EurAsian Journal of BioSciences
Eurasia J Biosci 5, 48-53 (2011)
DOI:10.5053/ejobios.2011.5.0.6

Research Note

Isolation of endophytic fungi from *Coscinium fenestratum* -a red listed endangered medicinal plant

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Abstract
Enumeration of the endophytic fungi from the red listed, critically endangered medicinal plant, *Coscinium fenestratum* was investigated for the first time. The ubiquitous presence of 41 endophytic fungi belonging to sixteen different taxa was identified from 195 samples of healthy leaves and stem using traditional morphological methods. The overall colonization rate of endophytes in both the leaf and the stem was found to be 21.02%.The stem showed low percentage frequency of colonization of the endophytic fungi when compared to leaf segments. Among the endophytic flora, *Phomopsis jacquiniana* was found to be the core-group fungus with a colonization frequency of 4.6%.
Keywords: Berberine, biodiversity, *Coscinium fenestratum*, endophytic fungi, *Phomopsis jacquiniana*.

Goveas SW, Madtha R, Nivas SK, D'Souza L (2011) Isolation of endophytic fungi from *Coscinium fenestratum* -a red listed endangered medicinal plant. Eurasia J Biosci 5: 48-53. DOI:10.5053/ejobios.2011.5.0.6

INTRODUCTION

Coscinium fenestratum (Gaertn.) Colebr. is a dioecious woody liana belonging to the family Menispermaceae. The 1997 IUCN red list of threatened plants recorded the status of *C. fenestratum* as highly endangered in India (Indian Institute of Forest Management 2001),vulnerable in Vietnam, rare in Singapore and indeterminate in Sri Lanka (Schippmann 1997, 2000). It is found in India, Malaysia, Vietnam, Myanmar, Singapore, Thailand and Sri Lanka. In India the distribution of the plant is restricted to isolated regions of the Western Ghats. The plant takes around fifteen years to mature and flower (Remashree et al. 2005). It is commonly known as tree turmeric (Tushar et al. 2008), The liana is facing a population reduction of 80% over a period of three generations, and is on the verge of extinction (Ravikumar and Ved 2000) since it is overexploited for its medicinal importance (Kumar et al. 2007, Ali et al. 2008).

The active chemical secreted in the plant is reported to be berberine, a natural isoquinoline alkaloid having wide variety of pharmacological activities (Piyanuch et al. 2007). Berberine is present in both vegetative and reproductive parts, indicating the secretion of berberine in all parts of the plant. The plant extracts act as an antimicrobial agent (Kumar et al. 2007), an antioxidant and is used in the ayurveda and siddha medicine (Punitha et al. 2005).

Endophytes are microorganisms living in the internal tissues of the plants without causing any overt symptoms (Stone et al. 2000). Hawksworth and Rossman estimated that nearly one million species of endophytes may exist in the unexplored plants (Strobel and Daisy 2003, Arnold 2005). Ever since the discovery of the rich diversity of the endophytic fungi, their population dynamics, their role in improving plant growth, plant health (Hallmann et al. 2007), their distribution in the plant, the metabolites they secrete and their potency to produce novel compounds within the plants (Tan and Zou 2001), have formed an important aspect of present day research.

The isolation of novel secondary meta-

Received: May, 2011
Accepted: July, 2011
Printed: August 2011

bolites from the endophytes is a progressive field in research. The endophytic fungi isolated from the traditional Chinese medicinal plants contained diverse bioactive compounds which were used for therapeutic purposes (Kumar and Hyde 2004, Huang et al. 2008). The isolation of endophytic fungi from the plant in *C. fenestratum* is the first such initiative and has not been reported earlier. The present study was carried out to determine the endophytic mycoflora in *C. fenestratum*, a widely used medicinal plant.

MATERIAL AND METHODS

Collection of plant material

The stem and the leaf of plant *Coscinium fenestratum* (Gaertn.) Colebr. (Menispermaceae) obtained from the foot hills of Western Ghats of Karnataka in the month of April-May (2010) was used for the investigation of endophytic fungal communities. The harvested samples were mature photosynthetic leaves, stem, randomly chosen from different parts of the plant present in the same vicinity. The leaves and the stem were 3-4 foot above the ground. The samples were brought to the laboratory in sterile bags and processed immediately to reduce the risk of contamination.

