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Diversity of cultivable actinobacteria in Nongkhyllem Wildlife Sanctuary, Meghalaya, and their antimicrobial and plant growth promoting potentials

DEBULMAN SYIEMIONG[1,2] AND DHRUVA KUMAR JHA[1*]

[1]*Microbial Ecology Laboratory, Department of Botany, Gauhati University, Guwahati-781014, Assam,*
[2]*Department of Botany, St. Edmund's College, Shillong-793003, Meghalaya*

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The diversity of actinobacteria in soil samples collected from a protected Nongkhyllem Wildlife Sanctuary, Meghalaya was studied using a culture-based approach and assessed their plant growth promoting and antimicrobial potentialities.Different sample pretreatment methods and selective media were used for isolation. The recovered isolates were characterized using morphological, biochemical, chemotaxonomic methods, amplified ribosomal DNA restriction analysis (ARDRA) profiling, and 16S rRNA gene sequence analysis. The isolates were screened for antimicrobial activity by agar well diffusion assay against Gram-negative, Gram-positive, and candidal strains. Presence of biosynthetic gene clusters related to the synthesis of antimicrobial compounds viz. PKS-I, PKS-II, and NRPS were also screened.Plant growth-promoting traits viz. indole-3-acetic acid and siderophore production, phosphate solubilization, growth in the nitrogen-free medium were also studied. Eighty-two (82) percent of the isolates were grouped into 24 phylotypes based on *Rsa*I restriction fragment profile, and they belonged to seven genera viz. *Streptomyces*, *Nonomuraea*, *Nocardia*, *Actinomadura*, *Kribbella*, *Streptosporangium,* and *Amycolatopsis* based on 16S rRNA gene sequence analysis. The diversity indices revealed low generic diversity due to dominance by *Streptomyces*(77%), and there were indications of rich species diversity based on ARDRA phylotypes and 16S rRNA gene sequence analysis.Few streptomycetestrains expressed antimicrobial activity against Gram-positive and candidal strains. Three strains PF-22, PF-31, and PF-48 exhibited broad-spectrum antimicrobial activity; 61% of the isolates were found to have the genetic potential to produce antimicrobial metabolites, but only 5% exhibited bioactivity, probably due to inappropriate screening and culture conditions. Several streptomycete strains showed plant growth-promoting traits and antagonistic activity against potential phytopathogens.The tropical forest soil of the study site in Meghalaya harbours potentially diverse actinobacterial species, with genetic potential for producing antimicrobial metabolites and plant growth promotion.

Key words: Pristine forest soil; actinobacteria diversity; antimicrobial activity; plant growth promotion

INTRODUCTION

Microorganisms play an important role in ecosystem functioning. In the soil, they are one of the important components of the biotic community, busy decomposing dead organic matter and transforming soil minerals into nutrients that are made available to the plants and other microbes. For that reason, they are the interface between plants and soil. Due to their interaction with plants and other microbes, they may need to either work together or compete against each other in nutrient acquisition and nutrient exchange to continue to sustain themselves in their ecological niche. For that matter, microorganisms produce a myriad of chemical compounds that may be beneficial or harmful to plants and each other. Scientists have exploited these compounds for many years for the development of clinically relevant antibiotics, other industrially important compounds, and the development of biofertilizers and biocontrol agents for the improvement of plant growth of agriculturally important crops.

Actinobacteria, a phylum of mostly Gram-positive bacteria with high DNA GC content, is of particular interest because they resemble both bacteria and fungi in their characteristics and are widely

*Correspondence Author: dkjhabot07@gmail.com

distributed in aquatic and terrestrial environments. Among microorganisms, they are one of the most widely exploited in the field of biotechnology, medicine, agriculture, and other important industries. Actinobacteria, notably *Streptomyces*, are the richest source of natural products (Goodfellow and Fiedler 2010). They have contributed 45% of the natural products derived from microorganisms, of which 80% came from *Streptomyces* alone (Goodfellow and Fiedler 2010). Actinobacteria contributetwo-third of the microbial-derived antibiotics and 80% of them came from *Streptomyces* alone (Harir et al. 2018). Other pharmaceutically important drugs can also be potentially obtained from actinobacteria such as anti-fungal, immunosuppressant, anti-helminthic (Barka et al. 2016), anti-cancer (Busi and Pattnaik 2018), and anti-viral (Li et al. 2018) drugs. Plant growth-promoting actinobacteria (PGPA) are also gaining importance in agricultural microbiological research due to their ability to enhance nutrient availability, regulate plant growth, fix atmospheric nitrogen, decrease environmental stress, control disease-causing phytopathogens, and improve soil texture (Hamedi and Mohammadipanah 2014; Solanki et al. 2016). Actinobacteria are also important sources for extracellular enzymes with potential applications in various industries (Salwan and Sharma 2018). However, all this is just a tip of the iceberg because only 10% of the natural products are believed to have been discovered so far (Goodfellow and Fiedler 2010) and there is still a lot to explore underneath the tip of the iceberg.

The state of Meghalaya is one of the eight states of the North Eastern Region of India and falls under the Indo-Burma biodiversity hotspot (Myers et al. 2000). With many under-explored pristine forest soil ecosystems in terms of microbial diversity, Meghalaya naturally becomes one of the important regions for an elaborate microbial diversity study. Work on actinobacteria from this region is also very sparse, with a few reports of actinobacterial strains isolated from the mining site, rock, caves, and medicinal plants (Baskar et al. 2009, 2011, 2016; Dochhil et al. 2013; Banerjee and Joshi 2014, 2016; Bhattacharjee et al. 2018; Syiemiong and Jha 2019a; Barman and Dkhar 2019, 2020; Syiemiong and Jha 2019b). Tropical forest soil is microbiologically very active, as organic matter decomposition and nutrient cycling are optimum to maintain nutrient balance for high biomass productivity . The diversity of microbes involved in the process is also understandably enormous. Therefore, tropical forest soil is a hotbed for microbial diversity analysis.

Actinobacterial diversity from forest soil in different parts of the world has been extensively studied (Hayakawa *et al*. 2010; Zhen-lin *et al.* 2010; Lee and Whang 2010; Luo *et al*. 2010; Varghese *et al*. 2012, 2014; Shiburaj and Preethi 2012; Das *et al.* 2018; Rai *et al.* 2018; Sharma and Thakur 2020; Soyer and Tunali 2020), and has generated information that has helped us understand the role they play in ecosystem functioning. It is about time that the under-explored forest ecosystems of Meghalaya also have a microbial diversity and functions assessment from this group of Gram-positive bacteria. Therefore, this work reports on the cultivable diversity of actinobacteria from a pristine under-explored forest soil of Meghalaya located in a protected forest at NongkhyllemWildlife Sanctuary and also look for the important functions of antimicrobial and plant growth-promoting activities from them.

