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Diversity and Identification of Endophytic Fungi Isolated from Different Marine associated Plants

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ABSTRACT

An increasing threat is attributed to diseases such as cancer and various infections globally, creating an urgent need for innovative and effective compounds that possess distinct mechanisms of action to combat these life-threatening illnesses. In the present work, we analyzed the diversity and identification of endophytic fungi isolated from the marine associated plants. Totally, 135 fungal isolates harboring inside the leaf tissues of 6 marine associated plants such as *Aegiceras corniculatum, Acanthus ilicifolius, Cyphostemma setosum, Excoecaria agallocha, Rhizophora mucronata* and *Suaeda maritima.* The maximum diversity colonies of endophytic fungi in *R. mucronata* was significantly higher in leaves as compared to other plants. Eight isolates representing the genera *Aspergillus, Beauveria, Fusarium, Macrophomina, Nigrospora, Pestalotiopsis, Pencillium* and *Rhizopus* were obtained. Diversity indices, specifically the Shannon and Simpson indices exhibited variation in relation to the plant leaf species. The richness of fungal endophyte diversity suggests that these organisms may significantly contribute to plant health, metabolic processes and medicinal properties. Fungal endophytes have also demonstrated various biological activities that can be utilized for promoting environmental and agricultural sustainability.

Keywords: Marine associated plants, Endophytic fungi, Diversity.

INTRODUCTION

Fungal endophytes represent a polyphyletic group primarily composed of ascomycetous fungi that reside within healthy host tissues for at least a portion of their life cycle, all while not inducing any observable symptoms of disease or adverse effects on their hosts (Rajesh *et al.,* 2019). The composition of the endophytic community typically varies and is influenced by a variety of factors, including the species and genotype of the host, the origin of the tissue, geographical location, nutrient availability and interactions with the host, along with other abiotic and biotic stresses (El-Bondkly *et al.,* 2021). These elements contribute to the host specificity observed in the endophytic mycobiota associated with similar host species. The vast majority of plants and marine invertebrates or vertebrates remain untested for their fungal endophytes, presenting significant opportunities for the discovery of unique fungal forms, taxa and biotypes (Dastogeer *et al.,* 2018).

Endophytic fungi possess significant biotechnological potential across a range of life science applications, including the development of anticancer medications such as taxol, L-asparaginase, L-glutaminase, tyrosinase, and methioninase (El-Bondkly *et al.,* 2020). They also serve as sources for antibacterial compounds such as essramycin, ayamycin, benzopyrone derivatives (El-Gendy *et al.,* 2018) and coumarin derivatives, as well as antifungal substances such as saadamycin and prodigiosin (El-Gendy and El-Bondkly, 2010). Additionally, these fungi are explored for their antiviral properties and their role in the biological control of various pests and contaminants (Bovio, 2019). The activities associated with these processes are linked to the distinctive products produced by endophytes, which encompass steroids, alkaloids, terpenoids, quaternions, isocoumarin and quinone derivatives (Santos *et al.,* 2019), as well as phenolic compounds, flavans, xanthones and peptides that have applications in the fields of pharmaceuticals and biotechnology (Tan and Zou, 2001).

Endophytic fungi inhabit the internal tissues of host plants without causing any apparent harm. The presence of these fungi can assist the host plant in coping with both biotic and abiotic stressors

(Potshangbam *et al.,* 2017). Certain plants have demonstrated tolerance to biotic stress, which has been linked to the natural products produced by these fungi. Although endophytic fungi are considered promising sources of innovative active compounds, biological activities and biotechnological advancements, their full potential has yet to be thoroughly investigated (Hamzah *et al.,* 2018). Certain researchers have demonstrated that endophytic fungal communities serve as significant reservoirs of novel bioactive secondary metabolites, enzymes, and biological control agents. Additionally, they are crucial sources of fungi that exhibit plant growth-promoting and antimicrobial properties (Yang *et al.,* 2020).