Isolation of endophytic fungi

Isolation of endophytic fungi was standardized and modified based on the method described by Hallman et al. (2007).The samples were washed with running tap water to remove dust and debris adhering to them and surface sterilized with 70% ethyl alcohol for one minute. Surface sterilization to remove the adhering microorganisms was done by immersion in 4% sodium hypochlorite (commercially available) solution for three minutes. They were then rinsed with 70% ethyl alcohol for a minute. They were finally rinsed with deionized sterile distilled water to remove the sterilents and blot dried on sterile tissue paper. The stem was cut into explants 1x1 mm in size and the leaves were cut into 5-10 x 5-10 mm^2 size with and without the midrib under aseptic conditions using a sterile scalpel. Five sterilized stem/leaf explants were cultured in Petri dishes containing potato dextrose agar medium (PDA) supplemented with 100 μg/mL of streptomycin. The Petri dishes were sealed with parafilm and incubated at $27 \pm 2^{\circ}C$ for 15 days under dark conditions and monitored every day. Fungi growing out of the plant explants were subcultured on separate PDA plates at room temperature and identified in their sporulation state by staining with lactophenol blue.The fungi which failed to sporulate were designated as "mycelia sterilia". For study the colony characteristic the mycelia were transferred onto three different media, PDA, SDA and MRBA agar media. The classification of the fungi is adopted from Global Biodiversity Information Facility (GBIF).

Analysis of results

The Colonization Frequency (CF) percentage and the dominant fungi percentage of the endophytyic fungi was calculated using the method of Petrini and Fisher (1988) (Kumar and Hyde 2004):

$$\text{Colonization frequency } \% = \frac{\text{No. of segments colonized by an endophyte}}{\text{Total no. of segments analyzed}} \times 100$$

$$\text{Dominant fungi } \% = \frac{\text{No. of isolates collected from the samples}}{\text{Total no. of leaf/stem samples}} \times 100$$

RESULTS

A total of 41endophytic fungi were isolated from 195 samples of both leaves and stem of *C. fenestratum* (Table 1). The overall colonization rate of endophytes in both the leaf and the stem was found to be 21.4%. Sixteen different taxa of fungi were isolated from the *Coscinium fenestratum* plant (Table 2). Most of the fungi belonged to Ascomycetes. The fungus *Phomopsis jacquiniana* was found to be the core-group fungus with the colonization frequency of 4.6%. The leaves of different maturity showed different rates of colonization; young leaves had 3.33%, mature leaves had 21.02% and old leaves had 53.33% of colonization. The result shows that there was higher colonization by endophytic fungi with increasing age of the leaves. The stem

however showed low percentage frequency of colonization of the endophytic fungi. The sterile mycelia isolated were of three types with a total colonization frequency of 6.8%.

The identified species of the endophytic fungi mainly belong to Ascomycota. The isolated genera of endophytes are; *Phomopsis jacquiniana, Alternaria alternata, Aspergillus tamarii, Aspergillus fumigatus, Drechslera, Fusicladiella, Penicillium senticosam, Cladosporium cladosporioides, Asperisoprium caricae, Staphylotrichum cocosporium, Nigrospora oryzae, Mycelia sterilia, Gliocladium roseum,* and *Cladosporium* sp.

Characterization of endophytic fungi

i) *Aspergillus tamarii*: The colonies were white in colour turning yellowish with serial yellow globose heads, prominently seen within three days. The average colony growth was 1.4 cm per day. The length of the conidia was 51.5 μm, and 46 μm in diameter. The hypae were 15 μm in thickness. The spores were 2.91 μm in diameter (Fig. 1a).

ii) *Aspergillus fumigatus*: The colony was pulvinate in appearance, yellow at the centre, with light yellow radial rays and white colour edges. The diameter of the colony was 1.05 cm/day. The conidiophores were 500-530 μm in length and 7.5 μm in width. The conidial heads were columnar, compact, about 15 μm in length and 13 μm in breadth from the line of the phyllade heads. The vesicles were flask shaped aseptate in nature (Fig.1b).