MATERIALS AND METHODS

Sample collection

The soil samples were collected from a reserved forest located at Nongkhyllem Wildlife Sanctuary, Ri-Bhoi District, Meghalaya (N25°55.577' E91°51.673'; Altitude 688m amsl). Soil samples were collected aseptically from five different locations inside the forest at a depth of 10-20 cm below the soil surface. The collected soil samples were brought to the laboratory and stored at 4±1°C until further use.

Determination of physicochemical characteristics of the soil

The temperature of the soil was measured from each sampling location using a soil thermometer. The moisture content of the soil was gravimetrically determined in the laboratory. Soil pH was determined with a pH meter using a soil-distilled water suspension at a ratio of 1:5 (w/v). Soil organic carbon was determined according to Anderson and Ingram (1993). Soil nitrogen was determined by the Kjeldahl method (Jackson, 1973). Soil available phosphorus and potassium were determined by the molybdate blue method (Allen *et al*., 1974) and flame photometer method (Jackson, 1973) respectively.

Soil pretreatment and isolation of Actinobacteria

The soil samples were initially dried at room temperature for a week and subsequently mixed in equal proportions before pretreatment. The pretreatment methods used were dry heat, wet heat, 1.5% phenol, and 0.2% humic acid treatments (Syiemiong and Jha, 2019a). The isolation media used were Actinomycete Isolation Agar (AIA), Starch Casein Agar (SCA), Streptomyces Agar (SA), Bennett's Agar (BA), and Humic acid Vitamin Agar (HVA). During the preparation of the media, they were supplemented with cycloheximide (50µg ml^{-1}) and rifampicin (20µg ml^{-1}) antibiotics. 1g dried unpretreated and pretreated soil samples were suspended in sterilized distilled water (1:9 w/v) and serially diluted. 100µl of the prepared soil-water suspensions were used to inoculate the above media by spread plate method, followed by incubation at 28±1°C for 3-4 weeks. Recovered isolates were maintained on Bennett's Agar at 4±1°C.

Morphological, biochemical, and chemotaxonomic characterization

Micromorphology of the recovered isolates were microscopically studied on ISP3 medium grown coverslip cultures (Cross, 1994). All the isolates were Gram-stained according to Smith and Hussey (2005). Casein, xanthine, hypoxanthine, and tyrosine decomposition tests were performed on the isolates following the method of Berd (1973). The isomers of a diagnostic cell-wall amino acid, 2,6-diaminopimelic acid (DAP) were also determined from the isolates by thin-layer chromatography following the method of Hasegawa *et al*. (1983).

Isolation of genomic DNA

Genomic DNA was extracted from seven-day-old cultures by microwave method adapted from Li *et al*. (2007) and Chen *et al*. (2016) or by an enzymatic method adapted from Nikodinovic *et al*. (2003) and Chen *et al*. (2016), depending on the suitability of the method for each isolate.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

Partial genes of 16S rRNA were amplified using primers 24F (5-AGAGTTTGATCCTGGCTCAG-3) and ACT878R (5-CCGTACTCCCCAGGCGGGG-3) (Farris and Olson, 2007). The amplification mixture of 25µl volume consisted of a reaction buffer containing 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.5µM of each primer, 0.625 U of Taq DNA polymerase, and 5µl genomic DNA. The primers were obtained from Integrated DNA Technologies (IDT) and the rest were obtained from Promega. Amplification conditions were an initial denaturation of 95°C/5 minutes, followed by 40 cycles of 95°C/45 seconds, 50°C/60 seconds, 72°C/60 seconds, and a final extension reaction of 72°C/7 minutes. The PCR products were digested by restriction enzymes *Rsa*I and *Cfo*I (Promega) following the manufacturer's instructions. The digestion by the two enzymes was performed separately. 10µl of digested DNA was loaded in 3% w/v agarose wells (added with 1µl/10ml v/v LabSafe™ Nucleic Acid Stain) along with 2µl of 6X loading dye and electrophoresed. A 100bp DNA ladder was used for size reference. Gel images were analyzed using PyElph software (Pavel and Vasile, 2012). Clusters generated from dendrograms by the Neighbour-Joining method (Saitou and Nei, 1987) were analyzed and all the investigated isolates were grouped into different phylotypes. Representative isolates from each phylotype were randomly selected for 16S rRNA gene sequence analysis. For those samples whose ARDRA analysis could not be performed, their 16S rRNA gene sequences were directly analyzed.

16S rRNA gene sequence analysis

16S rRNA gene was amplified using one of the four different primer pairs (Table 1) depending on which pair successfully amplified the 16S rRNA gene. The sequences of the primers used were 27F 5'-AGAGTTTGATCMTGGCTCAG-3', A3R 5'-CCAGCCCCACCTTCGAC-3', Sm5R 5'-GAACTGAGACCGGCTTTTTGA-3' (Monciardini *et al*.,2002), 1392R 5'- ACGGGCGGTGTGTRC-3' (Farris and Olson, 2007), and SPActi1339aA18 5'-TCWGCGATTACTAGCGAC-3' (Pfeiffer *et al*., 2014). Amplification reactions were performed in a 25µl reaction mixture as already mentioned above for partial amplification of the 16S rRNA gene for ARDRA analysis**.** Amplification conditions and PCR product sizes are ~~also~~ given in Table 1. The amplified products were observed in electrophoresed agarose gel (1.2% w/v agarose and 1µl/10ml v/v LabSafe™ Nucleic Acid Stain). 100bp DNA ladder was used as a size reference.

The PCR products were sequenced at Xcelris Labs Ltd., Ahmedabad, India. The partial 16S rRNA gene sequences obtained were mined for closely related type strains from the EzBioCloud database (Yoon *et al*., 2017) and the phylogenetic affinities among the isolates and with the closely related type strains were determined using MEGA 7 (Kumar *et al*., 2016). Phylogenetic trees were constructed using the Maximum Likelihood method with bootstrapping (Felsenstein, 1985) of 1000 replicates. The obtained partial 16S rRNA gene sequences were deposited in GenBank with accession numbers MN173036-MN173055, MN173058-MN173077, and MN173097-MN173108.

Table 1: PCR primers, product length, and amplification parameters for 16S rRNA gene

Primer pair	PCR product (bp)	Cycling parameters #
27F-A3R	1500	95/300-35×(95/45-51/45-72/90)-72-420
27F-Sm5R	1300	95/300-35×(95/45-49/45-72/90)-72/420
27F-1392R	1400	95/300-35×(95/45-50/45-72/90)-72/420
27F- SPActi1339aA18	1300	95/300-35×(95/45-47/45-72/90)-72/420

#Numbers before forward-slash refer to temperature (°C) and numbers after forward-slash refer to time (seconds) for each step.

