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Metagenomic analysis of active ectomycorrhizal zone of *Astraeus* using cloud computing approach from two seasons

Vineet Vishal[1,2], Geetanjali Singh[1] and Shalini Lal[1*]

[1]*Department of Botany, Dr Shyama Prasad Mukherjee University, Ranchi- 834008, Jharkhand*

[2]*Department of Botany, Bangabasi Evening College, Calcutta University, Kolkata-700009, West Bengal*

[*]*Corresponding author Email: shalini.kumari@gmail.com*

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ABSTRACT

Ectomycorrhizosphere is a hub where plant roots and soil microorganisms actively interact (ectomycorrhizal fungi and bacteria). However, the mechanisms that regulate interactions between mycorrhizal fungi, soil bacteria, and plant roots are poorly understood. Here we use a cloud-based computing approach for the identification, diversity and functional roles of hidden uncultured microbial communities around the active ectomycorrhizal zone of wild edible mushroom of *Astraeus* from dry deciduous Sal Forest of Jharkhand, India by targeting 16S rRNA gene amplicons. The soil samples were collected during the monsoon (July) and winter (November) seasons of 2021. The monsoon and winter ectomycorrhizosphere were found to have a high richness of 161 and 251 Operational Taxonomic Units (OTUs) at a >97% sequence identity. These two environments have markedly different bacterial community compositions. Across the seasons Proteobacteria, Acidobacteria, Actinobacteria and Nitrospirota were significantly abundant phylum, surprisingly in winter, the ectomycorrhizosphere had fewer Firmicutes, Bacteroidota and a higher Verruco-microbiota taxon. Sugar metabolism, amino acid metabolism, metabolism of cofactors and vitamins was the most abundant functional category in the ectomycorrhizosphere. The Alphaproteobacteria and Gammaproteobacteria were the most abundant classes of bacterial communities in the active ectomycorrhizal zone. Thus, the presence of nitrogen-fixing and phosphate-solubilizing bacteria in the ectomycorrhizosphere soil may influence the plant-fungus symbiosis' functioning.

Keywords: *Astraeus*, Ectomycorrhizosphere, Nephele, Metagenomics, PICRUSt

INTRODUCTION

The ectomycorrhizosphere is a distinct microbiota niche in which plant roots, mycorrhizal fungi, and bacteria interact in a variety of ways ranging from mutualistic to commensal to antagonistic or competitive. Because the hyphae are linked to autotrophic vascular plants, massive amounts of carbon fluxes and energy can be actively transported to the fungal tissue and then released in the form of droplets at the fungal tip, resulting in an enrichment of the bacterial population (Dighton, 2018). Sun *et al.* (1999) postulated that, this mechanism will favour the interaction of bacteria and other microbes surrounding the fungal tip at soil-mycelial junction, even though soil water potential is low. The interaction of ectomycorrhizal fungi with bacteria in the ecto-mycorrhizosphere may function as a nutritional hot-spot which benefits tree in number of areas such as bio-remediations, biocontrol, biofertilization and sustainable agricultural practices (Finlay and Thorn, 2019). Furthermore, quorum sensing, regulation of microbial gene expression, symbiosis, biofilm formation, antibiotic production, motility, conjugation, and virulence are the driving processes of ectomycorrhizosphere microbiome (Vishal *et al.* 2021b). Garbaye (1991) and Bonfante and Anca (2009) advised that mycorrhizae helper bacteria (MHB) promoted mycorrhizal formation, aids in fungal-plant recognitions system and receptivity of host plant root to the mycorrhizal fungi. In the presence of *Pseudomonas fluorescens* HR13, the mycorrhizal symbiosis between *Acacia auriculiformis* and *Pisolithus alba* can be enhanced up to 70%, according to Duponnois and Plenchette (2003). Using microcosms of *Paenibacillus* sp. EJP73 and *Burkholderia* sp. EJP67, Aspray *et al.* (2006) demonstrated the enhancement of mycorrhizal colonisation in the *Pinus sylvestris-Lactarius rufus* symbiosis. Later, utilising a partial 16S rRNA gene sequence, Uroz *et al.* (2007) identified 61 bacterial strains from the Oak-*Scleroderma citrinum* symbiotic mantle. Their group experimentally demonstrated that bacteria (*Burkholderia, Collimonas, Pseudomonas,* and *Sphingomonas*) and ectomycorrhizal fungi (*Scleroderma citrinum*) are collectively engaged in mineral weathering and solubilisation processes.

