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Seasonal Variations of the Endophytic Fungal Diversity Associated with the Liverwort *Plagiochasma appendiculatum* Lehm. & Lindenb.

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ABSTRACT

This study investigates the diversity, colonization patterns and the seasonal variations in the fungal microbiome colonies associated with the liverwort *Plagiochasma appendiculatum* Lehm. & Lindenb. Altogether 17 different taxa of endophytic fungi dominantly belonging to the phylum Ascomycota and class Sordariomycetes, have been identified from 333 fungal isolates recovered from the thallus well as the rhizoids of the liverwort. *Pyrenopolyporus tonngachengensis*, the most abundant species recovered from our liverwort in *P. appendiculatum* is reported as an endophyte for the first time and it has been recovered from both the thalli as well as the rhizoids. However, the colonization rate was higher in the thalli (75.1%) as compared the rhizoids (24.9%), which can be most likely attributed to the larger surface area and nutrient availability for their growth in the thallus, further reaffirmed by the higher values of the ecological and diversity indices for thallus than the rhizoids. Present study also revealed the seasonal fluctuations in the diversity of the endophytic fungi across the year. Highest species richness was observed during summers, while the greatest colonization frequency was documented during winters, validating that the environmental factors such as humidity, temperature and physiology of host greatly affect the fungal endophytic community associated with the host.

Keywords: Ascomycota, Sordariomycetes, Liverwort thallus, Ecological indices, Seasonal variability

INTRODUCTION

Endophytic fungi are microbes that live quietly inside plant tissues without causing any visible symptoms and form complex relationships with the plant host that significantly affect their ecological and physiological characteristics (Melo *et al*., 2014; Begum and Tamilselvi, 2016). By occupying the internal tissues without causing any apparent disease symptoms, these fungi play a crucial role in boosting their host's ability to withstand various environmental stressors, such as attacks from pathogens or from herbivores (Porras-Alfaro and Bayman, 2011; Gautam and Avasthi, 2019). There is a considerable increase in the interest in studies related to endophytic fungi these days due to their remarkable capacity to generate bioactive metabolites that could lead to their potential new uses in the field of pharmaceutical industry (Omomowo and Babalola, 2019). Additionally, these fungi may play a crucial role in the ecology of bryophytes, aiding their survival in a range of environments, from tropical areas to the Arctic (Rodriguez *et al*., 2009). They assist host plants in dealing with both biotic and abiotic stresses, like pathogen invasions and nutrient shortages, through various mechanisms. Endophytic fungus, for instance, can help plants grow by fixing atmospheric nitrogen, solubilizing macro- and micro-nutrients present in the soil, and by generating phytohormones (Rana *et al*., 2020). Liverworts, a unique category of bryophytes, are notable for their unique metabolic profiles and the presence of various microbial communities hosted by them. However, the research related to fungal endophytes is still relatively scarce (Joshi *et al*.,2023). Liverwort *Plagiochasma appendiculatum* is particularly known for its wide distribution due to its remarkable ecological adaptability and a rich metabolomic profile of bioactive constituents owing to its anti-bacterial, anticancer, and antioxidant potential (Adebiyi and Tedela, 2023; Joshi *et al*., 2023). Despite this, the hidden diversity of the endophytic microbiota and their seasonal dynamics within this liverwort is still unexplored, indicating a critical research gap in our understanding towards liverwort-endophyte interactions. Furthermore, the seasonal fluctuations throughout the year also affect and derive the diversity of fungal community and their metabolic products within the host due to varied environmental conditions like temperature, humidity, and availability of nutrients in the environment (Omomowo and Babalola, 2019). Despite the rising interest and recognition of the relevance of endophytic fungi in bryophytes, there is still a limited understanding of their diversity, distribution, and function. Thus, investigating the diversity of these endophytic fungi will deepen our understanding and harnessing their potential at the biotechnological and commercial levels (Melo *et al*., 2014).

The present study focusses primarily to investigate the endophytic fungal microbiome associated with the liverwort *Plagiochasma appendiculatum* during different seasons of the year to elucidate the ecological dynamics between the host liverwort and fungal microbiota residing in it to reveal their

seasonal variability and adaptability across the different seasons of the year.

MATERIALS AND METHODS

Study area and Sample Collection

The liverwort *Plagiochasma appendiculatum* (**Figure 1**) was collected every month at regular intervals throughout the year from the moistened walls of the Rock Garden of Chandigarh (Elevation 1151 feet, Latitude 30º 7' 18" N and longitude 76º 8' 51" E) under sterile conditions. The plants were collected in a standardized manner, from uniformly occurring patches and approximately at the same dates of each month. The fresh samples with intact reproductive organs were collected aseptically in sterile polythene bags along with a thin layer of soil and brought to the laboratory. After proper identification of the plants, the fresh samples were subjected to the procedure of the isolation of endophytic fungi within 48 hours of their collection. Until their processing, properly washed samples were sealed and kept in refrigerated conditions.

