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Metagenomic analysis decodes the fungal diversity of bio-dynamic preparations

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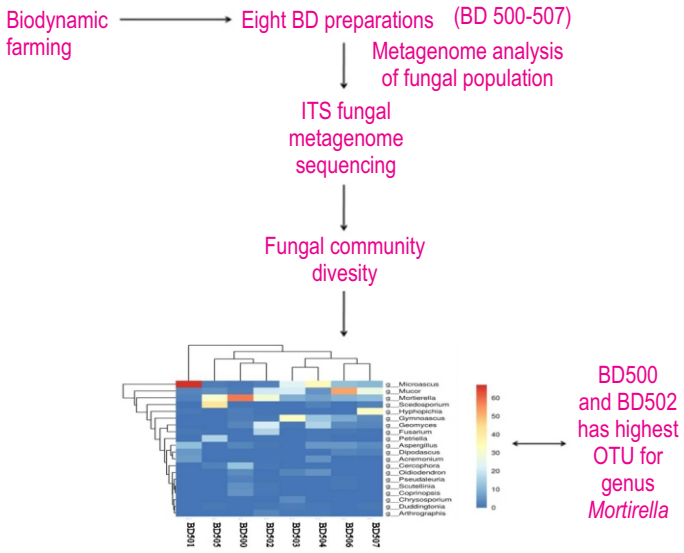
Abstract

Aim: Biodynamic farming system involves use of 8 different biodynamic preparations (BD 500-BD 507). Multi functionality of any ecosystem is due to its microbial diversity and community composition of microbes. So the present study was aimed to determine the total fungal population *viz.* unculturable ones, metagenomic analysis was done.

Methodology: In the present study, 18S rDNA sequencing of V3-V4 amplicon regions was performed to identify and characterize fungal diversity, which existed in these preparations.



Results: Alpha diversity was found to be maximum in BD506 with 868 OTU (operational taxanomic units) and minimum in BD507 with 254 OTU. At phylum level, the most abundant phylum was *Ascomycota* as recorded in 7 BD preparations with exception in the BD 500 (Unassigned). At genus level highest percentage of OTU abundance was observed for unassigned genus in all BD preparations, except *Mortierella* in BD 500 and BD 502; *Microascus* in BD 501 and BD504; *Gymnoascus* in BD503, *Scedosporium* in BD 505, *Mucor* in BD 506 and *Hyphopichia* in BD 507. On the basis of species diversity, BD502, 503 and 506 showed high percentage of OTU abundance for *Mucor* racemosus, while *Mortierella oligospora* was abundant in BD500, *Dipodascus geotrichum* in BD 501, *Kernia pachypleura* in BD504, *Petriella setifera* in BD505 and *Hyphopichia burtonii* in BD 507.

Interpretation: This indicated a unqiue class of fungus predominating each type of BD preparation. Furthermore, a large proportion of unassigned fungi at phylum and genus level were detected in metagenome analysis which might have specific roles in contributing for their overall effectiveness of each kind of BD preparations.

Key words: Biodynamic preparations, Fungal diversity, Metagenomic, Operational taxonomic units

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Introduction

Microbial diversity in organic manures composts and bio-fertilizers are the key constituents that are involved in changing the overall micro biome status of soil as well as the rhizosphere environment of plant system. Till date, the enrichment, augmentation, transformation and biomagnification of soil micro-flora by the application of organic amendments through organic farming has not been fully understood even though organic produces fetches maximum price in the market. Biodynamic farming system is a type of organic farming system which emphasizes on use of 8 biodynamic preparations (BD 500-BD 507). These biodynamic preparations have been successfully used in different crops that showed positive influence on plant growth promotion, pest and disease control (Carpenter *et al.,* 2000, Sharma *et al.,* 2012) besides improving the levels of soil organic matter soil microbial biomass and soil biological activities (Fliessbach *et al.,* 2007). Addition of biodynamic preparations to the soil not only enhances the soil physical properties but also improves the soil health through increased beneficial microorganism and soil nutritional status (Carpenter *et al.,* 2000, Sharma *et al.,* 2012, Brock *et al.,* 2019). However, the actual composition, nature of microbial diversity and its mechanism of action in soil and plant system have not been systematically studied.

