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Climatic dependency on the diversity and distribution of endophytic fungi from *Rauvolfia tetraphylla* L., of Western Ghats

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Abstract

In the present investigation, altogether 210 segments from plants of *Rauvolfia tetraphylla* were collected during winter and summer seasons from Whestern Ghats of Kerela and they were screened for the presence of endophytic fungi. A total number of 10 species viz., *Alternaria brassicae, Alternaria brassicola, Curvularia brachyspora, Drechslera sps, Nigrospora oryzae, Nigrospora spharica, Colletotrichum gloeosporioides, Colletotrichum graminicola, Pestalotiopsis versicola, Phyllosticta hymanaeae* and sterile forms were isolated and identified based on morphological characteristics of the fungal culture and spore with the help of standard manuals. The results revealed that the diversity of endophytic fungi was higher in leaves followed by stem and bark. Among these endophytes, the hypomycetes were found to be dominant. Higher colonization frequency and greater diversity of endophytes were observed during summer season than winter season.

Key words: Endophytic fungi, Climate, Colonization frequency, *Rauvolfia tetraphylla.*

Introduction

The microbes residing in the internal parts of plant tissues were 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., Aptroot, 2001), 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 roots, stems, leaves, barks 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 tetraphylla* 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 tetraphylla* were collected from winter and summer seasons. The plant parts leaves, stem, bark were collected for the isolation of endophytes. 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, surface sterilization was done by washing segments with 70% ethanol for 2 minutes, 2% sodium hypochloride for 1-2 minutes, 70% ethanol for 30seconds 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 the characteristics of the spores (Huang *et al*., 2008). All experiments and observation were repeated at least twice.

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)

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

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

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

Results

Isolation of Endophytic mycoflora of *Rauvolfia tetraphylla*

Colonization frequency

Two hundred and ten segments of plant parts viz., leaves, stem and bark of *Rauvolfia tetraphylla* screened for the presence of endophytic fungi. A total of 164 fungal isolates were obtained from the plant tissues. In winter season, 10 species of fungi belonging to 6 genera (6 Hypomycetes and 2 Coelomycetes) and two non sporulating sterile morphospecies were recovered from the leaves, stem, and bark tissues of *Rauvolfia tetraphylla*. In leaves, endophytic fungal colonization was dominated by *Alternaria brassicae, Nigrospora spharica* & *Pestaliopsis versicola* when compared to *Alternaria brassicola, Colletotrichum gloeosporioides*. The sterile form$_4$ showed minimum percentage of colonization in leaves. The colonization frequencies of *Alternaria brassicae, Nigrospora spharica* and *Pestaliopsis versicola* were 11.4%, 20% and 22.9% respectively. In stem, endophytic fungal

colonization was dominated by *Alternaria brassicae, Nigrospora spharica* and *Colletotrichum gloeosporioides* while *Curvularia brachyspora* and *Pestalotiopsis versicola.* The colonization frequency of *Alternaria brassicae, Nigrospora spharica* and *Colletotrichum gloeosporioides* were 14.3%, 17.1%, 11.4% respectively In Bark, *Drechslera sps* and Sterile form $_4$ showed maximum colonization frequency while *Nigrospora spharica, Colletotrichum gloeosporioides, Colletotrichum graminicola* showed minimum colonization frequency. The Colonization frequency of *Drechslera sps* and Sterile form $_4$were 11.4% and 11.4% respectively.

In summer, 8 species of fungi belonging to 6 genera (5 Hypomycetes and 2 Coelomycetes) and two non sporulating sterile morphospecies were recovered from the leaves, stem, and bark tissues of *Rauvolfia tetraphylla*. In leaves *Drechslera sps, Pestalotiopsis versicola and Curvularia brachyspora*, showed maximum percentage of colonization frequency, while *Alternaria brassicae Nigrospora oryzae, Nigrospora Spharica Colletotrichum gloeosporioides Phyllosticta hymanaeae*, sterile form$_4$ & sterile form$_5$. The colonization frequencies of *Drechslera sps, Pestalotiopsis versicola and Curvularia brachyspora* were found to be 28.6% , 17.1% and 14.3% respectively. In stem, endophytic fungal colonization was dominated by *Pestalotiopsis versicola, Alternaria brassicae*and *Drechslera sps* were found to be 20%, 14.3% and 14.3% respectively. *Curvularia brachyspora, Nigrospora oryzae, Colletotrichum gloeosporioides, Phyllosticta hymanaeae*, sterile form$_4$ & sterile form$_5$. In Bark *Curvularia brachyspora* (22.9%) and *Drechslera sps* (11.4%) showed maximum colonization frequency, while *Alternaria sps, Nigrospora oryzae, Pestalotiopsis versicola* and sterile form$_4$ (Table 1).