Figure 1: Liverwort *Plagiochasma appendiculatum* Lehm. & Lindenb.

Isolation of endophytic fungi

Freshly collected samples were first cleaned thoroughly under running tap water to remove all the external soil particles. The cleaned thalli of the liverwort were then dried properly under sterile conditions, cleaned and cut into small pieces of size 0.5-1.0 cm with the help of sterile surgical blade inside the laminar air flow. Rhizoids were also separated in a sterile petri-plate during the procedure. The cut explants and rhizoids were then subjected to superficial disinfection by immersing them gradually in 70% ethanol for 2 minutes followed by 3% Sodium hypochlorite solution for 3 minutes. These segments are then again soaked in 70% ethanol for 1-minute and finally rinsed with sterilised distilled water. The disinfected segments were cleaned and dried with the help of sterile filter paper under aseptic conditions inside the laminar air flow chamber. The sterilised explants were then transferred one by one onto the petri plates of PDA supplemented with 100 µg/mL chloramphenicol. These plates were incubated at 24°C for 15 days and with regular examination once in three days.

The fungal mycelium arising from different explants was transplanted carefully on fresh PDA plates and their subsequent sub culturing was done to obtain the pure cultures of the isolated endophytic fungi (**Figures 2 & 3**). To ensure effectiveness of the surface sterilization, spreading the final rinse water on PDA-containing plates was done and it was observed that no contamination appeared on these plates after incubation The pure cultures of the isolated endophytic fungi were stored in the slants for further use. (Li *et al.* 2007).

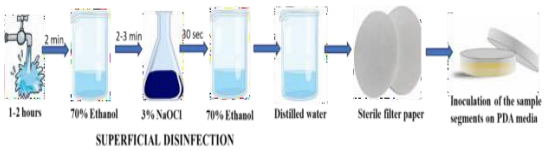

Figure 2: Procedure of isolation of the culturable endophytic fungi from the liverwort *Plagiochasma appendiculatum* Lehm. & Lindenb.

Morphological identification of the isolated endophytic fungi

The isolates growing on PDA plates at 24°C±1 for 15-20 days were observed morphologically to access the different cultural characteristics such the growth time of colony, its colour and shape from above and below for their identification. Furthermore, mycelial characters of the growing colony were also examined under the Nikon Eclipse E20 Microscope, for which the slides were prepared using Cotton blue and Congo red stains, the conidiophores and conidia were examined along with their size measurement using Nis element software.

Molecular identification of the isolated endophytic fungi

Molecular analyses of the morphologically identified endophytic fungi were also done to ensure proper identification. For molecular analysis, DNA was extracted from the freshly growing mycelium of fully-grown cultures using a Qiagen DNeasy Mini Plant Kit. The extracted DNA was visualized on 2% gel through gel electrophoresis. The internal transcribed spacers 1 and 2 and intervening 5.8S rRNA (ITS) gene region were amplified using primer pair ITS5 and ITS4 (White *et al.,*1990). Then PCR was done by using the following reagents: 9.5 µl of double-distilled water, 12.5 µl of 2 ×Taq PCR Master Mix with blue dye (Sangon Biotech, Shanghai, China), 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM) and 1µl of DNA template. The amplification condition for ITS gene region consisted of initial denaturation at 94°C for a duration of three minutes, which is followed by 35 cycles of denaturation at 94°C for 40 seconds, annealing at 50°C for 50 seconds, elongation at 72°C for one minute and a final extension period of 10 min at 72°C.The amplified amplicons were sequenced at PGIMER Chandigarh in India. The newly generated sequences were used to search the

NCBI GenBank database for matched sequences using nBLAST software. The ITS sequences were submitted to NCBI GenBank for accession numbers.

Statistical analysis

To assess the diversity and richness of fungal endophytes isolated throughout the year from the tissues of liverwort *Plagiochasma appendiculatum*, various indices were used, including Percentage frequency, Colonisation frequency, Colonisation rate, Relative abundance, Shannon-Wiener, Simpson's, and Evenness index.

Percentage frequency is based on the percentage ratio of the number of endophytes isolated from the plant tissue to the total number of endophytes isolated from the plant, whereas the Colonization frequency is the percentage of the number of segments colonized by each endophytic fungus of the total number of segments inoculated (Suryanarayanan *et al*., 2003).

Colonization rate is the ratio of the number of segments colonized by endophytes to the total number of segments inoculated and then multiplying with 100. Percent Isolation rate is based on the ratio of the number of endophytic fungi isolated to the total number of plant segments inoculated (Sunayana *et al*., 2014).

Shannon Weiner Index (Ronquist and Huelsenbeck, 2003) was used to evaluate the diversity of the fungal endophytic species and was calculated as **(H')** = $-\sum$ Pi×Ln (Pi), where Pi = ni / N, is the relative abundance of the endophytic fungal species, ni is the number of isolates of one species and N is the total species number of isolates present within each sample. Simpson's Diversity Index (Kusari *et al.,*2013) was calculated using formula **Ds** = $1-\Sigma Pi^2$ and Evenness Index (Jin *et al.,*2017) was calculated as **E** = H'/Ln(S), where S is the total number of the taxa present within each sample. Relative Abundance was calculated by numbering the total number of isolates of each taxon.

