

# Distribution of Endophytic Fungi in Leaves of *Azadirachta indica* A. JUSS. (Neem) of Panchmarhi Biosphere Reserve

R.K. Tenguria<sup>1</sup> and Firoz Naem Khan<sup>2\*</sup>

<sup>1</sup>Division of Microbiology, Department of Botany, Govt. Motilal Vigyan Mahavidhyalaya, Bhopal 462008, M.P. (INDIA)

<sup>2</sup>Immunology Laboratory, Centre for Scientific Research and Development, People's Group, Bhanpur, Bhopal 462037, M.P. (INDIA)

Article Info	Abstract
<b>Article History</b> Received : 17-02-2011 Revised : 25-03-2011 Accepted : 29-03-2011	Total 85 endophytic fungi of 10 genera were isolated from 200 segments of fresh <i>Azadirachta indica</i> leaves collected from Panchmarhi biosphere reserve. The endophytic fungi recovered belong to hyphomycetes (68.2%), coelomycetes (19.99%), ascomycetes and sterile mycelium (5.88%) each. The most dominant endophytes observed were <i>Trichoderma</i> (21.17%), <i>Pestalotiopsis</i> spp. (16.47%) and <i>Penicillium</i> (15.29%).
<b>*Corresponding Author</b> Tel : +91-9303829899 Fax : -- Email: <a href="mailto:firoznaemkhan01@gmail.com">firoznaemkhan01@gmail.com</a> ©ScholarJournals, SSR-SILAE	<b>Key Words:</b> Endophytes, Endophytic fungi

## Introduction

*Azadirachta indica* A. Juss. (syn. *Melia indica* Brandis; *Melia azadirachta* Linn.) is an indigenous medicinal plant in India and Africa, commonly known as Neem (Hindi) and Indian lilac (English). Neem is an evergreen tree growing in tropical to subtropical regions, semiarid to wet tropical regions, and from sea level to about 700m elevation, belongs to the family *Meliaceae* (order *Rutales*) [1]. It is widely used in Indian traditional medicine for various restorative purposes as well as the source of agrochemicals for many centuries. Based on the recent claims that the metabolites secreted from the endophytes recovered from medicinal plants play a key role as therapeutics, extracts has been scientifically investigated from the past two decades for anti-microbial, antipyretic anti-inflammatory effects and against malaria and cancer [2,3].

Endophytes are microbes that colonize the living internal tissues of plants without causing any immediate overt negative effects [4]. Fungal endophytes reside within the living tissues of higher plants without producing any apparent symptoms [5]. Although abundant, the extent of their contribution to fungal biodiversity remains unclear. Endophytic fungi had been previously isolated from leaves, stems and roots of a wide variety of plants in the temperate regions[6-8]. Where as, endophytic fungi are poorly known, especially in the tropics; therefore the current estimate of fungal species is probably conservative. Panchmarhi area was designated as Biosphere Reserve (BR) by Government of India vide notification no. J-220116/17/94-BR in March 1999. Panchmarhi BR lies in between latitude 22° 11' to 22° 56' N and 77° 47' to 78° 52' E longitude and falls under tropic region. It has a rich microbial diversity as of different climatic factors at various altitudes giving rise to well-off and luxuriant vegetation which is amongst the richest in Central India. The occurrence of biodiversity makes the area unique and the association of endophytic fungi with tropical plant species of this region has not yet been

explored[9]. Endophytes are constantly exposed to intergeneric-genetic exchange with the host plant. The practical applications of these endophytes are manifold; potent anticancer agent, taxol from *Pestalotiopsis microspora*, an endophyte of the yew tree (*Taxus brevifolia*) and anti-microbial agent Altersolanol A from *Phoma* sp. isolated from *Taxus wallichinia* suggests the potential of endophytes as a source of useful metabolites [10,11], as biocontrol agents in plant protection, sources of novel metabolites for therapeutics and as model systems for studying the host parasite interactions in natural ecosystems [12-14]. Therefore, the current study was carried out to isolate and identify fungal endophytes from leaf tissues of Neem (*Azadirachta indica* A. Juss) from Panchmarhi biosphere reserve.