iii) *Gliocladium roseum*: The centre of the colony was green in colour with radial rays from the centre and white edged margins. The diameter of the colony was 0.5 cm/day. The diameter of the spore was 6.2 μm, the phyllade was 1.0 μm in length, the metullae were 1.25 μm in length and the hypae were 0.25 μm in width. The phyllades of the conediophore group together and the spores form rosette like structure. The colour of the colony turns to powdery green on maturity (Fig.1c).

iv) *Penicillium senticosam*: Mycelium with grey colour at the centre and whitish edges. The colony secretes reddish orange colour into the media, and concentric grey colouration, each of the rings having different gradations of colour. Oil droplets are formed on the fifth day of the inoculation. The colony turned saffron-yellow in colour after six to seven days of inoculation. The hypae is septate, smooth walled; the length of the phyllades was 11.15 μm and the breadth 1.81 μm. The spores were circular in chains with length of each spore being 3.27 μm and breadth 1.86 μm (Fig.1d).

v) *Alternaria alternata*: White colour fuzzy mycelium with pulvinate appearance and a crater at the centre, with dark brown rings alternating with light brown ones with the white mycelium at the periphery at maturity. The colony diameter is observed as 1.33 cm/day. The conidiophores of these fungi are pale brown in nature. The length of the spore is 31.875 μm and the breadth at the broadest end is 8.125 μm. The conidiophores arise from substrate. The secondary conidia are shorter than the primary conidia (Fig.1e).

vi) *Nigrospora oryzae*: The colony is black in colour with sporulating dark conidia seen in the PDA media. The spores were ovoid in nature. The length of the spore was 12.5 μm

Table 1. Endophytic fungi isolated from different parts of *C. fenestratum*.

Site of isolation	Number of samples	No of fungi isolated	Frequency of colonization
Leaf	30	1	3.33%
	45	11	24.44%
	45	24	53.33%
Stem	75	5	11.11%
Total number	195	41	21.02

Table 2. Frequency of endophytic fungi isolated from the leaf and stem of *C. fenestratum*.

Endophytic fungi	Number of isolates	Frequency of colonization (%)		Dominant fungi (%)
		Leaf*	Stem*	
Phomopsis jacquiniana	9	4.6	-	21.95
Alternaria alternata	2	1.0	-	4.87
Aspergillus tamarii	1	0.5	-	2.43
Aspergillus fumigatus	1	0.5	-	2.43
Drechslera sp.	1	0.5	-	2.43
Fusicladiella sp.	5	2.6	-	12.19
Penicillium senticosum	1	0.5	-	2.43
Cladosporium cladosporioides	1	0.5	-	2.43
Asperisporium sp.	2	1.0	-	4.87
Staphylotrichum coccosporum	3	1.5	-	7.31
Nigrospora oryzae	2	1.5	-	4.87
Mycelia sterilia (white)	1	0.5	-	2.43
Grey mycelia	1	0.5	-	2.43
Brown mycelia	1	0.5	-	2.43
Gliocladium roseum	4		2.1	9.75

*195 segments were plated for frequency analysis.
The colonization of each fungus was calculated based on the number of segments colonized by a fungus over the total number of segments assessed and represented as percentage.

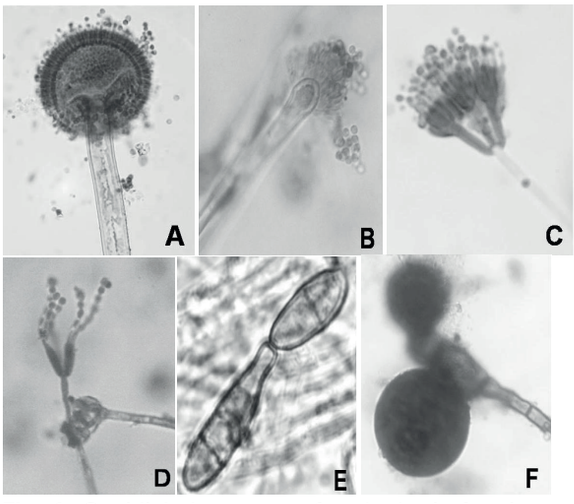

Fig. 1. Conidial characters of endophytic fungi isolated from the leaf and stem segments of *C. fenestratum*.
A-*Aspergillus tamari*, B-*Aspergillus fumigatus*, C-*Gliocladium* sp., D-*Penicillium* sp., E-*Alternaria alternata*, F-*Nigrospora oryzae*.