The wild edible mushrooms of *Astraeus* Phosri (family *Diplocystidiaceae*) found near the rhizosphere of *Shorea robusta* Garten. (**Fig. 1**) provide appealing commercial benefits to the tribal peoples of Jharkhand who rely on the forest (Phosri *et al.* 2007; Vishal *et al.* 2021a). This mush-room's annual productivity is exceedingly unpredictable and limited. Because it has yet to be successfully cultivated artificially. All *Astraeus* production is still based on wild collection from forests. Despite numerous studies on the taxonomy, phytochemistry, and nutraceutical properties of *Astraeus* (Trappe, 1967; Petcharat, 2003; Biswas *et al.,* 2011). There is relatively little known about the involvement of bacteria in *Astraeus* germination and development. Mycologists have successfully attempted to cultivate mushrooms such as *Agaricus, Pleurotus, Calocybe,* and *Volvariella* in recent decades (Gupta *et al.,* 2018). *Astraeus*, on the other hand, has yet to be artificially cultivated. The biology of this ectomycorrhizal fungus must be studied from the standpoint of its ecological interactions and co-occurrence with this fungi and other microorganisms, notably bacterial, for successful mushroom production and improved productivity. The primary goal of this study was to collect soil samples from a dry deciduous sal forest during two different seasons of the same year and analyse the abundance of microorganisms and their possible ecological function around the ectomycorrhizosphere of *Astraeus*. Using a cloud-based metagenomic approach to determine the optimal microbial content for proper nourishment of the taxon.



Fig. 1: *Astraeus asiaticus* - a wild edible mushroom growing in a Sal dominated forest. A-B. *Astraeus* partially buried in lateritic soil. C. *Astraeus* immature fruit bodies surrounded by white mycelium (Scale bar: A-B = 20 mm, C = 10 mm).

MATERIAL AND METHODS

Collection and sample preparation

Rhizospheric soil were collected from the village Bandgaon under Porahat forest division, West Singhbhum, Jharkhand, India (22.84°N, 85.35° E) from a dry deciduous forest of sal. The triplicate soil samples (10 x 10 x 10 cm) were taken at a depth of 5-10 cm during the monsoon (July) and winter (November) seasons of 2021 (**Fig. 1a**). The samples were collected into sterile falcon tubes and brought to the laboratory, where they were kept at 4°C for 12 hours before being processed (Vishal *et al.,* 2021b).

DNA extraction and 16S rRNA gene amplification

From a minimum of 50 mg of soil, genomic DNA was isolated from an environmental sample using the AllPrep DNA/RNA Mini Kit (Qiagen GmbH, Germany) following the manufacturer's protocol. DNA quality was assessed by Nanodrop and on agarose gel, quantified using QUBIT Flurometer 3.0. The extracted DNA was stored at -20°C. The library preparation was carried out by targeting the V3-V4 region of the 16S rRNA gene using the forward- V13F (5'-GAGTTTGATGMTGGCTCAG-3') and reverse- V13R (5'-TTACCGCGGCMGCSGGCAC-3') primers (Schloss *et al.,* 2011). The libraries were purified using Ampure XP beads and quantitated using the Qubit dsDNA High Sensitivity Assay Kit. Metagenomic 16S amplicon sequencing was performed by taking 1µg of DNA template using Illumina Miseq with 2 x 300 PE chemistry at Biokart India Pvt Ltd (Bengaluru, India) following the standard Illumina protocol.

Cloud-based metagenomic analysis

The NIH (National Institute of Health, USA) developed and maintains a cloud-based computing environment called Nephele (https://nephele.niaid.nih.gov/) (version 1.8), which is used for microbial bioinformatics analysis (Weber *et al.* 2019) (**Fig. 2)**. The Nephele includes tools for quality control and demultiplexing paired-end reads, such as FASTQC and QIIME 2 (Quantitative Insights into Microbial Ecology) for taxonomic assignment (Bolyen *et al.,* 2019). The QIIME 2 16S pipeline of Nephele has a preprocessing procedure that includes joining of forward and reverse reads as contigs using default parameters (% maximum difference of 25 and a minimum overlap of 10), exclusion of reads with an average Phred score of ≤ 20, exclusion of chimeric sequences using UCHIME (Edgar *et al.,* 2011) and a clustering algorithm followed by the Open Reference Method (Wang *et al.,* 2007). The reads were clustered using > 97% sequence identity. Taxonomic assignment was done using a SKLEARN trained classifier (Bokulich *et al.,* 2018) and the full length of Greengenes13 version 8 database (DeSantis *et al.,* 2006) with a default sequence similarity threshold of 0.97. The alpha diversity analysis (including Simpson, Shannon and Evenness) was performed using the Microbiomedb webserver tool (https://microbiomedb.org/mbio/app/) (Oliveira *et al.,* 2018).