## Materials and Methods

Leaf samples of Neem (*Azadirachta indica* A. Juss) were collected from the area of Panchmarhi Biosphere Reserve, during the monsoon of 2010 (July to September). The samples were placed in labeled sterile polyethylene bags and transported in ice box to the laboratory and placed in a refrigerator at 4°C. All samples were processed within 24 hours of collection. The collected leaves were washed thoroughly in running water and air dried. Leaf samples were 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 laminar airflow to ensure complete drying. 200 segments from leaf samples of 5x5 mm size were excised with the help of a sterile scalpel and the inner tissues are carefully placed on water agar plates. After several days of incubation, hyphal tips of the fungi are removed and transferred to Potato Dextrose Agar (PDA) supplemented with streptomycin (100 mg/l) to suppress bacterial growth. The efficacy of sterilization was confirmed by pressing the

sterilized leaf on to the surface of PDA medium. The absence of growth of any fungi on the medium confirms that the sterilization procedure was effective in removing the exogenous fungi [15,16]. The plates were incubated at  $25^{\circ}\text{C} \pm 1$  with 12 hours light and dark cycles for up to 6 weeks [17,18]. Periodically the colonies were examined and each colony that emerged was transferred to antibiotic-free Potato Dextrose Agar medium (PDA) for identification. Endophytic

isolates were identified on the basis of culture characteristics, morphology of fruiting body and spores.

The percent frequency of occurrence was calculated as the number of leaf segments colonized by a specific fungus divided by total number of segments plated x 100 and dominant endophytes were calculated as percentage colony frequency divided by sum of percentage of colony frequency of all endophytes x 100 [19,20].

## Results and Discussion

Table 1: Endophytic fungi isolated from leaf of Neem (*Azadirachta indica*)

Endophytic Fungi	No. of Endophytes	Colonization Frequency* (%)	Dominance (%)
Ascomycetes			
<i>Chaetomium globosum</i>	5	2.5	5.88
Coelomycetes			
<i>Pestalotiopsis</i> spp.	14	7.0	16.47
<i>Phoma</i> sp.	3	1.5	3.52
Hyphomycetes			
<i>Aspergillus flavus</i>	9	4.5	10.58
<i>Aspergillus niger</i>	8	4	9.41
<i>Alternaria alternata</i> (Fr.) Keissl.	6	3.0	7.05
<i>Fusarium</i> spp.	4	2.0	4.70
<i>Penicillium</i> spp.	13	6.5	15.29
<i>Trichoderma</i> spp.	18	9	21.17
Sterile mycelia	5	2.5	5.88
Total No. of isolates	85	42.5%	

\* Based on 200 segments for frequency analysis.

A total of 85 endophytic fungi were isolated from the leaf tissue of *A. indica* belonging to 10 genera. The colonization frequency was calculated 42.5% (Table 1). The fungal composition included 68.2% of hyphomycetes, 19.99% of coelomycetes, 5.88% each of ascomycetes and sterile mycelia. In the tropics, only a few studies have been carried out on endophytes of tree species [21]. In the previous endophytic fungi as *Fusarium* spp. and some sterile fungi had been recorded in the leaves of *A. indica*, and effect on endophyte assemblages and colonization by leaf tissue type, site and seasonality was shown [22]. We have recovered endophytic genera like *Alternaria* spp., *Fusarium* spp., *Phoma* sp. which are reported as endophytes. In this study *Trichoderma*, *Pestalotiopsis* spp. and *Penicillium* were the most dominant endophytes. *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 [23]. *Penicillium* spp. had been found to produce significant antibiotics, which deteriorate or exterminate bacteria and other organisms causing disease. But, the occurrence of endophytes seems to be influenced by environment and type of host tissue and is mainly influenced by seasonal variation [6,24]. Currently, we are pursuing fermentation of the endophytes recovered to obtain the secondary metabolites to facilitate screening against therapeutic targets.

### Acknowledgement

The authors are thankful to Shri. S.N. Vijaywargia and Capt. Ruchi Vijaywargia for providing laboratory facilities and Sarvajank Jankalyan Parmarthik Nyas, People's Group for

granting financial assistance to carry out the present research work.

### References

- [1] Okpanyi, S.N. and G.C. Ezeukwk. 1981. Anti-inflammatory and antipyretic activities of *Azadirachta indica*. *Planta Med.* 41: 34-49.
- [2] Chattopadhyay, R.R., R.N. Chattopadhyay, A.K. Nandy, G. Podder and S.K. Maitra. 1987. Anti-inflammatory activity of a fraction of fresh leaves of *Azadirachta indica* (Beng. Neem). *Bull. Calcutta Trop. Med.* 35: 6-8.
- [3] Wiyakrutta, S., N. Sriubolmas, W. Panphut, N. Thongon, K. Danwisetkanjana, N. Ruangrungrsri and V. Meevootisom. 2004. Endophytic fungi with anti-microbial, anti-cancer and anti-malarial activities isolated from Thai medicinal plants. *World J. Microbiol. Biotechnol.* 20: 265-72.
- [4] Bacon, C.W. and J.F. White. 2000. *Microbial Endophytes*, Marcel Dekker Inc., NY: pp 3-27.
- [5] Bills, G.F. 1996. Isolation and analysis of endophytic fungal communities from woody plants. In: Redlin, S. and L. M. Carris (Eds.), *Systematics, Ecology and Evolution of Endophytic Fungi in Grasses and Woody Plants*. APS Press, St. Paul, M.N.: pp 31-65.
- [6] Rodrigues, K.F. 1994. The foliar fungal endophytes of the Amazonian palm *Euterpe oleracea*. *Mycologia* 86: 376-85.
- [7] Wilson, D. and G.C. Carroll. 1994. Infection studies of *Discula quercina*, an endophyte of *Quercus garryana*. *Mycologia* 86: 635-47.
- [8] Fröhlich, J. and K.D. Hyde. 1999. Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic? *Biodivers. Conserv.* 8: 977-1004.