Microbial activities play key roles in successful bio-transformation of organic substrates in any composting treatment or soil system. Although bacterial communities in the composting system have been studied by many researchers (Tian *et al.,* 2017, Piceno *et al.,* 2017), the role of fungal communities has been sparsely characterized. Ascomycota and Basidiomycota represent the main fungal decomposers in livestock manure and agricultural waste composting (Neher *et al.*, 2013, Yu *et al.,* 2015). Classical culture-based methods have limitation of identification of only culturable fungi which contributes for only 0.1% to 1.0% of the total fungal diversity. This limitation is overcome by using culture-independent methods such as environmental cloning and next-generation sequencing. Though, few reports have stated about microbial diversity of biodynamic preparations (Giannattasio *et al.,* 2013; Hartmann 2006) but these did not critically ascertain the entire microbial ecology in the biodynamic preparations. Preliminary studies in our laboratory indicated that these biodynamic preparations contained fungal population greater than 10^8 CFU (Vaish *et al.,* 2021).

The total number of fungal isolates purified from all the eight biodynamic preparations (BD 500 - BD 507) was twenty seven (Vaish *et al.,* 2021). However, since only 0.1-1% of the microbes are culturable, the remaining are unculturable. Metagenomics has been used as an appropriate approach for understanding the diversity and related metabolic activities in any environmental sample without culturing the microbes. Over past few years, NGS platforms have been effectively utilized for metagenome analysis of different eco-systems and habitats across the globe, including soils (Tedersoo *et al.,* 2014) for characterization of fungal diversity. These advantages of metagenome analysis using 18S rDNA amplicon sequencing technique offers scope for studying the overall diversity. Fungi are an important component of any ecosystem due to their biochemical activity and engagement in nutrient cycling. In view of the above, present study aimed at characterization of biodynamic preparations using metagenome analysis.

Materials and Methods

Eight Biodynamic (BD) preparations in the series of BD 500–BD 507 were obtained from Supa Biotech, Nainital, Uttarakhand, India. BD500 was prepared from cowdung filled in cow horn, BD501 from silica filled in cow horn, BD 502 and 506 from fermented flower heads of yarrow (*Achilles mille folium*) and dandelion (*Taraxacum officinalis*) respectively, BD 503 from fermented chamomile blossom (*Matricaria recutila*), BD 504 from air dried leaves of stinging nettle fermented in the soil (*Utrica dioca*), BD 505 from fermented oak bark (*Ouercus robus*) and BD 507 form valerian flower extract (*Valerian officinalis*).

DNA isolation: Genomic DNA from all the eight biodynamic preparations was extracted by using genomic DNA isolation kit following the protocol described in the manufacturer's instructions manual (Chromus Biotech, Bengaluru). The quality of DNA was checked on 0.8% agarose gel and then by bioanalyzer. The concentration of DNA was quantified using Qubit (Thermo scientific), and high quality DNA was used for library preparation.

Library preparation and sequencing: For library preparations, about 25 ng of DNA was used to amplify ITS2 hyper variable regions of fungi using 100 nm final concentrations of forward and reverse primers (ITS3F: 5'CAHCGATGAAGAACGYRG-3' and ITS4R: 5'CCTSCGCTTATTGATATGC-3') harboring partial Illumina sequencing adapters as overhangs and KAPA HiFi Hot Start Ready Mix (Tedersoo *et al.,* 2015). PCR reaction was performed following the steps which include an initial denaturation of 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 45 sec and 72°C for 30 sec and a final elongation at 72°C for 7 min. The amplicons of 300 bp size were purified using Ampure beads and unused primers were removed. Libraries were quantitated using Qubit dsDNA High Sensitivity assay kit. QC passed libraries was then sequenced using Illumina Miseq 2000 with 2x300PE V3 sequencing kit.

