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A preliminary study on the diversity of endophytic fungi isolated from *Catharanthus roseus* (L.) G. Don.

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In the present investigation 341 endophytic fungal isolates representing 23 different endophytic species were obtained from a total 300 pieces of leaves, inner bark and stem of *Catharanthus roseus* growing in Pune and adjoining areas. Plant samples were collected from four different locations. The Hyphomycetes were found most dominant (49.26%) of the total isolates, followed by Coelomycetes (30.20%), and Ascomycetes (15.24%). Non sporulating isolates 5.27% were obtained which categorized as Mycelia sterilia. Endophytic colonization frequencies were greater in stem (34.60%) than leaf (32.84%) and bark (32.55%) samples. Locations A, Location B, Location C and Location D had yielded 26.39%, 25.80%, 26.97% and 20.82% respectively out of the total endophytic isolates. The bark samples from Loc B and Loc D had yielded equal number of endophytes (19). The dominant endophytic fungi obtained were *Colletotrichum gloeosporioides*, *Alternaria alternata*, *Chaetomium globosum*, *Nigrospora sphaerica*, *Cladosporum cladosporioides*, *C. tennuissimum*, and *Pestalotiopsis* sp. Genera like *Gonatobotryum* and *Scytalidium* were found as uncommon colonizers of the host tissues.

Key words: Endophytes, fungal diversity, India, medicinal plants

INTRODUCTION

The rising interest in biology of endophytic fungi has led to search for endophytic fungal strains from the medicinal host plants, which may have potential to produce bioactive metabolites from parent plants and such fungi could become an alternative sources for these metabolites (Stierle *et al*, 1993; Monaghan *et al.*, 1995; Rodrigues *et.al.*, 2000) Most recently, Gangadevi and Muthumary (2007) have isolated taxol from the organic extract of six endophytic cultures and showed strong *in vitro* cytotoxic activity towards BT 220, HI 16, Int 407, HL 251 and HLK 210 human cancer cells. The challenge for today's pharmaceutical and agrochemical industry lies in the discovery and development of new active molecules. Vast genetic diversity available in endophytic fungi offers a wealth of possibilities for the betterment of mankind in the production of medicines. It requires recovery of indigenous endophytic fungal flora from host plants of various ecological settings and their preservation.

Numbers of medicinal plants have been screened for endophytic assemblage in India (Singh and Waingankar 2004; Mahesh *et al*, 2005; Nalini *et al*, 2005; Raviraja, 2005; Tejesvi *et al*, 2005; Verma *et al.*, 2006; Gond *et al.*, 2007). At present more than 150 endophytic fungi have been reported from angiospermic plants of different ecological settings in India. Most of the studies were focused on isolating endophytic fungi from the individual plant species, but comprehensive approaches to screen different tissues of the individual plant species from different locations are rarely done (Verma *et al.*, 2006).

Catharanthus roseus (*Vinca rosea*) has been used in traditional medicine to cure diabetes. *Catharanthus* alkaloids comprise a group of about 130 terpenoid indole alkaloids. The most important one is Vinblastine, a well-known anticancer drug that has been on the market for more than 40 years (Robert *et al.*, 2004) .Decoctions of the plant have been used for maladies ranging from ocular inflammation, diabetes, hemorrhage to treating

insect stings and cancers. Hence the study was initiated to gather more information about the endophytic diversity from aerial tissues of *Catharanthus roseus* (Apocynaceae).

MATERIALS AND METHODS

Collection of plant samples : Four different locations were identified for sampling. Yawat (designated as Loc A), Pune (Loc B), Lonawala, a natural forest (Loc C) and Pimpri-Chinchwad, an industrial area (Loc D). Bark, leaf and stem were collected from individual plants at each location. Bark samples were collected from 2-3 years old stem stocks growing at each location. The Loc A is comparatively dry area, Loc B represented to the cultivated urban area, Loc C is a natural lush green forest and Loc D is industrial area which is considered as polluted areas.

