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Fungal endophytic communities in *Tribulus terrestris* L. collected from the delta region of Tamil Nadu, South India

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Key words: Ayurvedic medicine, Traditional medicine, Endophytic fungi, *Tribulus terrestris*, Morphological, Pharmaceutical

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Abstract

Tribulus (*Tribulus terrestris* L.) is an annual, silky herb plant belonging to the Zygophyllaceae family widely distributed around the world. Small woody Plant, leaves are compound and opposite and fruit have long, sharp and strong spines. The leaf, root and fruit of the plant have been utilized medicinally in traditionally Ayurvedic medicine, Siddha and Unani for their phytochemical and pharmacological activities. An assemblage of endophytic fungi was isolated from *Tribulus terrestris* L. collected from the Delta Region of Tamil Nadu, India. Fungal endophytes were isolated using leaf surface sterilization standard methods. Thirty-one species and 21 (genus) endophytic fungal strains were isolated from the leaves, petioles and stems of *Tribulus terrestris*. The identification of fungal strain through morphological observation from standard manuals and showed that fungal endophytes were associated with host plants belonging to a few taxons of *Alternaria*, *Cladosporium, Colletotrichum gloeosporioides*, *Curvularia*, *Fusarium* and *Phyllosticta capitalensis* fungi that were frequently isolated. The endophytic fungus *Fusarium oxysporum* was the dominant species in this study; it was isolated from host plant samples during wet and dry seasons.

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Introduction

Endophytes have been known about for a long time. The term endophyte was actually coined in 1884 by Heinrich Anton de Bary, who recognized that fungi and bacteria could dwell within plant tissues without causing any apparent harm. Endophytes are microbes that are bacteria, fungi and actinomycetes that live inside the tissues of plants for the entire or part of their life cycle without causing any disease to the host (Arpita *et al.*, 2022). These are ubiquitous microorganisms, reported in almost all the vascular plants and bryophytes studied to date (Hardoim *et al.*, 2015; Venkatesan and Mahalakshmi, 2022). Fungal endophytes have been isolated from lichens, moss, ferns and gymnosperms, monocotyledonous and dicotyledonous plants, growing in different environments (Petrini *et al.*, 1990). They are found in every part of plants, that is leaves, fruits, flowers, stems, roots and seeds and are transmitted by horizontal or vertical means (Bacon and White, 2000; Hartley and Gange, 2009). These microbes have potential applications in agricultural, pharmaceutical and other industries.

Endophyte constitutes an important component of microbial biodiversity. Diverse fungal community composition and isolation of endophytes are found in various host plants. The relationship of endophytes with single or multiple plant hosts can be described in terms of host specificity, host selectivity or host preference and host recurrence (Cohen, 2006). The endophytic fungal community confirmed host specificity at the species level but this specificity could be influenced by environmental conditions (Cohen, 2004). Differences in endophytic fungal assemblages in different tissue types have been reported in the same plant species, or even in different tissues of an individual plant which is a reflection of tissue specificity. Endophytic fungi can be found in every part of the plant such as the leaf, stem, root, flower, fruit, and seed.

Endophytic fungi can produce secondary metabolites that depend on their host plant. Those secondary metabolites protect the host plant itself and can act as antimicrobials and antivirus.

Moreover, the use of secondary metabolites of endophytes could also reduce the overexploitation of medicinal plants as the source materials of drugs and the production cost of medicines (Dhanalakhsmi *et al.*, 2013; Kursia *et al.*, 2018). Endophytic fungi isolated from medicinal plants are considered an attractive source of novel bioactive compounds (Strobel *et al.,* 2004; Kumar *et al.,* 2014). Various types of plants can be used as the hosts for endophytic fungi. The endophytic fungi from medicinal plants is a source of beneficial secondary metabolites. It also can produce bioactive compounds which have the potential to be materials for producing modern medicine or agrochemical applications (Widowati *et al.*, 2016; Praptiwi *et al.*, 2018). Its various parts contain a variety of chemical constituents that are medicinally important such as flavonoids, flavonol glycosides, steroidal saponins, and alkaloids. *Tribulus terrestris* L. is used in folk medicine as a tonic, aphrodisiac, palliative, astringent, stomachic, antihypertensive, diuretic, lithotriptic, and urinary disinfectant, antiurolithic, immunomodulatory, antidiabetic, absorption enhancing, hypolipidemic, cardiotonic, hepatoprotective, anti-inflammatory, analgesic, antispasmodic, anticancer, antibacterial, anthelmintic, larvicidal, and anticariogenic activities.

