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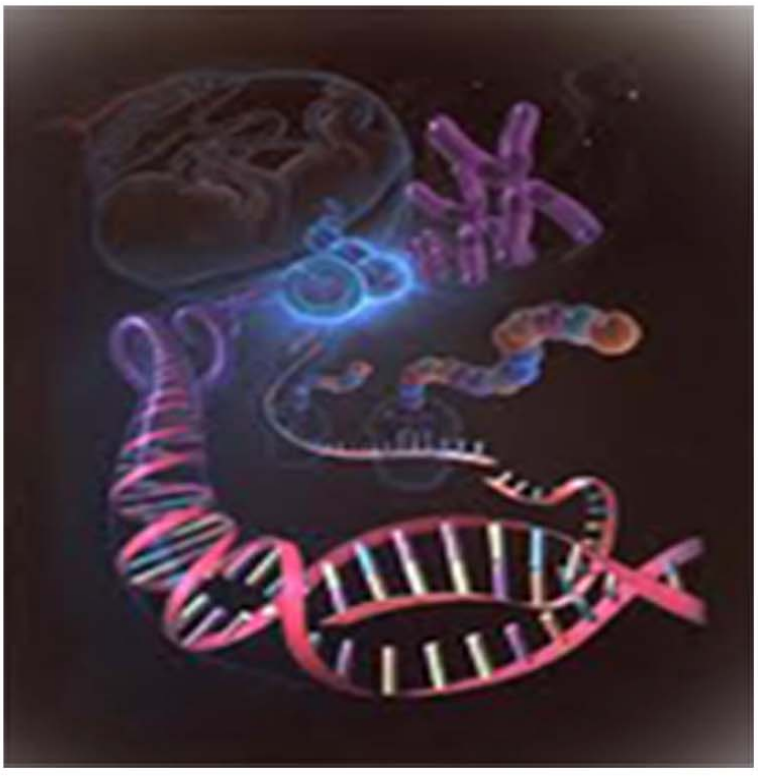

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Research Paper

ISOLATION AND CHARACTERIZATION OF ENDOPHYTIC FUNGI FROM ENDEMIC MEDICINAL PLANTS OF TIRUMALA HILLS

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Endemic medicinal plants of Tirumala hills of Seshachalam range falling under the Eastern Ghats of India, were exploited for endophytes as a possible source of bioactive secondary metabolites. Six hundread and ten (610) segments from *Boswellia ovalifoliolata, Pterocarpus santalinus, Shorea thumbuggaia, Syzygium alternifolium*, were processed for the presence of endophytic fungi. A total of 14 fungal species, viz., *Fusarium oxysporum, Colletotrichum falcatum, Pestalotiopsis species , Aspergillus fumigatus, Aspergillus flavipes, Sterile mycelia, Penicillium senticosum, Gliocladium roseum,Phomopsis jacquiniana, Nigrospora sphaerica, Leptosphaeria species, Phomopsis archeri , Alternaria alternata, Aspergillus niger* were isolated and identified based on the morphology of the fungal culture and characteristics of the spores. Sterile forms were common to all the host and few appeared to be host specific. The overall colonization rate of endophytes in both the leaf and the stem was found to be 3.44%. The stem showed low percentage frequency of colonization of the endophytic fungi when compared to leaf segments. Among the endophytic flora, *Colletotrichum falcatum* was found to be the core-group fungus with colonization frequency of 12.5%.