When compared to winter season, summer season showed maximum number of fungal endophytes *Alternaria brassicola* and *Colletotrichum graminicola* were recovered only during winter season. *Nigrospora oryzae* and sterile form were recovered only during summer season. *Alternaria sps, Curvularia brachyspora, Drechslera sps, Nigrospora spharica, Colletotrichum gloeosporiodes, Pestalotiopsis versicola, Phyllosticta hymanaeae* and Sterile form $_4$ were recovered as common and occurred in both seasons. *Alternaria brassicola* showed tissue specificity in leaves and bark tissues. *Colletotrichum graminicola* showed tissue specificity in bark tissues during winter season only. *Phyllosticta hymanaeae* and Sterile form$_5$ showed tissue specificity for leave and stem and not observed in bark.

Table – 1. Colonization frequency (CF%) and relative percentage occurrence (RPO%) of fungal endophytes isolated from the leaves, stem and bark of *R. tetraphylla*, sampled during Winter and summer season.

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** *Alternaria brassicae*	11.4	14.3	5.7	50	8.6	14.3	8.6	50
2.	*Alternaria brassicola*	5.7	0	8.6		0	0	0	
3.	*Curvularia brachyspora*	8.6	2.9	2.9		14.3	11.4	22.9	
4.	*Drechslera sps*	2.9	8.6	11.4		28.6	14.3	11.4	
5.	*Nigrospora oryzae*	0	0	0		5.7	8.6	5.7	
6.	*Nigrospora spharica*	20	17.1	2.9		11.4	0	0	
7.	**Coelomycetes** *Colletotrichum gloeosporiodes*	5.7	11.4	2.9	40	11.4	8.6	0	30
8.	*Colletotrichum graminicola*	0	0	8.6		0	0	0	
9.	*Pestalotiopsis versicola*	22.9	2.9	0		17.1	20	8.6	
10.	*Phyllosticta hymanaeae*	8.6	0	0		5.7	8.6	0	
11.	Sterile form $_4$	5.7	5.7	11.4	10	5.7	2.9	2.9	20
12.	Sterile form $_5$	0	0	0		2.9	8.6	0	
Total (CF %)		91.5	62.9	54.4		111.4	97.3	60.1	

L= Leaves S= Stem B= Bark

Periodicity of occurrence:

A total of 12 species were isolated from the leaves of *Rauvolfia tetraphylla* from winter and summer seasons. In leaves, *Alternaria sps, Curvularia brachyspora, Drechslera sps, Nigrospora spharica, Colletotrichum gloeosporiodes, Pestalotiopsis versicola, Phyllosticta hymanaeae* and Sterile form $_4$ were recorded as most common and occurred in 2 samplings. The remaining three species namely *Alternaria brassicola, Nigrospora oryzae* and Sterile form $_5$ were recorded as occasional and occurred in only one sample. In table 2, the stem, *Alternaria sps, Curvularia brachyspora, Drechslera sps,*

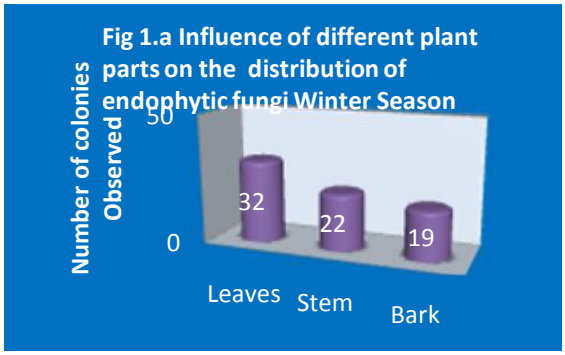
Colletotrichum gloeosporiodes and Sterile form $_4$ were recorded as most common and occurred in 2 samplings. *Nigrospora oryzae, Nigrospora spharica*, *Phyllosticta hymanaeae*, Sterile form $_5$ were recorded as occasional and occurred in only one sample. In bark *Alternaria sps*, *Curvularia brachyspora*, *Drechslera sps* and Sterile form $_4$ were recorded as most common and occurred in 2 samplings. *Alternaria brassicola*, *Nigrospora oryzae*, *Nigrospora spharica*, *Colletotrichum gloeosporiodes*, *Colletotrichum graminicola* and *Pestalotiopsis versicola* were recorded as occasional and occurred in only one sampling. *Alternaria brassicaae* and *Colletotrichum graminicola* were observed only in winter season.