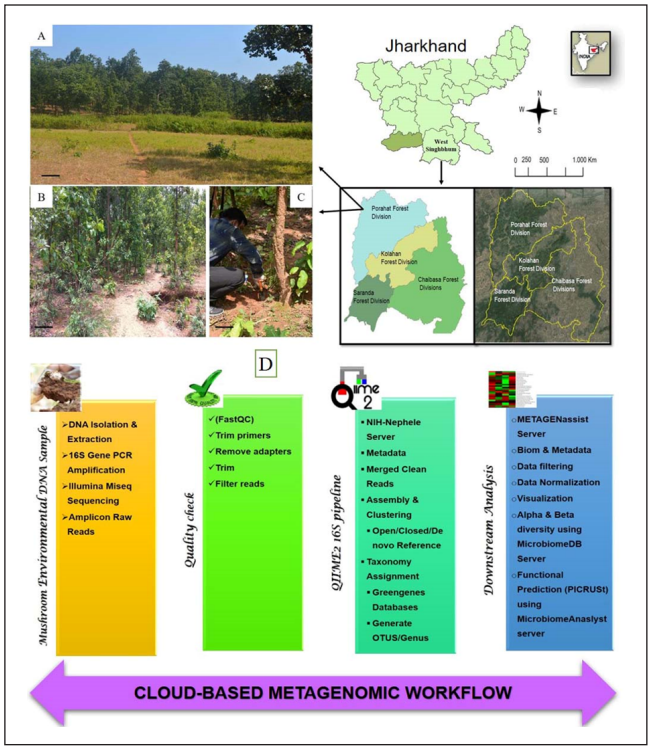


Fig. 2: Area of collection of soil samples. A-C. Map and satellite image of area of study from Porahat forest division, West Singhbhum, Jharkhand, India (22.84°N, 85.35° E), bar =20 mm. D. Cloud-based metagenomic workflow for microbial bioinformatics analysis.

METAGENassist (http://www.metagenassist.ca/METAGENassist/), a cloud platform with default parameter settings, was also used for data filtering, data normalisation, heatmap generation, and downstream analysis (Arndt *et al.,* 2012). Prediction of functional annotation by PICRUSt

(Phylogenetic investigation of communities by reconstruction of unobserved states) were performed using the Microbiomeanalyst web-server tool (https://www.microbiomeanalyst.ca/) (Dhariwal *et al.,* 2017; Chong *et al.,* 2020). The metagenome data are available at the NCBI - BioProject with accession number PRJNA825757 and PRJNA825754 for monsoon and winter ecto-mycorrhizosphere soil samples.

RESULTS

After preprocessing, demultiplexing, and normalization, a total of 64,872 reads with an average length of 300 bp and G+C% of 56±3 dataset were recovered from the monsoon season and 2,46,374 reads with an average length of 299 bp and G+C % of 58±3 dataset were recovered from the winter season in the active zone of the ectomycorrhizosphere by targeting the V3-V4 variable region of the 16S rRNA gene (**Table 1**). The quality processed pair end reads were mapped into OTUs by QIIME2 of Nephele. The pair end reads were mapped at the genus level to 162 OTUs in the monsoon and 251 OTUs in the winter using > 97 % sequence similarity (**Fig. 3a**). According to the alpha diversity indices (Simpson, Shannon, and Evenness), which estimate species richness and evenness based on the number of OTU, the winter ectomycorrhizosphere has more taxa diversity than the monsoon ectomycorrhizosphere (**Fig. 3b**).

Table 1: Overall statistics

Sample Id	Collection Month & Year)	16s Gene Target Variable region	Before QC (reads)	After QC (reads)	Mean Seq. Length (base pair)	Remove Chimera/a adapter	GC %	OUT/ Taxon
Monsoon	July 2020	V3-V4	1,68,326	64,872	300	1,03,454	56 ± 3	162
Winter	Nov 2021	V3-V4	3,89,374	2,46,872	299	1,42,547	58 ± 3	251

The taxonomic profiling revealed that the ectomycorrhizosphere of the monsoon season is classified and represented by 21 phylum, 52 classes, 88 orders, and 174 families, and 162 genera whereas the ectomycorrhizosphere of the winter season is classified and represented by 24 phylum, 79 classes, 148 orders, and 284 families and 251 genera.