The dried fruit of the herb is very effective in most of the genitourinary tract disorders. It is a vital constituent of Gokshuradi Guggul (it helps in treating renal calculi or kidney stones, it helps in treating urinary tract infections), a potent Ayurvedic medicine used to support the proper functioning of the genitourinary tract and to remove urinary stones.

Tribulus terrestris L. has been used for centuries in Ayurveda to treat impotence, venereal diseases and sexual debility. In Bulgaria, the plant is used as a folk medicine for treating impotence. In addition to all these applications, the Ayurvedic Pharmacopoeia of India attributes cardio tonic properties to the root and fruit. In traditional Chinese medicine, fruits are

used for the treatment of eye trouble, oedema, abdominal distension, emission, morbid leucorrhea, and sexual dysfunction. *Tribulus terrestris* L. is described as a highly valuable drug in the Shern - Nong Pharmacopoeia (the oldest known pharmacological work in China) in restoring the depressed liver, for treatment of fullness in the chest, mastitis, flatulence, acute conjunctivitis, headache, and vitiligo **(**Vitiligo is a disease that causes loss of skin color in patches). In Unani medicine, *Tribulus terrestris* L. is used as a diuretic, mild laxative, and general tonic (Gilman, 1971). One of the plant species that can be a host for endophytic fungi is *Tribulus terrestris* L.

This study was conducted to investigate the abundance and diversity of endophytic fungi inhabiting *Tribulus terrestris* collected from the Delta Region of Tamil Nadu, India.

Materials and methods

Study area

Tribulus terrestris L. medicinal plant species of the tropical herb, studied by endophytic fungi, this herbal plant is collected from Mannargudi town in Thiruvarur district in the South India state of Tamil Nadu. This city is 6 m above sea level and it's located at 10.6632° N 79.4482° E. As the temperature distinctively varies from 21°C to 38°C. Hence, the seasons vary from heavy rain and air humidity effects, year by year from October to December. The Northeast monsoon which starts in October to December contributes about 60 - 80% of the total annual rainfall. The Southwest monsoon rains from June to September and summer rains from March to June contribute about 20 to 40% of rainfall. *Tribulus terrestris* plant was collected from individuals of each host species and their fungal endophytes were isolated during both seasons such as the summer (April to June 2023) and winter (October to December 2023). It is native to warm temperate and tropical regions in southern Eurasia and Africa. Each plant sample was brought to the laboratory in sterile polythene bags and processed within 24 hours of collection.

Sterilization and culture protocols

The host plant was collected from Mannargudi, Thiruvarur (Dt.) Tamil Nadu. These stages of plant leaves were collected for the investigation. We have used culture- dependent approaches based on media culture. Leaf samples were collected from healthy plants. In these plants, leaves were randomly collected two hundred leaves a few plants and their one hundred and fifty tissue segments were cut from two hundred leaves. However, sterilization techniques were followed before cutting these segments. The plant leaves are washed thoroughly with running water and then the leaves are sterilized as follows. After the surface sterilization, the leaves were cut approximately into 0.5 cm (segments) of each leaf. The samples were washed in running water, dipped in 70% ethanol for 60 seconds immersed in 2.5% NaOCl for 90 seconds, and then washed in sterile water for 10 seconds (Suryanarayanan *et al*., 1998) or three times. The sterilized samples were placed on the PDA medium amended with antibiotics contained in Petri dishes.

The Petri dish was sealed with Parafilm™ and incubated in a light chamber at 26+1°C for 7 to 21 days (Bills and Polishook, 1992; Suryanarayanan *et al*., 1998). The light regimen given was 12 hours of light followed by 12 hours of darkness. Fungi that grew from the segments were periodically observed and the endophytes were identified.

Morphological identification of isolated endophytic fungi

Preliminary identification was done by studying the fungi's cultural characteristics such as colony growth, colour, shape, etc. The morphological characters were examined by growing cultures on PDA plates for 21 days. Microscopic observations are Conidiophores, conidia and mycelial characters were carried out by preparing slides stained with cotton blue and Congo red and observed under the compound microscope (Ellis, 1971; Subramanian, 1971; Barnett and Hunter, 1972; Sutton *et al*., 1981 and others).