Sequence data analysis and metagenomic characterization: Sequence obtained from NGS platform was assessed for their quality using FastQC (Babraham Bioinformatics – FastQC, 2017) and MultiQC (Ewels *et al.,* 2016) softwares. Quality read with QC threshold (R20>90%) were trimmed (20bp) from 5' end to remove the degenerate primers, further processed to remove adapter sequences and low quality bases using Trimgalore (Babraham Bioinformatics - Trim Galore, 2017). The final QC passed reads were imported into USEARCH (Edgar, 2010) and the paired ends were aligned to form contigs with penalty of maximum 10 mismatches. The contigs were screened for errors and high quality contigs were analyzed to generate unique sequences.

These unique sequences were then clustered at 97% sequence similarity into OTUs (Operational Taxonomic Unit) by detecting and removing chimeric contigs in parallel pipeline. OTUs with only one representative sequence named singletons were discarded. OTU clustering and chimera removal was carried out by the UNOISE algorithm (Edgar, 2016). OTUs were populated by mapping back all the filter passed contigs onto the representative sequence from which the abundance in each BD preparations were calculated and further OTUs were classified based on UNITE ITS fungal database version 7.2 (Kõljalg *et al.,* 2013). Metagenome raw data of eight biodynamic preparations sequenced in Illumina Mi Seq 2000 NGS platform by 18S ribosomal sequencing were submitted to NCBI and is accessible from the bioproject PRJNA564215. The study was carried out in ICAR- CISH, Lucknow.

Results and Discussion

Metagenome analysis is an efficient tool for assessing microbial diversity present in the biological samples. In the present study, fungal metagenome analysis was assessed in eight biodynamic preparations using 18S amplicon sequencing of V3-V4 ITS region and data analysis. By sequencing the eight libraries generated for each of the bodynamic samples, on an average 15 GB data was generated for each sample. The raw data submitted in NCBI analysis indicated that the maximum number of reads (194344) was obtained in BD 504 while the minimum number of reads (58104) in BD 507 (Table 1). The percentage of GC content was found to be highest in BD 501(55%) followed by BD 502 (51%) and BD 505 (51%) and minimum 40% was found in each biodynamic preparations. The true diversity of biodynamic preparations was detected by observing the species richness among the samples.

The rarefaction curve (Fig. 1) indicated the species richness observed in eight biodynamic preparations. The data was analyzed for annotating the V3-V4 region with known fungal genome data base and their OTU were classified and was critically analysed to assess the fungal diversity. It was observed that BD506 had the highest diversity followed by BD500, 502, 504, 503, 501 and BD505, while BD507 had the least diversity. Principal component analysis analysis was also performed to

Table 1: Brief statistics of metagenome data generated from 18S rDNA sequencing of eight biodynamic (BD) preparations

BD preparations	Number of quality reads	Average Read Length (bp)	GC%	% Bases with > Q20
BD 500	194096	301	46.5	99.37
BD 501	125694	301	55	98.05
BD 502	157746	301	51	99.01
BD 503	122232	301	50	99.17
BD 504	194344	301	50	98.86
BD 505	107960	301	51	99.08
BD 506	431072	301	50	99.39
BD 507	58104	301	50	90.13

Table 2: Comparative analysis of fungal diversity estimates of among eight different biodynamic (BD) preparations based on different indices

BD preparations	OTU's	Chao1	ACE	Shannon	Simpson	InvSimpson
BD 500	828	904.77	902.61	4.32	0.95	23.74
BD 501	504	601.12	593.57	3.86	0.93	16.04
BD 502	716	864.61	868.74	3.94	0.94	18.79
BD 503	564	724.11	767.70	3.72	0.92	12.64
BD 504	513	655.39	701.90	2.96	0.85	6.85
BD 505	472	516.28	529.055	3.65	0.92	13.86
BD 506	868	1029.83	994.66	3.64	0.92	13.79
BD 507	254	272.03	273.08	4.33	0.97	35.15

