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RESEARCH ARTICLE

Ecotype variations and endophytic fungal diversity of aquatic angiosperm: A case study with *Lagenandra toxicaria* Dalz.

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Abstract

A study on *Lagenandra toxicaria* ecotypes from Kottavayal, Pakkom and Mattilayam investigated the morphological variations in response to different pot sizes. The experiment employed a completely randomized design with three replicates to ensure reliable results. The researchers used statistical analyses to examine ecotype variations. The Duncan multiple range test calculated the mean separation at a 5% probability level. The study employed cluster analysis using the unweighted pair-group method of arithmetic averages and principal component analysis to identify and visualize the relationships among the samples. Cluster analysis revealed three distinct groups among the ecotypes, thereby shedding light on their relationships. The Kottavayal, Pakkom and Mattilayam ecotypes formed separate clusters, indicating their unique morphological characteristics. These findings provide valuable insights into the morphological diversity of *Lagenandra toxicaria* ecotypes and their adaptability to different environments. The endophytic fungal diversity details from this study revealed a high level of diversity, with 1408 isolates belonging to 12 families. The highest number of fungal isolates was observed during the summer. The present study revealed that the diversity was higher in the roots than in the leaves, higher than that of the rhizome of *Lagenandra toxicaria*. Colonization rate, Simpson's diversity index and Shannon-Wiener's diversity index were higher in root samples than in leaf and rhizome samples. MANOVA analysis revealed highly significant effects ($p < 0.01$) of individual factors, including location, season and plant species and their interaction effects. These results indicate that endophytic fungal diversity is significantly influenced by these factors and their interactions at a significance level of 1%.

Keywords

Lagenandra toxicaria; ecotype; biplot; endophytic fungi; diversity analysis; MANOVA

Introduction

Ecotype variations are common among plant species, allowing them to adapt to diverse environmental conditions. *Lagenandra toxicaria* exhibits ecotype variations that are influenced by habitat conditions of water availability, as well as environmental stressors. These variations may manifest as morphological differences and growth patterns across different ecological environments. Numerous species have observed phenotypic variability, including *Echinochloa crus-galli var. crus-galli* (L.) Beauv., which displays variations in growth habits and seed production in response to flooding and drought (1). *Convolvulus arvensis* L. exhibits differences in leaf morphology and stem elongation under varying light

conditions (2). *Eltrygia repens* L. exhibits variations in root architecture and tillering patterns in response to soil moisture and nutrient availability (3). *Cyperus esculentus* L. has adaptations in tuber production and vegetative growth under different temperature regimes (4). *Apocynum cannabinum* L. displays leaf shape and stem thickness variations in response to drought and competition (5). *Euphorbia esula* L. shows differences in root depth and leaf morphology under varying soil types and moisture levels (6). Other wetland or aquatic plants, such as *Typha latifolia* L. has adapted to rhizome growth and leaf morphology in response to water depth and nutrient availability (7) and *Spartina alterniflora* Loisel. has adapted to root growth and tillering patterns in response to tidal and sedimentation patterns (8).

The exploration of endophytic microbes has gained significant attention in the scientific community, as these elusive organisms have the potential to unveil a plethora of untapped natural resources. These organisms, which reside within the internal tissues of plants, possess the remarkable ability to establish symbiotic relationships with their hosts, often conferring beneficial effects. The isolation and characterization of these elusive microbes have become a growing interest among researchers, as they can unveil new avenues for biotechnological applications and environmental stewardship (9). Isolation of endophytic microbes involves a meticulous and systematic approach. It typically begins with the careful selection of plant samples, followed by surface sterilization techniques to eliminate any epiphytic (surface-dwelling) microorganisms. The internal plant tissues are then aseptically dissected and subjected to culture-dependent or culture-independent methods, depending on the specific research objective (10).

Culture-dependent approaches rely on the cultivation of endophytic microbes in specialized growth media, allowing for the identification and characterization of diverse bacterial and fungal species. These methods provide valuable insights into the physiological and metabolic properties of the isolated endophytes, flagging the way for their potential biotechnological applications. Alternatively, culture-independent techniques, such as metagenomic analyses, have revolutionized the field by enabling exploration of the entire microbial community within a plant, including those that are recalcitrant to cultivation. These approaches, which leverage advanced molecular biology tools, have revealed endophytic microbiomes' remarkable diversity and complexity, thereby shedding light on their ecological roles and potential functional contributions (11). The isolation and study of endophytic microbes have profound implications in various domains, including agriculture, environmental remediation and pharmaceutical development. Endophytes enhance plant growth, increase resistance to biotic and abiotic stresses and produce myriad bioactive compounds with therapeutic potential (12). By unlocking the secrets of these enigmatic microorganisms, researchers can potentially harness their capabilities to develop innovative solutions to pressing global challenges. The isolation of endophytic microbes represents a promising frontier in microbiology. Furthermore, the isolation of endophytic microbes provides valuable perceptions of the complex interaction between plants and their associated microbial communities. The application of rigorous methodologies and the integration of cutting-edge technologies to unravel the complexities of these intriguing microbes paves the way for groundbreaking discoveries and sustainable advancements in various domains.

Materials and Methods

Sites and sample procurement

Three specimen sites in the Wayanad district of Kerala were selected for this study (Fig.1). Site 1 was Kottavayal (11°34'52.2" N 76°06'22.7" E) surrounded by paddy fields, Site 2 was Pakkom (11°48'03.4" N 76°05'56.6" E) a dense forest area and Site 3 was Mattilayam (11°44'27.1" N 75°50'43.8" E), a hilly terrain region. Healthy and mature plants were randomly selected from each site and their plant parts, such as leaves, roots and rhizomes, were collected in triplicate in sterile bags. To ensure the endophytic nature of the isolates, the cut ends of the leaf, root and rhizome tissues were sealed with paraffin and transferred to pre-sterilized polythene bags. Upon collection, the plant samples were immediately transported to the laboratory in an icebox and subsequently stored at 4°C to maintain their integrity until further analysis. Sampling was performed at every study site in three seasons: summer, monsoon and winter in 2019-2021. Sampling was performed at the same sampling site on every occasion.

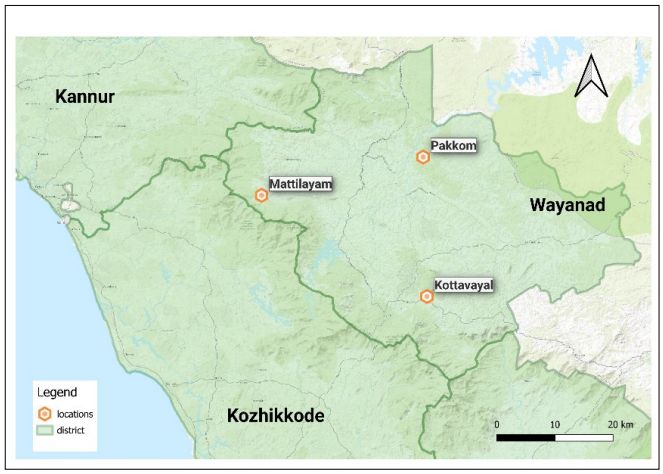

Fig. 1. Location map

Propagation of collected specimens

Plant samples were collected from 3 locations and were planted in 3 different-sized pots, 23×26.5 cm (Pot 1), 19×33.5 cm (Pot 2) and 25×41.5 cm (Pot 3) in height and width-wide pots and grown under normal conditions. Before planting, morphological features were recorded. Soil samples were also collected from potting sites. Basal fertilizer (1.0 g NPK/pot) was applied directly to each pot. The morphological variations of the potted plants were recorded after one year.