Statistical analysis

Colonization frequency

Colonization frequency (CF %) = {(Number of colonies)/ (Number of totals)}×100

The number of segments colonies and the number of totals segments colonized by each endophyte and total number of segments observed respectively Hata and Futai (1995).

Relative parentage of occurrence of each group of fungi (RPO)

The Relative Percentage of Occurrence (RPO) of each group (viz. Ascomycetes, Basidiomycetes, Coelomycetes, Hyphomycetes and Sterile like forms and Zygomycetes) of fungal species in each plant species was calculated as follows: Tedersoo *et al.* (2018).

RPO = ({Total colonization frequency of one group)/(Total colonization frequency for all the groups of fungi)}×100

Diversity index (Fisher's α)

The diversity index was calculated using the method of Fisher *et al.* (1943).

Species evenness index and species richness index (E5, R1)

The species evenness (E5, modified Hill's ratio) and species (R1, Margalefs index) were calculated as described by Ludwig and Reynolds (1998) using the software provided by the John Wiley and Sons, SPDIVERS.BAS.

Results and discussion

The *Tribulus terrestris* plant belonging to the Zygophyllaceae family was studied for their foliar endophyte assemblages during dry and wet seasons. This line inquiry was aimed at getting comprehensive knowledge of the endophyte's status in tropical plants. Isolation of fungal endophytes from the leaves of *Tribulus terrestris* during dry and wet seasons: One hundred and fifty leaf segments of *Tribulus terrestris* were screened for the presence of fungal endophytes.

The segments were cut from the entire portion of the basal leaves. Surface sterilized using ethanol and sodium hypochlorite (Sterilization and Culture Protocols) and screed as mentioned under Materials and Methods.

A total of 23 fungal species and 266 isolates were obtained during the dry season and 29 fungal species and 326 isolates were obtained during the wet season. *Fusarium oxysporum* showed the highest colonization frequency during both dry and wet seasons. A total of 23 fungal species and 266 isolates were obtained during the dry season and 29 fungal species and 326 isolates were obtained during the wet season. *Fusarium oxysporium* showed the highest colonization frequency during both dry and wet seasons. A total of 31 endophytic isolates were collected from 300 segments both during dry and wet seasons. Totally 31 endophytic isolates were categorized into 21 taxons, comprising 4 Ascomycetes genera *Chetomium globosum*, *Sporormiella* sp, *Talaromyces* sp., *Xylaria* sp., 1 Basidiomycetes genera *Rhizoctonia solani*, 4 Coelomycetes genera *Colletotrichum gloeosporioides*, *Phoma* sp., *Phomopsis* sp. and *Phyllosticta capitalensis*, 10 Hyphomycetes genera *Alternaria* spp., *Aspergillus* spp., *Aureobasidium pullulans*, *Curvularia* spp., *Fusarium* spp., *Nigrospora* sp., *Penicillium* spp., *Syncephalatrum recemosum*., *Trichoderma* sp. 1 Zycomyetes genera *Mucor* sp., and 4 sterile forms. All the leaf parts of plant tissues were found to harbor various endophytic fungal species with different colonization frequencies (CF %) and statistics analysis (PCO, CF%, E5, H1) (Tables 1 & 2) and the endophytic fungal pictures isolated from the plants are shown in (Fig. 1- 8).

The present work is entirely new and many descriptions and a few illustrations appear for the first time. The arrangement and numbering of genera are identical in numerous earlier works.

A survey of *Tribulus terrestris* plant species from the dry and wet seasons in the Delta region showed that the leaves of the host harbored fungal endophytes (Table 1).

Table 1. Fungal endophytes isolated from the leaves of *Tribulus terrestris* L. during dry and wet seasons\

