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# 'Traditional Sacred Groves', an ethnic strategy for conservation of microbial diversity

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'Sacred groves' represents a traditional effort to conserve biodiversity. They are rich patches of undisturbed forests and serve as a natural habitat for many endemic, rare, primitive and economically valuable organisms representing a micro-level biodiversity hotspots. In this study, *Bacillus* spp. and related genera characterized from native soils of the pristine sacred groves of Meghalaya, India revealed dominance of *Bacillus*, *Paenibacillus*, *Lysinibacillus* and *Viridibacillus*. All the isolates exhibited good plant growth promoting traits when screened for traits like phosphate solubilization, 1-aminocyclopropane-1-carboxylate deaminase and catalase activity, production of auxin, siderophores, hydrogen cyanide and ammonia. Bacteria native to the pristine niche of sacred groves showed better plant growth promoting activities as compared to isolates from disturbed forest as well as type strains implicating the importance of sacred groves and their potential role in microbial diversity conservation.

**Keywords**: Sacred groves, Meghalaya, Microbial diversity, Conservation, Plant growth-promoting

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'Sacred groves' refer to tracts of virgin forests of varying sizes which are community protected, and which usually have a significant religious connotation. Traditionally, 'Sacred groves' have been a means of biodiversity conservation which can be considered the ancient equivalent of natural sanctuaries where all forms of living creatures are given protection by a deity. They are characterized as relics of past vegetation and remnants of large ancient forest lands[1] representing repositories of organisms. Biologically, they are rich patches of undisturbed forests and serve as a natural habitat for many endemic, rare, vulnerable, threatened, primitive and economically valuable organisms representing a micro-level biodiversity hotspots[2,3]. Some of the richest groves in the country are found in the Khasi Hills of Meghalaya, where almost every village is said to have had a grove, known locally as the 'law kyntang'. The state Meghalaya, covering an area of 22 429km$^2$ lie 24°47'–26°10' N and 89°45'–92°47' E, is one of the species rich area under the mega biodiversity centers in North-east India[4]. The importance of 'Sacred groves' biodiversity, its inventorization and conservation has long been recognized[5,6]. Although there is some work done on the faunal and floral components of sacred forests, the microbial components especially the economically important group of bacteria belonging to members of the aerobic spore-forming *Bacillus* and related genera have not received any attention as yet.

The genus *Bacillus* represents one of the most diverse genera in the class bacilli. It includes aerobic and facultatively anaerobic, rod-shaped, gram-positive spore-forming bacteria[7]. 16S rRNA gene sequence analysis has revealed a high level of phylogenetic heterogeneity in this genus, on the basis of which a division into different genera was proposed: *Bacillus, Alicyclobacillus, Paenibacillus, Brevibacillus, Aneurinibacillus, Virgibacillus, Salibacillus* and *Gracilibacillus*[8]. Here the term "*Bacillus* and related genera" is used as an operational term to indicate these organisms. Plant growth-promoting bacteria (PGPB) including *Bacillus* and related genera represent a wide variety of soil bacteria which can stimulate growth of plants. The direct promotion by PGPB entails providing the plant with a plant growth-promoting substance like nitrogen source; iron through siderophores; facilitating the

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uptake of certain plant nutrients from the environment such as phosphorus via phosphate solubilization; synthesizing stimulatory phytohormones like indole-3-acetic (IAA), cytokinin and gibberellins; by the activity of 1-aminocyclopropane-1-carboxylate (ACC) deaminase. The indirect promotion of plant growth occurs when PGPB lessen or prevent the deleterious effect of phytopathogenic micro-organisms on plant growth or development and also from deleterious effects of environmental stressors, through the action of siderophores, Hydrogen cyanide (HCN) production, extracellular hydrolytic enzymes and catabolism of some molecule related with stress signaling such as bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme reduces plant ethylene level, which is increased by various unfavorable conditions and thus confers resistance to stress[9]. The PGP activity of some of the *Bacillus*[10] and related genera has been known for many years, resulting in a broad knowledge of the mechanisms involved[11,12]. There are a number of metabolites that are released by these strains[13], which strongly affect the environment by increasing nutrient availability of the plants[12]. Plant growth-promoting effects have been reported from species of *Bacillus*[10] and related genera, such as *Brevibacillus brevis* Migula[14] and *Paenibacillus lentimorbus* Dutky[15].