Processing, isolation and preservation of endophytes : The leaf and stem samples were kept in sterile polythene bags, and bark samples in sterile screw cap test tube. All the samples were washed properly in sterile water before processing. The plant samples were cut into 1-cm^2 pieces. Surface treatment was given to plant samples to eliminate surface microorganisms. The samples were treated in 70% ethanol for 1-2 min and then sterilized with aqueous sodium hypochlorite (4% available chlorine) for 2-3 min followed by immersion in 70% ethanol for 10 s. Sterilized segments (a total of 300 @ five segments per plate) were transferred onto the Potato Dextrose Agar medium incorporated with tetracycline (100 mg L^{-1}). The inoculated petri plates were then incubated in Bio Multi Incubator (Nippon Instrument, Japan) for 20 days at 12-h light/dark cycle at 25±1°C. The plates were checked routinely, and young hyphal tips of endophytically growing fungi were then transferred and sub-cultured. The recovered endophytic fungi were identified based on colony characters and microscopic characteristics. Standard taxonomic manuals were used to identify the fungal genera (Ellis, 1971, 1976; Sutton, 1971; Tulloch. 1972; Booth, 1977; Ames, 1969; Domsch *et al.*, 1980). All endophytic fungal cultures are maintained in live and pure forms in various storage conditions, including 15% glycerol, in lyophilized form stored at -70°C in a deep freezer (New Brunswick Scientific, Japan) and on agar slant. All the cultures are deposited in Agharkar Research Institute, Fungus Culture Collection (ARIFCC), Pune, India.

Analysis of data : The colonization frequency (CF) of an endophyte was calculated as the number of segments colonized by a single endophyte divided by the total number of segments observed x 100 (Hata and Futai, 1995). This is expressed as % CF = (Ncol /Nt) x 100; where Ncol:=: the number of plant segments colonized by each endophytic fungus, and Nt = the total number of segments. The dominant endophytes were calculated as the percent colony frequency of a given endophyte divided by the sum of the percentage of colony frequencies of all endophytes x 100 (Kumaresan and Suryanarayanan, 2002). The relative percentage of occurrence of different groups of endophytic fungi was calculated by dividing the number of segments colonized by a single group of fungi by a total numbers of segments colonized by all the groups of fungi. Statistical analysis of the data and graph were prepared with Sigma Plot (Ver. 10).

RESULTS AND DISCUSSION

A total of 300 collected tissue segments were processed and total 341 isolates representing 23 fungal species were recovered (Table 1). Among the total endophytic isolates. 49.26 % Hyphomycetes, 30.20 % Coelomycetes, 15.24% Ascomycetes and 5.27% Mycelia sterilia were found (Fig. 1). The maximum endophytic isolates were obtained from Loc C (26.97%), followed by Loc A (26.39%), Loc B

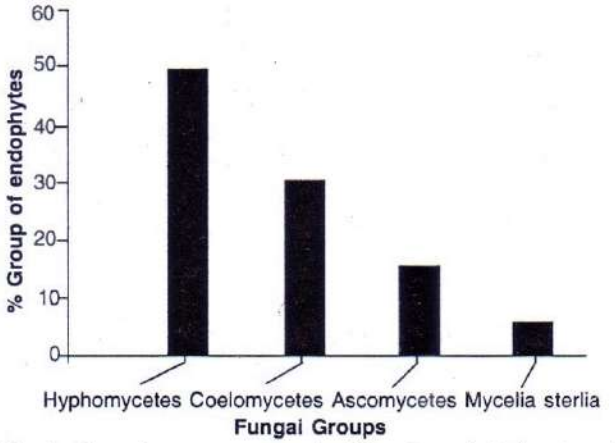

Fig. 1: Percentage recovery of different endophytic fungal groups from three aerial parts.

Table I : Isolation and occurrence of endophytic fungi from aerial tissues of *Catharanthus roseus* growing at four locations in Pune and adjoining areas

