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The Influence of geographical and seasonal factors on the endophytic fungal assemblage in the velamen roots of *Rhynchostylis retusa (L.) Blume*

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Abstract
A study was conducted to analyze the endophytic assemblage in the velamen roots of *Rhynchostylis retusa* in two geographical regions of Kerala. The study aimed to assess the influence of geographical and seasonal factors on the fungal communities. 264 fungal isolates were identified and categorized into 11 distinct morphotypes based on culture morphology. Ascomycotina was the dominant group, accounting for 97.34% of the isolates. *Lasiodiplodia theobromae* emerged as the predominant species, regardless of location or season. The colonization frequency of the isolates was highest during the monsoon season and lowest during the pre-monsoon season. The rates of isolation and colonization, along with the Shannon diversity index, were found to be significantly dependent on the sampling site and season. The study highlights the importance of geographical and seasonal factors in shaping these fungal communities.
Key words: Epiphytic orchids, *Rhyncostylis retusa*, Velamen root, Fungal endophytes, Geographical variation, Seasonal variation .

Introduction

Endophytic fungi are a ubiquitous group of microorganisms that reside within the internal tissues of plants without causing any apparent harm to their host (Akram et al., 2023; Schulz and Boyle 2005). These fungi form inconspicuous infections in the intercellular spaces, petioles, and leaves of healthy plants throughout or nearly throughout their life cycle (Vandana et al., 2024).

Endophytes have been isolated from a wide variety of plant species, including trees, grasses, algae, and herbaceous plants (Mane and Vedamurthy 2018). They belong to diverse taxonomic groups, including Ascomycota, Basidiomycota, Deuteromycota, and some Oomycetes (Park et al., 2003;Toghueo and Boyom, 2019). These endophytes may contribute to their host plant's growth and survival by producing a range of secondary metabolites that provide protection against pathogens, herbivores, and environmental stressors (Strobel et al., 2005).

Epiphytic orchids, which grow on the surface of other plants without deriving nutrients from them, represent a unique and understudied system for investigating the role of endophytic fungi (Glime, 2018). The velamen roots of these orchids, which serve as a protective layer, provide a specialized niche for endophytic fungal colonization (Mawinei and Paramitha 2024).

Understanding the diversity and function of endophytic fungi within the velamen roots of epiphytic orchids could shed light on the complex interactions between these fungi and their host plants, as well as their potential for producing novel natural products with valuable applications. The current study is an extension of the work published by Deepthi and Ray (2021). Reports regarding seasonal and geographic fluctuations in the endophytic fungal assemblage derived from the velamen roots of *Rhynchostylis retusa* are lacking. This study primarily aimed to identify, describe, and examine the spatial and temporal variability of the fungal endophytes found in the velamen roots of *Rhynchostylis retusa*.

Materials and Methods

Plant material

Medium sized healthy hanging Velamen roots of *Rhynchostylis retusa* (L.) Blume, commonly known as 'fox tail orchid' were collected for fungal isolation. The collected samples were placed in polyethylene bags, labelled and transported to the laboratory. All samples were processed within 24 h of collection.

Sampling Procedure

Velamen roots were collected from two distinct geographical regions of Kerala state, India such as the eastern high lands (HL) and central mid lands (ML) in three prominent seasons pre monsoon (PRM) (February – April), monsoon (MON) (June- August) and post monsoon (POM) (October – December). Ten samples were collected from each geographical region, in three seasons ie; 10×3 = 30 samples from each geographic region. Three samples collected from each site were combined to form the composite sample of that site. Altogether 60 composite root samples (2 geographic region X 30 samples) representing 20 different sample sites of the two geographic zones of the state (Figure 1) were collected for understanding endophytic fungal variability.

Isolation of Fungal endophytes

Isolation of the fungal endophytes was carried in accordance with the procedure described by Deepthi and Ray (2018).

Identification of Fungal endophytes

The morphological identification of the isolated fungi was carried out based on the fungal colony morphology and characteristics of the reproductive structures (Watanabe, 1937). For molecular identification, DNA isolation, PCR amplification and sequence analysis were carried out following the procedure described by Deepthi and Ray (2018).