The present study was aimed at exploring sacred groves of Meghalaya for native *Bacillus* and related genera. Morphological, biochemical and molecular characterization and inventorization of these bacteria were carried out to examine their diversity, phylogeny and also exploring the various potentials that these organisms have in plant growth, and were compared with human impacted disturbed forests to recognize the importance and potentials of sacred groves in *in situ* conservation of microbial biodiversity.

## Methodology

### Study sites

Soil samples were collected and analysed from 5 different sacred groves (Table 1). To understand the impact of disturbances, soil samples were also collected from disturbed forests sites under similar climatic zones which include the Mawsmai, Mawkadong and Tyrshi forests as described in our earlier study[4].

### Sample collection and soil parameters

Two hundred gm of root-free soil was sampled on each site from a depth of 10–30cm. To document maximum bacterial population and diversity, five replicates were selected in each location and the samples were pooled before analyses. The samples were kept in sterile containers and transported to the laboratory for processing. Soil temperature was measured on site by using a soil thermometer. Soil pH was measured using a soil-water mixture 1:5 (w/v) with a pH meter. Soil moisture content was determined gravimetrically by oven drying 10gm of fresh sieved soil for 24hrs at 105°C.

### Enumeration, isolation and preliminary characterization of bacteria

Colony forming units (CFU) were determined using a soil dilution plate-count technique. Soil was serially diluted with sterile distilled water and the dilutions were used as inoculum for spread plating on Nutrient agar (NA) plates and incubated at 35°C for 48hrs. Well-isolated colonies with different morphologies were chosen from each plate and checked for purity by re-streaking on plates of the same medium. The tests characterizing the *Bacillus* and related genera were carried out in triplicate and included: microscopic appearance, spore-forming, gram stain, catalase test, oxidase test, and reduction of nitrate to nitrite. These tests were performed following standard protocols[16]. *Bacillus* and related genera species were estimated by morphologies and physiology characteristics based on Bergeys' Manual of Systematic Bacteriology.

### Molecular characterization of bacteria and Phylogenetic analysis

Selected isolates were subjected to 16S rRNA gene sequence analysis for establishment of their genotypic position as described in our earlier study[4].

### *In vitro* screening for plant growth-promoting traits of the isolates

Isolates were screened for phosphate solubilization on Pikovskaya's agar plates[10]. Indole-3-acetic acid IAA production was detected by the method as described by Wahyudi *et al.*[10]. Siderophore production was tested qualitatively following the method of Schwyn & Neilands. ACC deaminase activity was screened adapting the method of Penrose and Glick[17]. Filter sterilized ACC solution (3mM) was spread over Dworkin and Foster (DF) minimal salts agar plates and allowed to dry fully and inoculated with bacterial strains and incubated at 30°C for 3 days. The ability of a strain to utilize ACC was

Table 1—Sampling sites and soil parameters of the studied sacred groves

| Sacred Groves (District) | Location | Elevation (m asl) | Humidity (%) | Soil Temperature (ºC) | Soil pH | Soil Moisture content (%) | CFU/g dry soil |
|---|---|---|---|---|---|---|---|
| Law Lyngdoh Mawphlang (East Khasi hills) | 25°25' N and 91°44' E | 1796 | 78 | 20 | 4.5 | 33.6 | $7x10^5$ |
| Diengsyiang (West Khasi hills) | 25°26' N and 91°16' E | 1427 | 84 | 21 | 4.7 | 34.1 | $2x10^6$ |
| Lum Sohpetbneng (Ri Bhoi) | 25°76' N and 91°49' E | 1430 | 72 | 25 | 5.1 | 31.6 | $4x10^4$ |
| Ialong (Jaintia hills) | 25°27' N and 92°15' E | 1013 | 79 | 15.5 | 5.8 | 33.2 | $8x10^4$ |
| Raliang (Jaintia hills) | 25°29' N and 92°23' E | 1336 | 73 | 16.5 | 5.6 | 29.8 | $3x10^4$ |

verified by maintaining the same strain in a control in the absence of any nitrogen source. HCN production was inferred by the qualitative method of Lorck. Ammonia production was tested in peptone water. Freshly grown cultures were inoculated in 10ml peptone water in each tube and incubated for 48–72hrs at 30°C. Nessler's reagent (0.5ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production. Catalase activity test was performed by taking a drop of 3% hydrogen peroxide was added to 48hrs old bacterial colony on a clean glass slide and mixed using a sterile tooth-pick. The effervescence indicated catalase activity.

