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Diversity of fungal endophytes from *Salacia* species in Western Ghats of Karnataka, India

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ABSTRACT

Endophytes are the microbes residing in the plant tissues without any harmful effect. Fungal endophytes were isolated from *Salacia* species in which it is having a wide range of therapeutic properties, such as antidiabetic, antiobesity etc. In this study, 474 endophytic fungi were isolated from 1200 plant tissue segments from three different seasons collected from four different species of *Salacia,* i.e *S. chinensis. S. oblonga, S. fruticosa, S. macrosperma.* Among them, *Fusarium oxysporum* was isolated as a dominant genus from *S. chinensis*, *Penicillium notatum* from *S. oblonga*, *Pestalotiopsis* from *S. fruticosa* and *Phoma* species from *S. macrosperma*. The sterile forms were dominated in *S. chinensis* and *S. oblonga*. The colonization frequency during all three seasons mentioned was observed as 57.33% from *S. chinensis*, 41.33% from *S. oblonga*, 42.67% on *S. fruticosa*, 16.67% on *S. macrosperma*.

Keywords: Fungal Endophytes, *Salacia*, Diversity index, Medicinal plant, anti-diabetic properties.

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INTRODUCTION

The Western ghats is evolved with innumerous amount of medicinal plants, The plant biodiversity is majorly based on the hotspots of Western ghats. These medicinal plants are endangered because of over exploitation [1]. India's biodiversity act 2002 aims for the convenience of the resources of India's biodiversity, and found around 4000 different species of higher plants of medicinal plants, in which 450 species are in extinct phase [2]. Plants that are found in these ecosystems are symbiotically associated with the fungal endophytes. The impression that is associated with the endophytes with their corresponding hosts is highly explosive in the field of biology[3].

Salacia Linn. (Hippocrateaceae) is a large genus of climbing or creeping shrubs or rarely small trees distributed mainly in the warmer parts of the world. The genus *Salacia* is morphologically and chemically distinct species distributed in Southern India and Western Ghats. *Salacia* species is an important medicinal plant, which is considered as a source of secondary metabolites with a wide range of pharmaceutical attributes [4]. Species of *Salacia* include *S. chinenesis, S. fructicosa, S. macrosperma, S. reticulata, S. oblonga* and *S. madagascariensis* etc., extensively in southern parts of India. The major chemical constituents of root bark include polyphenols such as salacinol, kotalanol, and mangiferin [5]. Different *Salacia* Species from western ghats of Karnataka and their medicinal uses were shown in Table 1.

Table 1: *Salacia* Species from western ghats of Karnataka and their medicinal uses.

Sl. No	Host Plant	Family	Collection site	Elevation	Medicinal uses
01	*S. chinensis*	Celastraceae	Western ghats, Karnataka	12.9095°N 75.588°E	antidiabetic, antiobese, hepatoprotective, hypolipidemic and antioxidant agent [5, 22].
02	*S. oblonga*	Celastraceae	Western ghats, Karnataka	12.9095°N 75.588°E	Anti-diabetic activity, Acute-glycemic Activity, Anti-hyperglycemic activity and cardiac fibrosis inhibition arthritis, Nephro-protective and antioxidant activities , Anti-microbial Activity [23].
03	*S. fruticosa*	Celastraceae	Western ghats, Karnataka	12.9095°N 75.588°E	Gonorrhea,Rheumatism,Obesity and skin diseases,antidiabetic,antihypertensive,hepatoprotective,anticancer potentials [24].
04	*S. macrosperma*	Celastraceae	Western ghats, Karnataka	12.9095°N 75.588°E	Root—decoction is given after parturition. Leaves—applied to eczema [25].

Endophytes might be bacteria, fungi or actinomycetes which are cropping up from the internal part of the plant tissues without causing any effects. These endophytes are associated in every part of the plant such as roots, leaves, stem, bark, twigs etc., [6]. The areas of higher plant indigenously possess particular endophytes that might develop gradually with the endemic plant species. The correlation of host parasite is also surveyed in the ecosystem [7]. These endophytes are the biological source for the production of secondary metabolites in which it plays a potential role in the field of medicine, agriculture, industries and prominently it is the source of natural products in which it is able to fabricate bioactive metabolites [8]. Endophytes used as sources of bioactive compounds that are less expensive when compared to the conventional method employing plants which is non-abundant and uneconomical. Endophytic Since, fungi present in a symbiotic mode within the host plants, replicate the chemistry of the host and incredibly they are able to produce the similar products like hosts [9]. Fungal endophytes develop antibacterial substances in which it shows the tolerance of host plants of biotic and abiotic stresses, in addition to metabolites, the symbiotic relationship results in the increased production of reactive oxygen species (ROS) in which it maintains mutualistic fungal-plant interaction and involves in various biosynthetic pathways [10]. The novel bioactive compounds in endophytes have led the path of new drug discovery. This study implies the diversity of the endophytes within the host species [11]. Virtually there are not much reports on the fungal endophytes associated with *Salacia* species.

