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Fungal endophytes from the three-leaved caper, *Crataeva magna* (Lour.) DC. (Capparidaceae)

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Abstract

Fungal endophytes were isolated from *Crataeva magna*, a medicinal plant growing along the streams and rivers, constituting riparian vegetation in Karnataka, southern India. Fresh bark and twig pieces were used for the isolation using standard methods. Ninety-six endophytic fungal isolates were isolated from 800 bark and twig segments. Mitosporic fungi represented as a major group (85%) followed by zygomycetes (10%) and ascomycetes (5%). Bark samples contained more endophytes than twig samples. *Verticillium, Nigrospora oryzae* and *Fusarium verticilloides* were the dominant fungal endophytes.

Key words: bark, *Crataeva magna*, fungal endophytes, medicinal plants, twig

Crataeva magna (Lour.) DC., commonly called three-leaved caper is a member of the family Capparidaceae. The plant is distributed along the riparian vegetation of India, Burma, Malaysia, China and Nepal. The bark and root extracts have been used to cure cough, obesity, blood disorders, rheumatoid arthritis and heart diseases [2]. In recent years, the quest for the isolation of new compounds from medicinal plants has become a fascinating area of research. Plants with ethnopharmaceutical importance are being exploited because of their healing properties. However, large scale harvesting of medicinal plants has already become a major threat to biodiversity. As an alternative, microbes which live inside such plants (endophytes) may offer tremendous potential sources of therapeutic compounds.

'Endophytes' are the microbes residing in the plant host tissues without causing any overt symptoms [8]. Biologically and ecologically, they represent diverse for nutritional requirements ranging from biotrophic parasites to facultative saprotrophs. They also represent a large reservoir of unexplored genetic diversity. They have been isolated from monocots, grasses [6], palms [7, 16, 23] rainforest plants [13] and few tropical plants [5, 14, 19–22]. The practical applications of these endophytes are manifold; as potential biocontrol agents, sources of novel metabolites for therapeutics, plant protection, other industrial applications, and as model systems for studying the host–parasite interactions in natural ecosystems [18].

Virtually very few reports are available on the association of endophytic fungi from tropical medicinal tree species. Therefore, this study provides first information on the isolation of fungal endophytes from *C. magna*. We are currently pursuing fermentation of these microbes to obtain the secondary metabolites to facilitate screening against cardiovascular targets.

Bark and twig samples of *C. magna* were collected from healthy trees inhabiting the riparian vegetation of Nanjangud, (12°07′N latitude and 76°44′E longitude) and Paschimavahini, Srirangapatna (12°25′N latitude and 76°40′E longitude), Karnataka during the monsoon season

of 2003 (July to September). Bark pieces (5.0 × 5.0 cm) from the trunk were cut 1.5 m above the ground level with the help of sterile machete. Twigs were sampled from the lower branches. The samples were placed in polyethylene bags, labelled, transported in ice box to the laboratory and placed in a refrigerator at 4 °C. All samples were processed within 24 h of collection.

Bark samples were halved, first immersed in 70% ethanol (v/v) for 1 min followed by second immersion in sodium hypochlorite (3.5%, v/v) for 3 min. The samples were rinsed three times in sterile distilled water and dried on sterile blotters under the airflow to ensure complete drying. Bits of 1.0 × 0.1 cm size were excised with the help of a sterile blade. Eight hundred bits from bark and twigs were plated on water agar (15 g agar/l) supplemented with the antibiotic streptomycin sulphate (100 mg/l). Ten segments were plated per plate. The plates were wrapped in Clean Wrap^{TM} cling film and incubated at 22 °C with a 12 h light and dark cycles for up to 6 weeks. Periodically the colonies were examined and each colony that emerged from segments were transferred to antibiotic-free potato dextrose agar medium (PDA) to aid identification. The fungus that did not sporulate was inoculated onto sterilized banana leaf bits (1 cm^2) impregnated on agar to ensure sporulation. The fungal identification was done based on the colony morphology and conidial characters. All the fungal isolates have been catalogued as DB# series and maintained at the culture collection of the department by cryopreservation on PDA overlaid with 15% glycerol (v/v) at −80 °C in a deep freezer.