Endophytic Fungi	Loc A			Loc B			Loc C			Loc D		
	Bark	Leaf	Stem	Bark	Leaf	Stem	Bark	Leaf	Stem	Bark	Leaf	Stem
Hyphomycetes												
Acremonium sp.	02	01	02	01	-	-	-	-	01	01	01	-
Alternaria alternata	-	04	02	-	05	05	-	05	-	-	04	02
Aureobasidium pululans	-	02	01	-	01	-	-	02	01	01	-	01
Cladosporium cladosporiodes	02	02	-	01	02	01	01	-	06	02	-	01
Cladosporium tenuisimum	01	03	01	01	-	01	02	01	01	01	02	04
Curvularia lunata	02	01	01	-	-	02	03	01	02	01	-	02
Fusarium graminearum	01	01	-	-	01	01	-	02	02	-	-	-
Fusarium oxysporum	02	-	01	-	01	02	-	-	-	01	01	01
Gliocladium sp.	02	-	01	01	-	02	02	-	01	02	-	02
Gonatobolryum sp.	01	-	02	-	-	-	03	-	02	-	-	01
Nigrospora sphaerica	03	03	-	02	03	04	-	-	01	02	-	03
Periconia sp.	02	-	-	-	-	-	03	-	-	-	01	
Scytalidium sp.	01	-	-	02	-	02	-	01	-	-	-	-
Coelomycetes												
Colletotrichum dematium	-	-	-	-	-	04	-	-	-	01	-	01
Colletotrichum gloeosporioides	02	05	-	-	04	01	03	-	-	06	02	04
Lasiodiplodia theobromae	-	-	-	-	-	01	-	-	-	01	-	
Myrolhecium verrucarium	01	01	01	-	-	-	-	-	-	-	-	-
Myrothecium sp. (sp. nov.)	-	-	-	01	-	-	03	-	01	-	-	01
Pestalolia sp.	05	-	-	02	-	-	07	-	03	-	-	-
Pestalotiopsis sp.	02	02	-	01	-	-	-	03	02	-	04	01
Phoma sp.	-	03	01	-	01	03	01	-	01	-	-	02
Phomopsis sp.	-	02	-	-	03	-	02	-	02	-	-	-
Phylosticta sp.	-	02	-	-	03	01	-	-	-	-	-	-
Ascomycetes												
Chaetomium globosum	02	-	-	01	-	03	06	03	05	-	-	04
Mycosphaerella sp.	01	-	-	-	02	-	-	-	-	-	01	-
Sordaria sp.	01	-	01	02	-	02	-	-	-	-	-	-
Xylaria sp.	01	04	03	-	06	01	-	01	-	-	02	-
Mycelia sterilia	01	02	-	04	01	-	02	01	03	-	03	01
Total Isolates	35	38	17	19	33	36	38	20	34	19	21	31

* Locations selected for study : Loc-A, Yawat, Loc-B Pune, Loc-C, Lonawala, Loc-D Pimpari-Chinchwad, Total isolates = 341. Total segments planted = 300