Morphological trait assessments and statistical analysis

Petiole height (PH), flag leaf length (FLL), flag leaf width (FLW), rhizome diameter (RD), cataphyll length (CL) and cataphyll width (CW) were assessed. Principal component analysis (PCA) was also performed to observe the *L. toxicaria* ecotype groups. The morphological dataset was analyzed using hierarchical clustering based on Euclidean distances through the UPGMA algorithm in NTSYS-pc version 2.1, yielding a dendrogram (13). Complementary principal component analysis facilitated

ecotype differentiation among the *L. toxicaria* ecotype groups. Square root transformation was used to transform the raw data before analysis. Data normalization was achieved through square root transformation. Subsequent ecotype classification employed the SHAN clustering module within the NTSYS-pc. The statistical significance of the growth traits was evaluated using ANOVA with three replicates. Post-hoc comparisons were performed using Duncan's multiple range test at α=0.05. Multivariate relationships were visualized through bi-plot analysis using SPSS version 16 and R version 2.15.1 (R Development Core Team 2009) software.

Surface sterilization and endophyte recovery

Before isolation, the collected plant samples were subjected to surface sterilization using a standardized protocol with some modifications proposed by Petrini (14). *Lagenandra toxicaria* Dalz., an aquatic herbaceous plant, was selected for this study. Initially, the plant samples were thoroughly cleaned by repeated rinses of running tap water, after which surface sterilization was performed using various sterilizing agents. Surface decontamination of plant materials typically employs antimicrobial agents such as dilute sodium hypochlorite (NaOCl) and ethanol (C_2H_5OH). However, the effective sterilization vary greatly depending on factors such as plant type, tissue thickness, texture and sensitivity. A standardized surface sterilization protocol was implemented, comprising sequential treatments: 1-minute exposure to 70% ethanol, followed by 7-minute treatment with 2% NaOCl for leaves, 4% for roots and rhizomes and a final 30-second ethanol rinse. The plant material was then rinsed extensively with sterile distilled water (5-6 times) to remove residual sterilants. Subsequently, the material was dried using a sterile filter paper and subjected to endophytic fungal isolation under strict aseptic conditions.

A total of 420 healthy, surface-sterilized explants (leaf, root and rhizome) were divided among 60 petri plates containing PDA medium with chloramphenicol (0.5mg/ml), with approximately seven segments per plate. The inoculated plates were incubated at 28 ± 2°C to promote endophytic growth. Regular monitoring revealed mycelial growth, prompting subculturing to achieve pure endophytic fungal cultures. Quality control measures included verifying the culture purity through repeated transfers to fresh PDA plates. Additionally, control plates containing sterilized rinse water were used to validate the surface sterilization protocol.

Fungal diversity analysis

The colonization rate was quantified as the percentage of tissue segments harboring one or more endophytic isolates relative to the total number of segments subjected to isolation. The diversity of the endophytic fungi was assessed using various statistical indices. The proportionality of individual taxa in the total endophytic isolates obtained was assessed by Colonization Frequency (CF) using the formula (15). Simpson's Diversity Index (D) was used to measure species diversity within the endophytic community, calculated as follows:

$$D = \Sigma n_i(n_i\text{-}1) / N(N\text{-}1)$$

The Simpson's Diversity Index (D) formula utilizes species-specific abundance (ni) and total population size (N). The resulting values ranged from 0 to 1, with higher values corresponding to reduced diversity and lower values indicating increased diversity and species heterogeneity.

The Shannon-Weiner Diversity Index, also known as the Shannon-Weiner Index (SWDI) (16), measures the diversity of species in a community.

The formulae were as follows:

$$H' = -\sum [(ni / N) \times \ln (ni / N)]$$

N = total population size, ln = natural logarithm, n = number of individuals.

The diversity of endophytic fungi in the two host plants was assessed using the Margalef Richness Index (R1), which provides a quantitative measure of species richness (17).

$$R1 = \frac{S-1}{ln\, N}$$

Where, S represents the overall species count and N denotes the cumulative total of isolates across all species.

The evenness index (E) quantifies the spread of populations among various species present.

$$E = \frac{H'}{\ln S}$$

Where (S) is the total population size.

The effects of season, geographical location and tissue type on endophyte diversity and distribution were examined using multivariate statistical analysis. MANOVA analysis was conducted using SPSS v16, with site, season and tissue type as independent factors. Dependent variables included colonization rate (CR), Shannon-Wiener Diversity Index, Simpson's Diversity Index, Species Evenness and Margalef Richness Index. To determine the relationships between endophytes and sampling variables, principal component analysis (PCA) was performed using R v2.15.1. This involved applying PCA to the correlation matrix of the species-by-sample matrix.

Identification and molecular analysis of recovered fungal endophytes

Morphological examination of the fungal isolates revealed 36 distinct morphotypes/Operational Taxonomic Units (OTUs), which were differentiated by characteristics such as Color, texture, shape, margin, elevation, exudates, reverse morphology and microscopic features. A camera-coupled Leica Binocular Light Microscope (DM750) facilitated detailed observations of cultural characteristics. Morphological identification and grouping of the fungal isolates were conducted according to standard taxonomic protocols and manuals. Following identification, representative cultures of each morphotype were preserved and stored in triplicates for future reference.

Molecular identification of endophytes

Genomic DNA extraction from the selected endophytic fungal isolates was performed according to the protocol outlined by Moller (18), with minor modifications. DNA concentration and purity were assessed spectrophotometrically using a NanoDrop spectrophotometer and absorbance was measured at 260 nm and 280 nm. A 1.8 absorbance ratio indicated DNA purity. To standardize the DNA concentrations, samples were diluted with TE buffer to 50 ng/µL for PCR amplification. Specific fungal DNA regions were amplified using ITS-based primers, specifically eukaryote-specific ITS1 and ITS4 primers (19). PCR amplification was performed on a Bio-Rad Thermal Cycler. The resulting products were sequenced and analyzed in the FASTA format using the NCBI nucleotide Basic Local

Alignment Search Tool (nBLAST). Sequence comparisons with the GenBank database facilitated the identification of the isolated fungal endophytes. All sequences were submitted to NCBI GenBank and accession numbers were obtained. For phylogenetic analysis, sequences were nBLAST searched against the NCBI database and trimmed to match the closest reference sequence. Multiple sequence alignments of 28 OTUs and the two closest named reference sequences per morphotype were performed using CLUSTAL MUSCLE (20). Evolutionary distances were computed using the Maximum Composite Likelihood method (21) and tree topologies were evaluated using bootstrap analysis (1000 replications). Phylogenetic analyses were performed using MEGA 11 software (22).

Results

Variabilities in plant characteristics among L. toxicaria ecotypes

Differences in plant parts under different conditions are presented in Table 1. One-way analysis of variance (ANOVA) was performed for the six morphological traits, with treatment as the factor. Post-hoc analysis using Duncan's Multiple Range Test (DMRT) was conducted to compare the mean differences between the four treatments. The significance level was set at $p < 0.05$. The results of the plant characteristics showed that Pot 1 and Pot 2 had similar effects on characters, but Pot 3 and habitat showed high variation in characteristics of plants.