Screening of isolates for antimicrobial activity and detection of biosynthetic gene clusters

The prepared culture filtrates of the isolates were assayed for antimicrobial activity against *Escherichia coli* MTCC 1669, *Pseudomonas aeruginosa* MTCC 4673, *Klebsiella pneumoniae* MTCC 10309, *Staphylococcus aureus* MTCC 9886, *Bacillus subtilis* MTCC 1305, *Micrococcus luteus* MTCC 1538, *Candida albicans* MTCC 7253 and *C. tropicalis* MTCC 184 by agar-well diffusion method (Syiemiong and Jha, 2019a). Detection of biosynthetic gene clusters (BGCs) related to the synthesis of antimicrobial compounds, viz. type I polyketide synthase (PKS-I), type II polyketide synthase (PKS-II), and Non-ribosomal peptide synthetase (NRPS) gene clusters were also performed by PCR according to Syiemiong and Jha (2019a).

Screening of isolates for plant growth-promoting activity

The isolates were assessed for their ability of indole-3-acetic acid (IAA) production, phosphate solubilization, siderophore production, and growth in a nitrogen-free medium according to the methods described by Syiemiong and Jha (2019b).

Screening of isolates for antagonistic activity against phytopathogens

Antagonistic activity of the isolates against potential phytopathogenic bacterial and fungal strains viz., *Xanthomonas campestris*pv. *campestris*Pammel (ITCC No. BH0004), *Xanthomonas oryzae*pv. *oryzae* Ishiyama (ITCC No. BB0013), *Ralstonia solanacearum* Smith (ITCC No. BI0001), *Fusarium oxysporum* f. sp. *zingiberi* Trujillo (ITCC No. 2698), and *Aspergillus niger* MTCC 4325, was assessed by dual culture. The ITCC cultures were obtained from the Indian Type Culture Collection, Division of Plant Pathology, IARI, New Delhi, India, and the MTCC cultures were obtained from Microbial Type Culture Collection and Gene Bank, CSIR-IMTECH, Chandigarh, India. Antagonistic activity assay against bacterial strains was performed by the cross-streak method as described by Vijayakumar *et al*. (2015) and against fungal strains, as described by Khamna *et al*. (2009).

Statistical analysis

Pair-wise comparison between means was analyzed by a two-sample t-test at $p<0.05$. Multiple comparisons between means were analyzed by ANOVA with Duncan's test at $p<0.05$. The software package XLSTAT 2019 was used to compute all the data.

RESULTS AND DISCUSSION

Soil characteristics of the sampling site

The climate at the sampling site during sample collection was moderately warm with an air temperature of 24.5±0.29 °C. Soil temperature was also moderately warm having a temperature of 23.5±0.29 °C. Moisture content was low at 15.77±3.67 %, pH was slightly acidic at 6.06±0.22 and organic carbon was high at 4.88±0.14 %. Nitrogen and available phosphorus were within the normal range at 0.16±0 % and 6.53±0.24 µg g^{-1} respectively. Available potassium was, however, higher than normally reported at 453.05±11.88 µg g^{-1}.

Isolation of actinobacteria

From the different pretreatments used, the highest actinobacterial population number was recorded

from samples treated with 0.2% humic acid (Fig. 1), which was significantly higher than the other treatments at an average of 137.8±37.41×10^{2}CFU g^{-1} soil. However, most of the pure cultures were recovered from wet heat and unpretreated air-dried samples which together contributed 56% of the total isolates. Dry heat and 1.5% phenol treatments even though recorded very low population numbers compared to 0.2% humic acid treatment, contributed a substantial number of pure cultures (35%). 0.2% ofhumic acid treatment contributed only 9% of the total pure cultures. From the different isolation media used, the HVA medium recorded a significantly higher population number than the other media (Figure 1) at an average of 82.67±33.2×10^{2}CFU g^{-1} soil. It also contributed to the highest number of pure cultures obtained with 33% of the total isolates. However, other media also substantially contributed pure cultures for this study, where 19%, 17%, 16%, and 15% of the pure cultures came from AIA, SA, SCA, and BA media respectively.

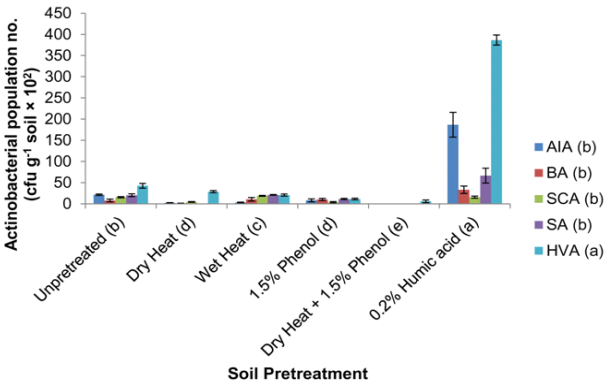

Fig. 1 : Influence of pretreatment and isolation medium on therecovery of actinobacteria

AIA, Actinomycete Isolation Agar; BA, Bennett's Agar; SCA, Starch Casein Agar; SA, Streptomyces Agar; HVA, Humic acid Vitamin Agar. Different lower-case letters within parenthesis were significantly different among different pretreatments and different isolation media at p<0.05. Vertical bars represent the standard error of the mean.

Characterization of isolates

The isolates were microscopically seen with substrate mycelia, aerial mycelia, or both. Numerous isolates belonging to the genus *Streptomyces* bore spore chains, with spiral, hooked or straight chains. All the isolates were found to be Gram-positive. For preliminary identification of the isolates, the biochemical characteristics and DAP isomers were used for identification. Out of the 94 isolates obtained, 75 were identified as *Streptomyces,* 9 as *Nocardia,* another 9 as *Actinomadura* and the identity of one isolate could not be ascertained. The biochemical and chemotaxonomic profile of the isolates is given in Table 2.