The colonization frequency (CF), expressed as percentage was calculated according to Kumaresan and Suryanarayanan [9] as follows:

$$\%CF = \frac{\text{Number of tissue segments colonized by a fungus}}{\text{Total number of tissue segments plated}} \times 100$$

Eight hundred bark and twig segments from *C. magna* yielded 96 endophytic fungal isolates. The isolates comprised of 15 genera of mitosporic fungi (85%), three zygomycota (10%) and two ascomycota (5%). The bark and twig samples differed in their endophytic fungal colonization (Table 1).

The studies at two locations differed in their endophytic fungal composition as indicated in Table 1. The percentage of mitosporic fungi recovered from the bark samples collected from Nanjangud was higher (56%) than of Paschimavahini (8%). However, the frequency of occurrence of endophytes on twig samples was lower. The incidence of zygomycete and ascomycete endophytes isolated was 10% and 5% from the bark and twig, respectively, from samples collected from Paschimavahini. These groups were entirely absent in samples collected from Nanjangud. *Fusarium, Acremonium* and *Verticillium* spp. were the commonly encountered genera of mitosporic fungi. *Fusarium* spp. accounted for 32 of the 96 isolates (33.3%) followed by *Verticillium* spp. (15%). *Fusarium chlamydosporum, Hansfordia* spp. and *Gliocladium delequescens* were some of the endophytic fungi isolated from the bark or twigs (Figure 1). The recovery of fungal taxa was more from Nanjangud area samples (65%) than Paschimavahini (35%) area. Among the endophytic fungi, species of *Isariopsis, Tharoopama* and *Tieghemiomyces* are the new reports.

Many plant species representing grasses, palms, conifers, pines, ferns, mosses and lichens have been studied world wide for the presence of endophytic fungi [18]. To date, very few medicinal tree species have been screened for their endophytic fungi. In our study, fungal endophytes were isolated from the bark and twigs of *C. magna*, the bark constituents of which is extensively used to cure various disorders viz., rheumatoid arthritis and heart diseases [2]. As the bark is attributed in healing of various disorders, an attempt was made to isolate the endophytic fungi residing in the bark by employing stringent surface sterilization techniques. Our studies yielded mitosporic fungi as the major group of endophytic fungi. They out-numbered other groups of fungi such as zygomycetes and ascomycetes. Mitosporic fungal isolations as endophytes are common among plants inhabiting temperate, tropical and rainforest vegetations [1]. A study of endophytic fungal assemblage in mangrove vegetations of coastal Karnataka and Picchavaram, Pondicherry showed that mitosporic fungal isolations were more than the ascomycetes [10, 19]. Some of the more frequently isolated taxa like *Fusarium*, *Acremonium* and *Verticillium* spp. are reported as endophytes of other host plants [3, 15].

In general, the colonization frequency of the endophytes was low in *C. magna* (Table 1). Only, *Verticillium* spp. (2.4), *Myrothecium verrucaria*

Table 1. Frequency of endophytic fungi isolated from bark and twigs of *Crataeva magna*

Endophytic fungi	Frequency of colonization from two locations (%CF)			
	BARK*		TWIG*	
	Nanjangud	Paschimavahini	Nanjangud	Paschimavahini
Mitosporic fungi				
A. strictum	0.6	–	–	–
Acremonium acremonium	0.6	–	–	
Aposphaeria spp.	0.6	–	–	–
Arthrobotrys conoides	0.2	–	–	–
Drechler				
Ascochyta rabei	0.2	–	–	–
Dithiorella spp.	0.2	–	–	
F. chlamydosporum	0.4	–	–	0.2
F. graminearum	0.6	–	–	–
F. oxysporum	0.6		–	–
Fusarium oxysporum var. subglutinans	0.8	–	–	–
F. solani	–	1.0	1.0	–
F. verticilloides	–	–	1.8	–
Gliocladium delequescens	–	0.4	–	–
Hansfordia spp.	0.8	–	–	
Isariopsis spp.	–	–	–	0.2
Myrothecium verrucaria	–	–	–	1.0
Nigrospora oryzae	–	–	–	0.4
Sporothrix spp.	0.4	–	0.6	–
Tharoopama trina	0.6	–	–	–
V. albo–atrum	1.0	–	–	0.4
Verticillium spp.	1.4	–	–	0.2
Zygomycetes	–			–
Absidia spp.	–	–	–	0.6
Cochlonema verrucosum	–	0.2	–	–
Tieghemiomyces spp.	–	–	–	0.4
Ascomycetes				–
Chaetomium globosum	–	0.8	–	0.4
Sclerotinia spp.	–	–	–	0.4
No. of isolates	45	12	17	22