Endophytes are typically bacteria or fungi that reside within a plant host in an endosymbiotic relationship, without inducing disease. These endophytes play a crucial role in promoting the growth of the plant host and facilitating nutrient uptake. Additionally, they improve the host's capacity to withstand abiotic stresses and reduce biotic stresses by bolstering the plant's resistance to infections (Ahmad *et al.,* 2022). Mangroves are halophytic plants that are unique to tropical and subtropical coastal regions, thriving in the transitional areas where marine and terrestrial environments meet (Revathy *et al.,* 2024). Secondary metabolites derived from medicinal plants are essential due to their bioactive properties, which can be harnessed in the pharmaceutical sector. Research indicates that the presence of endophytic fungi can enhance the production of these bioactive secondary metabolites in medicinal plants. A strong relationship between these endophytic fungi and their host plants can result in an increased yield of secondary metabolites (Toppo *et al.,* 2024).

Endophytic fungi are acknowledged for their diverse functions in the interactions between plants and microbes. These functions include the solubilization of nutrients within the plant rhizosphere, enhancement of plant growth, serving as biocontrol agents against pests and pathogens, inducing systemic resistance to both biotic and abiotic stresses and contributing to the biosynthesis of secondary metabolites (Jha *et al.,* 2023). There exists a restricted quantity of research concerning fungal endophytes that employs identical experimental methods, whether concentrating on a specific plant species or a particular ecosystem (Izolda *et al.,* 2024). Medicinal plants represent a natural resource that provides remedies for various ailments affecting living organisms (Manimegalai *et al.,* 2011). In the present study, an attempt has been made to evaluate isolation and identification of marine associated plant leaves endophytic fungi and thereby to carry out the analysis of Shannon and Simpson diversity indices.

MATERIALS AND METHODS

Study site

Mallipattinam is a coastal village in the Pattukkotai taluk of Thanjavur District, Tamil Nadu, India. It is located between Thiruthuraipoondi and Pattukkottai. Mallipattinam Latitude - 10°23'44.52"N and Longitude - 79°29'41.96"E (**Figure 1**).

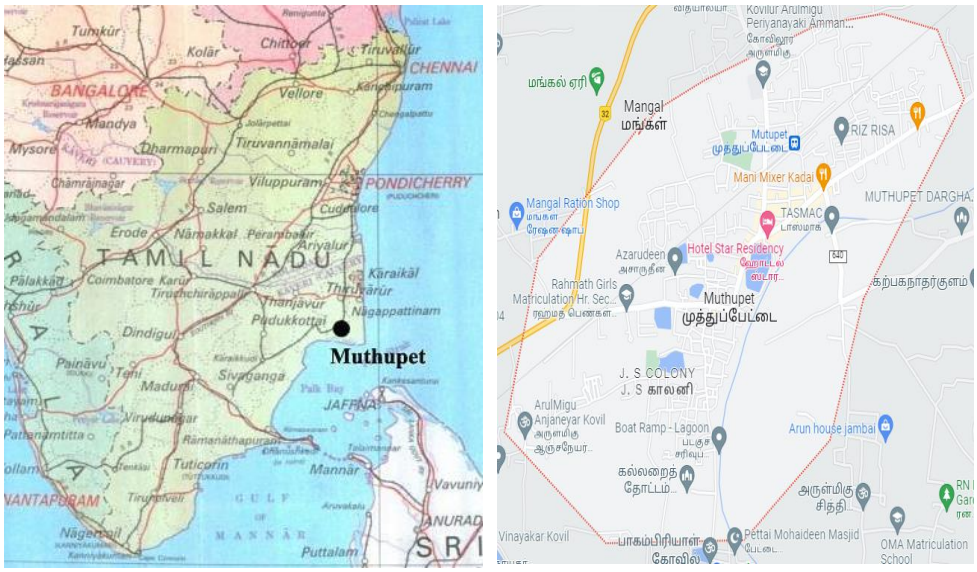


Figure 1: Study site.