Table 3: Description of percentage OTU abundance among eight biodynamic (BD) preparations at Kingdom level

Kingdom	BD500	BD501	BD502	BD503	BD504	BD505	BD506	BD507
Fungi	94.9	83.8	92	93.3	96.6	93.3	97.5	92.2
Chromista	4.65	11.5	6.43	6.08	2.65	6.39	2.26	0.832
Protista	0.333	0.273	0.216	0.269	0.0393	0.236	0.148	0.109
Plantae	0.0958	4.34	1.31	0.373	0.702	0.11	0.101	6.81
Protozoa	0.0103	0	0	0	0	0	0.000806	0

Table 4: Description of percentage OTU abundance among eight biodynamic (BD) preparations at Phylum level

Phylum	BD500	BD501	BD502	BD503	BD504	BD505	BD506	BD507
(Unassigned)	49.1	29.5	22.8	25.1	17.2	19.1	16.7	20.6
Ascomycota	29.2	61.7	55.5	39.5	50.4	57.8	44.9	49.2
Mortierellomycota	14.2	3.75	12.1	27.5	21.1	16.5	15.5	7.51
Chytridiomycota	2.67	0.0299	0.143	0.0355	0.0286	1.53	0.075	0
Basidiomycota	2.17	3.22	2.77	0.692	0.493	1.04	0.993	16
Aphelidiomycota	1.73	0.157	0.749	0.526	10.2	0.299	0.656	0.131
Rozellomycota	0.466	0.101	0.501	0.0237	0.0875	1.19	0.0621	0.0657
Cercozoa	0.333	0.273	0.216	0.269	0.0393	0.236	0.148	0.109
Mucoromycota	0.0929	1.22	5.07	6.21	0.402	2.21	21	6.35
Zoopagomycota	0.0575	0	0.00242	0.0798	0.00179	0.00628	0.0306	0
Ciliophora	0	0.0112	0.148	0	0.00536	0.0934	0.0113	0
Glomeromycota	0	0.0337	0	0	0	0	0	0

Table 5: Description of percentage OTU abundance among eight biodynamic (BD) preparations at genus level of fungal diversity

Phylum	BD500	BD501	BD502	BD503	BD504	BD505	BD506	BD507
(Unassigned)	93	69.1	74.5	70.4	90.8	47.1	60	57.6
Mortierella	3.39	0.584	7.13	2.61	0.548	15.7	3.89	2.54
Mucor	0.084	0.857	5.04	6.05	0.391	2.2	20.7	6.2
Aspergillus	0.161	3.12	0.206	0.0591	0.487	0.044	2.22	0.92
Gymnoascus	0.0988	0.0561	0.177	9.6	1.05	0.267	3.24	0.788
Microascus	0.0604	17.7	0.569	6.69	2.88	0.742	4.57	2.41
Fusarium	0.031	0.236	3.88	0.112	0.104	0.0346	0.336	0.0438
Penicillium	0.0206	0.251	0	0	0.00179	0.0314	0.00725	0.526
Acremonium	0	1.66	0.00242	0.127	0.421	0.00314	0.00484	0
Chrysosporium	0.0192	0.15	0.153	1.08	0.00893	0.00314	0.0105	0.0438

evaluate the genetic relatedness among the OTUs associate with specific organisms based on their taxonomical distribution. PCoA plot showed that all the eight samples were not similar with each other having different values on Axis 1 and 2, however, BD504 and BD506, and BD500, BD502 and BD503 were close on Axis 1 but dissimilar on Axis 2; like wise BD501, BD505 and BD507 were totally dissimilar on Axis 1 and 2 (Fig. 2). This indicated that all the biodynamic preparations had different fungal diversity. This could be probably due to varying methods used for preparing these biodynamics and also the native microbes that are recorded in the raw material used in biodynamic preparations and the type of microbial succession that could be taken over which would have become the basis of the composition of the fungal population in the biodynamic preparations.