This analysis aimed to explore the similarities among the four conditions based on six morphological factors (petiole height, flag leaf length, flag leaf width, rhizome diameter, cataphyll length and cataphyll width). Data were collected from triplicate observations across the three locations. Hierarchical clustering analysis using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) was performed, with Euclidean distance as the similarity measure. A dendrogram was generated to illustrate the clustering process and to identify similarities between the conditions (Fig. 2). The dendrogram revealed distinct clustering patterns among the four conditions. Pots 1 and 2 were the first to cluster, suggesting that they were the most similar among the six factors. These two conditions clustered at a lower distance, indicating high similarity. In contrast, Pot 3 and Habitat were more distinct, joining the cluster at higher distance levels, suggesting greater dissimilarity. This pattern indicates that Pots 1 and 2 share similar morphological characteristics, whereas Pot 3 and Habitat differ significantly.

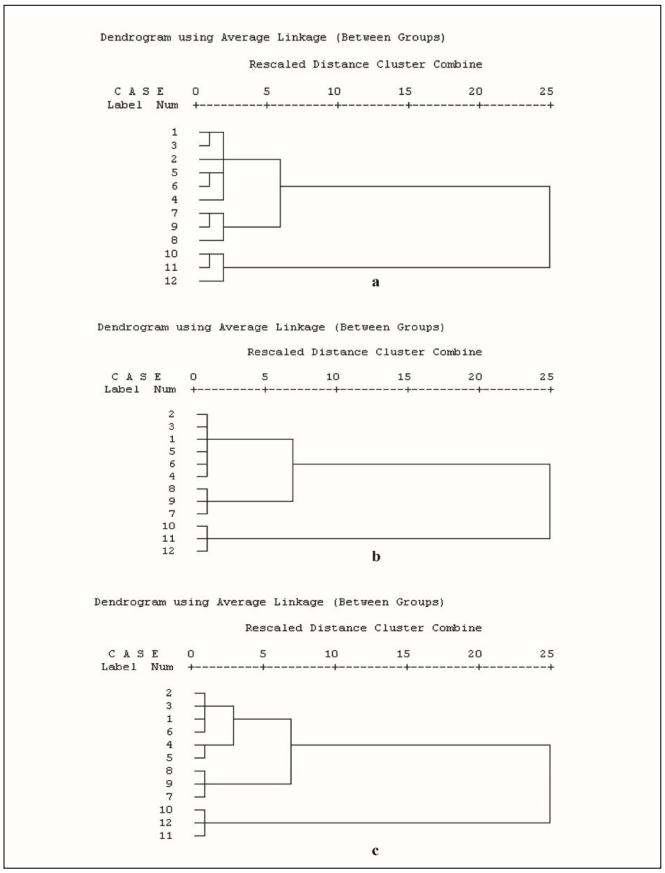

Fig. 2. Dendrogram from UPGMA clustering analysis using the Euclidian Distance coefficient of dissimilarity on *L. toxicaria* ecotypes from a. Kottavayal b. Pakkom c. Mattilayam (Note: 1,2,3: Pot 1 triplicates; 4,5,6: Pot 2 triplicates; 7,8,9: Pot 3 triplicates; 10,11,12: Habitat triplicates)

Principal component analysis was performed to identify the major components contributing to the variation in the data and to visualize the relationship between the treatments based on these components. In Kottavayal, the first two principal components (PC1 and PC2) explained 97.43% of the total variance, with PC1 accounting for 92.31% and PC2 for 5.12%, respectively. This indicates that a large portion of the variability in the data can be represented in two-dimensional space, making it possible to simplify the interpretation of the dataset. Similarly, in Pakkom, 91.08% and 8.42% of the variation was explained by the first two components. In Mattilayam, 92.7% of the variance was explained by PC1 and 6.05% was explained by PC2 (Table 2). The biplot between the first and second components showed that the individual plants present in the different pots had different characteristics. Plants in pots 1 and 2 showed similar characteristics. However, they were distinct from the other two

Table 1. Duncan's Multiple Range Test (95% confidence level) indicated no statistically significant differences among values sharing the same letter (Note: K- Kottavayal; P- Pakkom; M- Mattilayam; H- Habitat; P1- Pot 1; P2- Pot2; P3- Pot 3)

	PH	FLL	FLW	RD	CL	CW
KP1	6.13 ± 0.23^{a}	12.07 ± 1.53^{a}	3.4 ± 0.2^{a}	1.83 ± 0.21^{a}	7 ± 0.26^{ab}	1 ± 0.1^{a}
PP1	6.4 ± 0.82^{a}	12.77 ± 1.24^{a}	3.67 ± 0.46^{a}	3.27 ± 0.31^{c}	2.1 ± 0.36^{d}	0.35 ± 0.05^{b}
MP1	5.5 ± 0.5^{a}	13 ± 2.44^{a}	4.13 ± 0.25^{a}	2.77 ± 0.15^{bc}	6 ± 1.56^{c}	1.27 ± 0.15^{c}
KP2	4.23 ± 1.25^{a}	12.53 ± 1.38^{a}	5.6 ± 0.35^{b}	2.63 ± 0.15^{b}	7.4 ± 0.26^{b}	2.15 ± 0.05^{e}
PP2	6.03 ± 2.08^{a}	13.3 ± 0.92^{a}	3.77 ± 0.47^{a}	4.53 ± 0.25^{de}	6.73 ± 0.59^{abc}	1.53 ± 0.12^{d}
MP2	11.8 ± 2.07^{b}	18.33 ± 1.63^{b}	5.57 ± 0.45^{b}	4 ± 0.2^{d}	6.4 ± 0.1^{ac}	1.37 ± 0.12^{cd}
KP3	11.47 ± 1.06^{b}	19.87 ± 1.97^{b}	7.03 ± 0.46^{c}	4 ± 0.1^{d}	8.37 ± 0.15^{d}	1.4 ± 0.1^{cd}
PP3	22 ± 2.65^{c}	25.83 ± 1.97^{c}	6.93 ± 0.61^{c}	5.57 ± 0.25^{f}	12.8 ± 0.2^{e}	2.7 ± 0.1^{f}
MP3	18.33 ± 0.15^{d}	24.53 ± 2.05^{c}	8.73 ± 0.42^{d}	5.97 ± 0.42^{f}	11.8 ± 0.26^{f}	3.03 ± 0.15^{g}
KH	47.23 ± 1.12^{e}	30.77 ± 1.63^{d}	11.93 ± 0.76^{e}	4.83 ± 0.5^{e}	12.9 ± 0.44^{e}	3.57 ± 0.32^{h}
PH	49.23 ± 0.9^{e}	35.3 ± 2.72^{e}	14.67 ± 0.15^{f}	5.73 ± 0.29^{f}	13.07 ± 0.15^{e}	3.57 ± 0.06^{h}
MH	39.33 ± 1^{f}	29 ± 0.56^{d}	14.17 ± 0.4^{f}	6.07 ± 0.74^{f}	11.77 ± 0.25^{f}	3.13 ± 0.15^{g}