Data analysis

The colonization rate (CR) and isolation rate (IR) of endophytic fungi were calculated following the method of Frohlich et al., (2000) and Suryanarayanan et al., (2000). Relative frequency of isolation (RF%) was calculated as per Huang et al., 2008 . The colonization frequency (CF%) was calculated according to Kumaresan and Suryanarayanan et al., (2000). The percentage of dominant endophytes (D) was calculated based on the % CF divided by the total number of endophytes × 100 Kumaresan and Suryanarayanan et al., (2000). Species diversity indices such as Shannon diversity index (H'), Shannon evenness index (J') and Simpson diversity index (D) were calculated using the PAST software (Hammer et al., 2001). The diversity indices, colonization rate and isolation rate were subjected to ANOVA (Two way) to determine effects of location and season using SPSS 16.0 software.

Results

Endophytic fungal assemblage in the velamen roots of *Rhynchostylis retusa*

A total 264 fungal isolates were recovered from 1620 segments of the velamen roots of *Rhynchostylis retusa* could be assigned to 11 morphotypes based on culture morphology. The isolated morphotypes were grouped into 11 OTUs (operational taxonomic units referring to different morphotypes) belongs to 9 genera and 11 species (Table 1). Sequences of the identified taxa were submitted in Gen Bank and already received accession numbers (Table 1). 97.34% of isolated strains belongs to Ascomycotina and the remaining represented by Basidiomycotina (*Campanella sp*). The CF% of endophytic fungi ranges between 2.59% (*Campanella sp*) and 14.81% (*Lasiodiplodia theobromae*). *Lasiodiplodia theobromae* (5.61%) dominated the endophyte assemblage followed by *Trichoderma asperellum* (5.47%) (Table 1).

Average RF% ranges between 8.68 % (*Colletotrichum gloeosporioides*) and 17.72% (*Lasiodiplodia theobromae*). Among the 11 endophytic fungi isolated all except *M. caribica, C. gloeosporioides* and *N. sphaerica* had the RF more than 10%. (Figure 2)

Geographical and seasonal variation in the endophytic fungal assemblage in the velamen roots of *Rhynchostylis retusa*

Out of the 264 fungal isolates recovered 162 from the samples of midland region and 102 from the samples of highland region. Irrespective of the geographical locations studied maximum number of fungal isolates were obtained in the monsoon season (ML-75 and HL-50) and minimum from the pre monsoon season (ML-35 and HL-21). Out of the 11 fungal species identified all species except *N. sphaerica* and *Campanella sp* were common to the two geographical regions studied (Table 2). *Endomelanconiopsis endophytica* and *Trichoderma asperellum* were represented in all samples irrespective of season and location (Table 1). Colonization frequency (CF%) of isolates in the high land region ranged from 0.74% (*Myrozymma caribica*) to 2.96% (*Diaporthe eucalyptorum and Trichoderma asperellum*) and that of mid land region ranges between 1.7% (*Lasiodiplodia pseudotheobromae and Colletotrichum gloeosporioides*) and 5.55% (*Lasiodiplodia theobromae*). CF% of the isolates of samples from the premonsoon varies between 1.11% (*Trichoderma harzianum*) 3.7% (*Lasiodiplodia pseudotheobromae*); that of post monsoon varies between 1.11% (*Diaporthe eucalyptorum* and *Myrozymma caribica*) and 4.97% (*Lasiodiplodia pseudotheobromae*); that of monsoon ranges between 0.74% (*Myrozymma caribica*) and 5.55% (*Lasiodiplodia theobromae*). *Lasiodiplodia pseudotheobromae* has the highest CF% both in POM and PRM irrespective of the sampling locality (Table 1).

Colonization and isolation rate

Colonization rate (CR) was found to be highest in the midland region in monsoon season (23.70%) and lowest in the pre monsoon season of high land region (7.77%). Isolation rate (IR) was highest for samples collected from the mid land region in the monsoon season (0.16) and lowest for samples collected from the high land region in the pre monsoon season (0.07) (Table 2). Seasonal effect was prominent with maximum CR and IR occurs in monsoon season followed by post monsoon and minimum in pre monsoon season in the two geographical regions studied. Locations and seasons of sampling showed a significant influence on CR of fungal endophytes in *Rhynchostylis retusa* at $p < 0.001$. IR was also significantly influenced by season at $p < 0.001$and by location at $p < 0.01$. But season together

with location has no significant role in the CR and IR of fungal endophytes in *Rhynchostylis retusa* (Table 3).

