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Climatic dependency on the diversity and distribution of endophytic fungi from *Rauvolfia serpentina* of Westernghats

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Abstract

Altogether 210 segments from plant parts of *Rauvolfia serpentina* were collected during winter and summer seasons from Kerela and they were screened for the presence of endophytic fungi. A total of 10 species viz., *Aspergillus brevipes, Aspergillus sp.$_1$, Aspergillus sp.$_2$, Aureobasidium sps. Curvularia lunata, Fusarium moniliforme, Colletotrichum acutatum, Colletotrichum gloeosporioides, Phyllosticta hymanaeae* and sterile forms were isolated and identified based on morphology of the fungal isolates the nature of and spore with the help of manuals. In the present investigation, the diversity of endophytic fungi was higher in leaves followed by bark and stem. In this study, Hyphomycetes were found to be dominant. Higher colonization frequency and greater diversity of endophytes were observed during winter season than summer season.

Keywords: Endophytic fungi, Colonization frequency, *Rauvolfia serpentina.*

Introduction

The microbes residing in the internal parts of plant tissues was called “endophytes” which constitute a group of plant symbionts and are a component of microbial diversity. Endophytes offer plethora of unknown advantages to the host with immense application in agriculture and medicine (Clay *et al* 2005, Alvarez-Loayza *et al* 2011). Endophytic fungi have been found in all plant families so far investigated, which represent many species in different climatic regions of

the world (Spurr and Welty 1975, Petrini and Carroll 1981, Petrini *et al* 1992). Recent studies indicate that the diversity of fungi is much greater than that was previously thought and the same is true for several guilds of cryptic microfungi, including saprophytes (Lodge 1997, Frohlich and Hyde 1999), pathogens (Shivas and Hyde 1997) and fungal endophytes associated with leaves of woody plants (Lodge *et al* 1996).

Endophytic fungi colonize all plant parts such as root, stem, leaves, bark and floral organs and in some cases can affect both ecological and physiological process of their host (Petrini 1991). Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of pharmaceutical importance (Strobel *et al* 2004, Wiyakrutta *et al* 2004, Kumar *et al* 2005, Tejesviet *et al* 2007). The study of endophytic fungi apart from shedding light on diversity of fungal kingdom, offers a promising digression since some endophytes produce novel metabolites of pharmaceutical and agricultural value (Rajagopal *et al* 2010).

Materials and Methods

Plant materials and fungal isolates

In the present study mature and healthy leaves, stem and bark of *Rauvolfia serpentina* were collected in winter and summer seasons from Kerala. Geographically Kerala lies between North latitude $8^0.17^{'}.\ 30^{''}$ N and $12^0.47^{'}\ .40^{''}$ N and longitudes $74^0.\ 27^{'}.47^{''}$ E and $77^0.$ 37.12 E. The temperature can be between 18^0C -28^0C in winter season (Late part of November to middle of February) and average rain fall for winter season is 25mm. The temperature can be between 32^0C – 36^0C in summer season (towards end of February to till end of May) and average rain fall is 135mm.

Collection of Samples

Healthy plant tissues of *Rauvolfia serpentina* were collected from winter and summer seasons. Collected plant materials were washed under running tap water to remove the adhering particles. Plant tissues were cut into small segments (0.5cm-1.0cm) using sterile blade and surface sterilization was done by washing segments with 70% ethanol for 2 min, 2% sodium hypochloride for 1-2 min, 70% ethanol for 30 seconds followed by 2 or 3 rinses of sterile distilled water and allowed to surface dry under sterile condition. After drying each plant

segment was placed on petriplates containing potato dextrose agar medium (PDA) supplemented with streptomycin (100mg/l) to suppress bacterial growth. Petriplates were sealed with cling film and incubated at 30^0C in a light chamber for up to one week. They were monitored every day for growth of endophyte fungal colonies. Fungi growing out from the samples were subsequently transferred onto fresh PDA plates. The procedure of transferring to fresh PDA pates was carried out several times in order to isolate pure colonies.

Identification of endophytic fungal isolates

The identification of endophytic fungal strains based on the morphology of fungal culture colony (or) hyphae and the characteristics of the spore. All experiments and observation were repeated at least twice. (Huang *et al* 2008).

