World Journal of Agricultural Sciences 7 (5): 577-582, 2011
ISSN 1817-3047

Diversity of Fungal Endophytes from Two Endemic Tree Species *Artocarpus hirsutus* Lam. And *Vateria indica* Linn. of Western Ghats, India

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Abstract: It is well known that diverse endophytes colonize internal tissue of plants. Recently, endophytic fungi residing in medicinal plants have gained unequivocal attention, thus requiring their systematic identification and characterization. In this study 106 endophytic fungi from *Artocarpus hirsutus* Lam. and *Vateria indica* Linn. two endemic medicinal plants of Western Ghats, were documented using traditional morphological methods. The frequency dominant genera were *Coniothyrium* sp (96.5%), *Trichoderma* sp. (84.5%), *Mortierella* sp. (36.75%), *Phyllosticta* sp. (19%) and *Acremonium* sp. (21.5%). The other endophytes recovered belong to class *Ascomycetes* and *Hyphomycetes* i.e. *Aspergillus* spp. *Colletotrichum* spp. *Fusarium* spp. and *Penicillium* spp. Higher number of isolates was recovered from the bark of the plants than from twigs.

Key words: Endophytic fungi · Artocarpus hirsutus · Vateria indica · Western Ghats · Endemic trees

INTRODUCTION

Western Ghats is one of the 34 global hotspots of biodiversity with 4,780 plant species, amongst which 2,180 (0.7%) are endemic to this region [1]. About 500 plants are reported to have medicinal properties. Medicinal plants have been recognized as repository of fungal endophytes with novel metabolites [2, 3]. Studies on medicinal plants from Western Ghats report the presence of diverse community of endophytic fungi [4].

Artocarpus is a evergreen and deciduous tree belonging to the family Moraceae consisting of about 50 species. South-East Asia, Indonesia, Western part of Java and India uses a number of *Artocarpus* species in food and traditional folk medicines. Aerial and underground plant parts have been used in treatment of diarrhoea, diabetes, malarial fever, tapeworm infection and also have wound healing and antisyphilic properties. The pharmacological effect can be credited to the phenolic compounds like flavonoids, stilbenoids and arylbenzofurons [5] and Jacalin, a lectin present in the seeds of certain *Artocarpus* species [6, 7].

Vateria indica also known as the 'White Dammar' is an endemic tree of Western Ghats belonging to the family Dipterocarpaceae. The bark, seeds and resin find application in medicine. The resin which is extensively used in Indian medicine is credited with tonic, carminative and expectorant properties. It is also used for the treatment of throat troubles, chronic bronchitis, piles, diarrhoea, rheumatism, tubercular glands, boils, etc. A novel resveratrol octamer, vateriaphenol A [8] and two new stilbenoids, vateriaphenols A and B have been reported from the stem bark of *V. indica* [9] with anti-bacterial and anti-tumor effect [10].

Foliar fungal endophytes are ubiquitous in plant species examined to date [4]. Endophyte-host relationship could be termed as a balanced antagonism and the secondary metabolites could be a contribution of the endophytic partner to this mutualistic relationship [11]. They have been recognized as a repository of novel compounds with immense value in industry and medicine with properties as diverse as antibiotics, antimycotics, immunesuppressants and anticancer profiles [12]. Several reviewers have addressed the functional metabolites of endophytic origin with considerable potential in pharmaceutical arena [13]. A few examples, include Taxol® with antitumor activity isolated from the endophyte *Taxomyces andreanae* associated with yew, *Taxus brevifolia* [14]. Non-peptidal insulin mimetic L-783,281 from *Pseudomassaria* sp. another bioactive

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isolated from leaves of undetermined plant source prompted revolutionary diabetic therapy of oral administration of activator of human insulin receptor [15]. Another high potential anticancer molecule from endophyte is an alkaloid, camptothecin, a potent inhibitor of DNA topo-I used to treat colon, uterine, ovarian and cervical cancer was isolated from *Nothapodytes nimmoniana* [16].