Diversity estimation of endophytic fungi

Species evenness ranges between 0.95 to 0.99. Highest species evenness was observed in the samples from high land in post monsoon season (0.99) and lowest evenness was noted in mid land samples in pre monsoon season (0.95). Shannon –Weiner diversity index (H) of endophytic fungi ranged from 0.58 (HL, PRM) and 1.37 (ML, MON). Shannon –Weiner diversity index and simpson diversity index (1-D) was found to be highest in the monsoon season (HL-1.02, ML-1.37 ;HL-0.55, ML-0.71 respectively) and lowest in the pre-monsoon (HL-0.58, ML-0.83 ; HL-0.38, ML-0.50 respectively) irrespective of the geographic locations studied. Margalef species richness index was high in monsoon season (HL 1.48, ML 1.833) of the geographic locations studied (Table 4).

Sampling location has a significant role in determining the Simpson diversity index and Shannon diversity index of endophytic fungal taxa of *Rhynchostylis retusa* at $p<0.001$ and and species evenness at $p <0.05$. But Margalef species richness index was not significantly influenced by sampling location. Season of sample collection has significant role in determining species richness and Shannon diversity index at $p<0.05$. But sampling locations have more influence in determining Shannon diversity index of endophytic fungal taxa of *Rhynchostylis retusa* than season (Figure 3). Season and location together do not have any significant influence in determining the diversity of endophytic fungal taxa of *Rhynchostylis retusa* (Table 3).

Discussion

A systematic survey of the endophytic fugal assemblage in the velamen roots of *Rhynchostylis retusa* in Kerala has been done, with the goal of evaluating the influence of geographical and seasonal factors on these fungal communities. The common fungal genera are *Trichoderma* spp., *Lasiodiplodia* spp., *Diaporthe eucalyptorum*, *Endomelanconiopsis endophytica*, *Fusarium proliferatum* and *Nigrospora sphaerica*, most of these fungi seems to be endophytes in the previous studies (Sunayana et al., 2014; Gazis and Chaverri, 2010; Khoyratty, 2015; Rodrigues, 1994). The isolated strains were identified by morphological and molecular methods. Molecular techniques have become the most powerful tools for the identification of endophytic fungi (Ma et al., 2015). Most of the isolated members do not

produce reproductive structures in culture. ITS 1and ITS 2 regions of r DNA are most widely used markers for barcoding of fungi especially non sporulating forms (Schoch, 2012).

According to Chen et al., (2010), endophytic fungal colonization is more abundant in the roots of epiphytic orchids than in the leaves and stem, suggesting that velamen roots provide a better niche for microbial colonization (Tsavkelova et al., 2001, Deepthi and Ray, 2021). *Trichoderma* spp. and *Nigrospora* sp. have already been identified as endophytes in the roots of epiphytic orchids (Tan et al., 2012; Chen et al., 2010), which is consistent with our current study. Most of the isolated strains play specific biological roles in plants (Deepthi and Ray, 2018; Wary et al., 2022; Omomowo et al., 2023). According to Khoyratty et al., (2015), *Diaporthe* sp. and *Nigrospora* sp. help in the biotransformation of vanillin in *Vanilla planifolia*. *Trichiderma* spp. help to solubilize plant nutrients and make them available to the plants (Altomare et al., 1999). They also increase the availability of Fe and P in *Cucumber* spp. by increasing dry weight, shoot length and leaf area (Yedidia et al., 2001).