and the breadth was 13.75 μm (Fig.1f).

vii) *Phomopsis jacquiniana*: This is a slow growing, sporulating fungi. The colony looked dark in appearance with mycelia being immersed, branched, septate and brown in colour. The picnidia are formed at the top of the mycelial mat, were globose in nature. The mycelium secreted a black pigment on to the medium. The reverse side of the colonies was black in colour. The length of the ascocarp was 200 μm and the breadth 180 μm. The spore length was 10 μm and breadth was 5 μm.

DISCUSSION

Most of the isolated fungi belonged to anamorphic fungi belonging to Ascomycetes. The colonization of the endophytic fungi is ubiquitous yet selective in nature. This selective colonization of the endophyte may lead to the production of special compounds within the host plant (Huang et al. 2008). Fungi have been widely known as a source of bioactive compounds. An excellent example for this is the anticancer drug taxol, which was previously supposed to occur only in the plant tissues (Strobel and Daisy 2003).

C. fenestratum is a plant having a broad spectrum of medicinal properties (Punitha et al. 2005). Every part of the plant is used in one or the other types of medicines. The high demand for and the slow growth of this plant makes the plant vulnerable to poaching and the destruction of its habitat. The investigation of the endophytic fungi in various Chinese medicinal plants as a source of bioactive compound (Huang et al. 2008) which once were attributed only to the plant will aid the search for bioactive compounds from the endophytic fungi isolated from this plant.

Isolation of only sixteen taxa of endophytic fungi shows that the medicinal property of the plant has some role to play in the colonization of endophytic fungi. This low rate of colonization may be attributed to the secretion of the phyto-chemicals, since they contain certain antifungal and antibacterial components (Rajgopal et al. 2010). Out of the sixteen taxa three were mycelia sterilia with 6.8% of colonization. Endophytic fungi from tropical plants have recently gained importance in biological control of plant diseases and also as a source of pharmacologically active compounds. Only a few plant species have been investigated for their endophytic fungal population (Strobel and Daisy 2003). Therefore, any information and/or research on endophyte-plant symbiosis, such as in this study is of value. Effective extracts could provide potential leads towards the development of novel and environmental friendly biologically active agents.

ACKNOWLEDGEMENTS

The authors are thankful to Department of Biotechnology for funding through major research project and Mangalore Jesuit Educational Society for the financial assistance and to Dr. Karamchand Kishore for helping in the identification of the fungi.

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Kirmizi Listedeki Tibbi Bir Bitki Olan *Coscinium fenestratum*'dan Endofitik Mantarlarin Izolasyonu

Özet

Kirmizi listede olan ve nesli kritik derecede tükenme tehlikesi altinda bulunan tibbi bitki *Coscinium fenestratum*'da endofitik mantar sayimi ilk kez arastirildi. Geleneksel morfolojik metodlar kullanilarak, 195 saglikli yaprak ve gövde örneginde, 16 farkli taksona ait 41 endofitik mantarin yaygin olarak varligi tespit edildi. Endofitlerin yaprak ve gövdedeki genel kolonizasyon orani %21.02 olarak bulundu. Yaprak kisimlariyla karsilastirildiginda, endofitik mantarlarin gövdede, yüzde olarak, düsük kolonizasyon sikligi sergiledigi görüldü. Endofitik flora içinde, *Phomopsis jacquiniana*'nin %4.6 kolonizasyon sikligi ile çekirdek-grup mantar oldugu bulundu.

Anahtar Kelimeler: Berberin, biyolojik çesitlilik, *Coscinium fenestratum*, endofitik mantarlar, *Phomopsis jacquiniana*.