Strobel [17] reported that areas of high plant endemicity possess specific endophytes that may have evolved with the endemic plant species and could be a source of yet unknown novel molecules heralding drug discovery. As a part of ongoing efforts towards finding novel bioactives we have initiated the study of investigating two endemic tree species of medicinal importance *viz*. *A. hirsutus* and *V. indica* from Western Ghats of India for their endophytic profile. Virtually there are no reports on the fungal endophytes associated with the above indicated tree species. Hence, this study provides first information on the fungal endophytes associated with *A. hirsutus* and *V. indica*.

MATERIALS AND METHODS

Collection of Plant Material: Bark and twig samples of *Artocarpus hirsutus* and *Vateria indica* were obtained from Kigga and Kajre regions of Western Ghats in Karnataka, India. The bark pieces (5.0 cm x 5.0 cm) from the trunk were cut 1.5m above the ground level with a disinfected sickle. The samples were labeled and placed separately in polyethylene bags, transported to the laboratory and kept in a refrigerator at 4°C till processed.

Isolation, Identification and Preservation of Fungal Endophytes: The collected bark and twig samples were halved and washed thoroughly under running tap water before processing. The bark and twig pieces were surface sterilized by immersing them consecutively in 70% ethanol (v/v) for one minute and 3.5% sodium hypochlorite for 3 min and then rinsed thoroughly thrice with sterile distilled water. The samples were dried under laminar airflow. With a sterile blade, the outer tissues were removed and dissected into ~0.5 x 1.0 cm. Two hundred bits @ the rate of 10-15 pieces per plate were plated on water agar (15 $g.l^{-1}$) supplemented with chloramphenicol (100 $mg.l^{-1}$). The plates were incubated at 23°C at 12h light / 12h dark cycles for 6 weeks. The plates were observed periodically. Each colony grown from the segments were immediately transferred to potato dextrose agar medium (PDA). The morphology of the isolated fungal endophytes was studied using Zeiss Advanced stereo Discovery V20 Binocular Microscope. The fungal endophyte isolates were identified based on the morphological and conidial characters using standard identification manuals [18, 19]. All the endophyte isolates were catalogued and maintained with 15% glycerol at -80°C deep freezer at the Department of Biotechnology, University of Mysore, Mysore, India.

Table 1: Frequency of endophytic fungi isolated from bark and twigs of *Artocarpus hirsutus* and *Vateria indica*

	% of Colonization Frequency (%CF)					
	Artocarpus hirsutus		Vateria indica			
Endophytic fungi	Bark*	Twig*	Bark*	Twig*	Total	Relative Dominance
Acremonium sp.	4.0	0.50	9.50	7.50	21.5	7.63
Aspergillus sp.	-	-	2.0	-	2.0	0.70
Ascomycetes sp.	1.0	-	-	-	1.0	0.35
Aspergillus niger	-	-	2.50	1.50	4.0	1.41
Coniothyrium sp.	-	-	41.0	55.50	96.50	34.25
Colletotrichum sp.	-	-	1.50	-	1.50	0.53
Colletotrichum sp.	2.0	0.50	-	-	2.50	0.88
Fusarium sp.	-	3.50	-	-	3.50	1.24
Fusarium oxysporum	4.50	1.0	-	-	5.50	1.95
Mortierella sp.	33.25	3.50	-	-	36.75	13.04
Penicillium sp.	1.50	-	1.0	-	1.50	0.53
Phyllosticta sp.	19.0	-	-	-	19.0	6.74
Trichoderma sp.	40.50	44.0	-	-	84.5	29.99
Sterile mycelia	0.50	0.50	-	1.0	2.0	0.70
Total CF%	106.25	53.50	57.50	65.50	281.75	
Total no. of isolates recovered	54	32	28	25	139	
Species richness	9	7	6	4		
Shannon-Wierner diversity index	2.197	1.945	1.791	1.386		
Simpson diversity index	0.111	0.142	0.166	0.250		

* Based on 400 bits

*Simpson's index of diversity (D). #Total species richness is a measure of number of genera per location/ sample type

Data Analysis: The percentage of colonization frequency (% CF) was calculated according to Fisher and Petrini [20] as follows:

%CF = (Number of tissue segments colonized by a fungus/ Total number of tissue segments plated) x 100

The percentage of dominant taxa was calculated as the % CF divided by the sum of %CF of all endophytes x 100 [21]. The Jac-card's similarity indices *viz*. Simpson and Shannon diversity indices were calculated for the endophytes isolated from different sample types (bark and twig) of *A. hirsutus* and *V. indica* using the Shannon calculator. It was constructed based on the data provided in Table 1. The species present in the particular host (e.g. *A. hirsutus*, bark) was taken as 1 and the same species absent in the other sample type (e.g. *A. hirsutus*, twig) was taken as 0 and represented in Table 1.