Table 2. Principal component analysis among groups of *L. toxicaria*

Characters	Kottavayal		Pakkom		Mattilayam	
	PC1	PC2	PC1	PC2	PC1	PC2
Petiole height	-0.4121	0.1856	-0.4056	0.4467	-0.3934	0.6163
Flag leaf length	-0.4172	-0.2764	-0.4201	0.2011	-0.4197	0.0669
Flag leaf width	-0.4225	-0.0641	-0.3978	0.5088	-0.4072	0.4484
Rhizome diameter	-0.3910	-0.6367	-0.3993	-0.4998	-0.4101	-0.3228
Cataphyll length	-0.4209	0.1275	-0.4032	-0.4616	-0.4073	-0.4275
Cataphyll width	-0.3838	0.6806	-0.4225	-0.1949	-0.4110	-0.3568
Eigenvalue	5.539	0.307	5.465	0.506	5.565	0.362
Proportion (%)	92.31	5.12	91.08	8.42	92.75	6.04
Cumulative (%)	92.31	97.43	91.08	99.50	92.75	98.80

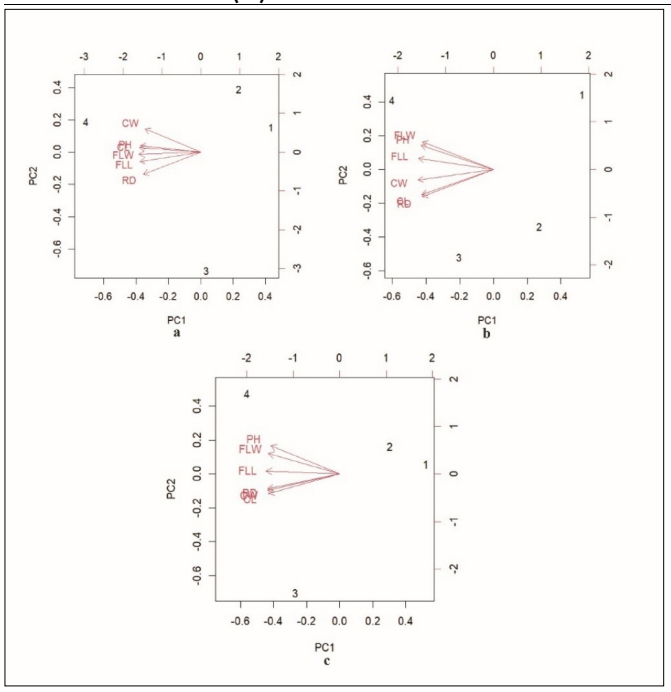

Fig. 3. The biplot illustrates the various parts of plant a. Kottavayal b. Pakkom c. Mattilayam

groups at three locations (Fig. 3). This study revealed distinct correlations between morphological traits in each *Lagenandra toxicaria* ecotype. In Kottavayal samples, petiole height and cataphyll length were closely related, indicating a strong link between these characteristics. In contrast, Pakkom exhibited two significant correlations: flag leaf width and petiole height were closely related and rhizome diameter and cataphyll length showed a strong relationship. This suggests that these traits are adaptively linked to this phenotype. The Mattilayam samples displayed a different pattern, with rhizome diameter, cataphyll length and cataphyll width being strongly intercorrelated. These correlations implied that specific morphological traits were connected in each ecotype, contributing to their unique characteristics and potential adaptations.

Diversity of endophytic mycobiota in Lagenandra toxicaria Dalz.

A total of 304 endophytic fungi were isolated from the leaf segments during the summer season, 196 during the winter season and 108 during the monsoon season. From the root segments, 499 fungi were in summer, 168 in winter and 115 in monsoon. 7 isolates in summer, 7 in winter and 4 in monsoon from the rhizome segments. A total of 1408 fungal isolates were obtained using the fragmentation method. In total, 810 endophytic fungi were isolated from all tissues in the summer, 371 in the winter and 227 in the monsoon season. Kottavayal holds 373 isolates from all plant parts in the summer, 175 in the winter and 93 in the monsoon. In Pakkom, 420 isolates were found in the summer, 154 in the winter and 94 in the monsoon season. 17 endophytic fungi were isolated from Mattilayam during the summer, 42 in winter and 40 during the monsoon season. No isolates were obtained from the Pakkom rizome samples in the monsoon season. Data for the isolated

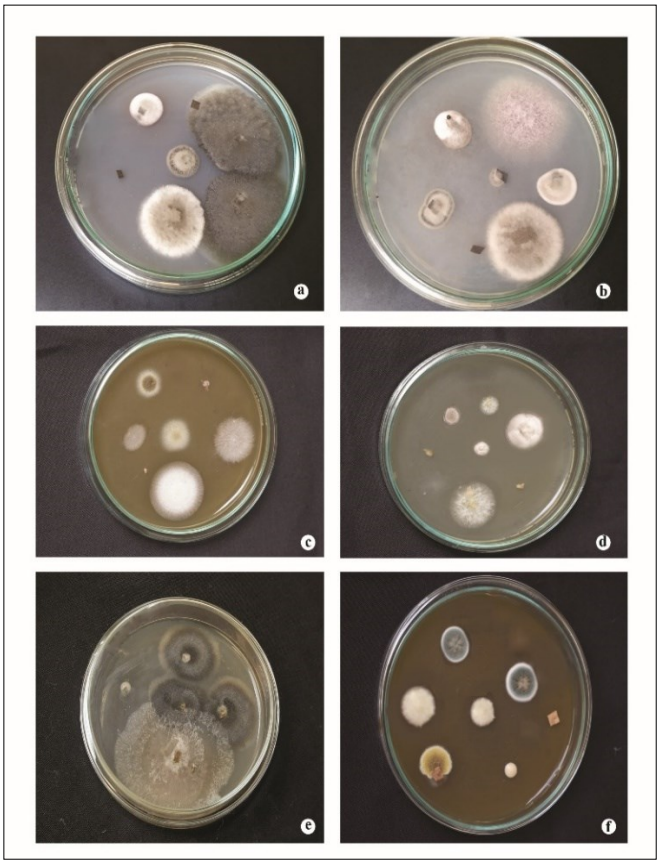

Fig. 4. Isolation plates of endophytic fungus a,b - Leaf; c,d - Root; e,f - Rhizome

Table 3. Endophytic fungi isolated from *Lagenandra toxicaria* Dalz. *S: Summer, W: Winter, M: Monsoon

		Locations											
		Kottavayal				Pakkom				Mattilayam			
		S	W	M	T	S	W	M	T	S	W	M	T
Plant parts	**Leaf**	119	80	40	239	179	85	46	310	6	31	22	59
	Root	250	93	51	394	239	66	48	353	10	9	16	35
	Rhizome	4	2	2	8	2	3	0	5	1	2	2	5
	Total	373	175	93	641	420	154	94	668	17	42	40	99

endophytic fungi are detailed in Table 3. Representative isolation plates are shown in Fig. 4.

The highest colonization rates were observed in 59.04% and 56.66% of the root samples from Kottavayal and Pakkom, respectively, in summer. Similarly, root samples from Kottavayal and Pakkom leaves showed the highest colonization rates of 22.14% and 20%, respectively, in winter. The lowest colonization rate was observed in the rhizome samples from Mattilayam, at 0.71% in the summer and winter. The rhizome samples from Pakkom in summer had the same colonization rate. The highest colonization rates observed in the root samples from Kottavayal and Pakkom during the monsoon season were 12.14% and 11.19%, respectively. The highest SWDI was observed in root samples from Pakkom in the summer season (3.44), followed by root samples from Kottavayal (3.41). The diversity index values of 0.23 and 0.59 were observed as the lowest in summer for the rhizome samples from Kottavayal and leaf samples from Mattilayam. In the monsoon season, 2.17 is the highest SWDI observed from root samples of Pakkom, followed by root samples from Kottavayal (1.94) and the lowest value was observed of 0.54 from leaf samples of Mattilayam. The SWDI of 2.58, followed by 2.36 and 2.23, are the highest diversity indices observed from leaf samples from Pakkom, Kottavayal and root samples from Pakkom in the winter season. The rhizome samples from Pakkom had the lowest SWDI (0.34) in winter, followed by the root samples from Mattilayam (0.67).