Colonization frequency

The colonization frequency (CF%) of a single endophytic fungal species in the Leaves, stem and bark segments was calculated by using the following formula (Hata and Futai 1995)

$$CF\% = \frac{\text{Number of segments colonized by an endophytic fungal species}}{\text{Total number of segments}} \times 100$$

Relative percentage occurrence (RPO%) of each group of fungi

Relative percentage occurrence (RPO%) of different group of fungi viz., Coelomycetes, hyphomycetes, xylariaceous and other fungi was calculated using the following formula

$$RPO\% = \frac{\text{Density of colonization of one fungal group}}{\text{Total density of colonization of all fungal group}} \times 100$$

Results

Isolation of Endophytic mycoflora of *Rauvolfia serpentina*

Colonization frequency

Two hundred and ten segments of plant parts viz., leaves, stem and bark of *Rauvolfia serpentina* were screened for the presence of endophytic fungi. A total of 185 fungal isolates were obtained from the plant tissues. In winter season, 10 species of fungi belonging to 5 genera (6 Hyphomycetes and 2 Coelomycetes) and two non sporulating sterile morphospecies were recovered from the leaves, stem, and bark tissues of *Rauvolfia serpentine*. In leaves, endophytic fungal colonization was dominated by *Aspergillus sps*$_1$, *Colletotrichum gloeosporioides* and *Curvularia lunata* while Sterile form $_3$ showed minimum percentage of colonization. The colonization frequencies of *Aspergillus sps*$_1$, *Colletotrichum gloeosporioides* and *Curvularia lunata* were 37.1 %, 31.4% and 22.9% respectively. In stem, endophytic fungal colonization was dominated by *Aspergillus brevipes,* while *Curvularia lunata* and Sterile Form$_2$ showed minimum percentage of colonization frequency. The colonization frequency of *Aspergillus brevipes*, was 14.3%. In Bark, *Aspergillus sps*$_1$ and Sterile form $_3$ showed maximum colonization frequency. The Colonization frequency of *Aspergillus sps*$_1$ and sterile form $_3$ were 22.9% and 14.3% respectively.

In summer, 8 species of fungi belonging to 5genera (3 Hyphomycetes and 3 Coelomycetes) and two non sporulating sterile morphospecies were recovered from the leaves, stem and bark tissues of *Rauvolfia serpentina*. In leaves, *Fusarium moniliforme, Phyllosticta hymanaeae* and *Colletotrichum gloeosporioides* showed maximum percentage of colonization frequency, while sterile form$_1$ and sterile form$_3$ showed minimum percentage of colonization. The colonization frequencies of *Fusarium moniliforme, Phyllosticta hymanaeae* and *Colletotrichum gloeosporioides* were found to be 34.3%, 31.4 %and 28.6% respectively. In stem, endophytic fungal colonization was dominated by *Aureobasidium sps,* and *Colletotrichum gloeosporioides.* Both species have same colonization frequency of 8.6%. In Bark, *Aspergillus sps*$_1$ showed maximum colonization frequency of 34.3%, sterile form$_1$ and sterile form$_3$ showed minimum percentage of colonization. (Table 1).

Table- 1 Colonization frequency (CF%) and relative percentage occurrence (RPO%) of fungal endophytes isolated from the leaves, stem and bark of *Rauvolfia serpentina,* sampled during Winter and summer seasons.

S.No		Winter season Nov 2013- Feb 2014				Summer season Mar 2014- Jun 2014			
		CF%			RPO %	CF%			RPO %
		L	S	B		L	S	B	
1.	Hyphomycetes *Aspergillus brevipes*	14.3	14.3	8.6		0	0	0	
2.	*Aspergillus sps*$_1$	37.1	8.6	22.9		20	5.7	34.3	
3.	*Aspergillus sps*$_2$	8.6	8.6	0	60	0	0	0	37.5
4.	*Aureobasidium sps*	17.1	0	5.7		22.9	8.6	0	
5.	*Curvularia lunata*	22.9	5.7	20		0	0	0	
6.	*Fusarium moniliforme*	17.1	0	2.8		34.3	2.8	0	
7.	Coelomycetes *Colletotrichum acutatum*	11.4	0	8.6		0	2.8	0	
8.	*Colletotrichum gloeosporioides*	31.4	0	0	20	28.6	8.6	8.6	37.5
9.	*Phyllosticta hymanaeae*	0	0	0		31.4	0	14.3	
10.	Sterile form Sterile form$_1$	0	0	0		5.7	5.7	2.8	
11.	Sterile form $_2$	8.6	2.8	5.7	20	0	0	0	25
12.	Sterile form $_3$	2.8	0	14.3		5.7	0	2.8	
Total (CF%)		171.3	40	88.6		148.6	34.2	62.8	

L= Leaves S= Stem B= Bark

When compared to summer season, winter season showed maximum number of fungal endophytes (104). Summer season showed minimum number of fungal endophytes (86). *Aspergillus brevipes Aspergillus sps*$_{2,}$ *Curvularia lunata* and sterile form $_2$ were recovered only during winter season. *Phyllosticta hymanaeae* and sterile form$_1$ were recovered only in summer season. Isolates of *Aspergillus sps*$_1$*, Aureobasidium sps, Fusarium moniliforme, Colletotrichum acutatum, Colletotrichum gloeosporioides,* and sterile form$_3$ were recovered as common and occurred in both seasons. Whereas *Aspergillus sps*$_1$ was the most dominant among all endophytes. *Aureobasidium sps* and *Fusarium moniliforme* showed tissue specificity towards leaves and stem in summer season. *Aspergillus sps*$_2$ showed tissue specificity in leaves and stem during winter season. *Aureobasidium sps Colletotrichum acutatum* and *Fusarium moniliforme* showed tissue specificity in leaves and bark tissues in summer season.