## Results

### Sampling sites, vegetation and bacterial population counts

The vegetation of the groves composed of important tree species like *Pinus kesiya* Royle ex Gordon, *Podocarpus neriifolius* Don, *Prunus jenkinsii* Hook & Thomson, *Castanopsis purpurella* NP Balakr, *Neolitsea cassia* (L.) Kosterm, *Engelhardtia spicata* Lech ex Blume, *Syzygium tetragonum* (Wight) Wall ex Walp, *Sarcosperma griffithii* Hook ex Clarke, *Fraxinus floribanda* Wall, *Acer laevigatum* Wallich, *Betula alnoides* Buchanan-Hamilton ex Don and bamboo species that constituted the forest canopy, while tree species like *Rhododendron arboretum* Sm., *Antidesma bunius* (L.) Spreng, *Diospyros kaki* L., *Helicia nilagirica* Bedd, *Myrica esculenta* Buchanan-Hamilton ex Don, *Wendlandia wallichii* Wight & Arn, *Erythroxylum kunthianum* Kurz, *Coffea khasiana* Hook, *Microtropis discolor* Wall. ex Meisn and *Sarcococca pruniformis* Lindl. constituted the sub- and under canopy layer in most groves. Shrub layer composed of a number of shrubs and saplings of tree species. Ground flora consisted of grasses, herbs, ferns and bryophytic species along with seedlings of the trees. The vegetation of disturbed sites comprised of *Pinus kesiya* Royle ex Gordon, *Rhododendron arboretum* Sm., *Sarcococca pruniformis* Lindl., *Coffea khasiana* Hook, *Erythroxylum kunthianum* Kurz, *Microtropis discolor* Wall. ex Meisn, grasses, herbs, few ferns and bryophytes. Variations were observed among the sampling sites in terms of soil temperature, soil pH and soil moisture content. The soil samples were slightly acidic for all the sites. The bacterial populations were enumerated as number of colony forming unit (CFU) which ranged from $3x10^4 – 3x10^6 g^{-1}$ dry soil (Table 1).

### 16S rDNA sequence analysis for bacterial identification and Phylogenetic analysis

DNA sequencing and phylogenetic analysis (Figs. 1& 2) revealed that the isolates showed 97–99% similarity to the sequences within GenBank (Tables 2& 3). The 16S rDNA nucleotide partial sequences were submitted to GenBank and accessions were assigned for all the 26 sacred grove isolates, from JX402416 to JX402441. Phylogenetic analysis based on the 16S rRNA gene in the present study showed that the 26 sacred grove isolates were divided into 4 different groups, viz. *Bacillus*, *Paenibacillus*, *Lysinibacillus* and *Viridibacillus* and represented by 19 different species. However, the disturbed forest isolates were divided into 3 different groups, viz. *Bacillus*, *Paenibacillus*, and *Viridibacillus* but are represented by only 6 different species.

### *In vitro* screening for some important plant growth-promoting traits

In this study, 69% sacred grove isolates were positive for phosphate solubilization as compared to

