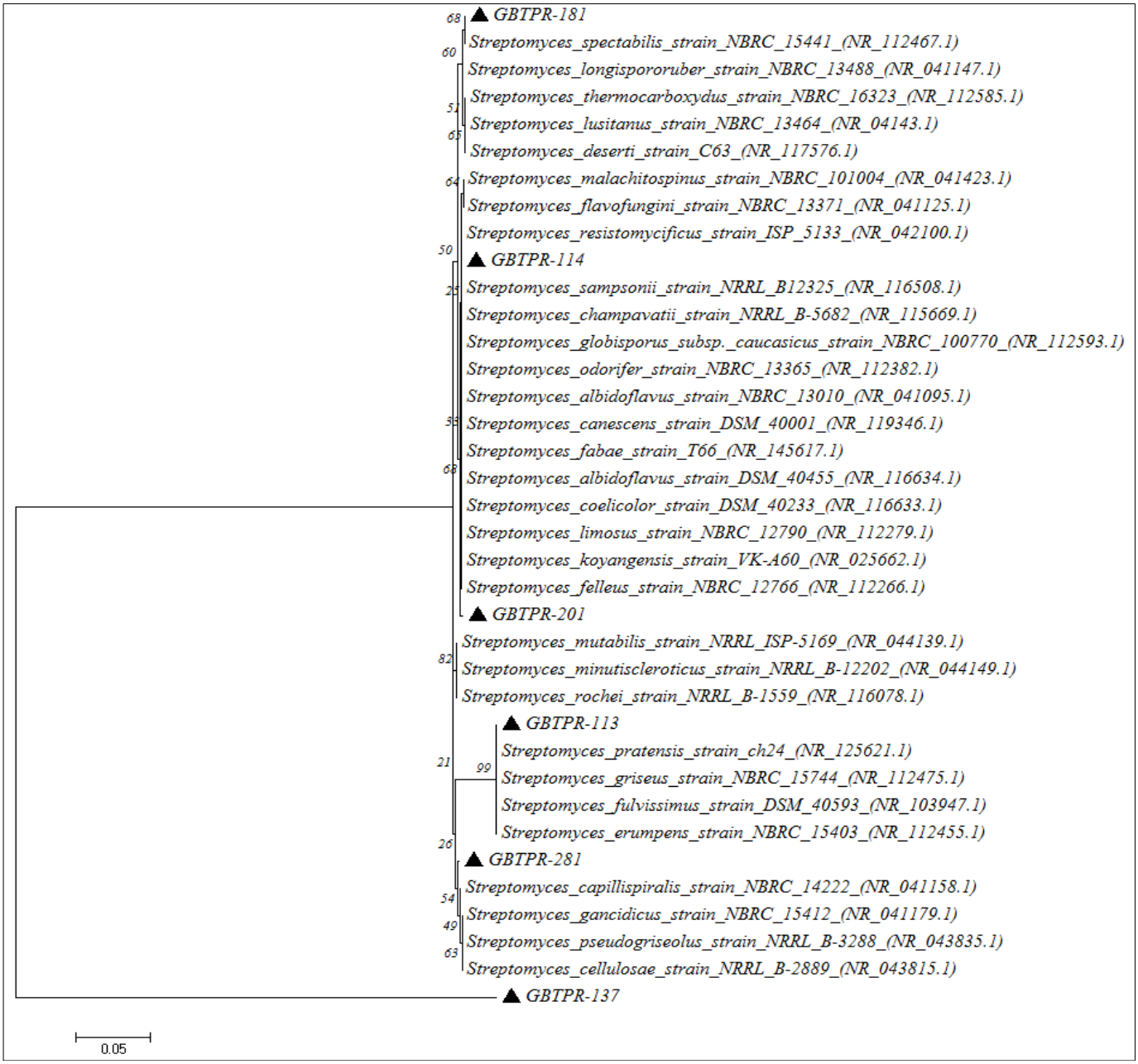

Fig 3: Based on 16S rRNA gene sequences, a phylogenetic tree was constructed using neighbor-joining method representing relationships between the representative actinobacterial strains and nearest strains of *Streptomyces*. A number of nodes indicate bootstrap values based on 1000 replicates. Bar 0.05 substitutions per nucleotide position.

Discussion

This study is conducted to examine the diversity of endophytic *Actinobacteria* associated with *P. roxburghii* and their potential to produce bioactive metabolites against human pathogens. For the last two decades, researchers have focused on medicinal plants for recovering endophytic *Actinobacteria* in the search of the new bioactive molecule [30, 31, 32, 33].

We explored endophytic *Actinobacteria* in order to classify their abundance, association and natal traits. As compared to terrestrial actinobacterial isolates, we recovered low diversity of endophytic *Actinobacteria* as is also reported in literature [34]. However using different isolating approaches, recovery of high rate of diversity in endophytic *Actinobacteria* can be obtained [35, 36, 37]. High diversity of isolates was obtained from roots followed by stem and leaves reason being the plant roots exudates attract microbes and help them to form association with tissue [38].

As reported in earlier studies, among vast diversity of genera isolated, *Streptomyces* was dominated by its presence in different plant samples and soil samples [39, 40]. In this study also, along with different rare genera including *Kitastaosporia*, *Actinomadura*, *Micromonospora*, *Streptosporangium*, *Microbiospora*, and *Nocardia*, *Streptomyces* sp. were predominant. Only few endophytic *Actinobacteria* were reported from *Pinus* earlier by Kataoka and Futai [6] while other isolates were reported as in endophytic association with other plants [35, 36]. On the basis of 16S rDNA phylogenetic analysis we propose that selected isolates GBTPR-113, GBTPR-137, GBTPR-181, GBTPR-201 and GBTPR-281 may represent new species within *Streptomyces* (Figure 3). Isolate GBTPR-114 belonged to genus *Streptomyces* with high sequence similarity to *Streptomyces sampsonii* NRRLD12325, however, DNA-DNA hybridization and sequence of other gene instead of 16S rDNA are essential for species determination [41].

A significant antimicrobial activity was observed against fungal (14%) and bacterial (47.7%) pathogens by endophytic *Actinobacteria*. A number of researchers represented 26%-

36.7% antibacterial activity of *Actinobacteria* from medicinal plants [42, 43]. Ranjan and Jadeja [44], isolated rare endophytic actinobacteria, *Micrococcus yunnanensis*, from *Catharanthus roseus* with antibacterial activity against Gram positive and Gram negative pathaogens. Similarly, Kataoka and Futai [6] reported that two genera of endophytic *Actinobacteria* associated with *Pinus thunbergii* i.e. *Microbispora*, and *Streptomyces* were having antifungal activity against *Cylindrocladium*.

Uttarakhand is a reservoir for rich biodiversity of flora, fauna and microbes. There have been reports to explore endophytic *Actinobacteria* associated with genus *Pinus*. The present study displayed diversity of endophytic *Actinobacteria* in association with the *P. roxburghii*. The isolates have the potential to produce bioactive compounds. Therefore, future evaluation of selected strains revealing their capability for production of bioactive secondary metabolites is required that may further be of importance in biotechnological industries.

Acknowledgements

We extend our gratitude to G. B. Pant Institute of Engineering & Technolgy, Ghurdauri, Pauri for providing the required facility to support the study and TEQIP-II for financial assistance and scholarship to one of the author. We also acknowledge Gujarat State Biotechnology Mission (DST, Gujarat Genomics Initiative), for providing the facility of molecular characterization.

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