SL	Endophytes	Total colonies	CF%	Total colonies	CF%
		Dry season		Wet season	
	Ascomycetes				
1	*Chaetomium globosum*	3	2.0	5	3.3
2	*Sprormiella* sp. 1	6	4.0	7	4.7
3	*Talaromyces* sp. 1	2	1.3	3	2.0
4	*Xylaria* sp. 1			2	1.3
	Basidiomycotes				
5	*Rhizoctonia solani*			3	2.0
	Coelomycetes				
6	*Colletotrichum gloeosporioides*	23	15.3	42	28.0
7	*Phoma* sp. 1	12	8.0	10	6.7
8	*Phomopsis* sp.1	16	10.7	19	12.7
9	*Phyllosticta capitalensis*	32	21.3	48	32.0
	Hyphomycetes		0.0		
10	*Alternaria* sp. 1	7	4.7	6	4.0
11	*Alternaria* sp. 2			2	1.3
12	*Aspergillus clavatus*	4	2.7		
13	*A. flavus*	2	1.3	11	7.3
14	*A. nidulans*			3	2.0
15	*A. niger*	6	4.0	15	10.0
16	*Aspergillus* sp. 1	2	1.3	1	0.7
17	*Aureobasidium pullulans*	3	2.0	5	3.3
18	*Curvularia lunata*	23	15.3	20	13.3
19	*C. cladosporioides*	5	3.3	9	6.0
20	*Curvularia* sp. 1			2	1.3
21	*Drechlera tripogonis*	2	1.3	4	2.7
22	*Fusarium oxysporium*	96	64.0	80	53.3
23	*F. solani*	5	3.3	2	1.3
24	*Fusarium* sp. 1	3	2.0		
25	*Nigrospora oryzae*	2	1.3	1	0.7
26	*Penicillium* sp.1	4	2.7	6	4.0
27	*Penicillium* sp.2	3	2.0	4	2.7
28	*Syncephalastrum racemosum*	1	0.7	7	4.7
29	*Trichoderma* sp. 1			5	3.3
	Zycomyetes				
30	*Mucor* sp. 1			2	1.3
	Sterile forms				
31	Unknown fungi	4	2.7	2	1.3
	Total No. of Isolates	23		29	
	Total No. of species	266		326	
	Total No. of CF%		177.3		206.0

Colonization Frequency (CF %)

Fig. 1. *Tribulus terrestris* plant growing in nature in the Delta region of Tamil Nadu, India

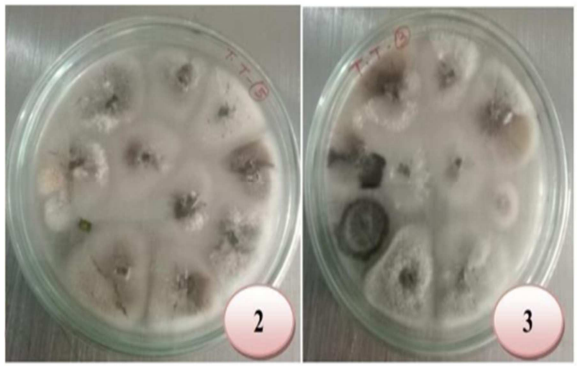

Fig. 2-3. Morphology of endophytic culture grown on PDA of representatives' fungal endophyte inhabiting the *Tribulus terrestris* province growth on PDA medium

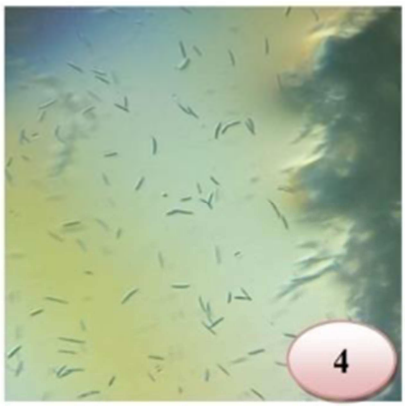

Fig. 4. Multicellular fungal endophytes of *Colletotrichum gloeosporioides* macro gonidia

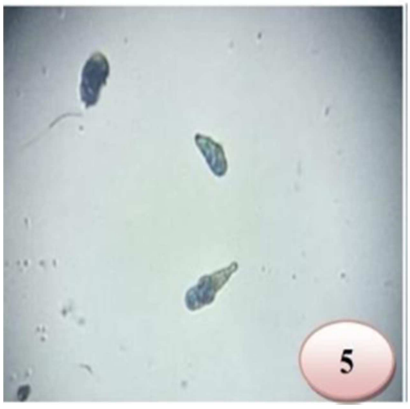

Fig. 5. Endophytic fungi conidia of *Alternaria* sp. 1

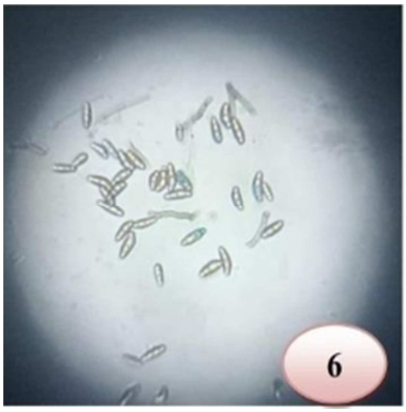

Fig. 6. Endophytic fungi and conidia of *Drechlera* sp.