Alpha diversity indices are extensively used to characterize microbial communities in any ecosystem (Schloss and Handelsman, 2006) which is composed of two components *viz.,* species richness and evenness indices. Species richness quantifies number of different species present in a certain niche, while, a measure of relative abundance of different species consisting of a community denotes eveness (Chawla *et al.,* 2012). The Alpha diversity indices *viz.,* Chao1, ACE showed that richness of BD 506 was highest while BD507 was lowest one as shown the Table 2, while the Shannon, Simpson, and Inv Simpson revealed the highest diversity and richness in BD507 and the lowest in BD504. However, Fisher index showed the highest in BD500 and the lowest in BD 507. Chaos1 and ACE are considered as abundance based estimator of species richness. Shannon-Weaver Index places a greater weight on species richness, whereas the Simpson index considers species evenness more than species richness in its measurement (Schloss and Handelsman, 2006; Schloss *et al.,* 2009). The ACE method divides observed frequencies into abundant and rare groups. The abundant species are those with more than 10 individuals in the sample, and the rare species are those with fewer than 10 individuals (Bo-Ra *et al.,* 2017).

Operational taxonomic unit (OTU) abundance at kingdom level revealed that the highest percentage of kingdom fungi was present in the BD506 (97.5%) followed by BD504 (96.6%), BD500 (94.9%), BD503 and BD505 (93.3%), BD502 and BD507 (92%) with the lowest in BD501 (83.8%) (Table 3). The OTUs which could not be identified using publically available data base were labelled as unassigned. Latter these represent novel fungi present in the biodynamic preparations. At phylum level, the percent abundance number of unassigned OTUs was highest in BD 500 (49.1%) followed by BD 501 (29.5%), BD503 (25.1%) and

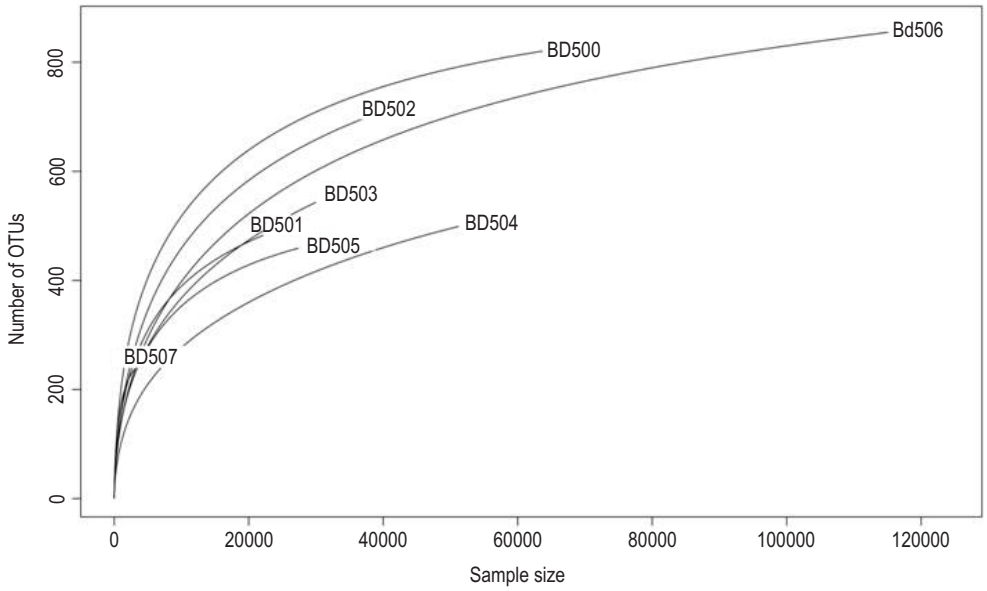

Fig. 1: Rarefraction curve indicated the species richness among eight biodynamic prepraration.