Collection of marine associated plants

Marine associated plants were gathered from the region of Muthupettai in the ecosystem of the east coast of India. The plant specimens were collected include *Aegiceras corniculatum, Acanthus ilicifolius, Cyphostemma setosum, Excoecaria agallocha, Rhizophora mucronata* and *Suaeda maritima* which were subsequently placed carefully into polythene zip lock bags. The leaf samples of these plants were promptly transported to laboratory. They were thoroughly rinsed with sterile water. The leaf samples were delicately pressed with sterile tissue paper to eliminate excess moisture. The removal of external microorganisms, the leaf samples underwent further sterilization facilitating the isolation of endophytic fungi.

Sample surface sterilization

The leaf samples from *Aegiceras corniculatum, Acanthus ilicifolius, Cyphostemma setosum, Excoecaria agallocha, Rhizophora mucronata* and *Suaeda maritima* underwent a sterilization protocol. The procedure commenced with a thorough washing under a continuous flow of fresh water followed by the segmentation of the leaves into smaller pieces. The surfaces were then treated with sterile distilled water, which had been subjected to autoclaving for 1 min. This was succeeded by 1 min exposure to 70% technical-grade ethanol to reduce the potential contamination. The samples were subsequently immersed in a 5.25% sodium hypochlorite solution for 5 min followed by another treatment with 70% technical-grade ethanol for 30 sec and rinsed 3 times with sterile distilled water. The final step involved drying the surfaces of the samples using Whatman filter paper (Wulandari *et al.,* 2022).

Isolation of endophytic fungi from marine associated plants

Approximately 1x1 cm in size of leaf was meticulously placed on separate petri dishes containing potato dextrose agar (PDA) medium from Merck. Specifically, 4 to 5 segments of plant samples were allocated to each petri dish, culminating in a total of 12 plates. The distribution of these plates was organized such that 2 plates were designated for leaf samples of each plant. This configuration facilitated the cultivation and isolation of fungi. The bacterial proliferation, streptomycin was incorporated into the PDA medium. The cultivated samples were then incubated at room temperature for a duration of 3-5 d. In order to characterize the fungal endophytes, several parameters were assessed including the overall colony appearance, color, edge morphology and surface elevation. These observations were instrumental in identifying and comprehending the characteristics of the fungal endophytes present in the samples (Basheer *et al.,* 2018).

Identification of fungi

The morphological characteristics of the purified fungal colonies were examined and staining with lactophenol cotton blue (LPCB) for subsequent observation under a light microscope. Identification of the fungi was conducted using established reference manuals including "Soil Fungi" by Gillman (1957), "Dematiaceous Hyphomycetes" by Ellis (1971), “A Manual of Penicillia” by Raper and Thom (1949) and “The Manual of Aspergilli" by Smith (1946). The identified fungal cultures were preserved in the laboratory on PDA medium, adhering to standard protocols and were subcultured at regular intervals. The isolated fungi were stained with lactophenol-cotton blue dye and examined microscopically.

Margalef’s diversity index (Clifford and Stephenson, 1975)

$$DMg = S-1/\ln N$$

where, N = population = the total number of individuals in the sample and S = the number of species recorded.

Menhinick’s diversity index (Whittaker, 1977)

$$DMn = S/\sqrt{N}$$

where N is the total number of individuals in the sample and S the species number.

Pielou’s Evenness (Pielou, 1975)

Species evenness was expressed by Pielou’s Evenness study.

Pielou’s Evenness Index e=H/logs

$$J = H'/H'max$$

where, H’max is the maximum value of diversity for the number of species present.

McIntosh diversity index (McIntosh, 1967)

The McIntosh index expresses the heterogeneity of the sample in geometric terms. It describes the sample as a point of a SS-dimensional hyper

volume and uses the Euclidean distance of this point from the origin.

Evenness: **E=N-UN/N-√S**

where, U is the distance of the sample from the origin in an S dimensional hyper volume.

Shannon index (Shannon and Weiner, 1949)

The statistic index which means it assumes all species are represented in a sample and they are randomly sampled.

$$\text{Shannon Index (H)} = -\sum_{i=1}^{S} pi \ln pi$$

where p is the proportion (n/N) of individuals of one particular species found (n) divided by the total number of individuals found (N), ln is the natural log, Σ is the sum of the calculations and s is the number of species

Simpson index (Simpson, 1949)

The dominance index calculated to common or dominant species.

$$\text{Simpson Index } (D) = \frac{1}{\sum_{i=1}^{s} pi^2}$$

where, p is the proportion (n/N) of individuals of one particular species found (n) divided by the total number of individuals found (N), Σ is still the sum of the calculations, and S is the number of species. S (number of species) N (total number of individuals) Σ (sum) of pi^2 (n/N) 2 Σ (sum) of pi ln pi.