Irrespective of geographical locations studied seasonal factors appeared to determine the CR, IR and diversity of endophytic fungal community as reported for other host species in tropical areas (Rodrigues, 1994; Jalgaonwala, 2015). In the present study CR, IR and diversity of endophytic fungal community found to be highest in monsoon season and lowest in premonsoon season. The low colonization during premonsoon season may be due to reduced fungal activity and in monsoon season endophytes were active (Frey et al., 1999). As rain showers help in the dissemination of fungal inoculum material isolation frequency of fungal endophytes increased during monsoon season (Jalgaonwala et al., 2011; Singh et al., 2017). High humidity and low temperature in the monsoon season support fungal spore germination and multiplication lead to high IR of endophytes (Singh et al., 2017). Earlier studies also reported that rain fall and climate of the investigated area has an important role in the diversity of endophytic fungal assemblage (Wilson and Carroll, 1994). The decreasing tendency of CR% and diversity indices of endophytic fungal community in the present study from MON to POM to PRM also agree with the findings of Singh et al., 2017. (Vaz et al., 2014) observed a significant difference in the colonization frequency of fungal endophytes with increase in the precipitation. On the other hand, Helander et al., (1994) reported colonization frequency of endophytes was not affected by season.

According to Collado et al., (1999), the environment and geographic location have an impact on the composition of endophytic fungus. In this study, two distinct geographical regions of Kerala were examined, each with a different endophytic fungal assemblage. Fungal diversity indices in *Rhynchostylis retusa* were analyzed in relation to the different sampling locations, and the results showed that the endophytic fungal community was relatively diverse in the root samples from the mid-land region. Promising endophytic fungal growth conditions are indicated by high diversity indices in the velamen roots of R. retusa found in the mid-land region.

As already reported for other hosts (Petrini et al., 1992; Collado et al., 1999; Gazis and Chaverri, 2010), environmental conditions affect the composition of the endophytic mycoflora, as the fungi are exposed to different selection pressures in different ecosystems (Gore and Bucak, 2007). This leads to the formation of very specific fungal populations at each location. The considerable differences in the diversity indices between the geographical locations of the study show that the endophytic fungal community varies according to region. Statistically significant differences were found in the colonization rate of the endophytic fungal community of *R. retusa* between the two geographic locations studied. "Site-specific factors" (Naik et al., 2008) have already been identified as factors contributing to variations in isolation and colonization rates in different geographic regions.

Conclusion

The study reveals a diverse assemblage of endophytic fungi in the velamen roots of *Rhynchostylis retusa*, with significant variations in colonization and isolation rates influenced by geographical location and seasonal factors, particularly highlighting the dominance of *Lasiodiplodia theobromae* during the monsoon season. These findings underscore the importance of environmental conditions in shaping. The variations observed across different geographical locations suggest that local climate, soil composition, and other ecological factors contribute significantly to the diversity of endophytic fungi. Overall, these findings not only enhance our understanding of the ecological dynamics within the velamen roots of this orchid species but also underscore the intricate relationships between plants and their associated microbial communities. This research could have broader implications for the conservation and management of orchid species, as well as for understanding the ecological roles of endophytic fungi in plant health and resilien

Table1. Seasonal and geographic variation in the Colonization frequency (CF%) of endophytic fungal isolates from the velamen roots of *Rhynchostylis retusa*

Name of the Isolate	**Gen Bank Accession Number (Deepthi, Ray 2021)**	**HL (CF%)**			**ML(CF%)**			**Total CF (%)**	**% D**
		PRM	POM	MON	PRM	POM	MON		
Diaporthe eucalyptorum	MH260584.1	1.11	1.11	2.96			2.22	7.4	2.80
Endomelanconiopsis endophytica	MH260585.1	1.48	2.59	2.59	1.48	1.48	1.85	11.47	4.34
Fusarium proliferatum	MH260586.1	1.48		1.98		2.52	2.82	8.8	3.33
Lasiodiplodia pseudotheobromae	MH259847.1 MH259862.1			1.48	3.7	4.97	1.70	11.85	4.49
Lasiodiplodia theobromae	MF100156.1 MH259842.1 MH260585.1 MH259863.1	1.31		2.4	2.59	2.96	5.55	14.81	5.61
Myrozymma caribica	MH259848.1		1.11	0.74	1.48	1.48	2.22	7.03	2.66