For the dry season, we recorded 266 endophyte isolates during the dry season and 326 endophyte isolates during the wet season from 150 leaf segments (each season) (Table 1). From the *Tribulus terrestris* medicinal plant, we recovered 24 endophyte isolates from 150 segments during the dry season and 29 isolates during the wet season (Tables 1 and 2). In most cases, each tissue segment was infected by more than one fungal species (multiple infections), substantiating the view that tropical plants have high rates of endophyte colonization (Suryanarayanan *et al*., 2002). Variation in endophyte assemblages among temperate hosts growing in allocation has been studied (Helander, 1994).

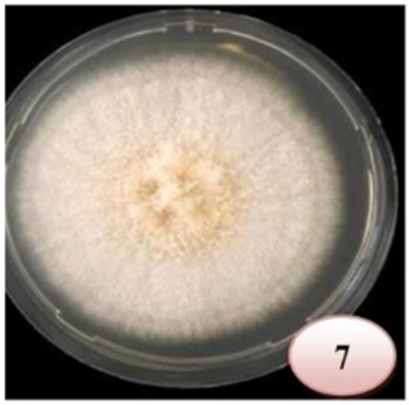

Fig. 7. Endophytic fungi and colony of *Fusarium* sp.

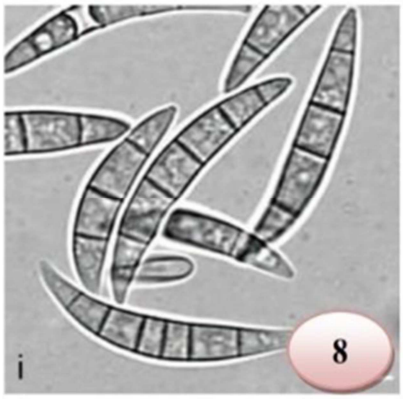

Fig. 8. Endophytic fungi and conidia of *Fusarium* sp.

We found that there was no significant difference between endophyte colonization between individuals. This was irrespective of the season (Table 1). The dominant fungi were the same for both seasons of a host. *Fusarium oxysporum* has shown that more endophyte isolates occur in leaves screened in the wet season than in the dry seasons.

In addition, in both seasons, dominant endophyte species had a higher CF % during the wet season. (Table 1), Thus bringing down the evenness index of the endophyte assemblages. A comparison of the endophyte assemblages of host the endophytes diversity during the wet season was more for the dry season due to higher mean annual rainfall.

Table 2. R1 and E5 values can range from 1.00 for complete similarity to 0.00 for complete dissimilarity

SL	Statistical analysis	Plant name code and seasons	
		Dry	Wet
		TT	TT
1.	Total no. of species (32 species)	24	29
2.	Total no. of segments	150	150
3.	Total no. of colonizes	266	326
4.	Total Colonization Frequency (CF %)	177.3	206.0
5.	Fisher's Alpha	6.40	7.69
6.	R1(Margalef's)	4.12	4.84
7.	E5 (Hill's Ratio)	0.52	0.61
8.	Relative Percentage of Occurrence (RPO) of each group of fungal species		
8.1	Ascomycetes (3 species / 3 genera)	9.67	12.90
8.2	Basidiomycotes (1species / 1 genera)	---	3.22
8.3	Coelomycetes (4 species / 4 genera)	12.90	12.90
8.4	Hyphomycetes (21 species / 10 genera)	51.61	58.06
8.5	Zygomycetes (1 species / 1 genera)	---	3.22
8.6	Sterile forms (- species / - genera)	12.90	6.45

However, the diversity index was almost the same for both seasons. However, the dry tropical forests do not support very high endophyte diversity (Suryanarayanan *et al.*, 2002; 2003) and the period is relatively dry. Earlier studies involving a few individual plant species revealed that precipitation and endophyte colonization of leaf tissues are positively correlated (Suryanarayanan *et al.*, 1998). Earlier studies reveal that Ascomycetes, Coelomycetes, Hyphomycetes and Sterile forms invariably constitute the endophyte assemblages trees; Basidiomycetes and Oomycetes are rarely encountered (Petrini, 1986). In dry tropical forests, rainfall is seasonal and the rest a relative of occurrence (RPO) (Fig. 9), also showed that the foliar endophytes of the wet season were different from those of the dry season.