The Simpson's diversity index of root samples from Kottavayal and Pakkom (0.96) showed the highest diversity in summer, followed by leaf samples from Pakkom (0.86) and Kottavayal (0.84) and the lowest index was shown by rhizome samples from Kottavayal of 0.16. In the case of monsoons, root samples from Pakkom and Kottavayal had the highest SDIs, 0.87 and 0.82), followed by Pakkom leaf samples (0.81). The lowest diversity was observed in the leaf (0.31) and root (0.49) samples of Mattilayam. The leaf samples from Pakkom showed the highest diversity index of 0.92 in winter, followed by 0.89 and 0.87 from Kottavayal and root samples from Pakkom. The Margalef richness index showed that the summer season showed the highest richness index from the root samples of Pakkom (8.16) and Kottavayal (7.91). In summer, leaf samples from Kottavayal and Pakkom also showed average richness of 3.76 and 3.62, respectively. The leaf and root samples from Mattilayam had a lower richness of 1.08 and 1.10, respectively. The lowest richness index was observed in rhizome samples from Kottavayal.

The highest monsoons were observed in the root samples from Pakkom and Kottavayal (3.15 and 2.59), followed by leaf samples of Pakkom (2.36) and Kottavayal (2.17). Comparatively, samples from Mattilayam had a lower richness. In the case of species evenness, complete evenness was observed in the rhizome samples from Kottavayal during winter. The root samples from Mattilayam during the monsoon season showed the highest value of 0.99, followed by the root and leaf samples from Pakkom at 0.95 and 0.93. The root samples from Pakkom and Kottavayal showed the highest evenness in the summer season (0.95 and the lowest 0.33 in the rhizome samples from Kottavayal. In addition to complete evenness in the winter season, the highest value was observed in the leaf samples from Pakkom at 0.96, followed by leaf samples from Kottavayal and Mattilayam at 0.93. The lowest species richness was observed in the Pakkom rhizome samples (0.50). The SWDI, SDI, Margalef richness index and evenness of rhizome samples from Kottavayal in the monsoon, Pakkom in the summer and Mattilayam in all seasons were zero.

Table 4. Results of multivariate ANOVA for endophytic fungal diversity

Effect	*df*	Pillai's trace	F	P
Location	2	0.935	6.673	0.000
Season	2	1.084	8.998	0.000
Plant	2	1.484	21.847	0.000
Location*Season	4	1.004	2.681	0.000
Location*Plant	4	1.267	3.710	0.000
Season*Plant	4	1.382	4.223	0.000
Location*Season*Plant	6	1.322	2.106	0.001

MANOVA on individual factors and interaction effects showed high significance (*p<0.01)* for location, season, plant, location*season, location*plant, season*plant and location*season*plant. From the observation, the endophytic fungal diversity is statistically significant at a 1% significance level (Table 4).

The effects of location, season and plant part on dependent characteristics were analysed. The effect of location on SDI, SWDI, evenness and Margalef richness was statistically significant from 1% to 5%. The CR and Margalef richness effects of all the factors were statistically significant. Instead, other dependents, such as the SDI and evenness, are not very significant in their impact. In the case of SWDI, location*plant and season*plant effects showed good significance.

For pairwise comparisons, CR showed a significant difference between the three locations at 5% significance level. No significant difference was observed between Kottavayal and Pakistan. In the case of SDI and SWDI, significance was observed in Mattilayam, Kottavayal and Pakkom. In addition, evenness did not show any significant difference according to location. The location-wise effect is that all characteristics except CR do not show any significant difference between Kottavayal and Pakkom. In the season, the SDI showed no significant difference, as the p-value was greater than 0.05. For evenness, only significance was observed between summer and winter. CR and Margalef richness varied significantly depending on the season. Among plant parts, CR and Margalef richness varied significantly concerning plant parts. The leaves and roots showed no significant differences in the SDI, SWDI and Evenness. However, rhizomes differ significantly between leaves and roots. The CR and other characteristics in all samples showed a significance of P<0.01 and also SWDI in the root with rhizome samples.

Principal component (PC) analysis was performed on the standardized dataset of fungi to reduce dimensionality and identify the key components explaining the variants in the data. The first two principal components explained > 99% of the variance. The loading of the first principal component was uniform across all the original variables. Each variable contributed similarly and positively to the variance captured by the first PC. In considering the second PC, CR contributed highly positively, followed by SDI and evenness, with a significant negative

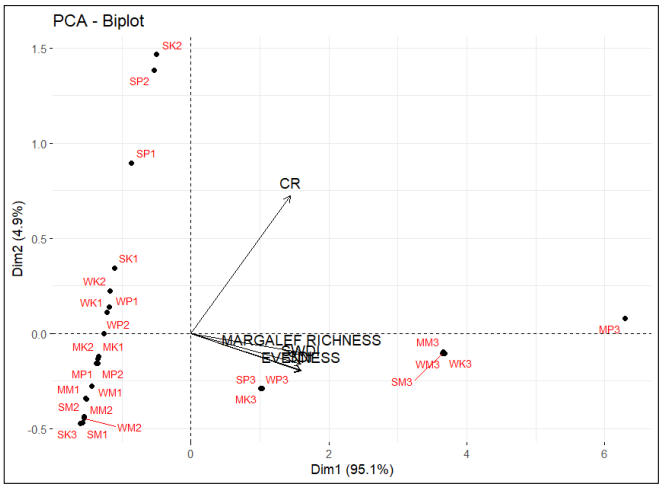

Fig. 5. The biplot illustrates the PCA performed on endophytic fungi

contribution. A biplot was generated to visualize the results of the PCA performed for each dataset (Fig. 5). The biplot simultaneously displays the scores of the observations and loadings of the original variables on the first PC. The first principal component (PC1) and the second principal component (PC2) are represented on the X- and Y-axes, respectively. PC1 accounted for 95% of the variance, whereas PC2 explained 4%, cumulatively capturing approximately 99% of the total variance in the dataset. Each point on the biplot represents an individual observation colored according to specific characteristics. The vectors represent the original variables. CR had a longer vector, indicating a significant influence on PC1. The remaining factors are highly positively correlated because they are close to each other. The similarity in vector length indicates uniformity in its influence. The direction of the vectors suggests that these variables are positively correlated as they point in similar directions. The standard deviation and variance for PCA are represented in Table 5. The eigenvectors for the principal component analysis are shown in Table 6.

The plot shows the projection of dependent variables (arrows) and observations (points) onto the principal component space. The variables are scaled to unit variance and the observations are centered. The lengths and directions of the arrows indicate each variable's contribution to the principal components, while the positions of the points reflect the relative similarity of the observations.