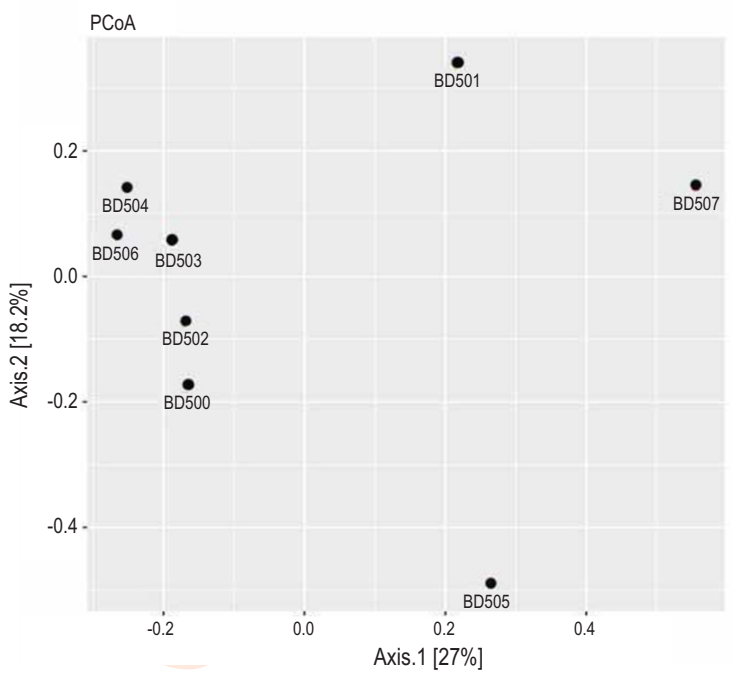

Fig. 2: PCoA plot represents diversity among eight biodynamic preparations.

22.8% in BD 502. At genus level, the percent abundance number of unassigned OTUs was highest in BD 500 (93%) followed by BD504 (90.8%), BD502 (74.5%) and BD BD 503 (70.4%). The top 10 genus identified in biodynamic preparations were (Unassigned), *Mortierella, Mucor, Aspergillus, Gymnoascus, Microascus, Fusarium, Penicillium, Acremonium* and *Chrysosporium* (Table 5). The species level analysis indicated dominance of unassigned sp. attributing to 98.6% in BD500, 93.5% (BD501), 93.2% (BD 502), 91.2% (BD503), 98% (BD504), 88.6% (BD505), 77% (BD506), 70.8% BD507. Heatmap