Proteobacteria (39.3% and 34.9%), were the most abundant phyla in the monsoon and winter ectomycorrhizosphere, followed by Firmicutes (24.2% and 4.8%), Actinobacteria (15.8% and 17.6%), Acidobacteria (6.9% and 18.5%), and Bacteroidetes (6.1% and 0.8%), with less than 0.1% of archaeal phyla were observed. In the active zone of ectomycorrhizosphere of monsoon and winter, the most abundant class were Alphaproteobacteria (21.5% and 39.8%), whereas Acidobacteria (3.8 and 17.5%), Actinobacteria (16.1% and 17.7%), Clostridia (15.8% and 0.6%) Gammaproteobacteria (9.6% and 4.1%), Betaproteobacteria (9.6% and 2.7%), Bacilli (9.4% and 6.1%) and Bacteroidia (6.2% and 0.4%) were significantly more frequent (**Fig. 2a**). Further analysis at genus level revealed that 42.4% of bacteria communities were common between monsoon and winter season of ectomycorrhizosphere (**Fig. 3**), out of which *Burkholderia* (15.6%), a gram negative, obligately aerobic, rod-shaped bacterium of Betaproteobacteria to be the most abundant genera observed in monsoon active zone of ectomycorrhizosphere whereas *Rhodoplanes* (32.3%), a phototrophic bacterium of Alphaproteobacteria to be the most abundant genera observed in winter. A number of significant reads fell under uncultured/unclassified OTUs.

The hypothetical functional prediction associated with these soil bacteria communities using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) identified 10 KEGG metabolism, 138 KEGG pathways, 191 KEGG modules and 21 clusters of orthologous groups of proteins (COGS) found in the ectomycorrhizosphere (**Fig. 4**). Sugar metabolism, amino acid metabolism, cofactor metabolism, and vitamin metabolism were all significantly enriched metabolisms, whereas valine, leucine and isoleucine biosynthesis (ko00290), limonene and pinene degradation (ko00903), biotin metabolism (ko00780), and D-glutamine and D-glutamate metabolism (ko00471) were highly enriched KEGG pathways, Additionally, leucine biosynthesis, 2-oxoisovalerate (M00432), isoleucine biosynthesis, pyruvate (M00535), pimeloyl-ACP biosynthesis, BioC-BioH pathway, malonyl-ACP (M00572) galactose degradation, Leloir pathway, galactose (M00632) were highly enriched KEGG modules, and carbohydrate transport & metabolism, cell wall membrane envelope biogenesis were highly enriched ortholog groups of genes of the ectomycorrhizosphere (**Fig. 5**).

DISCUSSION

Microbial communities are one of the prime driving forces in the active zone of the ectomycorrhizosphere of wild edible mushrooms *Astraeus*, which are mostly found in a dry deciduous forest of sal (**Fig. 1, 2A-C**). The soil is sandy, reddish-brown in colour, lateritic in texture, and slightly acidic to neutral (pH 5-6) in nature. During the monsoon season, providing an ideal environment for *Astraeus* germination and development (Vishal *et al.,* 2021b).

In this study, we explored the configuration, dynamics, and ecological functions of hidden uncultured bacterial communities in the mycorrhizosphere throughout two distinct seasons: monsoon, (June to August), when mushrooms germinate and winter (October to January) when mushrooms do not germinate in the same year. Such studies provide crucial information for future studies on the diversity and structure of bacterial communities, as well as how they cope with their physical environment. Their diverse biological and ecological resources determine their adaptability to climate change. Employing conventional techniques such as gas chromatography fatty acid methyl ester (GC-FAME) and amplified 16S rDNA restriction analysis (ARDRA), Khetmalas *et al.* (2002) demonstrated that bacterial are frequent dweller's of the mycorrhizosphere and that they are more abundant when compared to the bulk soil. With the evolution of cutting-edge technology, we adopt a cloud-based platform for microbial bioinformatic analysis in this study. In