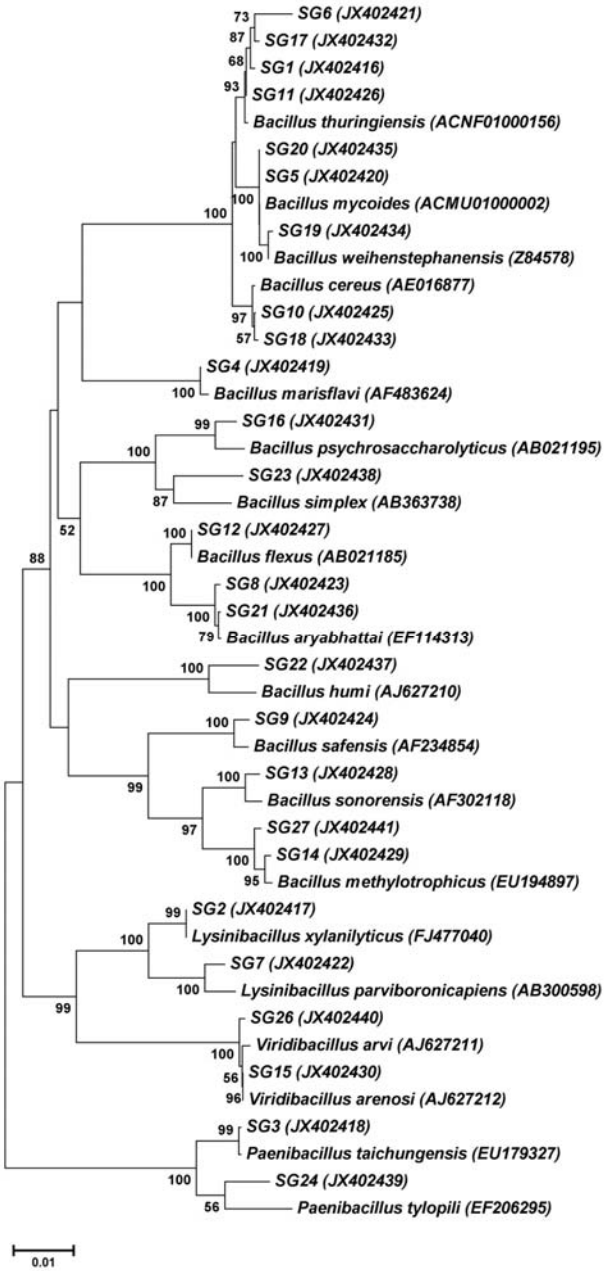


Fig. 1—Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence analysis for the sacred groves (SG) isolates. The scale bar represents the expected number of substitutions

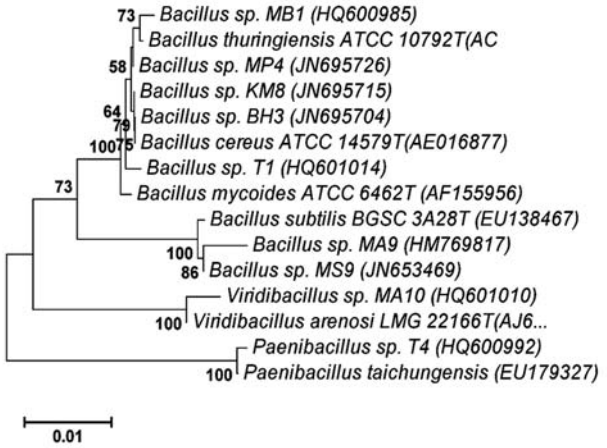


Fig. 2—Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence analysis for the disturbed forest isolates. The scale bar represents the expected number of substitutions.

only 44% isolates from disturbed forest. All the characterized sacred grove isolates showed production of siderophores, while 3 isolates from disturbed forest soils and one type strain lacked this property. 20 (76%) of the sacred grove isolates showed ACC deaminase activity, whereas, only 3 (33%) of the disturbed forest isolates showed this activity. All the isolates were positive for IAA production. Bacteria, having the ability to produce both ACC deaminase and IAA, are known to enhance root elongation and the dry weight to a greater extent than strains that produced solely ACC deaminase[18]. More than half (76%) of the isolates of the sacred grove showed production of both ACC deaminase and IAA, hence making them good plant growth promoters. On the other hand, only 33% of the isolates from disturbed forest showed the same properties. All the isolates were positive for Catalase and ammonia production activity with some isolates showing relatively higher activity than others. Only 23% of isolates of the sacred grove and none from the disturbed forest isolates could produce HCN (Tables 2 & 3).

## Discussion

Plant species and soil type are two important characteristics affecting the structure of the total bacterial community[19]. The variability in population densities of culturable soil *Bacillus* and related genera can be attributed to soil properties, physico-chemical conditions and vegetations which are among the most important factors that influence soil microbial growth, population density and diversity[20]. 16S rRNA gene sequences have been used in *Bacillus* classification as a framework for species delineation[21]. The ability of some microorganisms to convert insoluble P to an accessible form, like orthophosphate, is an important trait in a PGPB for increasing plant yields[22,23]. Phosphate utilizing bacteria could be a promising source for plant growth-promoting agent in agriculture[24]. Siderophore production is one of the most important attributes and biocontrol mechanisms of PGPB, including bacilli group[25]. Therefore, the