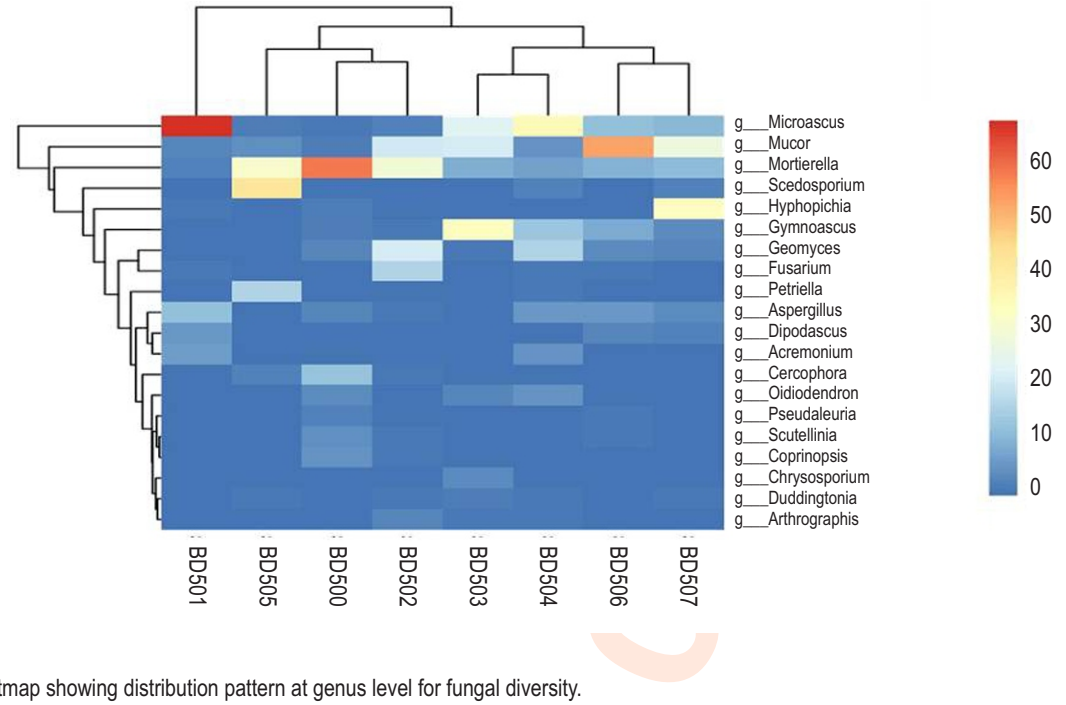

Fig. 3: Heatmap showing distribution pattern at genus level for fungal diversity.

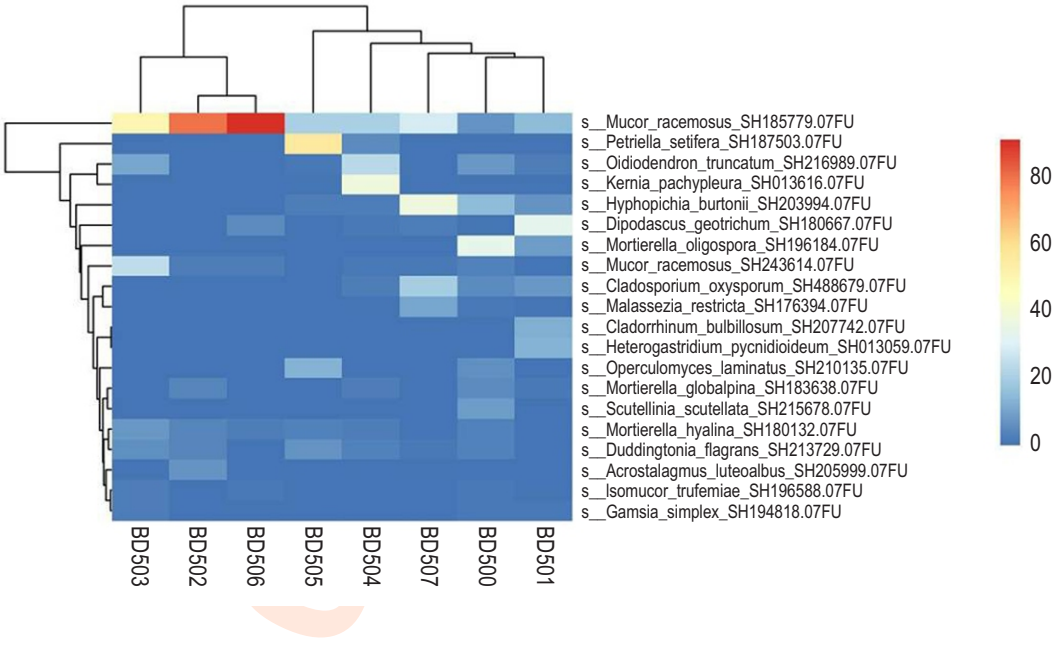

Fig. 4: Heatmap showing distribution pattern at species level for fungal diversity.