RESULTS

Species diversity

A total of three hundred and thirty-three (333) fungal isolates were obtained from the six hundred and forty-eight (648) surface-sterilized explants of liverwort *Plagiochasma appendiculatum*.The isolates were first characterized on the morphological basis for their identification coupled with their molecular analysis for their precise taxonomic placement and phylogeny. As a result, a total of seventeen (17) distinct fungal endophytic taxa were obtained mainly belonging to two phyla, i.e. Ascomycota and Basidiomycota. The isolated taxa which belonged to Ascomycota are *Alternaria alternata*, *Aspergillus aculeatinus*, *A. flavus*, *A. niger*, *A. piperis*, *Daldinia korfii*, *Epicoccum dendrobii, E.nigrum*, *Fusarium oxysporum*, *F. petroliphilum*, *Graphium basitruncatum*, *Pyrenopolyporus tonngachangensis*, *Stachybotrys chartarum*, *Talaromyces atroroseus*, *Trichoderma asperellum* and *T. koningiospis*. Only *Schizophyllum commune* belonged to Basidiomycota.

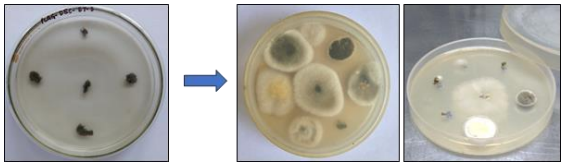

Figure 3. Isolation of different endophytic fungi from liverwort *Plagiochasma appendiculatum* Lehm. & Lindenb.

All the 17 taxa of the isolated endophytic fungi along with their percentage identity and NCBI accession numbers are given below in **Table 1**

Table 1. NCBI accession numbers and percentage identity of the endophytic fungi isolated from thallus of *Plagiochasma appendiculatum* Lehm. & Lindenb.

S. No.	Endophytic Fungi	Sequence number	NCBI accession numbers	Percentage identity
1	*Alternaria alternata*	SUB14728755 Seq6	PQ331202	100%
2	*Aspergillus aculeatinus*	SUB14728755 Seq5	PQ331201	100%
3	*Aspergillus flavus*	SUB14728755 Seq8	PQ331204	100%
4	*Aspergillus niger*	SUB14728755 Seq3	PQ331199	99.21%
5	*Aspergillus piperis*	SUB14728755 Seq15	PQ331211	100%
6	*Daldinia korfii*	SUB14728755 Seq7	PQ331203	99.13%
7	*Epicoccum dendrobii*	SUB14728755 Seq11	PQ331207	100%
8	*Epicoccum nigrum*	SUB14728755 Seq14	PQ331210	99.82%
9	*Fusarium oxysporum*	SUB14728755 Seq9	PQ331205	99.81%

S. No.	Endophytic Fungi	Sequence number	NCBI accession numbers	Percentage identity
10	*Fusarium petroliphilum*	SUB14728755 Seq17	PQ331213	100%
11	*Graphium basitruncatum*	SUB14728755 Seq16	PQ331212	99.81%
12	*Pyrenopolyporus tonngachangensis*	SUB14728755 Seq1	PQ331197	100%
13	*Schizophyllum commune*	SUB14728755 Seq10	PQ331206	99.84%
14	*Stachybotrys chartarum*	SUB14728755 Seq12	PQ331208	99.83%
15	*Talaromyces atroroseus*	SUB14728755 Seq13	PQ331209	99.81%
16	*Trichoderma asperellum*	SUB14728755 Seq2	PQ331198	99.05%
17	*Trichoderma koningiospis*	SUB14728755 Seq4	PQ331200	100%