** Aerial plant parts : Bark (B), Leaf (L) and Stem (S)

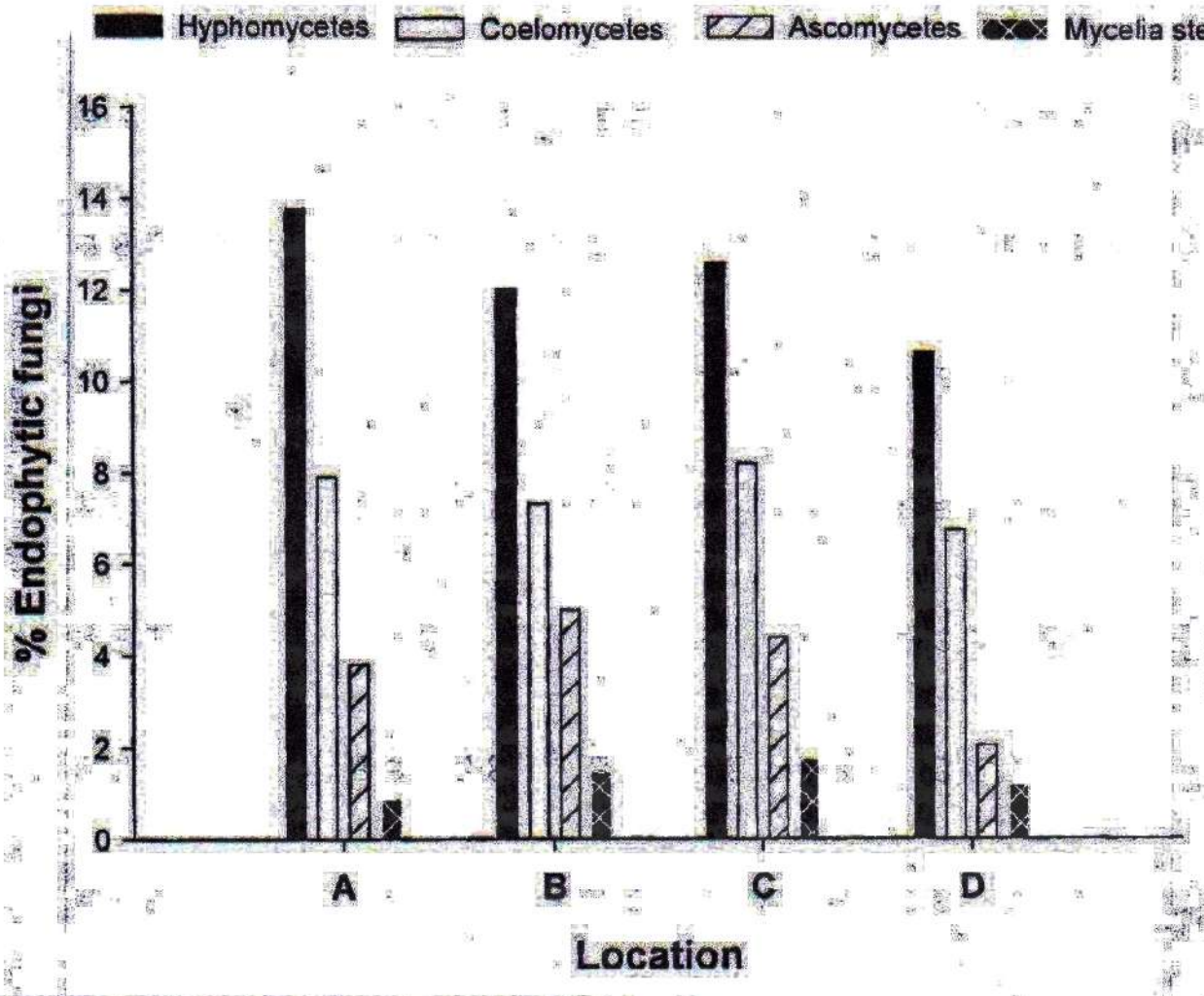

Fig. 2 : Per cent occurrence of endophytic group of fungi from four locations.

(25.80%) and Loc D (20.82%) (Fig. 3). In general endophytic colonization was greater in stem (34.60%) than leaf (32.84%) and bark (32.55%) samples. Hyphomycetes were found most dominant endophytes recovered from all the location (Fig. 2). At Loc C the per cent colonization of tissue samples by endophytes were found highest (30.65%) and lowest at Loc D (23.66%). The dominance of fungal endophytes isolated from stem at Loc A was found minimum (Loc AL - 4.98), while it was maximum (Loc AL -11.14) in the leaf at the same location (Table 3). The most diverse endophytic assemblage was found at Loc C. *Colletotrichum gloeosporioides*, *Alternaria alternata* and *Nigrospora sphaerica* were obtained as the most dominant endophytes (Table 2). These are ubiquitous and have been reported from several host plants (Brown. 1998; Singh and Waingankar, 2004). Despite the variations in the recovery of endophytes from different tissues and the locations, some overlaps in endophytic assemblage were also recorded. The bark samples from locations B and D yielded equal number of endophytes (19). Similarly, leaf samples of Loc A and bark samples of Loc C yielded equal numbers (38) of endophytes, which represented the maximum numbers isolated from individual sites and locations. In this study maximum fluctuations in recovery of endophytic isolates were observed at Loc B and Loc D (Fig. 3).

Kharwar *et al.* (2008) isolated 19 different species from leaf, stem and root. Out of which only *Alternaria alternata* , *Cladosporium cladosporioides*, *Curvularia lunata*, *Chaetomium globosum*, were recovered in this study. *Alternaria alternata* and *Fusarium oxysporum* recovered from *C. roseus* was also found to be common in other host plants according to previous reports. Recently, Zhang *et al.* (2000) reported an endophytic *Fusarium oxysporum* from the inner bark of *Catharanthus roseus* and successfully showed that the fungus could produce 'vincristine' which has an anticancer activity. Brady and Clardy (2000) reported a new pentaketide antifungal agent (CR377) from an endophytic *Fusarium* sp. obtained from *Selaginella pallescens*.