Keywords: Isolation, Ascomycetes, Coelomycetes, Endophytic fungi, Medicinal plants

INTRODUCTION

Endophytic fungi that live inside the tissues of living plants are under-explored group of microorganisms. Dreyfuss and Chapela (1994) estimated that there may be at least one million species of endophytic fungi alone. Recently they have received considerable attention. They were found to protect their host against insect pests, pathogens and even domestic herbivores (Weber, 1981, Malinowski and Belesky, 2006). Almost all the plant species (~400,000) harbor one or more endophytic organisms (Tan and Zou, 2001). To date, only a few plants have been extensively investigated for their endophytic biodiversity. Medicinal plants are reported to harbor endophytes (Strobel, 2002), which in turn provide protection to their host from infectious agents and

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also provide adaptability to survive in adverse environmental conditions. The occurrence of endophytic microorganisms in different plant species is extensively studied. The knowledge of their occurrence on the endemic plants of Tirumala hills in Eastern Ghats is not reported so far . The present study forms the first report on the endophyte diversity of endemic medicinal plants of Tirumala hills.

MATERIALS AND METHODS

Collection of Plant Material

Endophytic fungi were isolated from endemic medicinal plants of Tirumala hills by the procedure of standardized and modified method described by Hallman *et al.* (2007). The samples were rinsed gently in running water to remove dust and debris. After proper washing, stem samples were cut into long 0.5-1 cm pieces, whereas leaves were cut into 3-4 mm x 0.5-1 cm pieces under aseptic conditions. Surface sterilization was done by 1-13% sodium hypochlorite (NaOCl) according to the type of tissues. Each set of plant material was treated with 75% ethanol for 1 min followed by immersion in sodium hypochlorite and again in 75% ethanol for 30 s (Bills, 1996). They were finally rinsed with deionized sterile distilled water to remove the sterilants and blot dried on sterile tissue paper, 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±2°C for 15 days under dark conditions and monitored every day. Fungi growing out of the plant explants were sub cultured 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 colony chracteristics the mycelia were transferred in to PDA agar media. Colonization Frequency (CF) was calculated as described by Suryanarayanan *et al.* (2003).

$$\text{Colonization frequency of endophytes} = \frac{\text{Number of segments colonized by fungi}}{\text{Total number of segments analysed}} \times 100$$

MICROSCOPIC CHARACTERISTICS

Isolates were incubated for 7 days at 27°C. The experimental design was completely randomized with 6 replicates. Colonies were analyzed with respect to their average diameter (cm), the aspect of their borders, the coloration of the mycelium, the coloration of the reverse of the petri dish also and the coloration of the medium. The cultures were submitted to Fungal Identification Service, Mycology and Plant Pathology Group Agharkar Research Institute, G G Agarkar Road, Pune for confirmation.

RESULTS AND DISCUSSION

Total of 14 endophytic fungi were isolated from 610 samples of both leaves and stem of different parts of endemic plants (Table 1). Most of the fungi belonged to Ascomycetes. The fungus *Colletotrichum falcatum* was found to be the core-group fungus with the colonization frequency of 12.5%. The frequency of colonization in leaf samples was varied between 1.6 to 12.5%. The colonization frequency *Colletotrichum falcatum* was maximum 12.5 % followed by *Fusarium oxisporum* (6.66%), *Penicillium senticosum* (5%) *Aspergillus fumigatus* (4%). The frequency of *sterile mycelia* in leaf tissue was 3.33%, *Phomopsis archeri* exhibited lowest % of

Table 1: Name and Colonizing Frequency of Endophytic Fungi Isolated From Different Parts of Endemic Plants

S. No.	Species	Site of Isolation	Number of Samples	Fungi Isolates	Colonization Frequency%
1.	*Fusarium oxysporum*	Leaf	15	1	6.66
2.	*Aspergillus niger*	Stem	45	2	4.44
3.	*Colletotrichum falcatum*	Leaf	60	2	12.5
4.	*Pestalotiopsis*	Stem	40	1	2.5
5.	*Aspergillus fumigatus*	Leaf	25	1	4
6.	*Alternaria alternata*	Stem	35	2	5.7
7.	*Sterile mycelia*	Leaf	30	1	3.33
8.	*Aspergillus flavipes*	Stem	50	3	6
9.	*Penicillium senticosum*	Leaf	20	1	5
10.	*Gliocladium roseum*	Stem	55	2	3.6
11.	*Phomopsis jacquiniana*	Stem	70	1	1.4
12.	*Nigrospora sphaerica*	Leaf	40	1	2.5
13.	*Leptosph aerulina*	Stem	65	2	3.0
14.	*Phomopsis archeri*	Leaf	60	1	1.6
	Total Number		**610**	**21**	**3.44**