Statistical analysis

A total of two hundred sixty-eight (268) isolates belonging to 17 taxa were obtained from the thallus and sixty-five (65) isolates belonging to 6 taxa were obtained from the rhizoids. All these fungal isolates were categorised majorly into two phyla, of which 16 isolates belonged to Ascomycota and remaining one belonged to Basidiomycota. These isolates further belonged to four distinct classes, 8 of which dominantly belonged to Sordariomycetes followed by Eurotiomycetes (5 isolates), Dothediomycetes (2 isolates) and Agaricomycetes (1 isolate). Further taxonomic classification at the order level classified the isolates into six orders, given in in the decreasing order of their representation: Eurotiales (5 isolates), Hypocreales (5 isolates), Pleosporales (3 isolates), Xylariales (2 isolates), Agaricales (1 isolate), and Microascales (1 isolate). The remaining families, each represented by a single isolate were Hypocreaceae, Pleosporaceae, Schizophyllaceae, Stachybotryaceae, and Microascaceae. Genera wise distribution of isolated endophytic fungi (thalli and rhizoids) in terms of percentage is shown in the pie chart (**Figure 4**). Species of the genus *Aspergillus* (27%) were most common amongst the isolated endophytic fungi followed by those of the genus *Trichoderma* (14%) while those of the genera *Schizophyllum* and *Alternaria* were least found (4%). Relative abundance of the isolated endophytic species is depicted in the **Figure 5**. Percentage-wise distribution of the isolated endophytic fungi showed *Pyrenopolyporus tonngachangensis* as the most common isolate followed by *Alternaria alternata* and *Trichoderma asperellum*. Among all the isolated taxa *Alternaria alternata*, *Trichoderma asperellum* and *T. koningiospis* emerged as the most frequently occurring taxa. Also, the colonization frequency of the 17 isolated fungal taxa is depicted in the **Figure 6**. Endophytic isolate *P. tonngachengensis* has the highest colonization frequency followed by members of genus *Trichoderma* while taxa belonging to genus *Aspergillus* showed lower colonization frequencies.

The values of percentage frequency were 75.1% for thallus and 24.9% for rhizoids. Isolation rate was 18.8% for thallus and 17.1% for that of the rhizoids. Species richness was also more in the thallus (17%) than the rhizoids (06). Also, the percentage frequency, colonization rate and isolation rate and of the various endophytic fungi isolated from thalli and rhizoids are given in **Figure 7**. Thallus explants significantly depict higher values of all three parameters than the rhizoids. Percentage frequency of the thallus segments was much higher in the thallus (75.1%) than the rhizoids (24.9%). Same trend was followed by the colonization rate and isolation rate, where the thallus segments showed higher values (43%, 18.8%) as compared to the rhizoids (28.1%,17.1%), thus clearly indicating the thallus of the liverwort is more favorable for endophytic fungal colonization than the rhizoids. The investigations clearly conclude that the presence of fungal endophytes was more abundant in thallus than in the rhizoids of the liverwort.

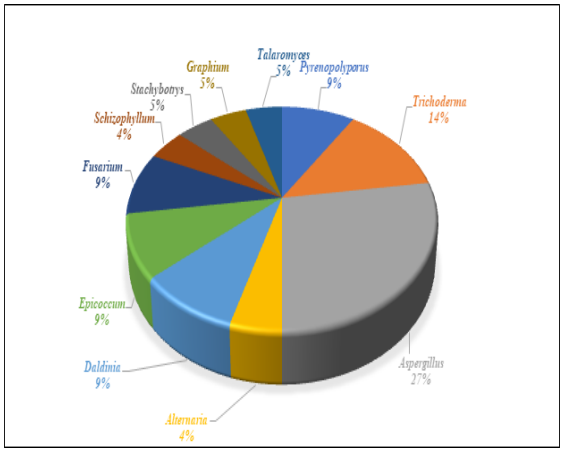

Figure 4. Genera-wise distribution of fungal endophytes obtained from *Plagiochasma appendiculatum* Lehm. & Lindenb.

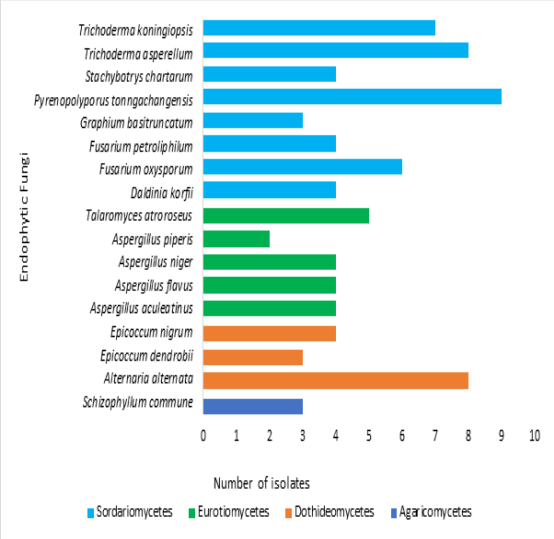

Figure 5. Relative abundance of endophytic fungi isolated from *Plagiochasma appendiculatum* Lehm. & Lindenb.

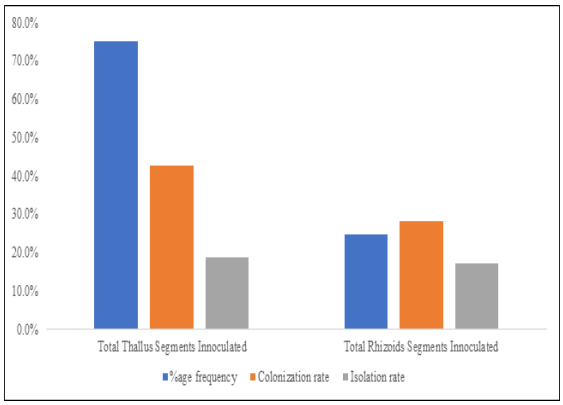

Figure 6. Colonization frequency of the endophytic fungi obtained from *Plagiochasma appendiculatum* Lehm. & Lindenb.