Nigrospora sphaerica	MH259869.1				1.85		1.85	3.7	1.40
Trichoderma asperellum	MH260587.1	1.48	2.22	2.96	1.85	2.22	3.7	14.43	5.47
Trichoderma harzianum	MH260588.1	1.11	2.22	2.59		3.7	2.59	12.21	4.63
Campanella sp	MH846120.1						2.59	2.59	0.98
Colletotrichum gloeosporioides	MF100149.1			2.4			1.70	4.1	1.55
		7.97	9.25	20.1	12.95	19.33	28.79		

HL- High land, ML- Mid land, CF- Colonization Frequency

PRM –Pre monsoon, POM – Post Monsoon, MON – Monsoon

Table 2 Seasonal and geographic variation in the CR, IR and total number of fungal isolates from the velamen roots of *Rhynchostylis retusa*

	HL			ML		
	PRM	POM	MON	PRM	POM	MON
CR	7.77 ± 4.43	9.26 ± 3.15	16.66 ± 5.86	12.96 ± 3.15	20.16 ± 8.10	23.70 ± 10.36
IR	0.07 ± 0.02	0.08 ± 0.04	0.13 ± 0.06	0.09 ± 0.02	0.11 ± 0.05	0.16 ± 0.05
Total Number of Isolates	21	31	50	35	52	75

HL- High land, ML- Mid land, PRM –Pre monsoon, POM – Post Monsoon, MON – Monsoon

Table 3. Summary of Two-way ANOVA of different diversity indices, CR and IR of endophytic mycobiota from the velamen roots of Rhynchostylis retusa.

Indices	Location (df 1)	Season (df 1)	Location x Season (df 2)
Simpson (1-D)	5.056^{*}	2.631^{ns}	0.247^{ns}
Shannon (H')	5.651^{*}	4.884^{*}	0.375^{ns}
Evenness	7.229^{**}	1.242^{ns}	1.7^{ns}
Species richness	1.423^{ns}	4.056*	0.214^{ns}
CR	21.55^{***}	11.904^{***}	1.014^{ns}
IR	5.229^{*}	11.099^{***}	0.838^{ns}

*** Significant at $p \leq 0.001$, ** Significant at $p \leq 0.01$, * Significant at $p \leq 0.05$, ns Non significant. Figures in the table were F values

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Table 4. Seasonal and geographic variation in the Diversity indices of fungal isolates from the velamen roots of *Rhynchostylis retusa*

	HL			ML		
	PRM	POM	MON	PRM	POM	MON
Dominance_D	0.619 ±0.273	0.579 ± 0.320	0.453 ± 0.312	0.493 ± 0.223	0.423 ± 0.239	0.282 ± 0.107
Simpson_1-D	0.381 ±0.273	0.421 ± 0.320	0.547 ±0.312	0.507 ± 0.223	0.577 ± 0.239	0.718 ± 0.107
Shannon_H	0.584 ±0.439	0.681 ± 0.527	1.018 ±0.643	0.832 ± 0.418	0.997 ± 0.486	1.370 ± 0.373
Evenness_e^H /S	0.978 ±0.029	0.994 ± 0.019	0.963 ±0.025	0.950 ± 0.045	0.966 ± 0.032	0.953 ± 0.030
Margalef	0.941 ±0.702	1.130 ± 0.861	1.481 ±0.890	1.191 ± 0.587	1.187 ± 0.649	1.833 ± 0.411