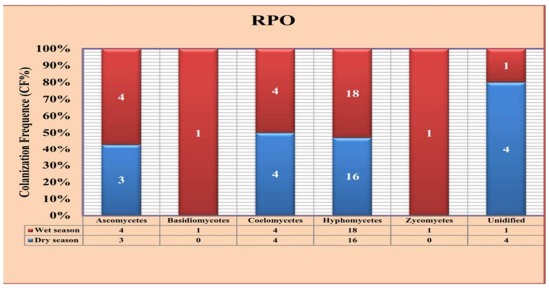

Fig. 9. Relative percentage occurrence of endophytes belonging to different groups of fungi during dry and wet seasons

The present work stated the presence of major foliar endophytic fungi mainly belonging to genera *Fusarium* spp. then *Colletotricum gloeosporioides*, *Phomopsis* sp.1 and *Phyllosticta capitalensis* were the second dominant endophytes in this plant. Several previous studies have reported the presence of these *Fusarium* spp. fungal genera as a dominant group of endophytes residing in association with different medicinal plants (PrabhaToppo *et al.,* 2024). Compared to the other endophytes, higher abundance rates and frequencies in the dry season as well as *Fusarium oxysporum* had higher abundance rates and frequencies in the wet season. While the number of isolated species described in each season fungi compared to those recorded in the species richness (Fig. 10-11). The Coelomycetes fungal group remained isolated in the *Tribulus terrestris* medicinal plant during the predominant frequency (Table 1). Endophytic fungal genera such as *Colletotrichum gloeosporioides*, *Phoma* sp. 1, *Phomopsis* sp.1, and *Phyllosticta capitalensis* had higher abundance rates and frequencies in both seasons. Hyphomycetes have more fungal diversity in both seasons (Table 1).

A survey of *Tribulus terrestris* plant species from the dry and wet seasons in the Delta region showed that the leaves of the host harbored fungal endophytes (Table 1). For the dry season, we recorded 266 endophyte isolates during the dry season and 326 endophyte isolates during the wet season from 150 leaf segments (each season) (Table 1). From the *Tribulus terrestris* medicinal plant, we recovered 24 endophyte isolates from 150 segments during the dry

season and 29 isolates during the wet season (Tables 1 and 2). In most cases, each tissue segment was infected by more than one fungal species (multiple infections) substantiating the view that tropical plants have high rates of endophyte colonization (Suryanarayanan *et al.*, 2002). Variation in endophyte assemblages among temperate hosts growing in allocation has been studied (Helander, 1994).

We found that there was no significant difference between endophyte colonization between individuals. This was irrespective of the season (Table 1). The dominant *Fusarium oxysporum* fungi were the same for both seasons of a host. *Fusarium oxysporum* has shown that more endophyte isolates occur in leaves screened in the wet season than in the dry seasons. In addition, in both seasons, dominant endophyte species had a higher CF % during the wet season. (Table 1), Thus bringing down the evenness index of the endophyte assemblages.

A comparison of the endophyte assemblages of host the endophytes diversity during the wet season was more for the dry season due to higher mean annual rainfall. However, the diversity index was almost the same for both seasons. However, other studies indicate that the dry tropical forests do not support very high endophyte diversity (Suryanarayanan *et al.*, 2002; 2003) and the period is relatively dry. Earlier studies involving a few individual plant species revealed that precipitation and endophyte colonization of leaf tissues are positively correlated (Suryanarayanan *et al.*, 1998). Earlier studies reveal that Ascomycetes, Coelomycetes, Hyphomycetes and Sterile forms invariably constitute the endophyte assemblages of trees; Basidiomycetes and Oomycetes are rarely encountered (Petrini, 1986).

In dry tropical forests, rainfall is seasonal and the rest a Relative Percentage of Occurrence (RPO) (Fig. 9), also showed that the foliar endophytes of the wet season were different from those of the dry season. The present work stated the presence of major foliar endophytic fungi mainly belonging to genera *Fusarium* spp. then *Colletotricum gloeosporioides*, *Phomopsis* sp.1 and *Phyllosticta capitalensis* were the second dominant endophytes in this plant. Several previous studies have reported the presence of these *Fusarium* spp. fungal genera as a dominant group of endophytes residing in association with different medicinal plants (PrabhaToppo *et al.*, 2024). Compared to the other endophytes, *Fusarium oxysporum* had higher abundance rates and frequencies in the dry season as well as *Fusarium oxysporum* had higher abundance rates and frequencies in the wet season. While the number of isolated species described in each season fungi compared to those recorded in the species richness (Fig. 10-11).