Morphological characteristics of fungal endophytes

Thirty-six fungal endophytes from *Lagenandra toxicaria* were selected based on their maximum frequency of occurrence, growth rate and differences in appearance. The selected fungal endophytes were analyzed by studying their macro-and micromorphological characteristics. Fungal isolates FRWS8, FLWW5, FLWW17, FRWM12, FLWS32, FRWW18, FRWM25, FRWW30, FLWS1, FRhWW3 and FLWW12 were identified as species of *Aspergillus*. FRhWS1, FRWM3, FRWS24, FRWW10 and FRWW11 are species of *Fusarium* and FLWW15, FRhWS2 and FRhWW4 are species of *Penicillium* based on their phenotypic characteristics. All the isolated endophytic fungi belong to the families Aspergillaceae (38.89%), Nectriaceae (16.67%), Glomerellaceae (8.34%), Trichocomaceae (5.56%), Didymellaceae (5.56%), Hypocreaaceae (5.56%), Botyosphaeriaceae (2.78%), Diaporthaceae (2.78%), Phanerochaetaceae (2.78%), Debaryomycetaceae (2.78%), Corynesporascaceae (2.78%), Mucoraceae (2.78%), Pleosporaceae (2.78%). A total of 97.22% of the isolates, representing 12 families, belonged to the phylum Ascomycota, whereas the remaining 2.78% (Mucoraceae) belonged to the phylum Mucoromycota.

Diverse colony colour ranges include white, yellow, green, grey and black. These colours may be uniform or exhibit concentric zoning, which adds to the complexity of identification. Texture also varied, with some isolates displaying a powdery, woolly, or cottony appearance. The colony shape and size are also important distinguishing features. Some isolates formed circular colonies, whereas others formed irregular or lobate colonies. The elevations of the colonies ranged from flat to raised, with some exhibiting wavy or undulated margins. The presence of liquid droplets or an oily appearance is another characteristic of certain isolate, further aiding its identification. Mycelium, which is the vegetative part of the fungus, also exhibits distinct colors and textures. These characteristics can be used in conjunction with colony features to ensure accurate identification. Most colonies were circular or irregular, whereas FLWW11 and FRWM1 had a radial pattern. The colonies were either slightly raised or raised from the surface. FLWW11 was raised with furrows, FLWS21, FRWW11 and FRWS9 were umbonate and FRhWW4, a bluish colony, was convex. The margins of colonies varied from strain to strain. A slightly wavy, smooth, or entire margin generally occurs in the colonies, whereas FLWW14 and FLWW6 have lobate margins. Fungal exudates were observed as liquid droplets in most strains. FLWW6, FLWW17 and FRWS4 did not produce exudate. The reverse morphology of each strain was slightly different from strain to strain; hence, it can be used as a key characteristic for identification.

The fungal isolates exhibited diverse characteristics, including hyphal structure, conidia, shape of the vesicle, conidiophore and color of conidia. Septate and aseptate hyphae are present, with septate being more common. Conidia shapes range from ellipsoidal, globose, ovoid, sub-globose, fusiform

Table 5. Standard deviation and variance of PCA

Importance of components	PC1	PC2	PC3	PC4	PC5
Standard Deviation	2.180	0.494	0.017	0.006	0.002
Proportion of Variance	0.951	0.048	0.000	0.000	0.000
Cumulative Proportion	0.951	0.999	0.999	1.000	1.000

Table 6. Eigenvectors for the principal component analysis

	PC1	PC2	PC3	PC4	PC5
CR	0.409	0.907	0.876	0.000	-0.000
SDI	0.455	-0.241	0.372	-0.091	0.766
SWDI	0.456	-0.201	-0.054	0.839	-0.208
Evenness	0.455	-0.246	0.425	-0.429	-0.605
Margalef Richness	0.457	-0.127	-0.818	-0.320	0.047

and crescent, while vesicle shapes include spherical, flask-shaped, pyriform and clavate. Conidiophores were mostly unbranched, with some showing sparse or branched structures. The colour of the conidia varied widely, featuring shades of brown, green, yellow, olive, blue-green, pinkish-white and hyaline. Some isolates, like FLWW14 and FRWM27 had aseptate hyphae and brown conidia, whereas FRWS8 had septate hyphae and dark green conidia. Conidia, the asexual reproductive structure, are spherical, ellipsoidal and fusiform. They are pale yellow to green in color. The vesicles in the fungal hyphae of the strains were isolated in different shapes, such as spherical, globose and flask-shaped. FLWW5 was characterized by flask-shaped vesicles and brown conidia. The unique combination of these characteristics allows the precise identification and classification of each fungal isolate, highlighting the vast diversity within this group of organisms. The conidiophores in which conidia are produced are either branched or branched.

Molecular taxonomical identification

The molecular taxonomy of the isolates was elucidated using genomic DNA extraction, ITS region PCR amplification, sequencing and sequence analysis. DNA quality was evaluated spectrophotometrically, with A260/A280 ratios ranging from 1.6 to 1.8. Agarose gel electrophoresis was used to verify DNA integrity. PCR amplification using ITS1 and ITS4 primers produced amplicons of approximately 540-600 base pairs in length (Fig. 6). Sequence assembly was performed using BIOEDIT v7.2.5, which generated nearly full-length contigs. This approach enabled the robust molecular characterization of the isolates. The sequences were analyzed using the BLAST alignment program of the GenBank database. To confirm the relationships among the 36 isolates, phylogenetic trees were constructed using MEGA 11 (Fig. 7). In the tree, each isolate was clustered in a strongly supported clade with a reference strain. Based on the similarity results, a comparison of ITS gene sequences and morphological characteristics confirmed the identity of the isolates. Among the identified isolates, *Aspergillus aculeatus*, *Aspergillus terreus*, *Pencillium oxalicum*, *Aspergillus niger* and *Fusarium odoratissimum* were the most frequently colonizing in various segments in different seasons of 16.7%,