- [9] Jain, A.N. 2002. Conservation strategy for sustenance of threatened bioresources of Panchmarhi forest Division (M.P). Environment and Forests, Government of India, New Delhi. Environ. Plan. Coordination Org. (EPCO): 2(2): 1-66.
- [10] Stierle, A., G.A. Strobel and D. Stierle. 1993. Taxol and taxane production by *Taxomyces andreanae*. Science 260:214-16.
- [11] Yang, X., G.A. Strobel, A. Stierle and W.H. Hess. 1994. Fungal endophytes-tree relationship: *Phoma* sp. in *Taxus wallachiana*. Plant Sci. 102(1): 1-9.
- [12] Strobel, G.A., B. Daisy, U. Castillo, and J. Harper. 2004. Natural Products from Endophytic Microorganisms. J. Nat. Prod. 67(2): 257-68.
- [13] Deshmukh, S.K. and S.A. Verekar. 2009. Fungal Endophytes: A potential source of anticancer compounds In: Carpinella, M.C. and M.K. Rai (Eds.), Novel Therapeutic Agents from Plants: Progress and Future Perspectives. Science Publishers Inc., Enfield USA: pp175-206.
- [14] Deshmukh, S.K., P.D. Mishra, A. Kulkarni-Almeida, S. Verekar, M.R. Sahoo, G. Periyasamy, H. Goswami, A. Khanna, A. Balakrishnan and R. Vishwakarma. 2009. Anti-inflammatory and anticancer activity of ergoflavin isolated from an endophytic fungus. Chem. Biodiv. 6(5): 784-9.
- [15] Strobel, G.A. 2002. Microbial gifts from rain forests. Can. J. Plant Pathol. 24:14-20.
- [16] Schulz, B., C. Boyle, and S. Draeger. 2002. Endophytic fungi: a source of biologically active secondary metabolites. Mycol. Res. 106: 996-1004.
- [17] Suryanarayanan, T.S., T.S. Murali and G. Venkatesan. 2002. Occurrence and distribution of fungal endophytes in tropical forests across a rainfall gradient. Can. J. Bot. 80: 818-26.
- [18] Bills, G., A. Dombrowski, F. Pelaez, J. Polishook and Z. An. 2002. Recent and future discoveries of pharmacologically active metabolites from tropical fungi. In: Watling, R., J.C. Frankland, A.M. Ainsworth, S. Issac, C.H. Robinson (Eds), Tropical mycology: micromycetes, New York: CABI Publishing, 2:165-94.
- [19] Fisher, P.J. and O. Petrini. 1987. Location of fungal endophytes in tissues of *Suaeda fruticosa*: A preliminary study. Trans. Br. Mycol. Soc. 89(2): 246-49.
- [20] Kumaresan, V. and T.S. Suryanarayanan. 2002. Endophyte assemblages in young, mature and senescent leaves of *Rhizophora apiculata*: evidence for the role of endophytes in mangrove litter degradation. Fungal Divers. 9: 81-91.
- [21] Fröhlich, J. and Hyde, K.D. 2000. Palm Microfungi. Fungal Diversity Research Series: 3, Fungal Diversity Press: Hong Kong, pp 1- 393.
- [22] Rajagopal, K. and T.S. Suryanarayanan. 2000. Isolation of endophytic fungi from leaves of neem (*Azadirachta indica* A. Juss.). Curr. Sci. 78: 1375-78.
- [23] Strobel, G.A., A.M. Metz, A. Haddad, J. Worapong, D.M. Long, E.J. Fodr and Hess, W.M. 2000. Induction of the sexual stage of *Pestalotiopsis microspora*, a taxol-producing fungus. Microbiol. U.K., 146: 2079-89.
- [24] Halmschlager, E., H. Butin and E. Donaubaue. 1993. Endophytische Pilze in Blättern und Zweigen von *Quercus petraea*. Eur. J. For. Pathol. 23: 51-63.