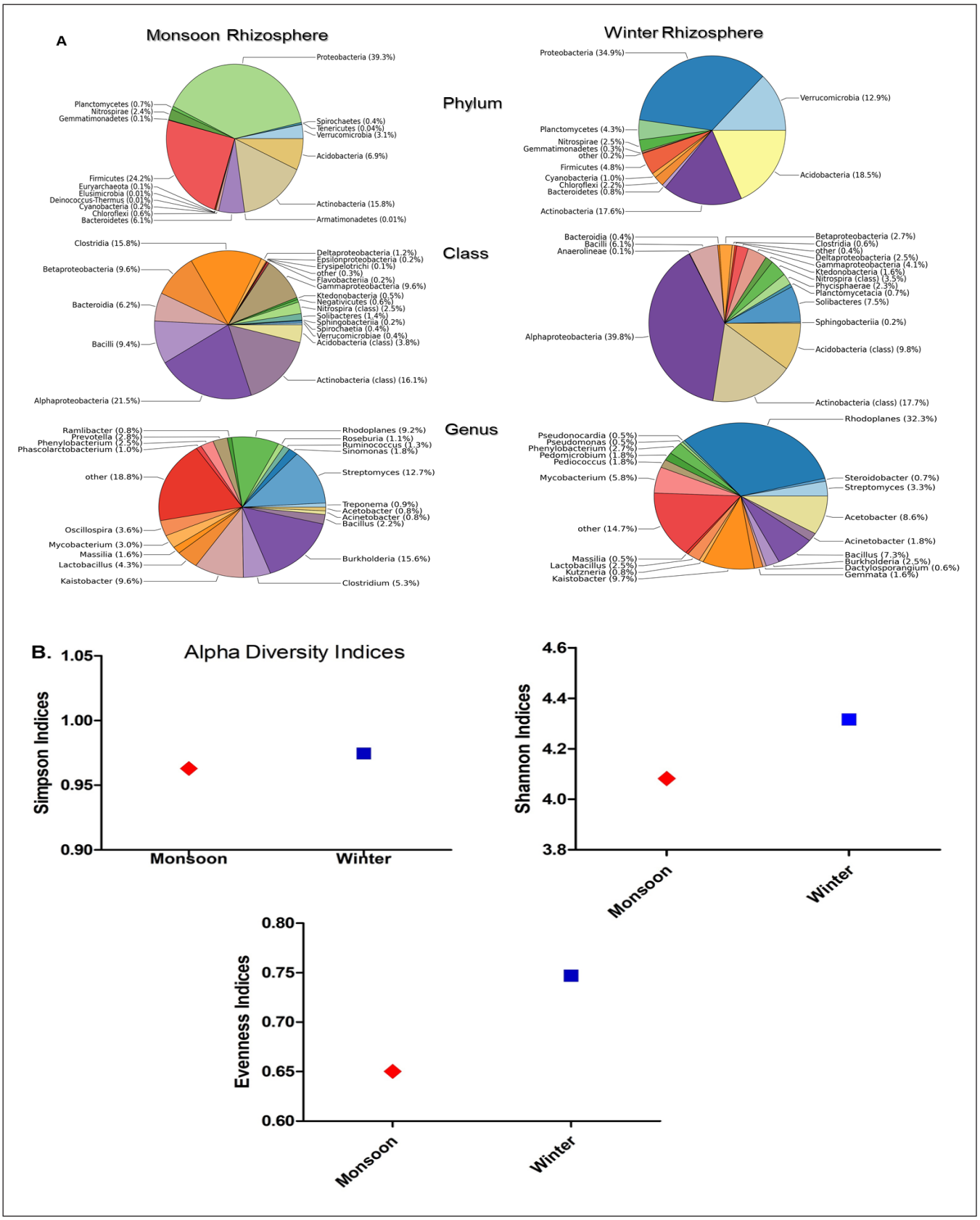


Fig. 3: Taxonomic profiling and alpha diversity analysis of monsoon and winter ectomycorrhizosphere soil. A. Pie-chart showing relative abundance of bacteria at phylum, class and genus level. B. Scatter-graph of alpha diversity analysis of ectomycorrhizosphere soil showed that winter soil samples had a more diverse bacterial community than monsoon soil.