RESULTS AND DISCUSSION

A total of ~1600 (~ 400 bark and ~ 400 twig) segments from *A. hirsutus* and *V. indica* collected were screened for fungal endophytes. A total of 106 isolates representing 13 fungal endophyte species were enumerated (Table 1). The total percentage of fungal endophytes recovered from the bark samples of *A. hirsutus* was 106.25 % and in twig samples it was 53.50 %. *Trichoderma* sp. (Fig. 1, a-b) was the most dominant isolate in bark (40.50%) and in twig (44.0%) followed by *Mortierella* sp. (Fig. 1, c-d) in bark (33.25%) and twig (3.5%). The other endophytes recovered were *Acremonium*, *Ascomycetes*, *Colletotrichum*, *Fusarium* spp. *Penicillium* and *Phyllosticta* species (Fig. 1, e-f). The total percentage of fungal endophytes recovered from *V. indica* includes 57.5% from bark and 65.5% from twig. Amongst them *Coniothyrium* sp. (Fig. 1, g-h) was the most dominant species isolated from bark (41.4%) and twig (55.50%). The other fungal endophytes were *Acremonium*, *Aspergillus* spp. *Colletotrichum* and *Penicillium* species.

Medicinal plants, especially tree species in the natural habitat are threatened due to their overexploitation as drug targets. The endophytes isolated from the medicinal plants have the potential as alternative source of bioactive molecules. It is hypothesized that endophytes could synthesize similar secondary metabolites as the host plant presumably due to horizontal gene transfer from the host plant to the residing endophytes [11]. One ubiquitous example is paclitaxel (Taxol®) isolated from inner bark of *Taxus brevifolia*, a relatively rare and slow growing tree species. The supply crisis with endangered status of the tree prompted search for paclitaxel producing microorganism. It resulted in abundant reports on endophytes from Yews producing this natural bioactive molecule [11, 14]. Others include non-yew sources *Periconia* sp. from *Torreya grandifolia* [22], *Pestalotiopsis guepinii* from *Wollemia nobilis* [23], *Colletotrichum gloeosporioides* from *Justicia gendarussa* [24] and *Chaetomella raphigera* isolated from *Terminalia arjuna* [25]. Thus, there is a great potential of finding new drugs from endophytes for treating humans [26].

