Endophytic mycoflora of inner bark of *Azadirachta indica* A. Juss

Endophytes are microbes that colonize the living internal tissues of plants without causing any immediate overt negative effects[1]. They are a largely unexplored component of biodiversity, especially in the tropics. Endophytic fungi have been isolated from leaves, stems and roots of woody plants in the temperate regions and the tropics[2–4]. They have a protective role against insect herbivory and many are potential producers of novel antimicrobial secondary metabolites[5]. Endophytes are constantly exposed to intergeneric–genetic exchange with the host plant. Isolation of a potent anticancer agent, taxol from *Pestalotiopsis microspora*, an endophyte of the yew tree and the phytohormone-producing fungus from rice plant, *Gibberella fujikuroi* suggests the potential of endophytes as a source of useful metabolites[6,7].

The current study was carried out to isolate and identify fungal endophytes from living symptomless inner bark tissues of neem (*Azadirachta indica* A. Juss), which is an indigenous medicinal plant in India and Africa. Neem is an evergreen tree of the tropics and sub-tropics belonging to the family Meliaceae. It is widely used in Indian traditional medicine for various therapeutic purposes as well as the source of agrochemicals for many centuries. The bark extract has been scientifically investigated from the past two decades for anti-bacterial, antipyretic[8,9], anti-inflammatory[10] effects and against skin diseases such as eczema, burns, ulcers, herpes, etc.[11]. Based on the recent claims that endophytic microbes may play a key role in therapeutic properties of plants, we postulate that the healing properties may be due to the secretion of metabolites from the endophytes residing in the bark.

Bark samples from a neem tree growing in Mysore were obtained by cutting the tree bark at 1.5 m above the ground level and 1–1.5 cm depth with ethanol-disinfected machete. Approximately 5×5 cm bark pieces were taken for the study. The samples were processed within 24 h of collection. Surface sterilization of bark sample was done by immersing the bark pieces in 70% (v/v) ethyl alcohol for 1 min and 3.5% (v/v) sodium hypochlorite for 2 min and rinsed three times in sterile distilled water for 1 min[12]. Excess water was blotted in an airflow chamber. The outer bark was removed and the inner portion containing the cortex was carefully dissected into bits (1.0×0.2 cm). 200 segments were plated on water agar medium (15 g l^{-1}) amended with streptomycin (100 mg l^{-1}) and incubated in a chamber for 21days at 12 h light/dark cycles at 22°C[13]. The plates were monitored regularly for the growth of endophytic fungi. The hyphal tips that grew on surface-sterilized bark pieces were isolated onto potato dextrose agar (PDA). Each fungus was assigned a number and stored at 4°C. Endophytic fungal strains were identified based on morphological characters using standard identification manuals. All the endophytic isolates were documented, maintained in cryovials on PDA layered with 15% glycerol (v/v) and stored in –80°C freezer (Cryo Scientific Pvt Ltd, Chennai) at the Department of Applied Botany and Biotechnology, University of Mysore.

The per cent frequency of occurrence[14] was calculated as the number of bark segments colonized by a specific fungus divided by total number of segments plated $\times$ 100 and dominant endophytes[15] were calculated as percentage colony frequency divided by sum of percentage of colony frequency of all endophytes $\times$ 100.

A total of 77 endophytic fungal isolates belonging to 15 genera were isolated from the inner bark of *A. indica*. The colonization frequency was 38.5% (Table 1). The fungal composition included 71.4% of hyphomycetes, 18.2% of coelomycetes, 6.5% of ascomycetes and 3.9% of sterile mycelia.

In the tropics, only a few studies have been carried out on endophytes of tree species[16]. Rajagopal and Suryanarayanan[17] have investigated the endophytic fungi in the leaves of *A. indica*. These studies have shown the effect of leaf tissue type, site and seasonality on endophyte assemblages and colonization. They recorded only *Fusarium* spp. and some sterile fungi. We have recovered endophytic genera like

Table 1. Endophytic fungi isolated from inner bark of neem (*Azadirachta indica*)

Endophytic fungi	No. of endophytes	Colonization frequency*	Dominant fungi
Ascomycetes			
Chaetomium crispatum	1	0.5	1.3
Chaetomium globosum	4	2.0	5.1
Coelomycetes			
Pestalotiopsis spp.	12	6.0	15.5
Phoma eupyrena	1	0.5	1.3
Phyllosticta spp.	1	0.5	1.3
Hyphomycetes			
Acremonium acremonium	1	0.5	1.3
Aspergillus flavus	4	2.0	5.1
Aspergillus niger	5	2.5	6.4
Aspergillus oryzae	1	0.5	1.3
Cladosporium acaciicola	1	0.5	1.3
Cladosporium cladosporioides	3	1.5	3.9
Cochlonema verrucosum	1	0.5	1.3
Curvularia lunata	1	0.5	1.3
Fusarium clamydosporum	1	0.5	1.3
Fusarium moniliformae var. *subglutinans*	2	1.0	2.6
Fusarium oxysporum	1	0.5	1.3
Fusarium solani	2	1.0	2.6
Gliomastix spp.	1	0.5	1.3
Nigrospora oryzae	2	1.0	2.6
Penicillium spp.	9	4.5	11.6
Trichoderma spp.	18	9.0	23.3
Verticillium albo-atrum	2	1.0	2.6
Sterile mycelia	3	1.5	3.9
No. of isolates	77	38.5%	

*Based on the 200 segments plated.