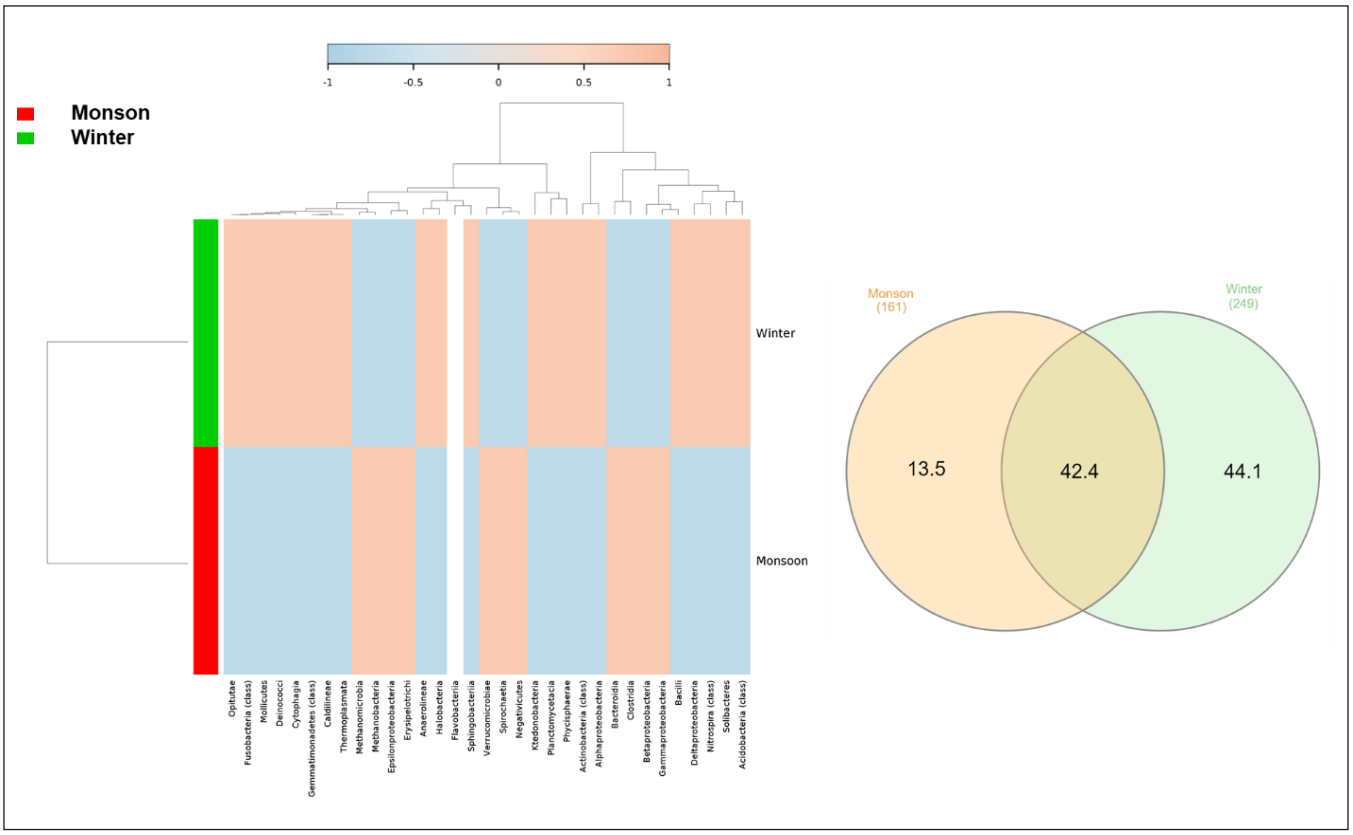


Fig. 4: Heat-map of monsoon and winter ectomycorrhizosphere soil at phylum level and Venn diagram depicting common and unique bacterial taxa of ectomycorrhizosphere.

our investigation, we detected well markedly different bacterial community compositions between the two seasons: monsoon (21 phylum, 52 classes, and 162 genera) and winter (24 phylum, 79 classes, and 251 genera) (**Table 1**). The richness and diversity of bacterial communities are estimated by using Simpson, Shannon and Evenness of alpha diversity indices were significantly higher in winter than those of monsoon corroborating the results obtained by Xiang *et al.* (2021) (**Fig. 3b**).

The superphylum Proteobacteria was the most prevalent phyla in both seasons, however it was much more abundant in the active zone of the monsoon ectomycorrhizosphere soil. Firmicutes and Bacteroidetes were also the most abundant phyla in the monsoon mycorrhizosphere, whereas Acidobacteria, Actinobacteria, and Verrucomicrobia were the most abundant phyla in the winter mycorrhizosphere (**Fig. 3a**, **4**). The Alphaproteobacteria were most abundant class across both the seasons but Alphaproteobacteria (*Rhodoplanes, Kaistobacter, Phenylobacterium, Bradyrhizobium* and *Novosphingobium*) and Deltaproteobacteria (*Desulfovibrio, Geobacter* and *Anaeromyxobacter*) were significantly higher in the active zone of the winter ectomycorrhizosphere soil indicating the enrichment of copiotrophic, symbiotic as well as free-living nitrogen fixing and sulphur-reducing microbial taxa (Van Insberghe *et al.,* 2015; Llado and Baldrian. 2017). Members of Betaproteobacteria (*Burkholderia, Salinispora, Acidovorax, Ramlibacter* and *Cupriavidus*) and methanotrophs of Gammaproteobacteria (*Pseudomonas, Succinivibrio, Legionella* and *Dyella*) were significantly overrepresented classes in the monsoon ectomycorrhizosphere soil. They advocated for the enrichment of methane and ammonia oxidizers (Isobe *et al.,* 2011; Long *et al.,* 2012) and as well as phosphate solubilizing (Vishal *et al.,* 2021b) bacterial taxa. Surprisingly members of the gram-positive Bacilli and Clostridia as well as gram-negative Bacteroidete bacterial classes were significantly abundant in monsoon while underrepresented in winter ectomycorrhizosphere soil. Members of Bacilli (*Bacillus* and *Paenibacillus*), Clostridia (*Clostridium*), Bacteroidetes (*Cytophaga* and *Bacteroides*), Actinobacteria (*Rhodococcus, Mycobacterium, Microbacterium,* and *Streptomyces*) and Betaproteobacteria (*Sphingomonas* and *Burkholderia,*) are thought to play important roles as lignin degraders (Bandounas *et al.,* 2011; Tian *et al.,* 2014), as do several white rot fungus, filamentous fungi, and yeast taxa (Durham *et al.,* 1984; Silva *et al.,* 2021). The significant prevalence of possibly lignolytic bacterial communities in the active zone of monsoon ectomycorrhizosphere soil led to the notion that these bacterial taxa were important in the germination and growth of wild edible mushrooms, *Astraeus*, which grew in a litter-rich environment. Furthermore, members of the Verrucomicrobia that are acidophilic methanotrophs and Acidobacteria were