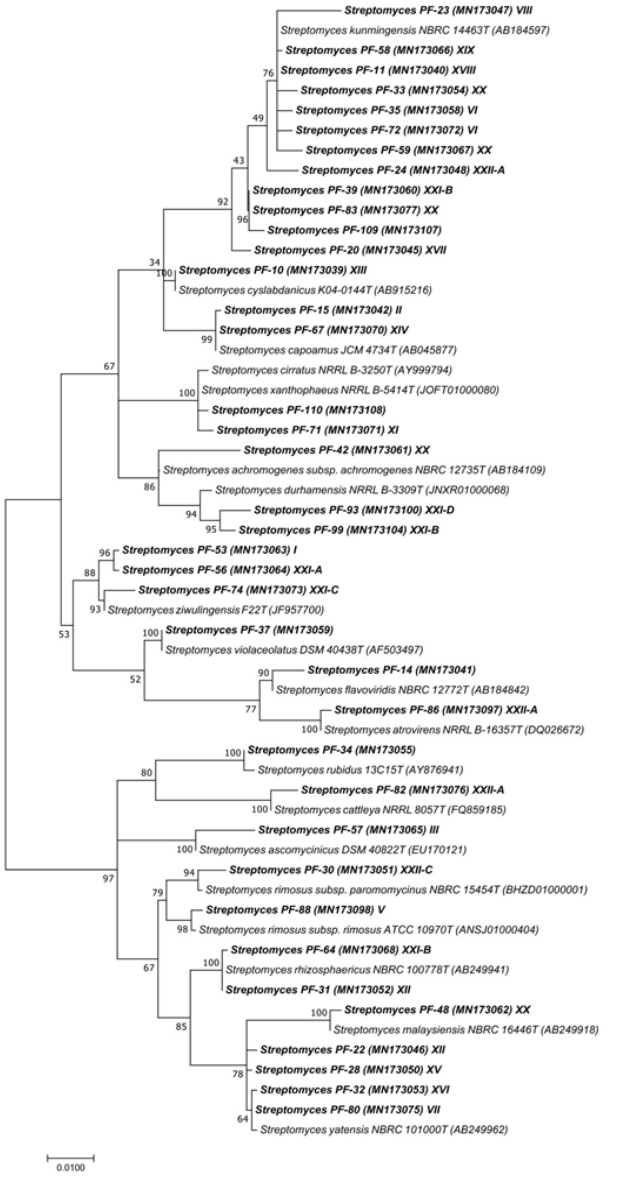

Fig. 2 : Maximum Likelihood Tree of 16S rRNA gene sequenced *Streptomyces* isolates along with type strains from EzBioCloud database.The numbers at the nodes are bootstrap percentages based on 1000 replicates. Bar equals 1% sequence divergence. ARDRA phylotypes are given in uppercase Roman letters after each isolate.

ARDRA analysis using restriction enzyme *Rsa*I classified 77 isolates into 24 phylotypes (Table 3), 17 of which belonged to *Streptomyces*, two belonged to *Actinomadura*, another two belonged

Table 2: Biochemical characteristics and DAP isomer of the isolates

Isolate	Biochemical characteristics				DAP
	Casein decomposition	Xanthine decomposition	Hypoxanthine decomposition	Tyrosine decomposition	
PF-01	+	+	+	+	LL
PF-02	+	-	-	-	meso
PF-03	-	-	+	-	meso
PF-04	+	-	+	-	meso
PF-05	+	-	+	-	LL
PF-06	-	-	-	-	meso
PF-07	-	-	+	-	meso
PF-08	+	-	+	-	LL
PF-09	+	+	+	+	LL
PF-10	+	+/-	-	-	LL
PF-11	+	+	+	+	LL
PF-12	+	+	+	+	LL
PF-13	+	-	-	-	LL
PF-14	+	+	+	+	LL
PF-15	+/-	+/-	+/-	+/-	LL
PF-16	+	-	-	-	meso
PF-17	+	-	+	-	meso
PF-18	+	-	+	-	LL
PF-19	+	+	+	+	LL
PF-20	-	+	+	+	LL
PF-21	-	-	-	-	meso
PF-22	+	-	-	-	LL
PF-23	-	-	-	-	LL
PF-24	-	+/-	-	-	LL
PF-25	+	-	+	-	LL
PF-26	+	-	+	+/-	LL
PF-27	+	-	+	-	LL
PF-28	+	-	-	+	LL
PF-29	+	-	-	-	LL
PF-30	+	-	+	-	LL
PF-31	-	+	+	+	LL
PF-32	+/-	-	+/-	+/-	LL
PF-33	+	+	+	+	LL
PF-34	+	-	-	-	LL
PF-35	+	+	+	+	LL
PF-36	+	-	+	+	LL
PF-37	+	+	+	+/-	LL
PF-38	+	+	+	+	LL
PF-39	+	+	+	+	LL
PF-40	+	+	+	+	LL
PF-41	+/-	-	-	-	LL
PF-42	-	+	+	-	LL
PF-43	+	-	-	-	LL
PF-45	+	+	+	+	LL
PF-48	+	+	+	+	LL
PF-49	+	+	+	+	LL
PF-51	+	+	+/-	-	LL
PF-53	+	-	-	-	LL
PF-56	+	+	+	-	LL
PF-57	-	-	-	-	LL
PF-58	+	+	+	+	LL
PF-59	+	+	-	+	LL
PF-60	+	+	+	+	LL
PF-61	+	-	+	+	LL
PF-63	+	-	-	-	LL
PF-64	-	-	-	+	LL
PF-66	+	+/-	-	-	meso
PF-67	+	+/-	-	+/-	LL
PF-68	-	-	+	-	LL
PF-69	+	+	+	+	LL
PF-71	+/-	+	+/-	+/-	LL
PF-72	+	+	+/-	+/-	LL
PF-73	+	+	+/-	+/-	LL
PF-74	+/-	+/-	+/-	+/-	LL
PF-75	+	+	+	+	LL
PF-78	+	+/-	+	+	meso

PF-79	+	+	+	+	LL
PF-80	+	-	+	+/-	LL
PF-81	-	-	-	+/-	meso
PF-82	-	-	-	-	LL
PF-83	+	+	+	+	LL
PF-84	-	-	-	-	meso
PF-85	+	-	+	-	LL
PF-86	+	+	+	+	LL
PF-88	+	-	+	-	LL
PF-89	+/-	+/-	+/-	+/-	meso
PF-90	+	+	+	+	LL
PF-91	+	-	-	+/-	meso
PF-92	+	-	-	+	meso
PF-93	+/-	+/-	+/-	+/-	LL
PF-94	+	-	+	-	meso
PF-95	-	+	+	+	LL
PF-96	-	-	-	-	meso
PF-98	-	-	+	+	meso
PF-99	-	+	+	+	LL
PF-100	+	-	+	-	meso
PF-101	+	+	+	-	LL
PF-103	+	+	+	+	LL
PF-105	+	-	+	+	LL
PF-107	+	-	-	-	LL
PF-108	+/-	+/-	+/-	+/-	LL
PF-109	+	+/-	+/-	+	LL
PF-110	+	+	+/-	+/-	LL
PF-111	+	+	+	+/-	LL

+ positive; - negative; +/- uncertain; DAP, 2,6-diaminopimelic acid

Table 3: ARDRA-based phylotyping of isolates using restriction enzyme *Rsa*I and 16S rRNA gene sequence comparison of representative isolates with type strains from EzBioCloud database