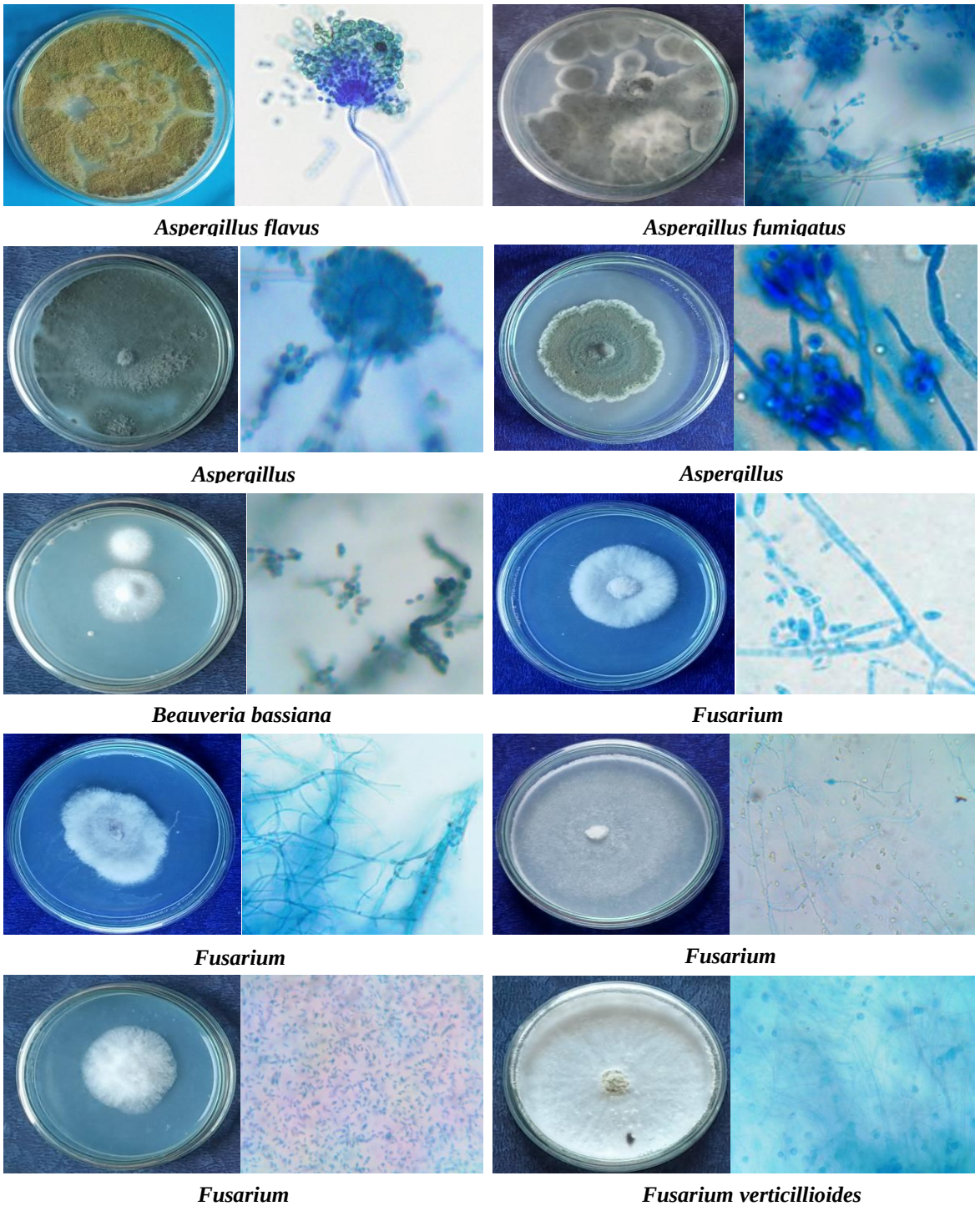
RESULTS AND DISCUSSION

In the current investigation, fungi obtained from 3 distinct locations of marine associated plants were noted in their occurrence at regular intervals. In this study, 135 colonies of endophytic fungi were isolated from 6 marine associated plants such as *Aegiceras corniculatum, Acanthus ilicifolius, Cyphostemma setosum, Excoecaria agallocha, Rhizophora mucronata* and *Suaeda maritima* by culturing them on PDA medium prepared with and without artificial salt. Totally, 22 endophytic fungal isolates were obtained from 8 different genera such as *Aspergillus, Beauveria, Fusarium, Macrophomina, Nigrospora, Pestalotiopsis, Pencillium* and *Rhizopus.* Interestingly, *Pencillium* was found to be dominant among the fungal isolates. The maximum fungal colonies 56 were observed from *Rhizophora mucronata* followed by 23 from *Excoecaria agallocha,* whereas minimum colonies 12 were observed from *Acanthus ilicifolius* were recorded (**Table 1, Figure 2 a,b**).

Table 1: Endophytic fungi from marine associated plants.