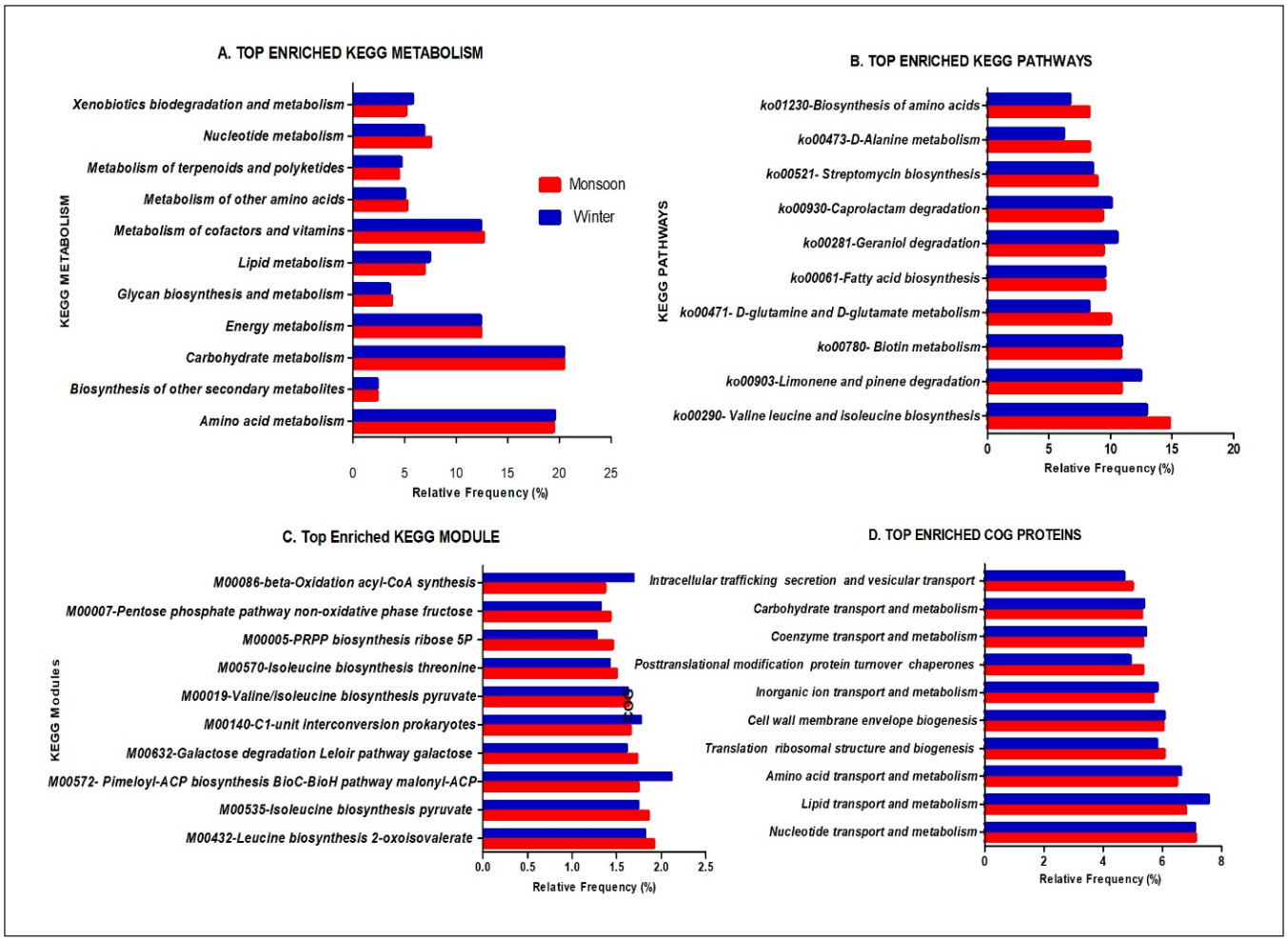


Fig. 5: Top 10 functional annotation categories: KEGG metabolism, KEGG pathways; KEGG module and ortholog groups of genes (COG) obtained from PICRUSt of monsoon (red) and winter (blue) ectomycorrhizosphere soil.