colonization frequency. Similarly the frequency of colonization in stem tissue of varied between 1.4 to 5.7% maximum being by *Alternaria alternate* and minimum *Phomopsis jacquiniana* (1.4%).

CHARACTERIZATION OF ENDOPHYTIC FUNGI

Fusarium oxysporum

Growth moderate, white, peach, to salmon pink or violet. Conidiogenous cells hyaline, enteroblastic, mono or polyphialidic. *Fusarium* species produce several types of conidia. Microconidia hyaline, 0-1 or septate, small, macroconidia hyaline, curved, phragmospores, with a foot cell bearing, some kind of heel. Chlamydospores may also be present, borne terminally or intercalary or on the macroconidia. Microconidia are oval to cylindrical or even curved and produced on simple, short phialides. Macroconidia 3-5 septate, 27 -60 X 3 - 5 μm.

Aspergillus niger

Colonies spreading rapidly with mycelium white to dark brown to black or purple brown conidial heads, conidial heads globose, radiate, conidiophores arising from the substratum varying from 200 μm to several millimeters long and 10-20 μm diameter, smooth, vesicle globose, phialides borne directly on the vesicles in some species, but metulae usually present, metulae varying in length from 10-15 μm, conidia small, more or less globose, rough, 4-5 μm in diameter.

Colletotrichum falcatum

Colonies grayish white, with sparse aerial mycelium and small dense felty patches, elsewhere reverse white to grey, conidial masses salmon pink. Some cultures have abundant greyish white aerial mycelium with poor sporulation and no distinct acervuli. Sclerotia absent from both races. Septae sparse. Conidia falcate, fusiform apices obtuse, 15.5-26.5 X 4-5 μ. Appresoria sparse, medium brown, clavate or circular, edge entire, 12.5-14.5 X 9.5-2.5 μ.

Pestalotiopsis species

Conidia clavate to fusiform, straight, rarely curved, equilateral, 5-celled, smooth walled, 23-29 X 80-95 (11) μm mean 25 X 90 μm. Apical and basal cell hyaline, apical hyaline cells long and broad cylindric, the basal hyaline cells Broad-conic. Median 3 cells colored, guttulate, together 16-20 μm long, slightly or hardly constricted at the septa, the lowest colored cell is light brown, apical appendage (2-3-4) divergent or recurved, hyaline, cylindrical with abtuse apices, 18 -40 μm long. Basal appendage hyaline, straight or slightly curved, 3-6 μm long.

Aspergillus fumigatus

The colony was pulvinate in appearance, yellow at the centre, with light yellow radial rays and white color 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.

Alternaria alternata

White color 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.

Mycelia sterilia

Many fungi do not produce any recognizable sexual/asexual conidia state in culture. Such forms are frequently classified for convenience in the *Mycelia sterilia.* This group is catchcall which may include a few well defined and easily recognizable genera, but more often is a repository for a large number of non descript mycelial isolates.

Aspergillus flavipes

Colonies white or silvery white, reverse yellow to orange brown or reddish brown. Conidial heads columnar in size. Vesicles globose to ovate, metulae fertile over entire vesicle, conidial heads splitting over age. Conidia smooth, globose, 2-3 μm in diameter.

Penicillium senticosum

Mycelium with grey color at the centre and whitish edges. The colony secretes reddish orange color into the media, and concentric grey coloration, each of the rings having different gradations of color. Oil droplets are formed on the fifth day of the inoculation. The colony turned saffron-yellow in color 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.