Periodicity of occurrence

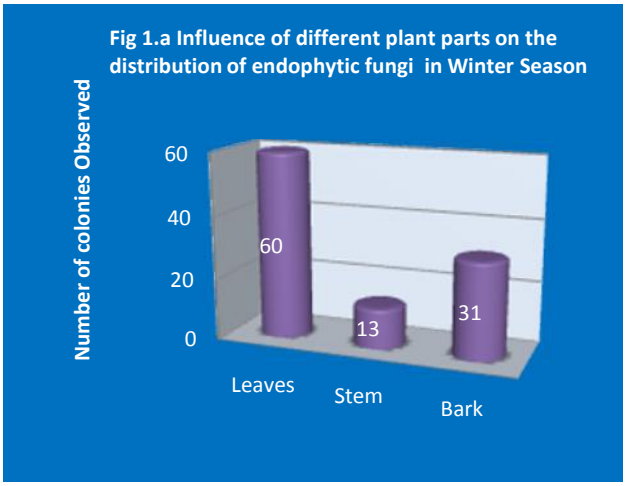
A total of 12 species were isolated from the leaves of *Rauvolfia serpentine* in winter and summer seasons. In leaves, *Aspergillus sps*$_1$*, Aureobasidium sps, Fusarium moniliforme, Colletotrichum gloeosporioides* and sterile form $_2$ were recorded as most common and occurred in 2 samplings. The remaining species namely *Aspergillus brevipes*, *Aspergillus sps*$_2$, *Curvularia lunata*, *Colletotrichum acutatum*, *Phyllosticta hymanaeae*, Sterile form $_1$ and Sterile form $_2$ were recorded as occasional and occurred in only one sample. In stem, *Aspergillus sps*$_1$ was recorded as most common and occurred in 2 samplings. *Aspergillus brevipes*, *Aspergillus sps*$_2$, *Aureobasidium sps*, *Curvularia lunata*, *Fusarium moniliforme*, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, sterile form$_1$, and sterile form$_2$ were recorded as occasional and occurred in only one sample. In bark, *Aspergillus sps*$_1$ and sterile form $_2$ were recorded as most common and occurred in 2 samplings. *Aspergillus brevipes*, *Aureobasidium sps*, *Curvularia lunata*, *Fusarium moniliforme*, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, *Phyllosticta hymanaeae*, sterile form$_1$, and sterile Form$_2$ were recorded as occasional and occurred in only one sampling. (Table 2).

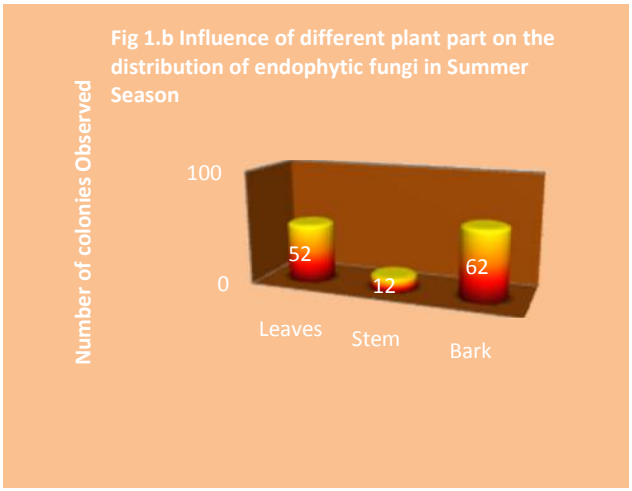
Table-2 Periodicity of occurrence of endophytic fungi recorded from leaf, stem and bark of *Rauvolfia serpentina* during winter and summer seasons