S.No	Name of the endophytic fungi	*Aegiceras corniculatum*	*Acanthus ilicifolius*	*Cyphostemma setosum*	*Excoecaria agallocha*	*Rhisophora mucronata*	*Suaeda maritima*
1.	*Aspergillus flavus*	-	-	-	01	01	-
2.	*A. fumigatus*	-	01	-	-	-	-
3.	*Aspergillus* sp.	-	01	-	-	-	-
4.	*A. sydowii*	-	-	-	02	13	01
5.	*Beauveria bassiana*	-	02	-	-	-	-
6.	*Fusarium solani*	-	-	-	05	02	04
7.	*F. lateritium*	-	-	-	03	-	-
8.	*F. moniliforme*	-	-	-	-	01	01
9.	*F. oxysporum*	01	-	02	-	-	-
10.	*F. verticillioides*	-	-	-	03	-	03
11.	*Fusarium* sp	-	01	-	-	-	-
12.	*Macrophomina phaseolina*	-	-	-	-	01	-
13.	*Nigrospora* sp	01	-	01	-	-	-
14.	*Pestalotiopsis* sp.	07	02	03	-	-	-
15.	*P. brevicompactum*	-	-	-	-	12	01
16.	*Penicillium citrinum*	-	-	-	01	04	02
17.	*P. chrysogenum*	01	02	01	-	-	-

S.No	Name of the endophytic fungi	*Aegiceras corniculatum*	*Acanthus ilicifolius*	*Cyphostemma setosum*	*Excoecaria agallocha*	*Rhisophora mucronata*	*Suaeda maritima*
18.	*P. crystallinum*	04	02	02	-	-	-
19.	*P. longibrachiatum*	-	-	-	03	22	03
20.	*P. verruculosum*	-	-	-	01	-	-
21.	*Rhizopus stolonifer*	01	01	05	-	-	-
22.	*R. microsporus*	-	-	-	04	-	-
Total number of fungi		06	08	06	09	08	07
Total number of colonies		15	12	14	23	56	15