HL- High land, ML- Mid land, PRM –Pre monsoon, POM – Post Monsoon, MON – Monsoon

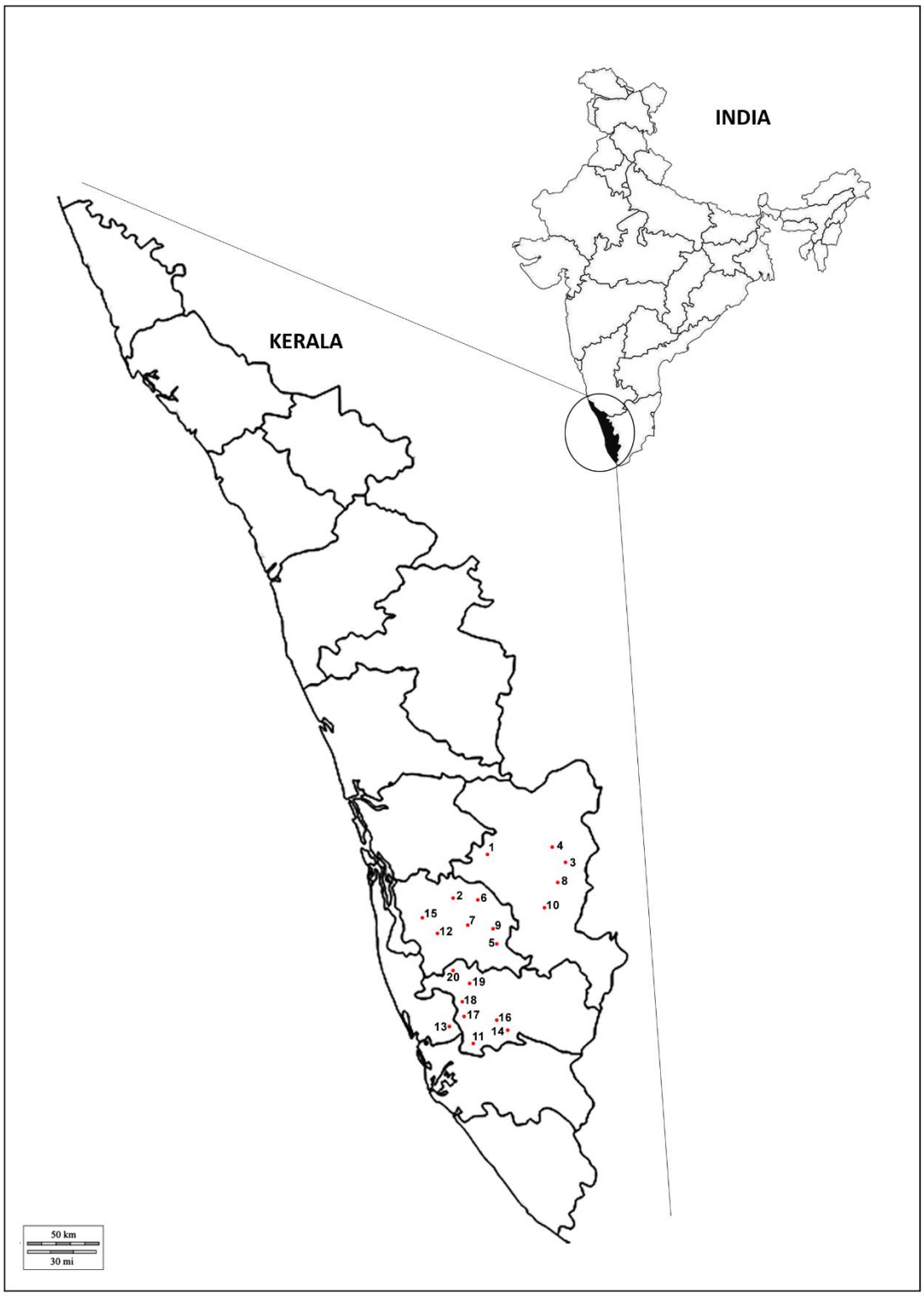

Figure 1. Study region showing 20 sampling sites.