Phylotype	Number of isolates	Representative isolates	Type strain with the closest match	% similarity
I	2	*Streptomyces* PF-53 (MN173063)	*Streptomyces ziwulingensis* F22T (JF957700)	99.49
II	2	*Streptomyces* PF-15 (MN173042)	*Streptomyces capoamus* JCM 4734T (AB045877)	99.91
III	1	*Streptomyces* PF-57 (MN173065)	*Streptomyces ascomycinicus* DSM 40822T (EU170121)	99.02
IV	1	*Streptosporangium*PF-78 (MN173074)	*Streptosporangiumanatoliense* N9999T (HQ157194)	99.07
V	1	*Streptomyces* PF-88 (MN173098)	*Streptomyces rimosus* subsp. *rimosus* ATCC 10970T (ANSJ01000404)	99.74
VI	5	*Streptomyces* PF-35 (MN173058)	*Streptomyces kunmingensis* NBRC 14463T (AB184597)	99.83
		Streptomyces PF-72 (MN173072)	*Streptomyces kunmingensis* NBRC 14463T (AB184597)	99.42
VII	3	*Streptomyces* PF-80 (MN173075)	*Streptomyces yatensis* NBRC 101000T (AB249962)	99.83
VIII	1	*Streptomyces* PF-23 (MN173047)	*Streptomyces kunmingensis* NBRC 14463T (AB184597)	98.96
IX	1	*Actinomadura* PF-100 (MN173105)	*Actinomadurameyerae* DSM 44715T (jgi.1107661)	98.99
X	1	*Actinomadura* PF-04 (MN173037)	*Actinomaduranapierensis* B60T (AY568292)	98.72
XI	3	*Streptomyces* PF-71 (MN173071)	*Streptomyces cirratus* NRRL B-3250T (AY999794)	99.92
XII	4	*Streptomyces* PF-22 (MN173046)	*Streptomyces yatensis* NBRC 101000T (AB249962)	99.66
		Streptomyces PF-31 (MN173052)	*Streptomyces rhizosphaericus* NBRC 100778T (AB249941)	100
XIII	1	*Streptomyces* PF-10 (MN173039)	*Streptomyces cyslabdanicus* K04-0144T (AB915216)	100
XIV	2	*Streptomyces* PF-67 (MN173070)	*Streptomyces capoamus* JCM 4734T (AB045877)	100
XV	1	*Streptomyces* PF-28 (MN173050)	*Streptomyces yatensis* NBRC 101000T (AB249962)	99.57
XVI	1	*Streptomyces* PF-32 (MN173053)	*Streptomyces yatensis* NBRC 101000T (AB249962)	99.58
XVII	1	*Streptomyces* PF-20 (MN173045)	*Streptomyces kunmingensis* NBRC 14463T (AB184597)	98.81
XVIII	1	*Streptomyces* PF-11 (MN173040)	*Streptomyces kunmingensis* NBRC 14463T (AB184597)	99.91
XIX	3	*Streptomyces* PF-58 (MN173066)	*Streptomyces kunmingensis* NBRC 14463T (AB184597)	99.83
		Streptomyces PF-33 (MN173054)	*Streptomyces kunmingensis* NBRC 14463T (AB184597)	99.57
XX	10	*Streptomyces* PF-42 (MN173061)	*Streptomyces achromogenes* subsp. *achromogenes* NBRC 12735T (AB184109)	98.54
		Streptomyces PF-48 (MN173062)	*Streptomyces malaysiensis* NBRC 16446T (AB249918)	99.75
		Streptomyces PF-59 (MN173067)	*Streptomyces kunmingensis* NBRC 14463T (AB184597)	99.49
		Streptomyces PF-83 (MN173077)	*Streptomyces kunmingensis* NBRC 14463T (AB184597)	99.39
XXI	13	These isolates were subjected to ARDRA analysis with a second restriction enzyme *Cfo*I. Refer Table 32B.		
XXII	16	These isolates were subjected to ARDRA analysis with a second restriction enzyme *Cfo*I. Refer to Table 32B.		
XXIII	2	*Streptosporangium* PF-91 (MN173099)	*Streptosporangiumpseudovulgare* DSM 43181T (X89946)	99.70
		Streptosporangium PF-98 (MN173103)	*Streptosporangiumanatoliense* N9999T (HQ157194)	99.08
XXIV	1	*Nonomuraea* PF-94 (MN173101)	*Nonomuraeabangladeshensis* 5-10-10T (AB274966)	100

GenBank accession numbers are given within parenthesis

Table 4: ARDRA-based sub-phylotyping of isolates using restriction enzyme *Cfo*I and 16S rRNA gene sequence comparison of representative isolates with type strains from EzBioCloud database

Sub-phylotype	Number of isolates	Representative isolates	Type strain with the closest match	% similarity
XXI-A	5	*Streptomyces* PF-56 (MN173064)	*Streptomyces ziwulingensis* F22^T (JF957700)	99.23
		Streptomyces PF-39 (MN173060)	*Streptomyces kunmingensis* NBRC 14463^T (AB184597)	99.43
XXI-B	4	*Streptomyces* PF-64 (MN173068)	*Streptomyces rhizosphaericus* NBRC 100778^T (AB249941)	100
		Streptomyces PF-99 (MN173104)	*Streptomyces durhamensis* NRRL B-3309^T (JNXR01000068)	98.99
XXI-C	1	*Streptomyces* PF-74 (MN173073)	*Streptomyces ziwulingensis* F22^T (JF957700)	99.38
XXI-D	2	*Streptomyces* PF-93 (MN173100)	*Streptomyces durhamensis* NRRL B-3309^T (JNXR01000068)	99.06
XXI-E	1	*Amycolatopsis* PF-02 (MN173036)	*Amycolatopsisrhabdoformis* SB026^T (KF779477)	99.41
XXII-A	5	*Streptomyces* PF-24 (MN173048)	*Streptomyces kunmingensis* NBRC 14463^T (AB184597)	99.38
		Streptomyces PF-82 (MN173076)	*Streptomyces cattleya* NRRL 8057^T (FQ859185)	99.57
		Streptomyces PF-86 (MN173097)	*Streptomyces atrovirens* NRRL B-16357^T (DQ026672)	99.84
XXII-B	3	*Nocardia* PF-07 (MN173038)	*Nocardia jiangxiensis* NBRC 101359^T (BAGB01000020)	99.84
XXII-C	1	*Streptomyces* PF-30 (MN173051)	*Streptomyces rimosus* subsp. *paromomycinus* NBRC 15454^T (BHZD01000001)	99.49
XXII-D	3	*Kribbella* PF-26 (MN173049)	*Kribbellasindirgiensis* FSN23^T (JN896614)	99.49
		Kribbella PF-105 (MN173106)	*Kribbellapodocarpi* YPL1^T (KM382222)	99.58
XXII-E	4	*Nonomuraea* PF-16 (MN173043)	*Nonomuraeakuesteri* NRRL B-24325^T (JOAM01000718)	99.83

GenBank accession numbers are given within parenthesis

Table 5: Isolates not phylotyped by ARDRA analysis along with closely related type strains from EzBioCloud database