Aspergillus flavus ***Aspergillus fumigatus***

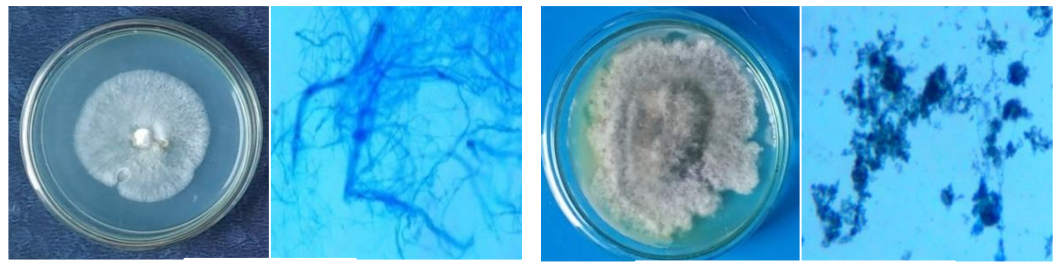
Aspergillus ***Aspergillus***

Beauveria bassiana ***Fusarium***

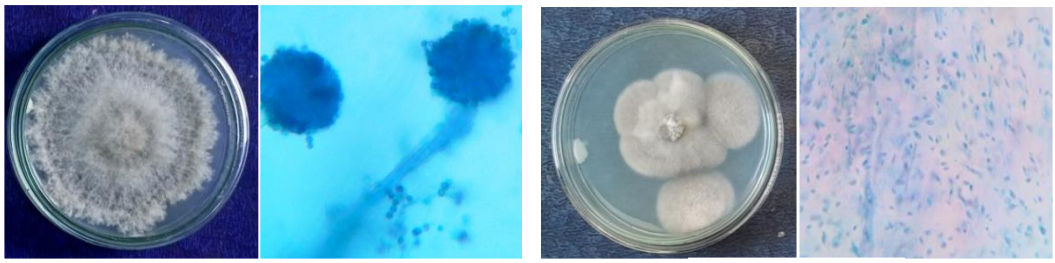
Fusarium ***Fusarium***

Fusarium ***Fusarium verticillioides***

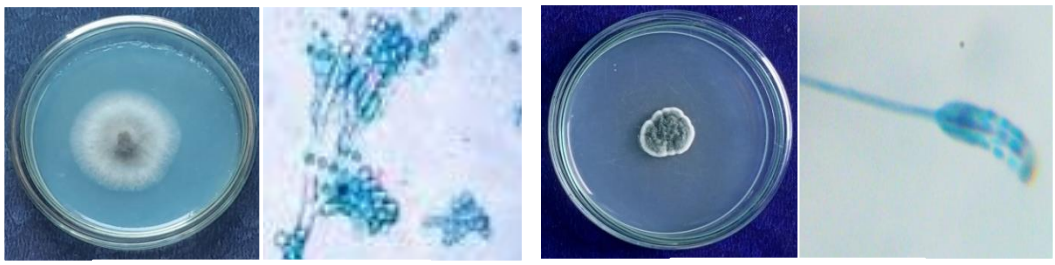
Figure 2a: Endophytic fungi from marine associated plants