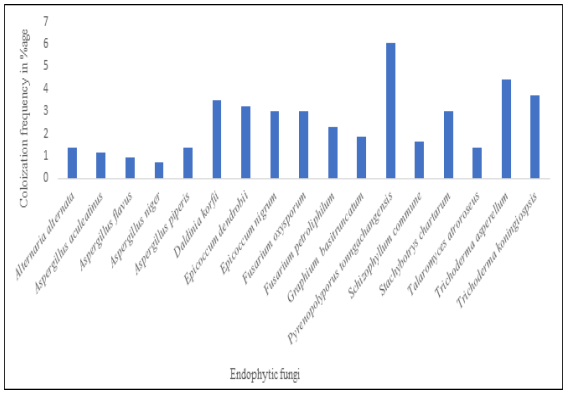

Figure 7. Percentage frequency, Colonization rate and Isolation rate of the endophytic fungi isolated from the thalli and rhizoids of liverwort *Plagiochasma appendiculatum* Lehm. & Lindenb.

Diversity indices including Shannon's Index, Simpson's Index and Evenness Index were also calculated (**Figure 8**). Shannon- Weiner's Index is the measure of species diversity including both abundance and evenness. The values of Shannon-Weiner's index for endophytes isolated from the thalli of the liverwort came out to be 2.688 and that from rhizoids came out to be 1.746, indicating high species diversity of endophytic fungi in the liverwort *Plagiochasma appendiculatum*. Simpson's Index is the measure of probability of two randomly selected species from a data set to be same. The values of Simpson's index for the endophytes isolated from thalli is 0.92 and that of rhizoids is 0.8, both of which are close to 1.00 indicating high species diversity. However, the species diversity of the endophytic fungi isolated from the thalli was more than the endophytic fungi isolated from the rhizoids. Evenness index is indicative of how evenly the species are distributed. Values of evenness index for thalli and for rhizoids were indicated that the species were evenly distributed.

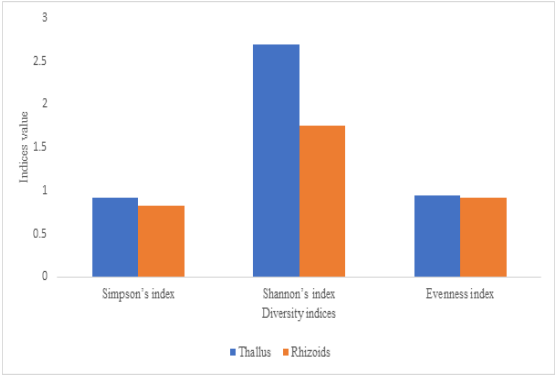

Figure 8. Diversity indices (Simpson's Index, Shannon's Index and Evenness Index) of the endophytic fungi isolated from the thalli and rhizoids of liverwort *Plagiochasma appendiculatum* Lehm. & Lindenb.

Seasonal variation of endophytic diversity isolated from *Plagiochasma appendiculatum* Lehm. & Lindenb.

The present investigations reveal that the colonisation of endophytic fungi found in *Plagiochasma appendiculatum* was significantly affected by variations in the seasons, as the environmental conditions such as temperature, humidity, rainfall, and host plant physiology change throughout the duration of the year. The maximum number of isolates were found in summer season (59), followed by autumn (56), spring (54), and winter seasons (36) (**Figure 10**). During summers, *Trichoderma asperellum* was the dominant species. In the autumn, a more diverse fungal population emerged, with the main taxa being *Alternaria alternata, Talaromyces atroroseus* and *Pyrenopolyporus tongachengensis*. *Trichoderma koningiopsis* was prevalent in spring, while *Aspergillus flavus* was the most common endophyte in winters. Table 2 shows the seasonal variations of the isolated endophytic fungi using various diversity indices.

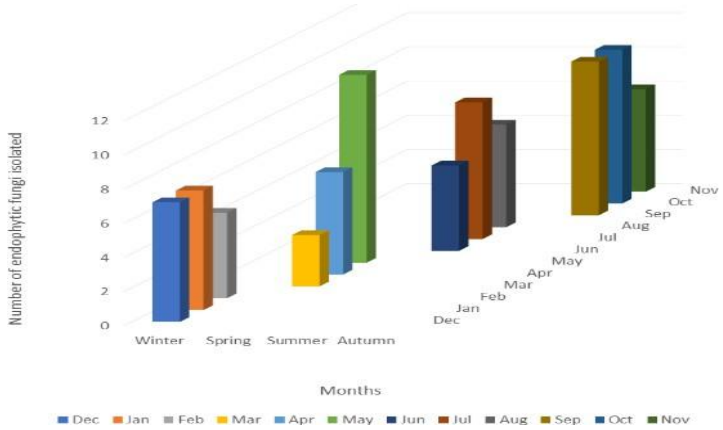

Figure 9. Monthly variations in the number of endophytes isolated from liverwort *Plagiochasma appendiculatum* Lehm. & Lindenb.