Table 2—Plant growth-promoting traits of the characterized isolates of the sacred groves

| Isolate | Closest species (% similarity) | Accession number | $PO_4$ solubilization[#] | Siderophore[#] | ACC | IAA | HCN | Catalase | $NH_3$ |
|---|---|---|---|---|---|---|---|---|---|
| SG1 | *Bacillus thuringiensis* Berliner (99.80) | JX402416 | 4 ± 0.5 | 6 ± 0.5 | + | + | - | + | ++ |
| SG2 | *Lysinibacillus xylanilyticus* Lee (100) | JX402417 | - | 3 ± 0.5 | + | + | + | + | + |
| SG3 | *Paenibacillus taichungensis* Lee (99.93) | JX402418 | 5 ± 0.5 | 4 ± 0.5 | + | + | + | + | + |
| SG4 | *Bacillus marisflavi* Yoon (99.80) | JX402419 | 5 ± 0.5 | 4 ± 0.5 | - | + | - | + | + |
| SG5 | *Bacillus mycoides* Flugge (99.93) | JX402420 | 3 ± 0.5 | 3 ± 0.5 | + | + | - | + | ++ |
| SG6 | *Bacillus thuringiensis* Berliner (98.92) | JX402421 | 3 ± 0.5 | 4 ± 0.5 | + | + | + | + | + |
| SG7 | *Lysinibacillus parviboronicapiens* Miwa (99.12) | JX402422 | - | 3 ± 0.5 | + | + | - | ++ | + |
| SG8 | *Bacillus aryabhattai* Shivaji (99.80) | JX402423 | 5 ± 0.5 | 4 ± 0.5 | - | + | - | + | ++ |
| SG9 | *Bacillus safensis* Satomi (99.16) | JX402424 | 6 ± 0.5 | 3 ± 0.5 | + | + | - | ++ | + |
| SG10 | *Bacillus cereus* Frankland and Frankland (99.66) | JX402425 | 3 ± 0.5 | 4 ± 0.5 | + | + | - | + | + |
| SG11 | *Bacillus thuringiensis* Berliner (99.93) | JX402426 | 3 ± 0.5 | 4 ± 0.5 | + | + | - | ++ | ++ |
| SG12 | *Bacillus flexus* ex Batchelor (100) | JX402427 | - | 3 ± 0.5 | - | + | - | + | + |
| SG13 | *Bacillus sonorensis* Palmisano (99.50) | JX402428 | 5 ± 0.5 | 3 ± 0.5 | + | + | - | ++ | + |
| SG14 | *Bacillus methylotrophicus* Madhaiyan (99.65) | JX402429 | 4 ± 0.5 | 4 ± 0.5 | + | + | - | ++ | + |
| SG15 | *Viridibacillus arenosi* Heyrman (99.93) | JX402430 | - | 3 ± 0.5 | + | + | + | ++ | + |
| SG16 | *Bacillus psychrosaccharolyticus* Priest (99.65) | JX402431 | - | 3 ± 0.5 | - | + | - | + | + |
| SG17 | *Bacillus thuringiensis* Berliner (99.73) | JX402432 | 4 ± 0.5 | 6 ± 0.5 | + | + | + | + | + |
| SG18 | *Bacillus cereus* Frankland and Frankland (99.73) | JX402433 | 3 ± 0.5 | 4 ± 0.5 | + | + | - | ++ | + |
| SG19 | *Bacillus weihenstephanensis* Lechner (99.45) | JX402434 | - | 5 ± 0.5 | + | + | ++ | + | + |
| SG20 | *Bacillus mycoides* Flugge (100) | JX402435 | 3 ± 0.5 | 4 ± 0.5 | + | + | - | + | + |
| SG21 | *Bacillus aryabhattai* Shivaji (99.93) | JX402436 | 4 ± 0.5 | 3 ± 0.5 | - | + | - | + | + |
| SG22 | *Bacillus humi* Heyrman (98.30) | JX402437 | 4 ± 0.5 | 3 ± 0.5 | + | + | - | + | + |
| SG23 | *Bacillus simplex* Priest (97.29) | JX402438 | 3 ± 0.5 | 3 ± 0.5 | - | + | - | ++ | + |
| SG24 | *Paenibacillus tylopili* Kuisiene (97.78) | JX402439 | - | 3 ± 0.5 | + | + | - | + | + |
| SG26 | *Viridibacillus arvi* Heyrman (99.73) | JX402440 | - | 3 ± 0.5 | + | + | - | + | ++ |
| SG27 | *Bacillus methylotrophicus* Madhaiyan (99.38) | JX402441 | 4 ± 0.5 | 8 ± 0.5 | + | + | - | ++ | + |