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MATERIALS AND METHODS

Collection of plant material

Samples were collect for one year (2013-2014) during the months March to May (summer season), June to October (Monsoon season) and November to February (winter season) for four *Salacia species* medicinal plants viz., *S. chinensis*, *S. oblonga*, *S. fruticosa* and *S. macrosperma.* Stem tissue samples were collected from healthy plant from the western ghats of Karnataka. Samples were tagged, labeled and placed in separate sterile polythene bags, brought to the laboratory and processed within 24 h of collection.

Isolation, identification and preservation of fungal endophytes.

Samples were washed thoroughly in running water before processing. Stem pieces were surface sterilized by sequential washes in 75% (v/v) ethanol (1 min), 4% (v/v) NaOCl (4 min) and rinsed with sterile water to remove surface sterilizing agents and allowed to surface dry under sterile conditions [12]. One hundred stem segments of 0.5×1.0 cm from each plant collected at different seasons were dissected and placed on water agar (WA) (15 g/L) medium amended with Streptomycin (100 mg/L) contained in 9 cm diameter Petri dishes. Ten segments were placed on 20 ml WA medium in each Petri dish and incubated in a light chamber for 2 weeks at 12 h light/dark cycles at 23 °C [13]. After incubation for 15 days, individual fungal colonies were picked from the edge with a sterile fine tipped needle and transferred onto potato dextrose agar (PDA) without antibiotic. The identification of endophytic fungal strains was carried out based on the morphology of the fungal culture colony or hyphae, the characteristics of the spores and reproductive structures were identified using Zeiss Advanced stereo Discovery V20 Binocular Microscope and standard manuals ([14]. Isolates were maintained in the cryovials with 15% glycerol at -80˚C deep freezer at the Department of Biotechnology, University of Mysore, Mysuru, Karnataka, India.

Data Analysis:

Colonization rate (CR) was calculated as the total number of plant tissue segments infected by fungi divided by the total number of segments incubated [15-16]. Isolation rate (IR) was determined as the number of obtained from plant segments divided by the total number of segments incubated [17]. Colonization frequency (CF) was calculated isolate as the number of plant segments colonized by a single endophyte divided by the total number of segments observed × 100 [18] Simpson index (D), Shannon [19], Berger-Parker Dominance Index (d) [20] and Margalef's species richness index (R) [21] were used to assess and quantify endophytic fungal diversity from four host plants.

Simpson's index of Diversity (D) was calculated using the following equation

$$D = \frac{\Sigma n(n-1)}{N(N-1)} \qquad \text{..... (1)}$$

where, n = the total number of organisms of a particular species
N = the total number of organisms of all species.

Shannon diversity index (H) was calculated using the following equation

$$H = -\sum_{i=1}^{k} p_i \log p_i \qquad \text{.....(2)}$$

where, H = symbol for the diversity in a sample of S species or kinds
k = the number of species in the sample
Pi = relative abundance of i^{th} species or kinds measures= n/N
N = total number of individuals of all kinds

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Ni = number of individuals of i^{th} species

Berger-Parker Dominance Index (d) was calculated using the following equation

$$d = \frac{N_{max}}{N} \quad \text{.....(3)}$$

where, N_{max} = number of individuals in the most abundant species
N = total number of individuals in the sample.

Margalef Richness Index (R) was calculated using the following equation

$$R = \frac{S-1}{\ln N} \quad \textbf{.....(4)}$$

where, S = total number of species.
N = the total number of isolates of all species.

RESULTS

A total of four hundred seventy four (474) (39.50%) endophytic isolates were collected from 1,200 plant tissue samples of stem collected from four different Salacia species in three different seasons namely summer, monsoon and winter. 474 endophytic isolates were categorised as 21 species viz., *Alternaria alternata* (4.85%), *Aspergillus niger* (4.85%), *Aspergillus terreus* (5.49%), *Botryosphaeria rhodina* (0.84%), *Cladosporium herbarum* (7.17%), *Colletotrichum* species (5.27%), C*oriolopsis caperata* (1.69%), *Curvularia* species (1.69%), *Diaporthe perjuncta* (5.70%), *Drechslera* species (4.01%), *Fusarium oxysporum* (9.07%), *Gliocladium roseum* (0.84%), *Lasiodiplodia theobromae* (0.42%), *Myrothecium verrucaria* (7.38%), *Penicillium notatum* (10.13%), *Pestalotiopsis* (6.33%), *Phoma* species (7.59%), *Sterile* species (8.02%), *Trichoderma longibrachiatum* (4.43%), *Trichophyton mentagrophyte* (2.95%) and *Xylaria* species (1.27%).