Isolate	GenBank Accession No.	Type strain with the closest match	% Similarity
Nocardia PF-03#	--	--	--
Streptomyces PF-08#	--	--	--
Streptomyces PF-14	MN173041	*Streptomyces flavoviridis* NBRC 12772^T (AB184842)	99.32
Nonomuraea PF-17	MN173044	*Nonomuraeabangladeshensis* 5-10-10^T (AB274966)	99.34
Streptomyces PF-25#	--	--	--
Streptomyces PF-29#	--	--	--
Streptomyces PF-34	MN173055	*Streptomyces rubidus* 13C15^T (AY876941)	99.75
Streptomyces PF-36#	--	--	--
Streptomyces PF-37	MN173059	*Streptomyces violaceolatus* DSM 40438^T (AF503497)	100
Streptomyces PF-45@	--	--	--
Streptomyces PF-63#	--	--	--
Actinomadura PF-66	MN173069	*Actinomadurasyzygii* GKU 157^T (KF667496)	99.41
Nocardia PF-84#	--	--	--
Nonomuraea PF-96	MN173102	*Nonomuraeasyzygii* GKU 164^T (KF667499)	98.85
Streptomyces PF-107#	--	--	--
Streptomyces PF-109	MN173107	*Streptomyces kunmingensis* NBRC 14463^T (AB184597)	99.22
Streptomyces PF-110	MN173108	*Streptomyces xanthophaeus* NRRL B-5414^T (JOFT01000080)	99.83

#Isolates lost due to contamination
@16S rDNA amplification failed with all available primers

to *Streptosporangium* and one belonged to *Nonomuraea*.Twenty-nine isolates from two phylotypes XXI and XXII were subjected to a second ARDRA analysis using restriction enzyme *Cfo*I and were each further classified into five sub-phylotypes

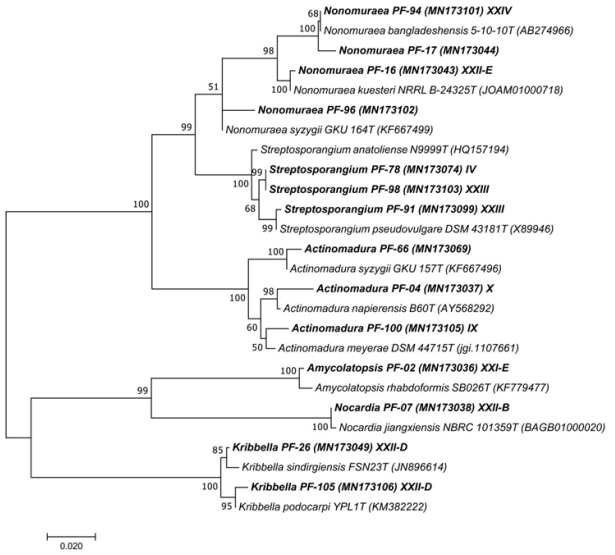

Fig. 3 : Maximum Likelihood Tree of 16S rRNA gene sequenced non-streptomycete isolates along with type strains from EzBioCloud databaseThe numbers at the nodes are bootstrap percentages based on 1000 replicates. Bar equals 1% sequence divergence. ARDRA phylotypes are given in uppercase Roman letters after each isolate.

(Table 4), six of which belonged to *Streptomyces*, and one phylotype each belonged to *Amycolatopsis, Nocardia, Kribbella* and *Nonomuraea*. Eight isolates could not be characterized by ARDRA analysis and their 16S rRNA genes were directly sequenced, of which five were found to be *Streptomyces*, two were *Nonomuraea* and one was *Actinomadura*(Table 5). Nine isolates were neither ARDRA-characterized nor sequenced due to amplification failure or loss due to contamination.Phylogenetic trees of the 16S rRNA gene sequenced isolates are given in Figs. 2 and 3.

Based on morphological, biochemical, chemotaxonomic, ARDRA and 16S rRNA gene sequence analysis, the 94 isolates were grouped into seven genera, with *Streptomyces* being the largest group of 72 isolates, followed by *Nonomuraea*(7), *Nocardia* (5), *Actinomadura*(3), *Kribbella*(3), *Streptosporangium*(3) and *Amycolatopsis*(1).Diversity indices were calculated using software called "PAleontologicalSTatistics (PAST)".The diversity indices reveal a relatively low generic diversity due to the dominance of *Streptomyces* (Simpson 1-D index 0.4; Shannon H index 0.93; Evenness $e^{H/S}$ index 0.36; Menhinick index 0.72; Margalef index 1.32; Equitability J index 0.48).

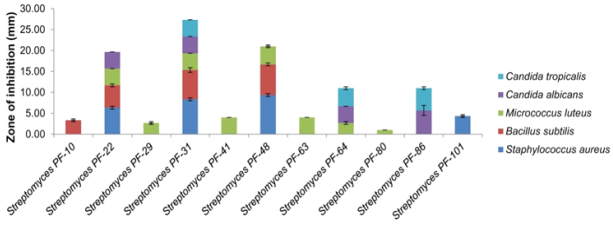

Fig. 4 : Antimicrobial activity of bioactive isolates Vertical bars represent standard error of mean

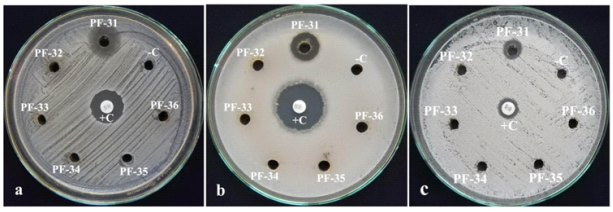

Fig. 5 : Agar well diffusion assay plates showing bioactivity of *Streptomyces* PF-31 a, lawn of *Staphylococcus aureus*; b, *Bacillus subtilis*; c, *Candida tropicalis*; -C, negative control (uninoculated broth); +C, positive control (vancomycin disc, 30µg / amphotericin-B disc, 20µg).

Antimicrobial activity and biosynthetic gene clusters

Ten isolates exhibited antibacterial activity against Gram-positive bacteria and only four exhibited anticandidal activity. These bioactive isolates were attributed only to *Streptomyces*. The streptomycete isolates PF-22, PF-31 and PF-48 showed potent antibacterial activity against all three Gram-positive bacteria (Fig 4). Isolates PF-31, PF-64, and PF-86 showed potent anticandidal activity against both the candidal strains. *Streptomyces* PF-31 exhibited the broadest spectrum of activity and its bioactivity in agar well diffusion assay plates is shown in Figure 5.Sixty-one percent of the isolates were found to harbor at least one of the three biosynthetic gene clusters (BGCs) related to the synthesis of antimicrobial compounds. However, only 5% of them manifested bioactivity in the agar well diffusion assay. It was also noted that 6% of the isolates manifested bioactivity, with no detection of any of the BGCs, and notably,*Streptomyces* PF-22 which manifested broad-spectrum activity. All the BGCs were more prevalent in the genus *Streptomyces*.