Gliocladium roseum

The centre of the colony was green in color 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 conidiophore group together and the spores form rosette like structure. The color of the colony turns to powdery green on maturity.

Phomopsis jacquiniana

This is a slow growing, sporulating fungi. The colony looked dark in appearance with mycelia being immersed, branched, septate and brown in color. The pycnidia 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 color. The length of the ascocarp was 200 μm and the breadth 180 μm. The spore length was 10 μm and breadth was 5 μm.

Nigrospora sphaerica

Colonies white later brown to black when sporulation is abundant. *Conidiophores micronematous*, branched, flexous, colorless to brown, smooth, conidia solitary, acregenous, simple, spherical or broadly ellipsoidal, compressed dorsiventrally, black , shining, smooth, 0-septate, 10-16 μm diameter.

Leptosphaeria species

A filamentous ascomycetous fungus that produce dark colored pseudesthesia. The asci of *Leptosphaerulina* are shortly clavate to saccate and have bitunicate. Bitunicate asci are characterized by an inner extensable wall. Ascospores are hyaline to brown in color and ellipsoid, cylindrical or oblong.

Alternaria porri

Conidiophores dark, septate, sometimes inconspicuous, simple or branched, bearing conidia at the apex, spores solitary or more often produced in acropetal succession to form simple or branched chains, darkly pigmented, ovate to obclavate, tapering abruptly or gradually towards the distal. Overall conidial dimensions are 15-20 μm.

DISCUSSION

Medicinal plants which form the backbone of traditional medicines in the last few decades have been the subject for very intense pharmacological studies. Every plant on earth is known to harbor at least one endophytic microbe. Endophytic fungi are one of the most unexplored and diverse group of organisms having symbiotic associations with higher life forms and produce beneficial substances for host (Weber, 1981). However only a few plants have been studied for their endophyte biodiversity and their potential to produce bioactive compounds. Several studies have been carried out about the endophytic bio diversity, taxonomy, reproduction, host ecology and their effort on host (Petrini and fisher, 1980; Clay and Schardl, 2002; Selosse and Schardl, 2007). Endophytes, are now considered as an outstanding source of bioactive natural products, because they occupy unique biological niches as they grow in so many unusual environments (Strobel and Daisy, 2003). Fungi have been widely investigated as a source of bioactive compounds, an excellent example is anticancer drug taxol, which had been previously to occur only in the

plants (Strobel and Daisy, 2003). In the present study, altogether 14 fungi were isolated as endophytes from the leaves, and stem parts of endemic medicinal plants collected from Tirumala hills. Some hyphomycetous forms, viz., *Alternaria porri Aspergillus niger, Aspergillus flavipes, Fusarium oxysporum, Nigrospora sphaerica* (Blodgett *et al*., 2000; Suryanarayanan *et al.,* 1998, 2002) were isolated as endophytes in the present study. A significant variation was detected in the colonization frequency of endophytic species, even though all are belongs to Ascomycota division. In this investigation low rate of colonization of endophytic fungi may be attributed due to the secretion of the certain antifungal and antibacterial components (Rajagopal *et al*., 2010). The occurrence of sterile mycelia as endophytes demand the use of molecular techniques, for classification and induction of sporulation is suggested by means of incubation under near UV or low temperature (Bills, 1996). Previous studies reported distinct endophyte community compositions in different host plants suggesting host preference (Cannon and Simmons, 2002; Cohen, 2006). This study shows such a trend was apparent with the leaves, stem parts of different medicinal plants. 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.

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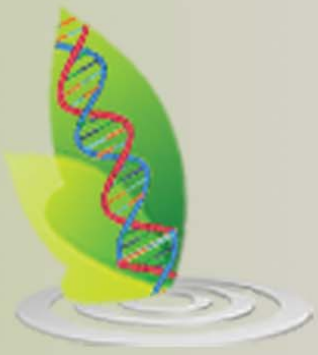

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