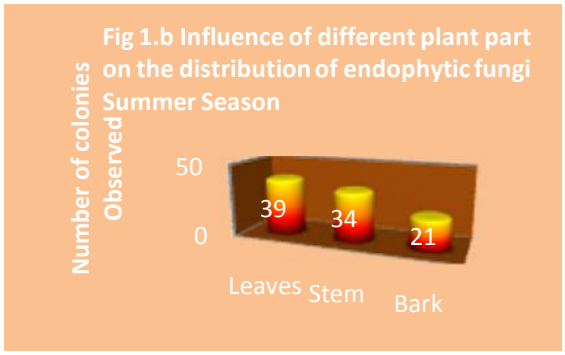
Table -2. Periodicity of occurrence of endophytic fungi recorded from leaf, stem and bark of *Rauvolfia tetraphylla* during winter and summer seasons

Leaf	Stem	Bark
Most Common: 51-100% *Alternaria brassicae* (2) *Curvularia brachyspora* (2) *Drechslera sps* (2) *Nigrospora spharica*(2) *Colletotrichum gloeosporiodes* (2) *Pestalotiopsis versicola* (2) *Phyllosticta hymanaeae* (2) Sterile form $_4$(2)	**Most Common: 51-100%** *Alternaria brassicae* (2) *Curvularia brachyspora* (2) *Drechslera sps* (2) *Colletotrichum gloeosporiodes* (2) Sterile form $_4$(2)	**Most Common: 51-100%** *Alternaria brassicae* (2) *Curvularia brachyspora* (2) *Drechslera sps* (2) Sterile form $_4$(2)
Occasional 1-50% *Alternaria brassicola*(1) *Nigrospora oryzae* (1) Sterile form $_5$ (1)	**Occasional 1-50%** *Nigrospora oryzae* (1) *Nigrospora spharica*(1) *Phyllosticta hymanaeae* (1) Sterile form $_5$ (1)	**Occasional 1-50%** *Alternaria brassicola*(1) *Nigrospora oryzae* (1) *Nigrospora spharica*(1) *Colletotrichum gloeosporiodes*(1) *Colletotrichum graminicola* (1) *Pestalotiopsis versicola* (1)

Relative Percentage Occurrence (RPO%)

The RPO (%) of Hyphomycetes, Coelomycetes and sterile morphospecies were 50%, 40%, 10% respectively in winter season. The RPO (%) of Hyphomycetes, Coelomycetes and sterile morphospecies were 50, 30%, 20 % respectively in summer season.





Discussion

Medicinal plants are the one of the oldest forms of health care known, every plant on earth is known to harbour at least one endophytic microbe. These are one of the unexplored and diverse groups of organism having symbiotic association with higher life forms and may produce beneficial substances for host (Weber, 2007)**.** The colonization frequency (CF %) of fungal endophytes in this study was within the plant of studied in the tropics *Rauvolfia tetraphylla.* Total of 164 fungal isolates were obtained from the plant tissues like stem, leaf and bark in both winter and summer seasons. Summer season showed maximum number of fungal endophytes when compared to winter seasons and leaf also has high number of endophytic fungal colonies while compared to bark and stem. The difference in colonization by endophytes between the plant parts might be due to substrate, secondary metabolites and the physiological state of the host plants. This type of difference in winter and summer season suggests that colonization by endophytes is correlated with climatic factors (Wilson & Carroll 1994). These factors may determine spread and germination success of endophytic fungal spores (Schulthess & Faeth 1998). The *Colletotrichum gloeosporioides* was isolated from both summer and winter season. Similarly, *Colletotrichum* spp. has the ability to express different symbiotic life styles based on host genotypes (Redman *et al.* 2001). Most of the leaf samples finding more number of endophytic diversity in the examined plants. One of these possible reasons 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) have reported that the colonization rate in the leaves was found to be significantly higher than those in other parts studied of the host. The mycelia sterile fungi were also isolated in all the three parts of the present plants. This results is lined with the results of Arnold et al. 2000; Frohlich et al. 2000. Both of these two author reports that mycelia sterilia have been isolated as endophytes from a wide range of host plants.

The present study gives evidence that *R. tetraphylla* harbours endophytic fungi of beneficial activity. The results revealed that these fungi may be helping the plants in protecting from pathogenic fungi. The screening for endophytic fungi in some medicinal trees described here helps to gain knowledge on their symbiotic fungi. The role of these fungi through mutual interaction in antagonism against phytopathogens or insect herbivores could be speculated . We are currently investigating secondary metabolites from fungi isolated in the present study both to understand their ecology and to determine potential as therapeutic targets.

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