Plant growth-promoting activity

The isolates produced low levels of IAA and the highest recorded production was only 11.44±0.81

µg ml^{-1} by *Streptomyces* PF-51 which was below the moderate production range (Jog *et al*. 2016). Fifteen isolates solubilized phosphate above 350µg ml^{-1}, a level comparable to other reported plant growth-promoting rhizobacteria (Jog *et al*. 2016),with *Streptomyces* PF-35 showing the highest ability at 843.81±11.08 µg ml^{-1}.Four isolates mostly *Streptomyces*, showedthe highest siderophore activity not significantly different from one another. Fifteen isolates showed siderophore activity (above 25 mm halo diameter) which was at a range reported by other workers.Twenty-seven isolates could grow optimally on a nitrogen-free medium and increased its pH, suggesting that they were probable diazotrophs.

Antagonistic activity against phytopathogens

Three streptomycete isolates showed antagonism against both*Xanthomonas oryzae*and *X. campestris*, out of which PF-56 was the most prominent (Fig 6a, 7a). Ten isolates, mostly *Streptomyces*, showed antagonism against *Aspergillus niger*and *Fusarium oxysporum*, out of which, six were against *A. niger*and eight were against *F. oxysporum*(Fig 6b).Four streptomycete isolates showed antagonism against both *A. niger*and *F. oxysporum*, one of whose (*Streptomyces* PF-48) antagonistic dual culture assay is shown in Figure 7b and c.It is noteworthy that the bioactive isolates showed specificity against the target microorganism. Almost all the antibacterial isolates were not antifungal and vice versa.

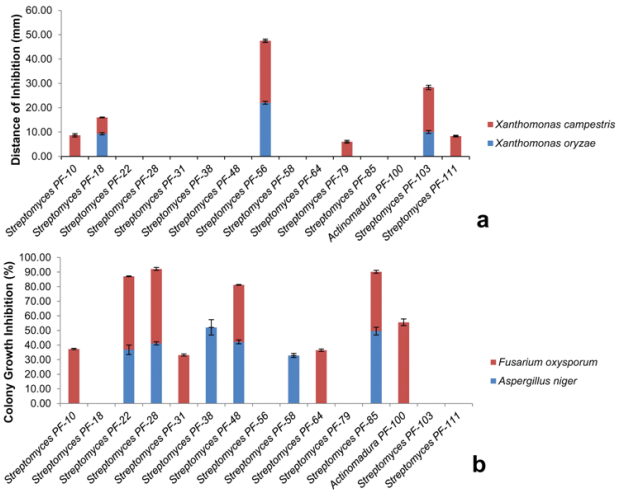

Fig. 6 : Antagonistic activity against phytopathogens a, antibacterial activity; b, antifungal activity; Vertical bars represent standard error of mean

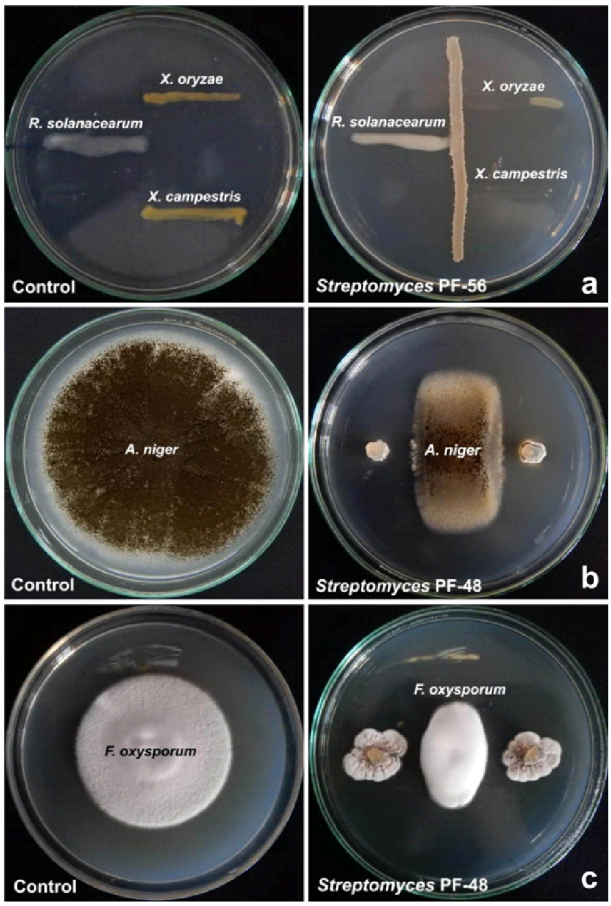

Fig. 7 : Antagonistic activity of (a) *Streptomyces* PF-56 against *X. oryzae and X. campestris*, (b-c) *Streptomyces* PF-48 against *A. niger* and *F. oxysporum*.

The moderately warm climate at the sampling site was due to its altitude (688m amsl) which makes it a tropical region of semi-evergreen vegetation. The soil physicochemical characteristics were more or less the same as previously reported from the same location by Thapa *et al*. (2011) and from similar other locations of Meghalaya forests (Tripathi *et al*. 2009).

Humic acid pretreatment of the soil samples before isolation, helped in the maximum recovery of actinobacteria because it activates spore germination of spore-formers and increases their recovery. However, this pretreatment method was impractical to obtain pure cultures due to overcrowding of actinobacterial colonies. Other pretreatment methods fared better in recovering pure cultures. HVA was a good medium for isolation of actinobacteria and has been reported to help recover diverse actinobacteria from the soil and found to suppress the growth of non-filamentous bacteria (Yamamura *et al*. 2003). However, as evident from the isolation results, the other media

used for isolation in this work also fared well in the recovery of pure cultures. They have also been previously reported to have been successfully used in the isolation of actinobacteria (Abidin *et al*. 2016; Syiemiong and Jha 2019a).