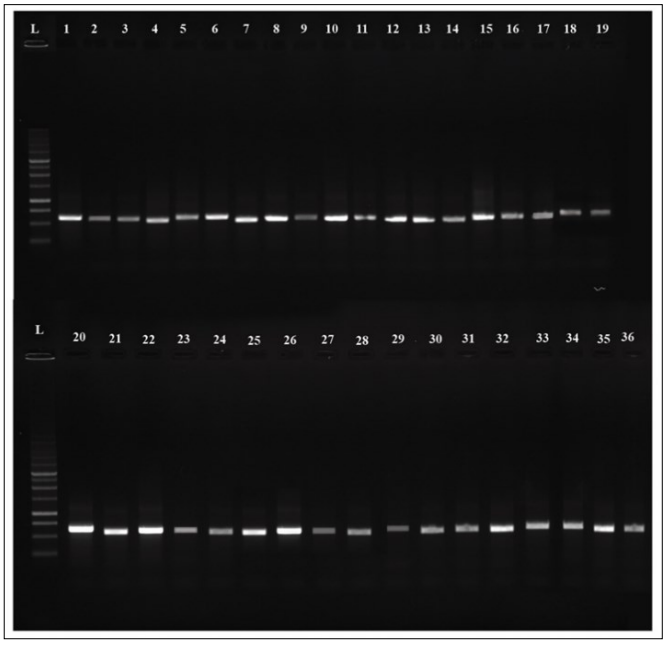

Fig. 6. ITS region amplification of endophytic fungal DNA

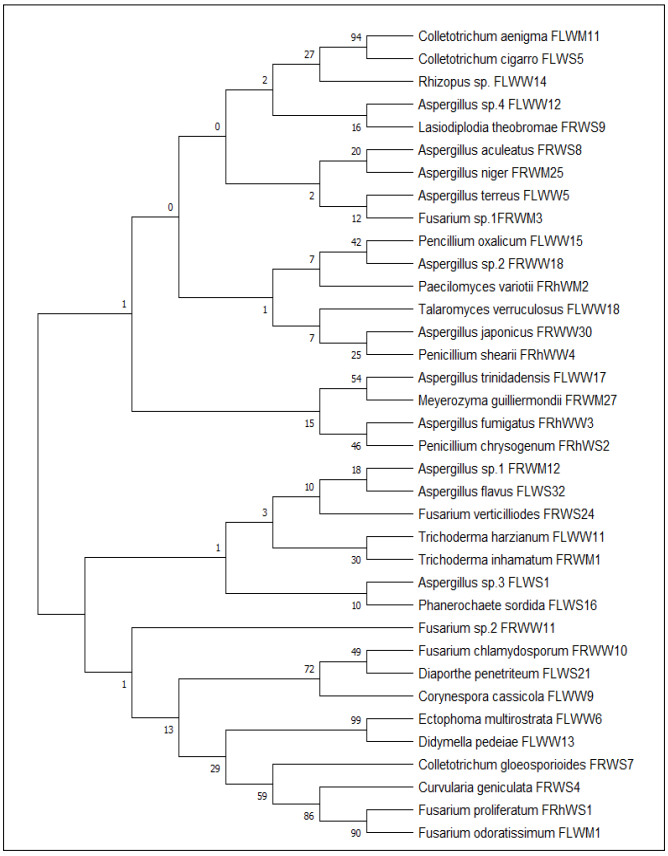

Fig. 7. Phylogenetic analysis using Maximum Likelihood methods revealed evolutionary relationships between the isolated endophytic fungi and their nearest taxonomic counterparts

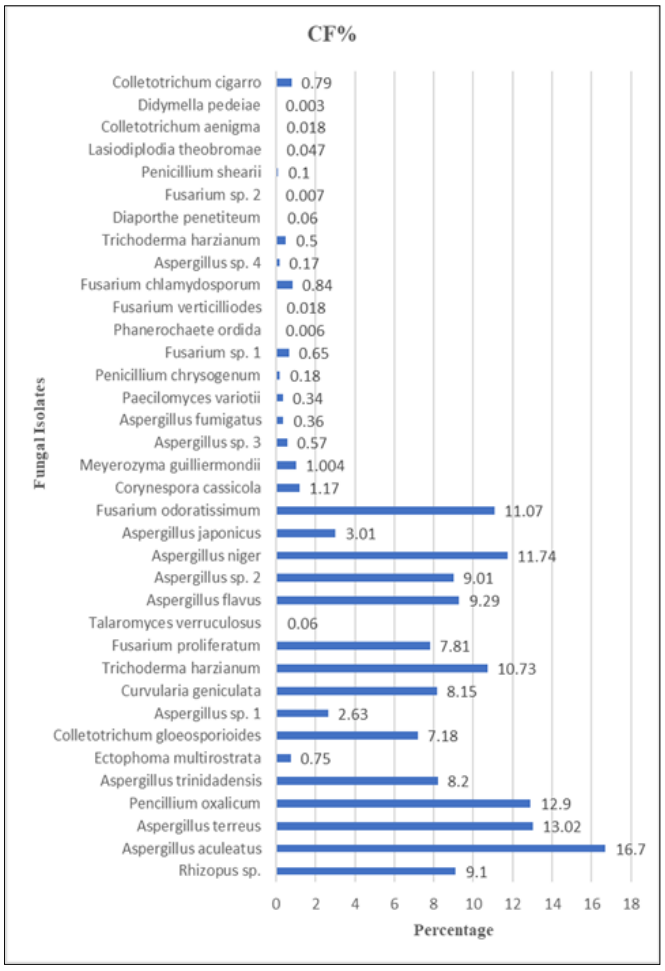

Fig. 8. Colonization frequency of selected endophytic fungal isolate

Table 7. Identification of selected endophytic fungal isolates

Sl No.	Strain code	% of similarity	Identity	Family	Accession No.
1	FLWW14	98.65	*Rhizopus sp.*	Mucoraceae	PQ165882
2	FRWS8	95.66	*Aspergillus aculeatus*	Aspergillaceae	PQ165881
3	FLWW5	99.49	*Aspergillus terreus*	Aspergillaceae	PQ197657
4	FLWW15	98.66	*Penicillium oxalicum*	Aspergillaceae	PQ197655
5	FLWW17	98.29	*Aspergillus trinidadensis*	Aspergillaceae	PQ197648
6	FLWW6	93.94	*Ectophoma multirostrata*	Didymellaceae	PQ219763
7	FRWS7	94.63	*Colletotrichum gloeosporioides*	Glomerellaceae	PQ219473
8	FRWM12	99.66	*Aspergillus sp.* 1	Aspergillaceae	PQ197647
9	FRWS4	98.99	*Curvularia geniculata*	Pleosporaceae	PQ166595
10	FLWW11	95.07	*Trichoderma harzianum*	Hypocreaceae	PQ166527
11	FRhWS1	94.82	*Fusarium proliferatum*	Nectriaceae	PQ197636
12	FLWW18	92.29	*Talaromyces verruculosus*	Trichocomaceae	PQ197638
13	FLWS32	99.66	*Aspergillus flavus*	Aspergillaceae	PQ168921
14	FRWW18	95.31	*Aspergillus sp.* 2	Aspergillaceae	PQ219309
15	FRWM25	98.71	*Aspergillus niger*	Aspergillaceae	PQ168922
16	FRWW30	94.85	*Aspergillus japonicus*	Aspergillaceae	PQ197427
17	FLWM1	97.67	*Fusarium odoratissimum*	Nectriaceae	PQ181486
18	FLWW9	98.59	*Corynespora cassicola*	Corynesporascaceae	PQ168926
19	FRWM27	83.76	*Meyerozyma guilliermondii*	Debaryomycetaceae	PQ181464
20	FLWS1	99.83	*Aspergillus sp.* 3	Aspergillaceae	PQ168931
21	FRhWW3	95.17	*Aspergillus fumigatus*	Aspergillaceae	PQ168932
22	FRhWM2	99.34	*Paecilomyces variotii*	Trichocomaceae	PQ197586
23	FRhWS2	99.16	*Penicillium chrysogenum*	Aspergillaceae	PQ181462
24	FRWM3	97.86	*Fusarium sp.* 1	Nectriaceae	PQ181461
25	FLWS16	97.19	*Phanerochaete sordida*	Phanerochaetaceae	PQ181460
26	FRWS24	96.90	*Fusarium verticilliodes*	Nectriaceae	PQ197587
27	FRWW10	99.11	*Fusarium chlamydosporum*	Nectriaceae	PQ181298
28	FLWW12	94.29	*Aspergillus sp.* 4	Aspergillaceae	PQ181297
29	FRWM1	92.03	*Trichoderma inhamatum*	Hypocreaceae	PQ197588
30	FLWS21	96.47	*Diaporthe penetriteum*	Diaporthaceae	PQ168940
31	FRWW11	93.32	*Fusarium sp.* 2	Nectriaceae	PQ181291
32	FRhWW4	98.97	*Penicillium shearii*	Aspergillaceae	PQ181289
33	FRWS9	93.04	*Lasiodiplodia theobromae*	Botryosphaeriaceae	PQ219310
34	FLWM11	98.65	*Colletotrichum aenigma*	Glomerellaceae	PQ168941
35	FLWW13	95.76	*Didymella pedeiae*	Didymellaceae	PQ361983
36	FLWS5	97.93	*Colletotrichum cigarro*	Glomerellaceae	PQ168942

13.02%, 12.9%, 11.74% and 11.07% respectively. The lowest percentage of colonization frequency was observed in the isolates *Didymella pedeiae* (0.003%), *Phanerochaete ordida* (0.006%) and *Fusarium sp.* 2 (0.007%) The colonization frequency of identified fungal isolates is represented in Fig. 8. The details of the isolates, including strain code, similarity index, identity, family and accession number, are shown in Table 7.