significantly more abundant in winter than those of the monsoon ectomycorrhizosphere soil, indicating that the presence of higher levels of methanotrophic and oligotrophic bacterial taxa in winter is due to changes in organic matter content and nutrient availability (Llado *et al.,* 2017). The genus-level comparative analysis reveals that 42.4% of bacterial communities were shared by monsoon and winter ectomycorrhizosphere soils, while 13.5% (monsoon) and 44.1% (winter) were unique bacterial communities around the active zone of the ectomycorrhizosphere (**Fig. 4**). The most abundant genera of monsoon ectomycorrhizosphere soil was gram-positive rod-shaped saprophytic bacterium *Burkholderia*. The absence of these bacteria has a negative impact on hyphal elongation and branching in the arbuscular mycorrhizal fungus *Gigaspora decipiens*, as demonstrated by Levy *et al.* (2003). Despite this, these bacteria actively participate in the degradation of cellulose and lignin as well as mineral-weathering in a litter-rich deciduous forest (Llado *et al.,* 2017). Another rod shaped, gram-negative, bacterium *Rhodoplanes* abundant genera of winter ectomycorrhizosphere soil. The members of phototrophic bacteria are potential nitrogen fixers and chemotrophs in nature (**Fig. 2a**) (Buckley *et al.,* 2007; Srinivas *et al.,* 2014). Anthony-babu *et al.* (2014) reported the presence of bacterial communities not only in the active zone of the ecto-mycorrhizosphere of edible *Ascomycetes* truffles (*Tuber melanosporum*), but also in its sporocarp and peridium, namely Proteobacteria, Bacteroidetes, Actinobacteria, Verrucomicrobia, Firmicutes, Acidobacteria, and those of gleba includes Proteobacteria, Bacteroidetes and Actinobacteria taxa. From October to January, the diversity of the environment changes significantly.

The soil microbiome linked with the monsoon and winter ectomycorrhizosphere induced an array of biological functioning pathways like quorum sensing, regulation of microbial gene expression, symbiosis, biofilm formation, antibiotic production. We discovered that nucleotide metabolism, cofactor and vitamin metabolism, glycan biosynthesis and metabolism, and xenobiotics biodegradation and metabolism, as well as lipid metabolism, were the most enriched metabolisms of monsoon and winter ectomycorrhizosphere soil, highlighting the gradual change of biological roles from one season to the next. Valine, leucine and isoleucine biosynthesis (ko00290), D-glutamine and D-

glutamate metabolism (ko00471), D-Alanine metabolism (ko00473), Streptomycin biosynthesis (ko00521) and biosynthesis of amino acids (ko01230) were significantly enriched KEGG pathway of monsoon ectomycorrhizosphere. Meanwhile, the winter ectomycorrhizosphere's most enriched KEGG pathways were limonene and pinene degradation (ko00903), geraniol degradation (ko00281), and caprolactam degradation (ko00930) (**Fig. 5**).

Previous investigations of the mycorrhizosphere metagenome from Douglas fir root tip (Burke *et al.,* 2008), pine forest (Kataoka *et al.,* 2012), and oak forest (Uroz *et al.,* 2012) reveal different taxa of bacteria that benefit the host plant and fungi as a symbiont. Other factors, such as temperature and moisture, organic matter content, and nutrient availability, frequently alter or interact in natural environments to shape and modulate the activities of soil microbial populations (Cruz-Paredes *et al.,* 2021). This may help to explain the taxonomic and functional diversity among them.

CONCLUSION

The amplicon-based metagenomic analyses of the ectomycorrhizosphere associated with wild edible mushrooms of *Astraeus* revealed a unique and diverse assemblage of bacteria in two seasons: monsoon and winter. Proteobacteria, Firmicutes, and Bacteroidetes are abundant in the monsoon ectomycorrhizosphere soil, whereas Acidobacteria and Verrucomicrobia are abundant in the winter ectomycorrhizosphere soil. Other microbial associations with a large number of unassigned taxa, on the other hand, show that little is known about the diversity of ectomycorrhizospheric microorganisms.

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