The colonization frequency (%) of endophytic fungi differed significantly between monsoon, winter and summer seasons of four salacia species plant. Maximum colonization was observed in winter followed by summer and monsoon.

The Maximum colonization frequency was observed as 42 % on *Salacia chinensis* and minimum colonization frequency was observed as 13 % on *Salacia macrosperma* during summer season.

The Maximum colonization frequency was observed as 37 % on *Salacia fruticosa* and minimum colonization frequency was observed as 8 % on *Salacia macrosperma* during monsoon season.

The Maximum colonization frequency was observed as 80 % on *Salacia chinensis* and minimum colonization frequency was observed as 22 % on *Salacia macrosperma* during winter season.

The colonization frequency during all three seasons mentioned was observed as 57.33% on *Salacia chinensis*, 41.33% on *Salacia oblonga*, 42.67% on *Salacia fruticosa*, 16.67% on *Salacia macrosperma*.

Fusarium oxysporum was isolated as a dominant genus from *Salacia chinensis*, *Penicillium notatum* from *Salacia oblonga* , *Pestalotiopsis* from *Salacia fruticosa* and *Phoma* species from *Salacia macrosperma*. The sterile forms dominated in *Salacia chinensis* and *Salacia oblonga*. The colonization rate and colonization frequency of above mentioned endophytes were tabulated (Table 2 -6).

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Table 2: Isolation and colonization rate of endophytic fungi from *Salacia* species

Host plant	Total No. of segments incubated	No. of segments yielding endophytic fungi			No. of isolates	Isolation rate	Colonization rate
		Summer	Monsoon	Winter			
S. chinensis	300	42	36	80	172	0.57	0.53
S. oblonga	300	26	27	49	124	0.41	0.34
S.a fruticosa	300	33	37	45	128	0.43	0.38
S.macrosperma	300	13	8	22	50	0.17	0.14

Table 3: Diversity and Seasonal distribution of endophytic fungi isolated from *S. chinensis* bark

Endophytes	Summer	Monsoon	Winter	Total	Colonization frequency(%)
Alternaria alternate	6	4	8	18	6.00
Aspergillus niger	1	0	3	4	1.33
cladosporium herbarum	4	3	0	7	2.33
colletotrichum species	1	0	3	4	1.33
Curvularia species	0	2	4	6	2.00
Diaporthe perjuncta	1	2	5	8	2.67
Drechslera species	2	6	3	11	3.67
Penicillium notatum	4	7	8	19	6.33
Myrothecium verrucaria	3	1	7	11	3.67
Gliocladium roseum	4	0	0	4	1.33
Fusarium oxysporum	**7**	**10**	**16**	**33**	**11.00**
Phoma species	6	2	6	14	4.67
Sterile species	6	5	9	20	6.67
Trichophyton mentagrophyte	0	0	8	8	2.67
Xylaria species	2	0	3	5	1.67
					Total 57.33

Table 4: Diversity and seasonal distribution of endophytic fungi isolated from *S. oblonga* bark

Endophytes	Summer	Monsoon	Winter	Total	Colonization frequency (%)
Botryosphaeria rhodina	0	0	4	4	1.33
Cladosporium herbarum	2	4	8	14	4.67
Trichoderma longibrachiatum	0	4	0	4	1.33
Aspergillus terreus	1	2	6	9	3.00
Fusarium oxysporum	2	1	6	9	3.00
Aspergillus niger	5	6	2	13	4.33
Lasiodiplodia theobromae	0	0	2	2	0.67
Penicillium notatum	**8**	**5**	**14**	**27**	9.00
Phoma species	4	1	6	11	3.67
Sterile species	3	6	9	18	6.00
Coriolopsis caperata	0	5	3	8	2.67
Pestalotiopsis	5	0	0	5	1.67
					Total 41.33

Table 5: Diversity and seasonal distribution of endophytic fungi isolated from *S. fruticosa* bark

Endophytes	Summer	Monsoon	Winter	Total	Colonization frequency (%)
Drechslera species	3	1	4	4	2.67
Curvularia species	0	1	1	14	0.67
Cladosporium herbarum	3	7	2	4	4.00
Pestalotiopsis	**5**	**12**	**8**	**18**	**8.33**
Trichoderma longibrachiatum	4	5	0	9	4.67
Aspergillus terreus	5	2	7	9	4.67
Colletotrichum species	2	4	8	13	6.33
Diaporthe perjuncta	6	4	9	2	8.00
Myrothecium verrucaria	10	5	9	27	0.33
Xylaria species	1	0	0	11	3.00
					Total 42.67