# Radius of halozone in mm; (-) Not detected ;(+) moderate activity; (++) strong activity

Table 3—Plant growth-promoting traits of the characterized isolates of disturbed forest sites and MTCC strains

| Isolate | Closest species (% similarity) | Accession number | $PO_4$ solubilization[#] | Siderophore[#] | ACC | IAA | HCN | Catalase | $NH_3$ |
|---|---|---|---|---|---|---|---|---|---|
| MB1 | *Bacillus thuringiensis* Berliner (99.66) | HQ600985 | 3 ± 0.5 | 4 ± 0.5 | + | + | - | + | + |
| BH3 | *Bacillus cereus* Frankl and and Frankland (100) | JN695704 | - | 3 ± 0.5 | - | + | - | + | + |
| KM8 | *Bacillus cereus* Frankland and Frankland (100) | JN695715 | - | 3 ± 0.5 | - | + | - | + | + |
| MP4 | *Bacillus cereus* Frankland and Frankland (99.9) | JN695726 | 3 ± 0.5 | 4 ± 0.5 | - | + | - | + | + |
| T1 | *Bacillus mycoides* Flugge (99.5) | HQ601014 | - | 3 ± 0.5 | - | + | - | + | + |
| MA9 | *Bacillus subtilis* Cohn (99) | HM769817 | - | - | + | + | - | + | + |
| MS9 | *Bacillus subtilis* Cohn (100) | JN653469 | 3 ± 0.5 | - | - | + | - | + | + |
| T4 | *Paenibacillus taichungensis* Lee (99.79) | HQ600992 | 4 ± 0.5 | 3 ± 0.5 | - | + | - | + | + |
| MA10 | *Viridibacillus arenosi* Heyrman (99.3) | HQ601010 | - | - | + | + | - | + | + |
| | *Bacillus subtilis* Cohn MTCC 8141 | | 5 ± 0.5 | - | + | + | + | ++ | + |
| | *Bacillus thuringiensis* Berliner MTCC 8996 | | - | 4 ± 0.5 | + | + | - | + | + |
| | *Paenibacillus polymyxa* Ash MTCC 9489 | | - | 6 ± 0.5 | + | + | - | ++ | + |
| | *Bacillus cereus* Frankland and Frankland MTCC 10211 | | - | 5 ± 0.5 | + | + | - | + | + |

low availability of Fe in the environment would suppress the growth of pathogenic organisms including phytopathogens. ACC deaminase hydrolyzes plant ACC, the immediate precursor of the phytohormone ethylene, and thereby prevents/reduce the production of plant growth inhibiting levels of ethylene and also helping to withstand stress (biotic or abiotic)[17]. IAA (indole-3-acetic acid) is a member of the group of phytohormones and is generally considered the most important native auxin[26,27]. Certain hydrolytic enzymes protect bacteria and their hosts from deleterious effects of environmental stressors such as UV irradiation, extreme temperatures, and exposure to chemical and biological contaminants. Studies on inoculation of plants with diazotrophs have shown that their oxidizing enzymes contribute to stimulation of plant growth and also enhance oxidative stress tolerance[28]. The agent responsible for this heightened stress tolerance was found to be a suite of antioxidant enzymes including superoxide dismutases, peroxidases, and catalases that neutralize and thus control free radical formation of reactive oxygen species[28].

As evident from the present findings, the bacterial isolates native to the pristine niche of sacred groves showed better plant growth promoting activities as compared to disturbed forest isolates and type strains implicating the importance of sacred groves and their potential role in microbial diversity conservation. This suggests that the pristine niche of sacred groves carry enormous significance to understand their diversity and exploring various potentials in agricultural applications and also recognizing the importance of tropical sacred groves in conservation of microbial diversity.

## Conclusion

Sacred groves are of great research value in *in situ* conservation of biodiversity. Understanding the potential roles played by sacred groves in *in situ* biodiversity conservation, it would have greater appeal if efforts are diverted to inventorize the existing biodiversity, especially the species that might be of economic importance or facing extinction. The present study is the first report on the indigenous aerobic endospore-forming *Bacillus*

and related genera from the pristine and undisturbed sacred groves prevalent in the tropical belt of Meghalaya, India. The observations lead to the conclusion that *Bacillus* and related genera isolates obtained from the sacred groves exhibits stronger activity and more PGP traits than the disturbed forest isolates indicating their strong potential as candidates for promoting plant growth in agriculture and also the important contribution of sacred groves as a rich source of economically valuable microbes hence signifies the relevance of ethnic strategy and efforts for conservation of these pristine forests.

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