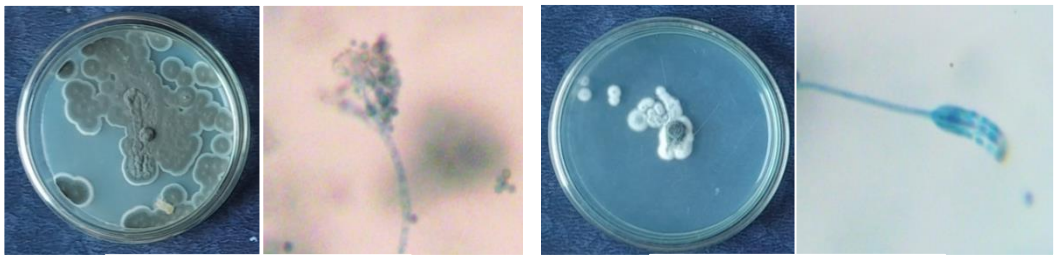
Fusarium ***Macrophomina***



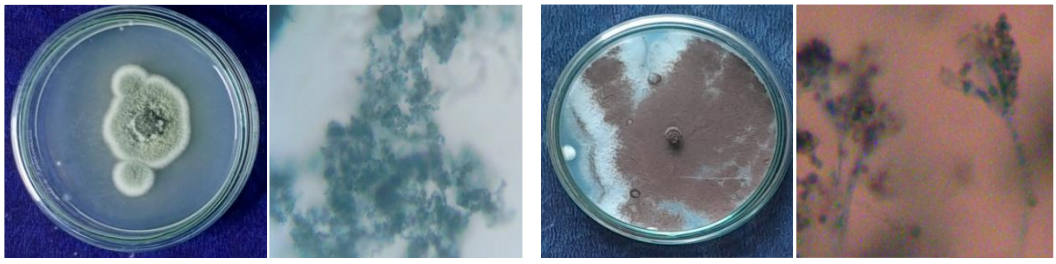
Nigrospora ***Pestalotiopsis***



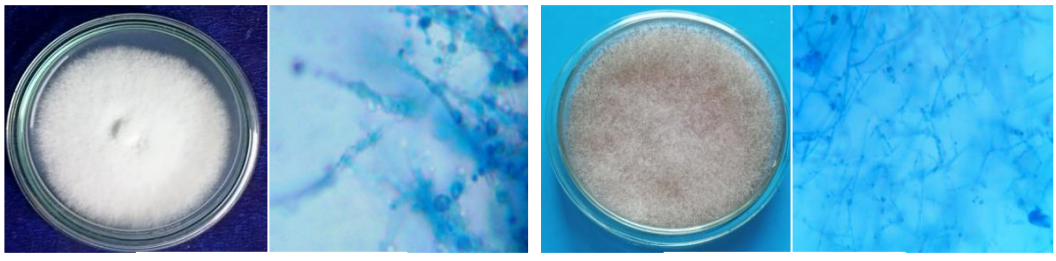
Penicillium ***Penicillium***



Penicillium ***Penicillium***



Penicillium ***Penicillium***



Rhizopus ***Rhizopus***

Figure 2b: Endophytic fungi from marine associated plants.

Marine organisms account for nearly 50% of the planet's overall biodiversity and represent a significant reservoir for anticancer treatments. Within this diverse group, fungi stand out as a biotechnologically important source of bioactive secondary metabolites; however, they have been less extensively studied than their terrestrial counterparts. Limited research has focused on the cytotoxic compounds derived from their associated endophytic fungi, including gliotoxin, cytochalasin B and demethoxyfumitremorgin (Kamat *et al.*, 2020). Numerous studies have highlighted the diversity and bioactivity of endophytic fungal secondary metabolites from the Rameswaram region, noting their insecticidal, antibacterial, and antioxidant properties; however, the potential anticancer activity has yet to be investigated (Thirunavukkarasu *et al.,* 2012).

In the current study, the isolated fungi in marine associated plants include *Aspergillus flavus, A. fumigatus, Aspergillus* sp., *A. sydowii, Beauveria bassiana, Fusarium solani, F. lateritium, F. moniliforme, F. oxysporum, F. verticillioides, Fusarium* sp., *Macrophomina phaseolina, Nigrospora* sp., *Pestalotiopsis* sp., *P. brevicompactum, Penicillium citrinum, P. chrysogenum, P. crystallinum, P. longibrachiatum, P. verruculosum, Rhizopus stolonifer* and *R. microsporus* (**Table 1, Figure 2a, b**). The fungal isolates recorded belonged to 8 genera as follows *Aspergillus* (18.18%), *Beauveria* (4.54%), *Fusarium* (27.27%), *Macrophomina* (4.54%), *Nigrospora* (4.54%), *Pestalotiopsis* (9.09%), *Pencillium* (22.72%) and *Rhizopus* (9.09%). *P. longibrachiatum* was mostly occupied fungal species in the leaves of *Rhizophora mucronata*.

Fungal colonies were isolated on PDA agar plates and incubated at room temperature (27°C) for a duration of 5 d. A total of 15 endophytic fungi were successfully isolated from various marine associated medicinal plants. The endophytic fungi were extracted from marine medicinal plants including *Acanthus ilicifolius, Aegiceras corniculatum, Avicennia marina, Ceriops decandra, Excoecaria agallocha* and *Rhizophora mucronata.* The identified endophytic fungi comprised *Aspergillus flavus, A. fumigatus, A. niger, A. terreus, A. ochraceous, Chaetomium* sp., *Fusarium* sp., *F. moniliforme, F. oxysporum, Penicillium chrysogenum, P. citrinum, P. janthinellum, P. purpurrescens* and *R. stolonifera*. Oviya and Madhanraj (2023) reported the maximum number of fungal colonies (48) in *Acanthus ilicifolius* plant, while the minimum number (16) in the leaves of *Rhizophora mucronata.*