* 200 segments were plated for frequency analysis.
Fungal endophytes were isolated from fresh bark and twig samples of *C. magna* collected from southern India on 1.5% water agar. The colonization frequency of each fungus was calculated based the number of segments colonized by a fungus over the total number of segments assessed and represented as percentage.

(1.0) and *F. verticilloides* (1.8) were isolated in greater numbers as indicated by their %CF. The occurrence of some coprophilous fungi as an endophyte is rather intriguing, but not uncommon [11]. *Chaetomium globosum* isolated as an endophyte is reported to be coprophilous. The frequency of isolations from bark was greater than of twig as some of the fungi were found to be specific for the bark. Since the trees were sampled from the riparian vegetation, the conditions of such a habitat might have suited the adaptation of fungi to such environment. Such vegetation represents natural ecosystem where disease epidemics are rare.

So far, only few publications had reported the isolation of fungal endophytes from bark of tree species [4, 17]. The Taxol®-producing fungus *Taxomyces andreanae* was isolated from the bark of Pacific yew, *Taxus brevifolia* [17]. Bills and Polishook [4] isolated 69 fungal species from the bark of a single *Carpinus caroliniana* tree, which

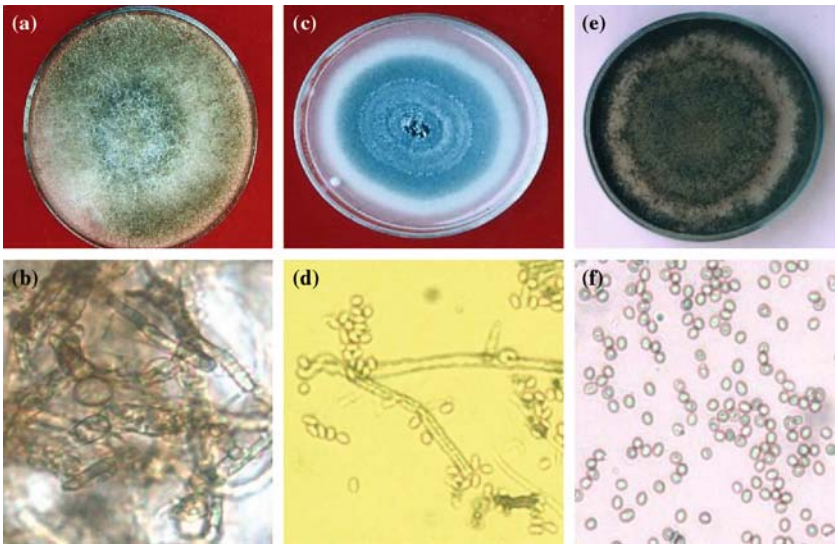

Figure 1. Colony morphology and conidial characters of endophytic fungi isolated from the bark and twigs of *Crataeva magna*. (a, b) Floccose, ochre mycelia of *Fusarium chlamydosporum* Wr. & Rg. with globose, intercalary chlamydospores (c, d) Grey-pigmented mycelia of *Hansfordia* spp. bearing globose sympodulospores (e, f) Blackish green fruiting colony of *Gliocladium delequescens* Sopp bearing greenish, elliptical conidia.

suggested the enormous extent of fungal diversity associated within a single plant. The exploration of woody perennials for organisms that might produce microbial metabolites for use as therapeutic agents needs much attention as it necessitates careful identification and selection of species unique to a particular host before the screening of metabolites for desired industrial applications.

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