Discussion

An effective experimental design considers the critical interplay between plant growth and pot size, allowing researchers to draw reliable and meaningful conclusions that foster a deeper understanding of plant biology and ultimately drive advancements in this field. A comprehensive meta-analysis revealed that doubling pot size yielded an average 43% increase in biomass. This growth enhancement is primarily driven by increased net photosynthesis, which is hindered in smaller pots. Notably, the plant mass per unit rooting volume, rather than the pot size, plays a critical role. Researchers should be aware that high plant mass per pot volume restricts growth and risks skewing the relative differences between treatments. To mitigate these effects, it is essential to select pots that could accommodate plant growth throughout the experiment. As the best practice, we recommend maintaining a plant biomass-to-pot volume ratio below one g/L and avoiding ratios exceeding 2 g/L. By adhering to these guidelines, researchers can minimize potential bias and ensure more accurate and reliable results (23). Research has consistently demonstrated that pots can constrain plant growth. To investigate this phenomenon further, a study examined the effect of pot size on the transpiration response of maize (*Zea mays* L.) and soybean (*Glycine max* L.) plants under water-deficit stress. Surprisingly, the results revealed that while pot size significantly influenced plant growth, it did not substantially

alter the relationship between transpiration and water deficit in these plants, suggesting that pot size effects on growth do not necessarily translate into altered physiological responses to stress (24). The analysis showed that rhizomatous plants showed many variations in plant characteristics.

Endophytic fungal diversity data from our study revealed a high level of diversity. This is consistent with other studies that found a high level of endophytic fungal diversity in various plant species (25). This study suggests that summer is the best season for isolating endophytic fungi from aquatic plants. These data are supported by a previous study that found a higher diversity of endophytic fungi in the summer (26). The colonization rate, Shannon-Wiener diversity index and Simpson's diversity index were higher in root samples than in leaf and rhizome samples, suggesting that roots may be a preferred habitat for endophytic fungi in aquatic plants (27). Interestingly, no fungal isolates were detected in the rhizome samples collected from Pakkom during the monsoon season. Overall, the fungal diversity in the rhizome was found to be extremely low, approaching negligible levels. This may be due to the rhizomes producing chemical compounds toxic to fungi, such as alkaloids, glycosides, or terpenoids, which can inhibit fungal growth and colonization. These chemical compounds can be produced in response to environmental cues, such as the monsoon season. They can help the rhizome maintain a balanced microbiome that favors beneficial bacterial endophytes over fungal endophytes.

Diversity indices of endophytic fungi found in aquatic plants are used to assess the variety and distribution of fungal species within a given environment. A higher Shannon-Wiener Index indicates a more diverse community with a balanced distribution of species, indicating that the endophytic fungal community is rich and even. In aquatic plants, a higher index suggests a complex fungal community that may contribute to the health and stability of the plant ecosystem (28). The Shannon-Wiener diversity index (SWDI) and Simpson's diversity index (SDI) values in our study were higher than those reported in other studies (29). Our Margalef richness index values were also higher than those reported in other studies (30). The evenness index values from our study are consistent with those of other studies that found a high level of evenness in endophytic fungal communities (31). The present investigation revealed that the diversity is higher in roots than leaves which is higher than that of the rhizome of *Lagenandra toxicaria*. This finding coincides with the findings of Kohout (32) studies carried out on aquatic plants in Norway and Kandalepas, who studied the leaves of two freshwater plant species collected from Louisiana wetlands. It could be observed that plant parts of the same site had a high species similarity. In addition, isolates from the plant parts of leaves and roots in the same plant and different plant samples from the same location showed similarity in species distribution and the isolates from the locations Kottavayal and Pakkom showed similar species.

A study conducted by You *et al*. identified that the major fungal genera isolated from the roots of aquatic plants (33) were *Aspergillus, Fusarium, Penicillium* and *Talaromyces*. They sequenced the fungal isolates' internal transcribed spacer (ITS) regions and conducted phylogenetic analysis. This study concluded that fungal diversity was influenced by environmental conditions and host plant species in both wetlands. In the present study, the isolated endophytic fungal genera were similar to those identified by You *et al*. and the ITS region was sequenced for identification. The most dominant genera identified in this study were *Aspergillus, Fusarium* and *Penicillium*. This is also consistent with previous studies that have found these genera are dominant in aquatic plants (31).

The MANOVA analysis revealed highly significant effects ($p < 0.01$) of individual factors, including location, season, plant species and their interaction effects. These results indicate that endophytic fungal diversity is significantly influenced by these factors and their interactions, with a significance level of 1%. This suggests a complex interplay between location, season and plant species in shaping the endophytic fungal communities. Principal component analysis (PCA) revealed that CR disproportionately influenced the first principal component (PC1), as evidenced by its longer vector. In contrast, the remaining factors exhibited a high degree of positive correlation, as indicated by their proximity to each other in the vector space. Notably, the similar lengths of their vectors suggest that these factors have a relatively uniform impact on PCA, with no single factor dominating the others.

Similarly, Arnold and Lutzoni (34) and Wilson and Carroll (35) demonstrated that endophyte colonization is shaped by a combination of factors, including geographic location, climate, seasonality, host plant identity and specific host tissues. These factors profoundly affect the colonization patterns and diversity of endophytic microorganisms. Singh *et al*., (36) conducted a similar study on endophytic fungi diversity using MANOVA, revealing significant differences ($p \leq 0.001$) in diversity measures across locations, seasons and tissue types. Their analysis of the Shannon-Wiener index (H') and species richness data showed significant variations in endophytic fungal diversity across these factors. Additionally, the interactive effect of location, season and tissue type was found to affect the dominant endophytes ($\%D \geq 0.5$), with tissue type having the most potent impact, as evident from the tissue-type-based grouping of isolates in the biplot analysis. This study underscores the importance of considering multiple factors when analyzing endophytic fungal diversity.

Conclusion

PCA effectively reduced the dimensionality of the dataset, allowing to represent the variability of the two principal components. Pots 1 and 2 showed strong morphological similarities, whereas habitat differed distinctly from the others. Petiole height and flag leaf length were identified as the main drivers of variability. *Lagenandra toxicaria*, an aquatic plant, harbors endophytic microorganisms significantly contributing to its growth and development. A comprehensive study involving isolation and diversity analysis of endophytic fungi from three plant parts (leaves, roots and rhizomes) across three locations (Pakkom, Kottavayal and Mattilayam) and three seasons revealed a rich diversity of microorganisms with varied potential activities. Notably, root tissues yielded more endophytes during the summer season in Pakkom and Kottavayal. Although leaf and root isolates shared some similarities, fungal isolates from the rhizome were scarce and even absent in some seasons.

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Authors' contributions

PP designed the study and performed the lab work and statistical analysis. LKE carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. NSP conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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