Curvularia, *Cochlonema*, *Gliomastix* and *Verticillium* spp., which are reported as endophytes. *Trichoderma*, *Penicillium* and *Pestalotiopsis* spp. were the most dominant endophytes isolated in this study. Endophytic genera such as *Phomopsis*, *Phyllosticta* and *Xylaria* are commonly isolated from tropical and temperate regions[12]. Some species of *Fusarium* are pathogenic to crops, since some phytopathogenic fungus can be modified by mutation to grow as a non-pathogenic endophyte[18].

Suresh *et al.*[19] reported the presence of limonoids in the leaf of neem as antifungal and perhaps this is the reason for a low score of endophytes, as reported by Rajagopal and Suryanarayanan[17]. The occurrence of endophytes seems to be influenced by seasonal variation[20]. The occurrence of fungal endophytes is mainly influenced by environment and type of host tissue[2]. Fungal species like *Trichoderma* are reported to have growth-promoting activity when cultivated with rice seedlings[21]. *Penicillium* spp. have been found to produce important antibiotics, which weaken or kill bacteria and other organisms that can cause disease. *Pestalotiopsis* spp. obtained as endophytes in the Himalayan yew (*Taxus wallichiana*) produce taxol, an important chemotherapeutic drug used in the treatment of breast and ovarian cancers[22]. We are currently pursuing fermentation of these microbes to obtain the secondary metabolites to facilitate screening against therapeutic targets as well as against economically important plant pathogens.

1. Bacon, C. W. and White, J. F., *Microbial Endophytes*, Marcel Dekker Inc., NY, 2000, pp. 3–27.
2. Rodrigues, K. F., *Mycologia*, 1994, **86**, 376–385.
3. Wilson, D. and Carroll, G. C., *Mycologia*, 1994, **86**, 635–647.
4. Fröhlich, J. and Hyde, K. D., *Biodivers. Conserv.*, 1999, **8**, 977–1004.
5. Arnold, A. E., Maynard, Z. and Gilbert, G. S., *Mycol. Res.*, 2001, **105**, 1502–1507.
6. Strobel, G. A. and Long, D. M., *ASM News*, 1998, **64**, 263–268.
7. Stierle, A., Strobel, G. and Stierle, D., *Science*, 1993, **260**, 214–216.
8. Okpanyi, S. N. and Ezeukwk, G. C., *Planta Med.*, 1981, **41**, 34–49.
9. Khattak, S. G., Gilani, S. N. and Ikaram, M., *J. Ethnopharmacol.*, 1985, **14**, 45–51.
10. Chattopadhyay, R. R., Chattopadhyay, R. N., Nandy, A. K., Podder, G. and Maitra, S. K., *Bull. Calcutta School Trop. Med.*, 1987, **35**, 6–8.
11. www.eco-logic-systems.com/Arishtia/Specs/ Neem foundation E1.pdf supplemented result.
12. Petrini, O., *Microbiology of the Phyllosphere* (eds Fokkema, N. J. and van den Huevel, J.), Cambridge University Press, Cambridge, 1986, pp. 175–187.
13. Suryanarayanan, T. S., *The Mycologist*, 1992, **6**, 144.
14. Fisher, P. J. and Petrini, O., *Trans. Br. Mycol. Soc.*, 1987, **89**, 246–249.
15. Kumaresan, V. and Suryanarayanan, T. S., *Fungal Divers.*, 2002, **9**, 81–91.
16. Fröhlich, J. and Hyde., K. D., *Palm Microfungi*, Fungal Diversity Research Series: 3, Fungal Diversity Press, Hong Kong, 2000, p. 393.
17. Rajagopal, K. and Suryanarayanan, T. S., *Curr. Sci.*, 2000, **78**, 1375–1378.
18. Freeman, S. and Rodriguez, R. J., *Science*, 1993, **260**, 75–78.
19. Suresh, G., Narasimhan, N. S., Masilamani, S., Partho, P. D. and Geetha, G., *Phytoparasitica*, 1997, **25**, 33–39.
20. Halmschlager, E., Butin, H. and Donaubauer, E., *Eur. J. For. Pathol.*, 1993, **23**, 51–63.
21. Mishra, R. C., Singh, R., Singh, H. B. and Dikshit, A., *Trop. Agric.*, 2000, **77**, 205–206.
22. Metz, A. M., Haddad, A., Worapong, J., Long, D. M., Ford, E. J., Hess, W. M. and Strobel, G. A., *Microbiology*, 2000, **146**, 2079–2089.

ACKNOWLEDGEMENTS. We thank the Department of Biotechnology, New Delhi for financial support. We thank Dr S. Lokesh, Department of Applied Botany, University of Mysore, for help in identifying fungal isolates.

Received 20 July 2004; revised accepted 30 September 2004

B. MAHESH[1]
M. V. TEJESVI[1]
M. S. NALINI[1]
H. S. PRAKASH[1,*]
K. R. KINI[1]
VEN SUBBIAH[2]
H. S. SHETTY[1]

[1]*Department of Studies in Applied Botany and Biotechnology, University of Mysore, Manasagangotri, Mysore 570 006, India*
[2]*PhytoMyco Research Private Limited, KIADB Industrial Park, Nanjangud 571 302, India*
**For correspondence.*
e-mail: legume@sancharnet.in