Variations in ecological and diversity indices were used to analyse the effect of seasonal variations on the diversity and colonization of endophytic fungi associated with the liverwort *Plagiochasma appendiculatum* **(Table 2, Figure 9).**

Table 2. Seasonal variations of isolated endophytic fungi isolated from *Plagiochasma appendiculatum* Lehm. & Lindenb. during different seasons of 2022 and 2023.

INDEX	SEASONS			
	SPRING	SUMMER	AUTUMN	WINTER
Percentage Frequency	**75.93**	**79.66**	**75.00**	**71.05**
Species richness	**13**	**14**	**11**	**13**
Isolation Rate	**19.42**	**17.43**	**20.69**	**18.45**
Colonization Frequency	**38.81**	**43.12**	**36.21**	**52.43**
Simpson's Index	**0.94**	**0.92**	**0.94**	**0.91**
Shannon Index	**2.04**	**2.13**	**2.10**	**2.41**
Evenness Index	**0.72**	**0.75**	**0.74**	**0.85**

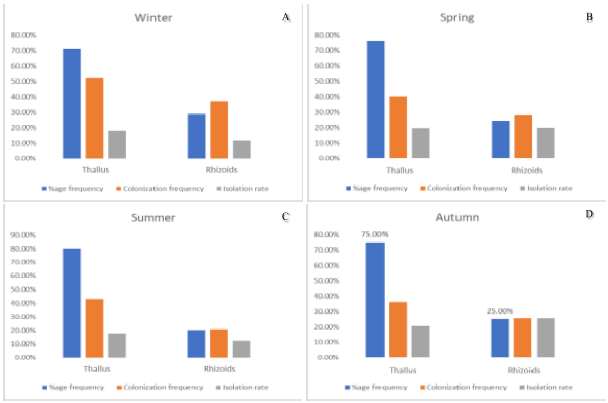

Figure 10. Percentage frequency, colonization frequency and isolation rate of the endophytic fungi isolated from liverwort *Plagiochasma appendiculatum* Lehm. & Lindenb. across the four seasons of the year: A) Winter B) Spring C) Summer D) Autumn.

Percentage frequency, which is the representative of the proportion of samples in which endophytic isolates were detected, remained relatively high across all seasons, ranging from 71.05% in winter to 79.66% in summer**,** indicating a consistently high presence of fungal population within the host. Species richness was highest in summer (14 species) and lowest in autumn (11 species**)**, suggesting a seasonal fluctuation in the colonization of endophytic microbiota. Isolation rate also varied slightly across seasons, with the highest value observed in autumn (20.69%) and the lowest during summers (17.43%), indicating that more successful endophytes colonization during autumn season.

Colonization frequency peaked during winters (52.43%), suggesting that endophytic colonization was most extensive during colder months, while the lowest colonization frequency was recorded in autumn (36.21%) (**Figure 10**).

Diversity indices like Simpson's Diversity Index, as depicted in **Figure 11**, also showed values ranging from 0.91 (in winter) to 0.94 (in spring and autumn)**,** indicating high diversity with no single species overwhelmingly dominant. Shannon's Diversity Index was recorded highest during winters (2.41) and lowest during spring season (2.04) suggesting greater species diversity and a more balanced distribution in the colder months. Evenness Index was also highest in winter (0.85) and lowest in spring (0.72)**,** further supporting the idea that fungal communities in winter were more evenly distributed compared to other seasons.

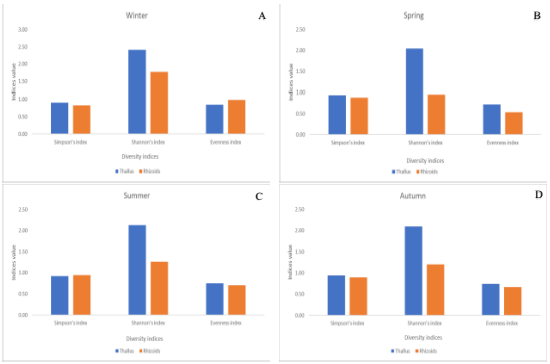

Figure 11. Diversity indices (Simpson's Index, Shannon's Index and Evenness Index) of the endophytic fungi isolated from the thalli and rhizoids of liverwort *Plagiochasma appendiculatum* Lehm. & Lindenb. across the four seasons of the year: A) Winter B) Spring C) Summer D) Autumn.

Overall, these indices highlight the seasonal variability of the endophytic fungi colonizing the liverwort showed that the highest colonization frequency and diversity was during winters, while summer exhibited the greatest species richness. So, the findings suggest that environmental conditions play a significant role in shaping fungal endophytic community within *P. appendiculatum*.