representing distribution of genus and species among eight biodynamic preparations has been depicted in Fig. 3 and 4, respectively. Overall perusal of data indicated that *Ascomycota* was the dominant phylum in all biodynamic preparations, except BD 500 as shown in Table 4. This was also ascertained with culturable microbial identification results (Vaish *et al.,* 2021), which verified the presence of fungi (belonging to ascomycota) *Aspergillus fumigates* strain NG14 (Accession number: Mn272432), *Penicillum rubens* strain NG10 (Accession Number: MN272400), *Penicillium citrinum* strain NG12 (Accession Number: MN272406) and *Alternaria brassicae* strain NG13 (Accession Number: MN272425) all belonging to phylum

Ascomycota. Latter has been reported to dominate the composting process upto 95.8% (Huhe *et al.,* 2017; De Gannes *et al.,* 2013). Ascomycota act as key decomposers in agricultural soils have the ability to produce a wide variety of extracellular enzymes (Vaish *et al.,* 2021). Mortierellomycota was observed to be the second highest occurring phylum in BD503 and BD504 preparations, while in BD506 mucoromycota was present.

One of the dominating genus identified in biodynamic preparations was *Mortierella*. Latter has been reported to assist crops and mycorrhizal fungi in phosphorus acquisition (Tamayo-Velez and Osorio, 2016), plant litter and aromatic hydrocarbon and soil toxin degradation (Osono 2005; Ellegaard-Jensen *et al.,* 2013) leading to improvement in soil (Fang Li *et al.,* 2018). At genus level, the presence of Mucor in the preparation of biodynamics (BD 500-507) was observed. It has been reported to utilize a wide range of carbon sources by producting degrading enzymes(Botha and Preeze, 1999). Fungi are known to participate in nitrogen fixation, hormone production, biological control against root pathogens and protection against drought (Baum 2015; El-Komy *et al.,* 2015). They also play an important role in stabilization of soil organic matter and decomposition of residues (Treseder and Lennon, 2015). The present study clearly indicates varying level of fungal population diversity among the eight biodynamic preparations, which could be attributed to preparation procedures and substrate composition favouring specific population dynamics as well as microbial succession leading to diversity in the population.

Biodynamic preparations are claimed to vitalize the soil, enrich it with macro and micronutrients, enhance seed germination, root formation, improve photosynthesis activity, strengthened plant immune system and better quality of fruits and seeds (Reeve *et al*., 2011). Yet the principal behind their bioenhancing power has still not been much worked out. Our previous study has indicated that culturable microbes present in BD preparations has high plant growth promoting and hydrolases (pectinase, cellulase, amylase) activities. Since, in any ecosystem only 1% of microbes are culturable. The study of unculturable microbes can provide explanation to metabolic functional properties including carbohydrate metabolism, Xenobiotic degradation, membrane transport function etc. The present study reports fungal biodiversity of biodynamic preparations, using 18S rDNA sequencing technology, possibly contributing for effectiveness and success of biodynamic farming. The alpha diversity indexes clearly indicate the diversity and species richness of various biodynamic preparations with respect to fungal population. The OTU abundance data at phylum, genus and species level reflects the presence of a large diverse fungal population yet unidentified and unavailable in public database. The dominance of unassigned fungi in BD preparations may contribute to the bioefficacy of organic preparations in soil.

Availability of data and material: Sequencing were submitted to NCBI and is accessible from the bioproject PRJNA564215

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Add-on Information

Authors' contribution: N. Garg: conceived, planned and designed the experiments. **S. Vaish:** performed the experiments and is a part of her PhD thesis work. **I.Z. Ahmad:** helped in sampling, and manuscript writing. **M. Manoharan:** assisted bioinformatics analysis and helped in statistical interpretations as well as preparation of manuscript.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable; Material is non pathogenic beneficial microbes.

Conflict of interest: Authors have no conflict of interest.

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Consent to publish: All authors have approved the manuscript for publication in *Journal of Environmental Biology*.

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