Phomopsis and *Phyllosticta* isolated in the present study were constantly associated with internal leaf tissues of many plants (Stone *et al.*, 1996; Suryanarayanan *et al.*, 1998; Girivasan and

Suryanarayanan, 2004). Bills and Polishook, (l992) classified these fungi as 'almost exclusive endophytes.' The wide host range of the common genera of Coelomycetes suggests that these fungi are well adapted to an endophytic mode of life. Suryanarayanan *et al.* (2004) characterized a melanin pigment, 1-8, dihydroxynaphthalene from a endophytic *Phyllosticta capitalensis* which was considered to enhance survival capabilities of fungi in stress full conditions. Among the *Myrothecium* isolates obtained, the morphology of the one isolate from stem (at Loc D) was found distinct from previously known species of this genus (Tulloch. 1972; Bohin, 1993). It readily produced perfect state in agar culture. Many other fungal genera isolated were also reported from other plant species. Genera like *Gonatobotryum, Scytalidium* and *Gliocladium* were found as uncommon colonizers of the host tissues. Among the endophytic ascomycetes, *Chaetomium* was the prevalent and maximum isolates were obtained from Loc C. *Xylaria* was the second most dominant colonizer.

The xylariaceous forms of endophytes are often encountered from tropical hosts (Dreyfuss and Petrini, 1984). Recovery of rare *Sordaria* sp., in the present study, exclusively from bark and stem samples at two locations (Table 1) with comparatively high humidity might be considered as incidental. *Mycosphaerella* isolated from leaf samples and only once from the bark samples was generally considered to be a foliar pathogen. The teleomorph state was observed represented by abundant small pseudothecia. *Mycosphaerella* was however, linked to approximately 23 anamorph genera including both Coelomycetes and Hyphomycetes (Crous *et al.*, 2000; Corlett, 1995; Crous, 1998; Crous & Braun, 2003; Aptroot 2006; Schubert & Braun, 2005).

Table 2 : The percentage colonization and dominance of endophytic fungi from all isolates obtained from the total plant segments studied

Endophytic fungi	Total isolates of each endophyte	Percentage (%) CFb	Dominance of fungi
Hyphomycetes			
Acremonium sp.	09	3.0	2.64
Alternaria alternata	27	9.0	7.92
Aureobasidium pullulans	09	3.0	2.64
Cladosporium oxysporum	18	6.0	5.28
Cladosporium teneussisum	18	6.0	5.28
Curvularia sp.	15	5.0	4.04
Fusarium graminearum	08	2.6	2.29
Fusarium oxysporum	09	3.0	2.64
Gliocladium sp.	13	4.3	3.79
Gonatobotryum sp.	09	3.0	2.64
Nigrospora sphaerica	21	7.0	6.16
Periconia sp.	06	2.0	1.79
Scytalidium sp.	06	2.0	1.79
Coelomycetes			
Colletotrichum dematium	06	2.0	1.79
Colletotrichum gloeosporioides	27	9.0	7.92
Lasiodiplodia theobromae	02	0.7	0.61
Myrothecium verrucarium	03	1.0	0.88
Myrothecium sp.	06	2.0	1.76
Pestalotia sp.	17	5.6	4.93
Pestalotiopsis sp.	15	5.0	4.40
Phoma sp.	12	4.0	3.52
Phomopsis sp.	09	3.0	2.64
Phylosticta sp.	06	2.0	1.79
Ascomycetes			
Chaetomium globosum	24	8.0	7.04
Mycospherella sp.	04	1.3	1.14
Sordaria sp.	06	2.0	1.76
Xylaria sp.	18	6.0	5.28
Mycelia sterilia	18	6.0	5.28