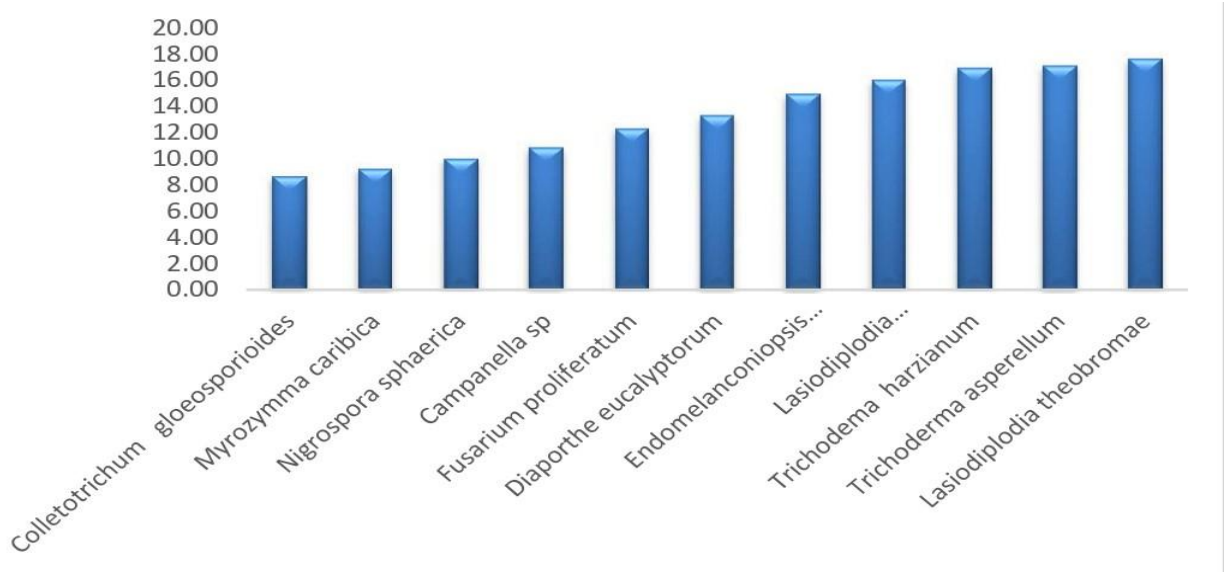

Figure 2. Overall Relative Frequency of endophytic fungal isolates in the velamen roots of *Rhynchostylis retusa*.

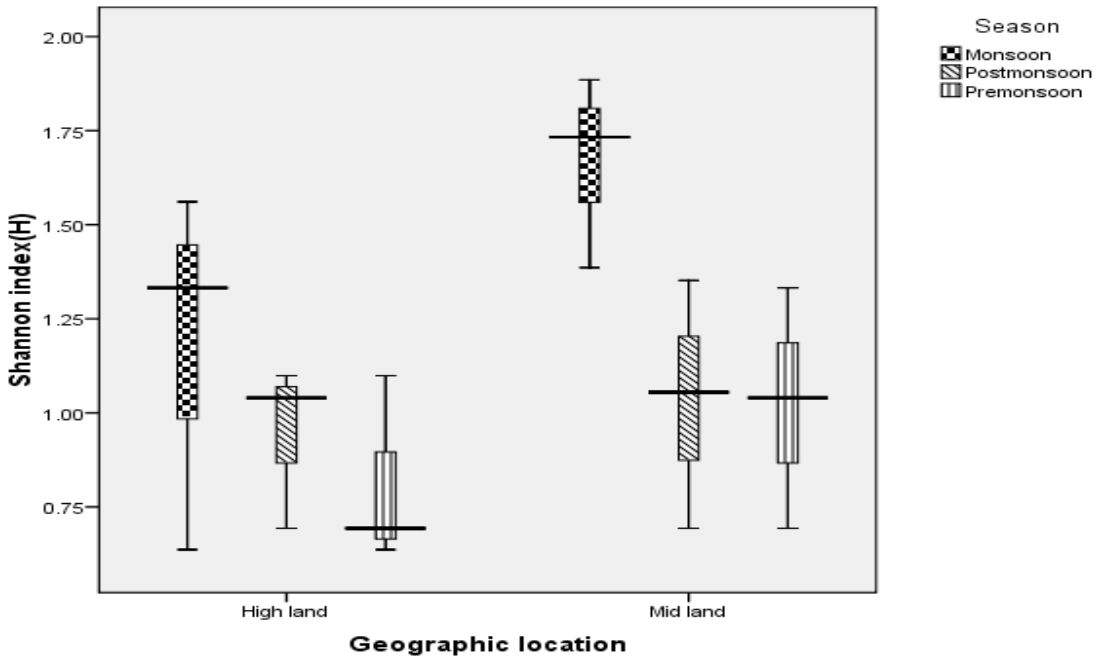

Figure 3. Boxplot representation of variability of species diversity (H') with respect to location, and season in the velamen roots of *Rhynchostylis retusa*.

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