In the current work, true diversity or the effective number of types, pertains to the number of equally abundant types of endophytic fungi necessary to attain the average proportional abundance of types were observed in the analyzed dataset. Various generalized means were calculated, including the Margalef index (d), Menhinick's species richness (SR), Pielou's evenness (J), McIntosh evenness (McE), Shannon diversity (H) and Simpson's index of dominance (D) for the leaf segments of *Acanthus ilicifolius, Aegiceras corniculatum, Avicennia marina, Ceriops decandra, Excoecaria agallocha* and *Rhizophora mucronata*. The total Shannon (1.6048 H) and Simpson (5.6253 D) index values of endophytic fungi isolated from plants were recorded (**Table 2**).

Table 2: Diversity indices of endophytic fungal species of different marine associated medicinal plants.

Name of the marine associated medicinal plants	No. of species	No. of individuals	Richness		Evenness		Diversity	
			Margalef index (d)	Menhinick's species richness (SR)	Pielou's Evenness (J)	McIntosh Evenness (McE)	Shannon's diversity (H)	Simpson's index dominance (D)
Aegiceras corniculatum	15	06	7.8135	6.1237	0.0162	0.6124	0.2441	0.9285
Acanthus ilicifolius	12	08	5.2899	4.2426	0.0179	0.6673	0.2151	0.9090
Cyphostemma setosum	14	06	7.2554	5.7155	0.0167	0.6289	0.2350	0.9230
Excoecaria agallocha	23	09	10.0126	7.6667	0.0131	0.5207	0.3015	0.9545

Name of the marine associated medicinal plants	No. of species	No. of individuals	Richness		Evenness		Diversity	
			Margalef index (d)	Menhinick's species richness (SR)	Pielou's Evenness (J)	McIntosh Evenness (McE)	Shannon's diversity (H)	Simpson's index dominance (D)
Rhisophora mucronata	56	08	26.4494	19.799	0.0065	0.4377	0.3650	0.9818
Suaeda maritima	15	07	7.1946	5.6695	0.0162	0.6124	0.2441	0.9285
Total	**135**	**44**	**64.0154**	**49.217**	**0.0866**	**3.4794**	**1.6048**	**5.6253**

The previous research indicated that the Shannon index and Simpson index for the *Acanthus ilicifolius* were H=2.6439 and D=0.9456, respectively (Oviya and Madhanraj, 2023). The values for the Shannon and Simpson's indices were determined to be 3, 2.5 and 2.58 for green algae, and 15.77, 9.45, and 9.46 for brown and red algae, respectively. This indicates that the diversity of fungal endophytes in red and brown algae is significantly greater than that observed in green algae. Furthermore, the species richness of endophytic fungi was found to be maximum in red algae, with a count of 6, while brown and green algae exhibited similar levels of richness (Sahoo *et al.*, 2021).

CONCLUSION

In the present study, we examined the diversity and identification of endophytic fungi obtained from marine associated plants. We isolated a total of 135 fungal specimens residing within the leaf tissues of 6 marine associated plant species, namely *Aegiceras corniculatum, Acanthus ilicifolius, Cyphostemma setosum, Excoecaria agallocha, Rhizophora mucronata*, and *Suaeda maritima*. Diversity indices, particularly the Shannon and Simpson indices, demonstrated fluctuations in accordance with the different species of plant leaves. The abundance of fungal endophyte diversity indicates that these organisms could play a crucial role in enhancing plant health, influencing metabolic processes, and providing medicinal benefits. The marine ecosystem has been recognized as a rich reservoir for various specific niches and marine organisms that harbour significant endophytic fungal diversity, which holds promise for the development of pharmaceutical and medicinal compounds. It is essential to understand that the production of these biologically active compounds, which possess various chemical structures, by marine organisms and their associated fungi results from intricate and dynamic interactions within the environment, either directly or indirectly. Endophytic fungi can be widely exploited up to molecular/gene level to obtain sustainable and low-cost bio-resources for useful biologically active substances at large scale for various purposes.

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