*Based on 300 segments processed.1 Colonization frequency

Kaneko *et al.* (2003) reported endophytic *Mycosphaerella buna* from *Japanese* beech (*Fagaus crenata*) in which living leaves were infected by ascospores of pseudothecia produced on fallen leaves. Overall significant differences in species recovery from different locations were not observed. This result is in concurrence with the recent report by Verma *et al.* (2006). Similarly, no tissue specific endophytes could be isolated from any of the locations studied. Much variation in endophytic colonization of plant tissues of all locations was observed e.g. Location C (Lonawala; constitutes a natural forest) had showed maximum diversity probably due to the moist and humid conditions, which favoured establishment of endophytic colonization. In the present study Loc D had yielded least endophytic diversity, represented by comparatively dry location and exposed to industrial pollution. Overall observations indicated that maximum fluctuations in recovery of endophytic fungi were found at Loc B and D. Loc A and Loc C had showed almost stable recovery of endophytes in general.

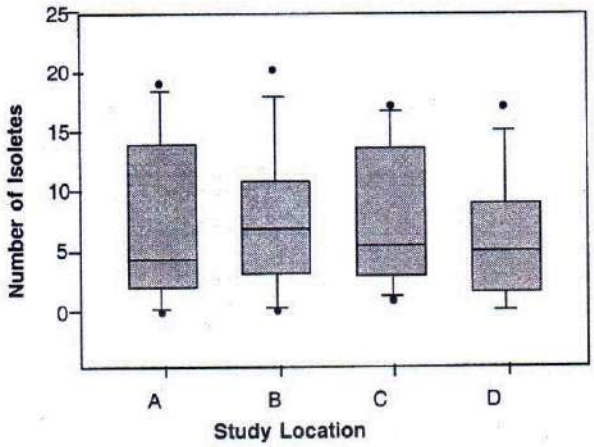

Fig. 3. Box plot showing location wise (four locations) distribution of endophytic fungi. The line between the boxes shows median value, the bar lines above and under the boxes show maximum and minimum values of endophytes isolated at each location, while the box itself represent the semi-quartile range

Table 3. Dominant endophytic fungi and per cent colonization of *C. roseus* at four locations.

Sampling Location		Total Isolates	Percentage (% cf)	Dominance of fungi
Loc A	Bark	35	11.66	10.26
	Leaf	38	12.66	11.14
	Stem	17	05.66	04.98
Loc B	Bark	19	06.33	05.57
	Leaf	33	11.00	09.68
	Stem	36	12.00	10.56
Loc C	Bark	38	12.66	11.14
	Leaf	20	06.66	05.86
	Stem	34	11.33	09.97
Loc D	Bark	19	06.33	05.57
	Leaf	21	07.00	06.16
	Stem	31	10.33	09.09

Endophytic fungi are a rich source of natural products displaying a broad spectrum of biological activities. *Aureobasidium pullulanse* influence the seed germination of certain plants by producing phytohormones. Endophytic fungi are known to offer the great potential for biocontrol programme as these are the integral part of the host plant. Rubini *et al.* (2005) report up to 70% reduction in incidence of deadly Witches' Broom Disease caused by a *Crinipellis perniciosa* in cacao seedlings by an endophytic *Gliocladium* sp. Stinson *et al.* (2003) have, also reported an endophytic *Gliocladium* sp. from *Eucryphia cordifolia*, producing a mixture of volatile antimicrobial organic compounds lethal to plant pathogenic fungi such as *Pythium ultimum* and *Verticillium dahliae*.

The fungal endophytes have been recovered from all plant species examined until now including grasses represent a ubiquitous component of the terrestrial plant community. This is probably because endophytes get nutrition and protection from the host plant and in turn they produce some active metabolites which provide variety of benefits to the host plants like enhance the host fitness and resistance against stresses. Perusal of literature on endophytes shows that endophytic genera obtained in the present study are also common in other host plants, which produce one or other bioactive compounds of pharmaceutical and agrochemical importance. Endophytes are abundant source of bioactive and chemically novel compounds and are reported to have various natural products and their potential like antibiotics, antiviral, antioxidants, anticancer, antidibetics, immunosuppressive compounds, etc. (Strobel, 2002; 2003).

In India various facets of endophytic fungal diversity and biology are being investigated. Currently focus is being given at characterization of their metabolites. This is the first comprehensive report of endophytic mycoflora associated with *Catharanthus roseus*. The endophytic fungi obtained in this study would be screened for their potential.

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