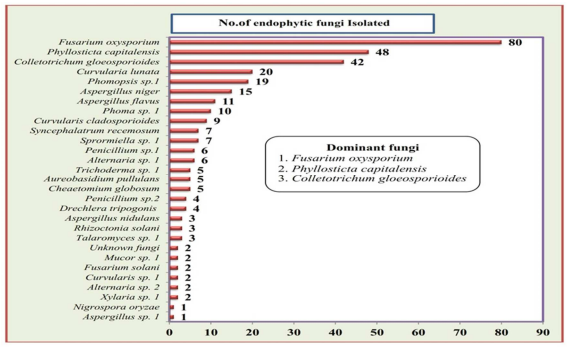

Fig. 10. A histogram that shows the diversity of isolated endophytic fungi during the dry season

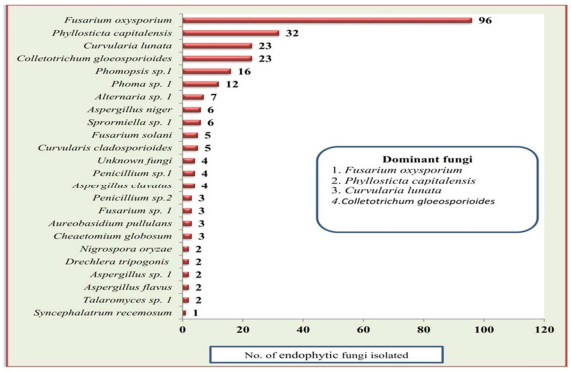

Fig. 11. A histogram that shows the diversity of isolated endophytic fungi during wet season

Next, species of *Colletotrichum* sp., *Phomopsis* sp., and *Phyllosticta* sp., appeared as second dominating. Here we present the results that have been found in *Syncephalastrum recemosum* were dry season and *Aspergillus* sp.1, *Nigrospora oryzae* which were also

in the lowest numbers but gradually decreased from the wet season. The Coelomycetes fungal group remained isolated in *Tribulus terrestris* medicinal plant during the predominant frequency (Table 1). Endophytic fungal genera such as *Colletotrichum gloeosporioides*, *Phoma* sp. 1, *Phomopsis* sp.1, and *Phyllosticta capitalensis* had higher abundance rates and frequencies in both seasons. Hyphomycetes have more fungal diversity in both seasons (Table 1).

Some authors have demonstrated that fungal endophytes community may be influenced by diverse biotic and abiotic factors, such as the type of plant tissues; a heterogeneous profile of microhabitats; and different substrates, climate and vegetation changes (Koide *et al*., 2017; Shen *et al*., 2007). Soil, airborne fungal spore concentrations and their diversity vary with the season of the year, geographical region, soil, air, meteorological parameters, presence of local resources, and vegetation. A few fungi that failed to sporulate were designated as "mycelia sterile", and can be identified later with different incubations such as sporulation in UV, so for colony characteristics, the mycelia were transferred into PDA agar media (Table 2). Similarity coefficients between the Number of species, total number of colonization Frequency (CF %) of the most dominant endophyte in the host during dry and wet seasons, Relative Percentage of Occurrence (RPO), Species richness (R1), species evenness (E5) and species diversity (Fisher's α) of the endophyte assemblages of the tree hosts during dry and wet seasons.

Conclusion

The global diversity of species-rich taxa such as leaf fungi is through investigation from *Tribulus terrestris* sampling. The most promising several are endophytic fungi, becoming an important source of bioactive chemicals for many applications in industry, agriculture, and medicine. In the current study, fungal endophytes were isolated, identified, and characterized using morphological, leaf and stem explants of the medicinal plant *Tribulus terrestris*. In the past few decades, many researchers have mainly focused on the investigation of fungal endophytes for diversities and their relationships with their host plants. Because of this, we investigated the fungi in the plant tissues of a few significant and widely utilized plants from the Tamil Nadu's Delta region and found a huge diversity of fungal species.

Acknowledgments

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