DISCUSSION

Although, a diverse array of microorganisms had been earlier isolated from various other liverwort species. Presently, the endophytic fungal community associated with *P. appendiculatum* is explored for the first time. No prior research has been conducted on this aspect of selected liverwort. The investigations were focussed to explore the fungal diversity and the seasonal fluctuations in the liverwort *P. appendiculatum*, across the year, highlighting its potential as a reservoir of unique microbial biota. *P. appendiculatum* flourishes usually under moist conditions, so several other surface microorganisms may also be a source of contamination during the fungal isolation process. Therefore, proper surface sterilization procedures were required to be optimized to do all the experiments and inoculations were performed with extensive care under sterile conditions (Romero *et al*., 2001). Appropriate concentrations and exposure time to the sterilizing agent are important for the isolation of significant number of endophytic fungi from the host (Schulz *et al*., 1997; Lodge *et al*., 1996; Arnold *et al*., 2000; Kohout *et al*., 2012), since the thallus as well as the rhizoids of the liverwort are prone to damage due to its soft and delicate structure. Presently, the treatment with 70% ethanol for 2 minutes followed by 3% sodium hypochlorite solution for 3 minutes proved to be the most effective method, ensuring insignificant contamination while maintaining the retention of endophytic fungi.

A total of three hundred and thirty-three (333) fungal isolates were obtained from the six hundred and forty-eight (648) surface-sterilized explants of liverwort *Plagiochasma appendiculatum*, of which two hundred sixty-eight (268) isolates belonging to 17 taxa were obtained from the thallus and sixty-five (65) belonging 6 taxa from the rhizoids. The isolated fungi belonged prevalently to the phylum Ascomycota, particularly to class Sordariomycetes which aligns with many previous studies (Davis and Shaw, 2008; Joshee *et al*., 2009; Rodriguez *et al*., 2009; Li *et al*., 2016; Park *et al*., 2017; Sarma *et al*., 2020) showing this group of fungi as the dominant group of endophytic fungi found in plants. Liverworts can exchange nutrients with Ascomycota fungi by trading photosynthates for phosphorus or nitrogen (Humphreys *et al*., 2010; Kowal *et al*., 2018; Field *et al*., 2019; Rich *et al*., 2021) that explains as to why members of the group Ascomycota are dominantly found associated with these plants. Phylum Basidiomycota was represented by a single taxon *Schizophyllum commune* indicating the rarity of endophytic fungi of this phylum in line with Crozier *et al.* (2006) and Davis and Shah, (2008). Many studies (Sandberg *et al*., 2014; You *et al*., 2015; Al-Bulushi *et al*., 2017; Ibrahim *et al*., 2017; Chen *et al*., 2020) reported Ascomycota and Basidiomycota being present as the dominant taxa of endophytic fungi associated with bryophytes and higher plants.

Deeper taxonomic analysis at the generic and species level revealed that *Pyrenopolyporus tonngachangensis*, a member of Xylariales, is reported as an endophyte for the first time and is the most abundant endophyte found in *P. appendiculatum.* Earlier, Davis *et al.* (2003) reported several other members of Xylariales as endophytic fungi from other liverworts as well as higher plants. Presently, *Alternaria alternata*, *Trichoderma asperellum* and *T. koningiopsis* are the

most frequently occurring species suggesting their strong colonization ability within the liverwort *P. appendiculatum*. *Alternaria alternata* has been reported as an endophyte from other lichens, mosses, grasses, and medicinal plants also (Rosa *et al*., 2009; Tripathi *et al*., 2014; Tripathi and Joshi, 2019; Khalmuratova *et al*., 2021). Wang *et al*. (2022), investigated its role in nutrient uptake wherein it helps in promoting plant growth. Similarly, species of *Trichoderma* such as *T. asperellum* as well as *T. koningiospsis* have also been recovered as endophytes from various plants (Hermosa *et al*., 2012; Brotman *et al*., 2013). *Trichoderma* species are mainly used as biocontrol agent (Kamal *et al*., 2018) and have promising role in the inhibition of the pathogenic fungi such as *Rhizoctonia solani*, *Pythium ultimum*, *Botrys cinerea*, *Colletotrichum* species etc (Mukhopadhyay and Kumar, 2020; Yao *et al*., 2023), none of which have been found in the present liverwort. Additionally, the widespread occurrence of the three different species of *Aspergillus* (27%) within the liverwort signifies their better ability to survive as endophyte in lower plants. *Aspergillus* endophytes are known as prolific producers of many bioactive secondary metabolites such as terpenoids, flavonoids and polyketides possessing agricultural, commercial, and pharmacological applications (White *et al*., 2019; Cui *et al*., 2024).

Many of the fungi isolated from *P. appendiculatum* have also been reported as endophytes in many other higher plants of medicinal value such as *Catharanthus roseus* (Dhayanithy, 2019) and in several other bryophytes such as *Marchantia polymorpha*, *Polytrichum commune*, and *Hylocomium splendens* (Gao *et al*., 2019; Nelson and Shaw, 2019). *Aspergillus niger* also forms association as an endophyte with the liverwort *Heteroscyphus tener* and this endophyte was further explored for its novel bioactive chemicals (Li *et al*., 2016; Rana *et al*., 2019).