Leaves	Stem	Bark
Most Common: 51-100% *Aspergillus sps*$_1$ (2) *Aureobasidium sps* (2) *Fusarium moniliforme* (2) *Colletotrichum gloeosporioides*(2) Sterile form $_3$ (2)	**Most Common: 51-100%** *Aspergillus sps*$_1$ (2)	**Most Common: 51-100%** *Aspergillus sps*$_1$ (2) Sterile form $_3$ (2)
Occasional 1-50% *Aspergillus brevipes* (1) *Aspergillus sps*$_2$ (1) *Curvularia lunata* (1) *Colletotrichum acutatum* (1) *Phyllosticta hymanaeae* (1) Sterile form $_1$ (1) Sterile form $_2$ (1)	**Occasional 1-50%** *Aspergillus brevipes* (1) *Aspergillus sps*$_2$ (1) *Aureobasidium sps* (1) *Curvularia lunata* (1) *Fusarium moniliforme* (1) *Colletotrichum acutatum* (1) *Colletotrichum gloeosporioides* (1) Sterile form $_1$ (1) Sterile form $_2$ (1)	**Occasional 1-50%** *Aspergillus brevipes* (1) *Aureobasidium sps* (1) *Curvularia lunata* (1) *Fusarium moniliforme* (1) *Colletotrichum acutatum* (1) *Colletotrichum gloeosporioides* (1) *Phyllosticta hymanaeae* (1) Sterile form $_1$ (1) Sterile form $_2$ (1)

Relative Percentage Occurrence (RPO%)

The RPO (%) of Hyphomycetes, Coelomycetes and sterile morphospecies were 60%, 20%, 20% respectively in winter season. The RPO (%) of Hyphomycetes, Coelomycetes and sterile morphospecies were 37.5%, 37.5%, 25% respectively in summer season.





Diversity index of endophytic mycoflora of *Rauvolfia serpentina.*

The relationship among the endophytic fungal isolates was studied by calculating the different diversity of leaves, stem and bark tissues in *R.serpentina*. Simpson, Shannon, Brillouin and Margalef diversity indices were found to be highest in leaf tissues during winter season. Dominance, Evenness, Equitability and Berger_Parker diversity indicies were found to be highest in stem during winter season. Menhinick and Fisher_alpha diversity indices were found

to be highest in bark tissues during winter season. Simpson, Shannon and Brillouin diversity indices showed maximum diversity indices in leaf tissues during summer season. Evenness, Menhinick, Margalef, Equitability and Fisher_alpha diversity indices showed highest diversity indices in stem tissues during summer season. Dominance and Berger_Parker diversity indices were found to be highest in bark tissues during summer season. Maximum diversity indices were found in stem during summer season while, leaf and stem tissues during winter season. (Table-3)

Table -3 Diversity indicies of endophytic fungi associated with leaves, stem and bark tissues of *Rauvolfia serpentina*.

Diversity Indicies	Winter Season			Summer Season		
	L	S	B	L	S	B
Dominance _ D	0.14	**0.24**	0.17	0.18	0.19	**0.37**
Simpson_ 1-D	**0.86**	0.76	0.83	**0.82**	0.81	0.63
Shannon_ H	**2.13**	1.49	1.90	**1.79**	1.71	1.22
Evenness	0.84	**0.89**	0.83	0.86	**0.92**	0.68
Brillouin	**1.89**	1.15	1.59	**1.60**	1.25	1.0
Menhinick	1.29	1.34	**1.44**	0.97	**1.73**	1.06
Margalef	**2.20**	1.52	2.04	1.52	**2.01**	1.29
Equitability	0.92	**0.93**	0.91	0.92	**0.95**	0.76
Fisher_alpha	3.43	2.78	**3.49**	2.18	**4.78**	2.02
Berger_Parker	0.22	**0.36**	0.26	0.23	0.25	**0.55**

L- Leaf S- Stem B-Bark. Bold number indicates maximum diversity.

Discussion

Endophytic fungi are the unexplored and diverse group of organisms having symbiotic association with higher life forms and may produce beneficial substances for host (Weber 2007)**.** *Colletotrichum gloeosporioides* is a worldwide pathogen that infects many plant species (Brown *et al* 1998; Jeger and Bailey 1992). *Colletotrichum gloeosporioides* was found as endophytes in banana, but these fungi also cause anthracnose of banana fruits (Photita *et al* 2001). In the present study, *Colletotrichum gloeosporioides* was isolated during both summer and winter seasons. During winter season host plant exhibited highest endophytic association than the summer season. Endophytes isolated from leaf samples exhibited greater diversity and high colonization frequency compared to the endophytes of the other plant parts examined. Leaf endophytes are indeed more species rich in the tropics than the temperate region (Arnold *et al* 2007). Most of the leaf samples had more number of endophytic fungal diversity. One of these possible reason for the differences in the colonization rates between plants is the structure and substrate which influence the colonization and distribution of endophytic fungi (Okane *et al* 2001). Similarly, Kumar and Hyde (2004) reported that the colonization rate in the leaves was found to be significantly higher than those in other parts of the host.

In the present study clearly revealed that the number of endophytic fungi was higher in leaves followed by bark and stem. Hyphomycetes were found to be dominant. Higher colonization frequency and greater diversity of endophytes were observed during winter season than summer season.

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