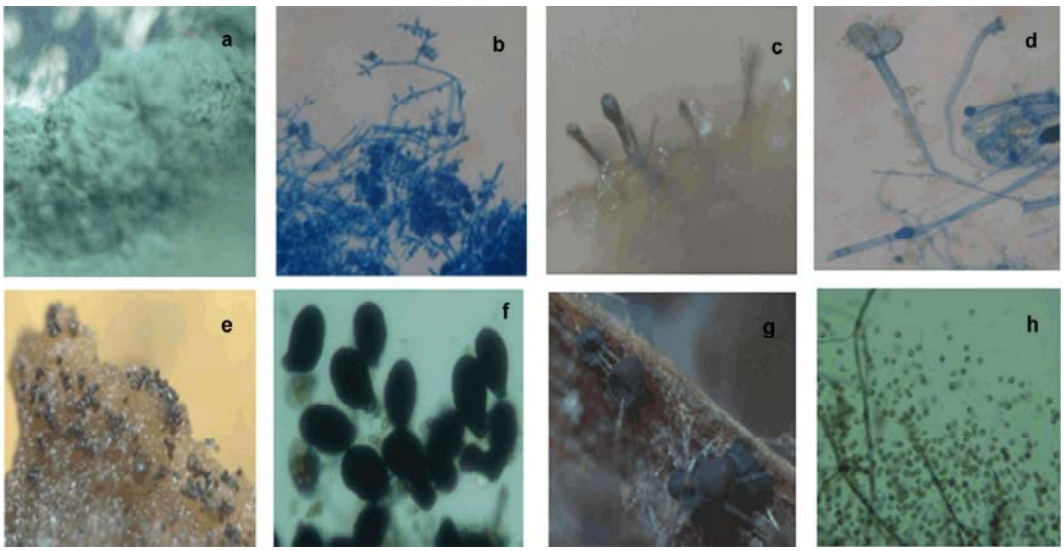

Fig. 1: *Trichoderma* sp. colony (a) and conidial characteristics (b); *Mortierella* sp. colony (c) and conidial characteristics (d); *Phyllosticta* sp. colony (e) and conidial characteristics (f); *Coniothyrium* sp. colony (g) and conidial characteristics (h)

Earlier studies from our laboratory have reported endophytic fungi from medicinal plants, *Terminalia arjuna*, *Azadirachta indica* and *Crataeva magna*. *Pestalotiopsis* (54.5%), *Chaetomium* (10.5%) and *Myrothecium* (9%) were the most predominant endophytes [27]. The bark tissues of neem (*Azadirachta indica* A. Juss) was colonized predominantly by *Trichoderma*, *Penicillium* and *Pestalotiopsis* spp. [28]. *Crataeva magna* bark was colonized by mitosporic fungi represented by *Verticillium*, *Nigrospora oryzae* and *Fusarium verticilloides* [29]. The distribution and density of some endophytic taxa in bark segments was more compared to twigs.

In the present study, total species richness as a measure of number of genera per sample (bark, twig) was recorded. The distribution of some endophytic taxa and their density in bark segments was more compared to twigs of *A. hirsutus* and *V. indica*. Jaccard's similarity indices and Simpson and Shannon diversity were calculated for endophytes from bark and twig samples using the Shannon calculator. Simpson and Shannon diversity indices differed between different samples (Table 1). Endophytes from *A. hirsutus* bark and twigs showed the highest diversity index (Species richness: 9, 7; Simpson diversity: 0.111, 0.142; Shannon diversity: 2.197, 1.945 for bark and twig respectively). Similarly in *V. indica* the species richness was: 6, 4; Simpson diversity: 0.166, 0.250; Shannon diversity: 1.791, 1.386 for bark and twig respectively. The frequency of isolations of endophytes from bark greater than from twig could indicate their affinity for different tissue types and their capacity for utilizing or surviving within a specific substrate and/or identifying a conducive habitat for their adaptation and growth.

Endophytes, especially those from medicinal plants have become the focus of research for bioactive secondary metabolites with pharmaceutical applications. The reason for this is high diversity of endophytes, easy to apply statistics, easy to study and easy to scale up [30]. Endophytes appear to be a microbial factory for bioactive molecules with relatively untapped bioresources *viz*. pestacin and isopestacin from *P. microspora* [31], taxol from *Colletotrichum gloeosporioides* [24] and deacetylisowortmin and wortmannin isolated from *Trichoderma* sp. [32] with potent antioxidant, anti-cancer and anti-microbial activities respectively.

CONCLUSION

Explorations on the fungal endophytes associated with tropical evergreen trees are confined. When a plant species disappears, so too does its entire suite of associated endophytes [12] (Strobel and Daisy, 2003). The conservation of plant hosts and their indigenous microbial flora is thus of vital importance in the future search for new drugs. Endophyte species could be unique to a particular host requiring their documentation and securing the information. It could help in establishing information on the available biodiversity and at the same time could add to the national collections of microorganisms from these areas *viz*. Western Ghats. We are currently pursuing fermentation of these endophytes to get a wide array of secondary metabolites to facilitate screening against therapeutic targets.

ACKNOWLEDGEMENTS

The authors acknowledge the recognition of University of Mysore as Institution of Excellence and financial support from Ministry of Human Resource Development (HRD) and the University Grants Commission, New Delhi. RK and SS acknowledge the receipt of Project fellowship and Post Doctoral fellowship from this grant.

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