On statistical level, the comparative analysis of fungal colonization between different tissues of the liverwort, i.e., the thallus and rhizoids revealed a significantly greater presence of fungal endophytes in the thallus than in the rhizoids. The percentage frequency (75.1%), isolation rate (18.8%) and species richness (17) were higher in the thallus compared to those in the rhizoids (24.9%, 17.1% and 6, respectively), suggesting that the thallus of the liverwort acts as more favourable niche for the colonization of endophytic fungi possibly due to the availability of more nutrients and more surface area. Additionally, the rhizoids being directly in contact to the soil may experience harsher environmental conditions, leading to the limited colonization of the fungi, while the thallus may provide a more stable environment to the proliferation of the endophytic fungi. Diversity indices also highlight the same observations. Shannon-Weiner's Index, which depicts both the species abundance and evenness was found to be higher in the thalli (2.688) as compared to the rhizoids (1.746). Similarly, Simpson's Index values, 0.92 and 0.8 for the liverwort thallus was 0.92 and the rhizoids, indicates that the diversity of fungal community within the thallus is more than that in the rhizoids. The Evenness Index, which accounts for the uniformity of the species distributed within a community supports the balanced colonization of the endophytic fungal community within the different tissues of the liverwort.

Although, no clear co-relation between the sampling time and the composition of the endophytic fungal community was observed in the present study, the seasonal variations in the community of endophytic fungi associated with the liverwort were certainly detected. The variations in the endophytic fungal populations with the fluctuations in the seasons across the year are due to the variable atmospheric factors such as temperature, moisture and light intensity and nutrient availability in the soil (Osono *et al*., 2008; Mishra *et al*., 2012; Singh *et al*., 2017). The endophytic fungal communities of the samples collected from the same site for two consecutive years showed similar pattern of the certain fungal taxa. Since the effect of time and seasonal changes on the endophyte populations are still understudied, certain studies have demonstrated that the fungal population residing within the host can vary significantly over the years or even within the span of one month (Cordier *et al*., 2012; Davey *et al*., 2013; Junker *et al*., 2012; Peršoh, 2015). Sharp *et al*. (2017) also reported different colonization patterns with the different changing seasons. Presently, the colonization patterns of the endophytic fungi in the liverwort exhibited seasonal variations in line with the outcomes of their ecological and diversity indices. The percentage frequency of endophytic fungi remained consistently high across all the seasons. Summer showed the highest percentage frequency (79.66%) which was lowest (71.05%) in winter season, suggesting that these endophytic fungi are constantly present in the host plant throughout the year but their presence and colonization dynamics are influenced by the variations in different environmental factors such as temperature, humidity, and the host's physiological state. The species richness peaked during the summer season leading to the isolation of 14 species and was the lowest in autumn when 11 species of the endophytic fungi were recovered from the host. The higher species richness in summer may be attributed to favourable growth conditions, including increased temperature and humidity, which enhance fungal proliferation. The slight decline in the population of endophytic fungi in autumn may be due to the transition to cooler conditions, affecting fungal sporulation and

colonization efficiency. The isolation rate with the highest value recorded in autumn (20.69%) and the lowest in summer (17.43%), is suggestive of lower species richness, but the fungal propagules were more abundant or more readily culturable during this period. Colonization frequency, reflecting the extent of fungal colonization within plant tissues, was highest during winter season (52.43%), indicating that despite lower percentage frequency and species richness, fungal endophytes were more extensively distributed across plant segments during colder months. This could be due to a reduced competition with epiphytic microbes or host-driven physiological changes that favour endophytic colonization.

The diversity indices also supported the seasonal shifts in the colonization of fungal community within the plant in present studies. The Simpson's Index remained consistently high across all the seasons (0.91–0.94), implying that no single species of the endophytic fungi dominated the microbiota associated with the liverwort. The Shannon's Diversity Index and the Evenness Index were highest in winters (2.41 and 0.85, respectively) and lowest in spring (2.04 and 0.72, respectively), indicating that fungal species were more evenly distributed and diverse in winter compared to other seasons.

Overall, this work emphasises the seasonal adaptation of endophytic fungi in *P. appendiculatum* and gives important information about their ecological interactions with thalloid liverworts. Through our present findings it can be concluded that fungal endophytes can be recovered throughout the year ever after employing the finest sterilization processes. However, the colonization frequency and isolation rates may vary due to the role of seasonal environmental fluctuations which influence the richness and distribution of endophytic fungi in *P. appendiculatum*. Nevertheless, these findings need further validation through research across different geographical regions to establish a comprehensive dataset. Also, further investigations on the metabolic and bioactive potential of isolated endophytic fungi may provide a clearer understanding of the unique environmental factors influencing them.

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