Based on morphological, biochemical, and chemotaxonomic characteristics, all the isolates were grouped into three genera viz., *Streptomyces*, *Nocardia,* and *Actinomadura*, with a large dominance of *Streptomyces* (80%). ARDRA and 16S rRNA gene sequence analyses further refined the groupings into seven genera with *Nonomuraea*, *Kribbella*, *Streptosporangium,* and *Amycolatopsis* as additional genera, with *Streptomyces* being similarly dominant (77%). Due to the single domination of *Streptomyces*, the generic diversity was relatively low as revealed by the different diversity indices. Other workers have also reported the dominance of *Streptomyces* from forest soils including rhizosphere soils from different parts of the world through cultivation-based approaches (Hayakawa *et al*. 2010; Lee and Whang 2010; Varghese *et al*. 2012, 2014; Rai *et al.* 2018; Sharma and Thakur 2020). *Streptomyces* was also recently found to be the dominant genera from endophytic actinobacteria isolated from six different plant species from tropical deciduous forests of Meghalaya (Barman and Dkhar 2020). The dominance of *Streptomyces*using cultivation-based approaches is due to the versatility of the genus in nutrient acquisition froma wide range of synthetic media(Maciejewska *et al*. 2016). However, there are reports where rare actinobacteria have also been frequently recovered using specialized enrichment methods and media favoringthe growth of rarer genera (Talukdar *et al*. 2012). The diversity, although seemed low at the generic level, may not be the case at the species level due to the large number of phylotypes generated from ARDRA analysis (Simpson 1-D index 0.9; Shannon H index 2.66; Evenness $e^{H/S}$ index 0.59; Menhinick index 2.74; Margalef index 5.3; Equitability J index 0.84), as well as due to the numerous species of closely related type strains retrieved from EzBioCloud database against the 16S rRNA gene sequenced isolates (Simpson 1-D index 0.92; Shannon H index 3.05; Evenness $e^{H/S}$ index 0.7; Menhinick index 4.16; Margalef index 7.34; Equitability J index 0.9). Nevertheless, more pretreatment and enrichment methods, selective media, and growth conditions need to be incorporated in the isolation of actinobacteria to recover a more diverse generic composition, with possible chances of harnessing more diverse beneficial phenotypes from them. Additionally, due to the recent advent of next-generation sequencing technologies, culture-independent approaches of microbial diversity analyses is now the gold standard due to its higher accuracy, and should also be routinely used in actinobacterial diversity analyses of such pristine forest soil ecosystems of Meghalaya.

Using the agar well diffusion assay method, the bioactive isolates manifested antimicrobial activity against Gram-positive and candidal strains. The lack of bioactivity against Gram-negative bacteria was most probably due to the resistant nature of their cell wall and other antibiotic resistance mechanisms. Several workers have also similarly reported on stronger antibacterial activity of actinobacteria against Gram-positive than against Gram-negative strains(Nimaichand *et al*. 2015; Maciejewska *et al*. 2016; Hamedi *et al*. 2019). Similar to our work, *Streptomyces* as the most prevalent antagonistic genus, have also been reported by others (Nimaichand *et al*. 2015; Ahmad *et al*. 2017; Charousová *et al*. 2017). Nevertheless, the broad-spectrum antimicrobial isolates PF-22, PF-31, and PF-48 from our work have been further investigated against more indicator strains and characterization of their bioactive compounds is underway. From the screening of isolates for the presence of biosynthetic gene clusters (BGCs), only 5% expressed bioactivity out of the 61% isolates detected with at least one of the gene clusters.One of the reasons could be due to the method of antimicrobial screening used. The agar well diffusion assay method adopted here for the screening of antimicrobial activity is flawed as it caters to detect only those isolates which constitutively produce the bioactive metabolites, irrespective of whether the target microorganism is in the vicinity or not. Several potential bioactive isolates expressing antimicrobial activity only in co-culture (Bertrand *et al.* 2014) would have been ignored by this screening method. Another reason for the low percentage of bioactivity could be due to inappropriate culture conditions which have failed to activate the metabolic pathways synthesizing antimicrobial compounds (Chiang *et al*. 2011). Many previous workers have also shownpieces of evidenceon the presence of silenced or under-expressed antibiotic gene

clusters while working on antimicrobial screening of actinobacteria (Li *et al*. 2012; Nimaichand *et al.* 2015; Bundale *et al.* 2018; Wei *et al*. 2018). Nevertheless, with proper culture conditions and screening methods, the percentage of antimicrobial phenotypic expressions could be substantially increased, especially becausenumerous actinobacterial species are found to harbor more than 20 biosynthetic gene clusters (BGCs) for the production of bioactive metabolites (Goodfellow and Fiedler 2010). The bioactive isolates not detected with any of the BGCs could be due to failure of amplification during PCR which might have arisen due to sequence variation within the primer binding site or due to the presence of PCR-inhibiting compounds in the sample (Watson and Blackwell 2000). Other BGCs not tested here might also have been responsible for the bioactivity.

Most of the plant growth-promoting (PGP) isolates were from the genus *Streptomyces*. IAA production by the isolates was low (highest level of 11.44±0.81 µg ml^{-1}) asother workers have reported IAA production of as high as 197 µg ml^{-1}(Dochhil *et al*. 2013). The forest vegetation of the study site was abundant and at their optimal growth, and they probably produce enough endogenous IAA for their normal growth and development. They probably did not put up much of a demand for IAA from external sources such as the vicinity of soil microbes. Even though the phosphate solubilizing ability of the isolates from this work were comparatively lower to other highly cited PGPRs (Jog *et al*. 2016), some other workers have reported phosphate solubilization levels comparable to our isolates, ranging from 100µg ml^{-1} to 680µg ml^{-1}(Dastager and Damare 2013; Gangwar and Kataria 2013; Palaniyandi *et al*. 2013; Paviæ *et al.* 2013; Gangwar *et al*. 2014; Saif *et al*. 2014; Gangwar *et al.* 2015; Tamreihao *et al.* 2017). The siderophore activity of the isolates from this work wasalso comparable with reports by previous workers (Khamna *et al.* 2009; Ruanpanun *et al*. 2010; Misk and Franco 2011; Goudjal *et al.* 2016; Singh *et al*. 2017; Lasudee *et al.* 2018). There were also many isolates with the potential diazotrophic ability which could be assessed only qualitatively. The tropical semi-evergreen forest at the study site with abundant vegetation demands a lot of phosphorus, iron, and nitrogen from the soil, and the soil microbes, including the PGP actinobacteria, help in making these nutrients available to the plants to maintain a stable ecosystem function. As seen in the screening results, several isolates were also found to have biocontrol potential, as they showed antagonism against bacterial and fungal phytopathogens in dual culture. Previous workers have also reported on the biocontrol potential of forest soil actinobacteria against various phytopathogens (Crawford *et al.* 1993; Sacramento *et al*. 2004; Lehr *et al.* 2008; Ningthoujam *et al*. 2009; Evangelista-Martinez 2014). The specificity of the antagonistic isolates towards their target microorganisms reveals the existence of different metabolic pathways for the synthesis of different antimicrobial compounds specific in action against particular groups of target microorganisms. A few promising isolates with PGP and biocontrol potential have been selected and their *in vivo* assessment of promoting plant growth in tomato is currently being carried out under greenhouse conditions.

CONCLUSION

The pristine forest soil of Nongkhyllem Wildlife Sanctuary, Meghalayathough harbors a diverse array of species but the cultivable generic composition was primarily dominated by *Streptomyces*, thus depicting a low generic diversity. Only a few of the recovered isolates, including*Streptomyces*, showed broad-spectrumantimicrobial activity against Gram-positive as well ascandidal strains. Many of them, however, were though havingthe genetic potential for expressing antimicrobial activity but due to some reasons couldn't express it. Some*Streptomycete* isolates also exhibited plant growth promoting traits.

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