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Table 6: Diversity and seasonal distribution of endophytic fungi isolated from *S. macrosperma* bark

Endophytes	Summer	Monsoon	Winter	Total	Colonization frequency (%)
Aspergillus terreus	2	0	1	3	1.00
Cladosporium herbarum	0	0	1	1	0.33
Colletotrichum species	0	2	5	7	2.33
Penicillium notatum	0	2	0	2	0.67
***Phoma* species**	**2**	**1**	**8**	**11**	**3.67**
Trichoderma longibrachiatum	4	0	4	8	2.67
Trichophyton mentagrophyte	3	0	3	6	2.00
Alternaria alternate	4	1	0	5	1.67
Aspergillus niger	0	2	4	6	2.00
Fusarium oxysporum	0	1	0	1	0.33
					Total 16.67

Simpson dominance index is comparatively higher in *Salacia fruticosa* (0.130) whereas *Salacia macrosperma*, *Salacia oblonga* and *Salacia chinensis* sharing index values 0.120, 0.111 and 0.093 respectively. Shannon index indicates that the foliar endophytic diversity is more with index value 3.6 and 3.296 for *Salacia chinensis* and *Salacia oblonga* which is due to occurrence of more number of endophytic species than *Salacia macrosperma* and *Salacia fruticosa* sharing relatively similar index value 3.022 and 3.008 respectively. Despite the similar occurrence of less number endophytes in both host plants, the overall species richness was greater in endophytic fungi colonizing stem tissues of *Salacia chinensis* (2.7), whereas other host plants differ in their values (Table 7).

Table 7: Diversity indices of endophytic fungi from *Salacia* plant

Plant Species	Total No. of isolates	Simpson's index (D)	Shannon Diversity index (H)	Berger-Parker Dominance Index (d)	Margalef Richness Index (R)
S. chinensis	172	0.093	3.6	0.19	**2.7**
S. oblonga	124	0.111	3.296	0.217	2.282
S. fruticosa	128	0.130	3.008	0.195	1.855
S. macrosperma	50	0.120	3.022	0.22	2.301

Simpson dominance index for three different seasons for four *Salacia* species host plants is comparatively higher in winter for *Salacia fruticosa* (0.1561) and *Salacia macrosperma* (0.1858) whereas summer for *Salacia oblonga* (0.1905) and monsoon for *Salacia chinensis* (0.1243). Seasonal Shannon index for individual host plants indicates that the endophytic diversity is more in monsoon with index value 2.5 and 2.878 for *Salacia macrosperma* and *Salacia fruticosa*, summer for *Salacia chinensis* and winter for *Salacia oblonga* with index value 3.416 and 3.024 respectively, the species richness was observed in monsoon for *Salacia macrosperma, Salacia fruticosa* and *Salacia oblonga* whereas summer for *Salacia chinensis* (Table 8).

Table 8: Dominance and richness of species diversity of endophytic assemblage of from *Salacia* species during three different seasons

Plant Species	Season	Total No. of isolates	Simpson's index (D)	Shannon Diversity index (H)	Berger-Parker Dominance Index (d)	Margalef Richness Index (R)
S. chinensis	Summer	47	0.08502	3.416	0.1522	**3.134**
	Monsoon	83	0.1243	2.953	0.25	2.701
	Winter	42	0.1011	3.377	0.2162	2.788
S. oblonga	Summer	30	0.1905	2.506	0.381	2.299
	Monsoon	60	0.1267	2.839	0.24	**2.485**
	Winter	34	0.1258	3.024	0.2414	2.217
S. fruticosa	Summer	39	0.1395	2.848	0.2857	2.25
	Monsoon	48	0.1241	2.878	0.2333	**2.352**
	Winter	41	0.1561	2.657	0.2195	1.885

S. macrosperma	Summer	15	0.09524	2.236	0.2857	2.056
	Monsoon	26	0.07143	2.5	0.25	**2.404**
	Winter	09	0.1858	2.421	0.3478	1.914

CONCLUSION

This study investigated the fungal diversity of the endophytic isolates in stem portion at different seasons of the year. In many cases stem sampled during the winter season harboured (cherished) more endophytes than those screened during the summer and monsoon season. It was found that the colonization frequencies and the colonization rates of endophytic fungi are greatly affected by seasonal and spatial factor. This may be due to changes in the environmental (abiotic) factors with the season. In this regard, investigations of the interactions of this plant and its symbiotic fungi would be the next direction for future research.

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