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Endophytic fungal communities associated with two ethno-medicinal plants of Similipal Biosphere Reserve, India and their antimicrobial prospective

S.K. Jena and *K. Tayung
Department of Botany, North Orissa University, Takatpur, Baripada-757003, India.

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ABSTRACT

To study endophytic fungi associated with two plant species used as ethno-medicines by aboriginal tribes of Similipal Biosphere Reserve and evaluation for their antimicrobial potentials against some clinically significant human pathogens. A total of 458 endophytic isolates were obtained from leaf, stem and fruit tissues of *Solanum rubrum* and *Morinda pubescence*. The dominant endophytic fungi belong to genera *Aspergillus, Colletotrichum, Curvularia* and Mycelia sterilia. Maximum endophytic isolates were obtained from leaves segments followed by stem and fruit tissues. In both the plants class hypomycetes were dominant over other fungal classes. Shannon-Weiner and Simpson indexes showed rich diversity of endophytic fungi suggesting even and uniform occurrence of various species. The endophytic isolates showed varying degree of antimicrobial activity against 9 human pathogens. In *S. rubrum* 20% and 10% of the isolates inhibited all the Gram-positive and Gram-negative bacteria and 35% of the isolates displayed antifungal activity against all the test fungal pathogens. One of the isolate showed considerable antimicrobial activity against all the test pathogens. Endophytic isolates of *M. pubescence* showed 24% antibacterial activity against Gram-positive bacteria and 28% antifungal activity against all the test fungal pathogens. The study revealed that medicinal plants associated endophytes could be a rich source of antimicrobial agents.

INTRODUCTION

Medicinal plants have been playing important role for treatments of various human ailments since time immemorial. According to the World Health Organization, over 80% of the world's population, or 4.3 billion people, rely upon such traditional plant-based systems of medicine to provide them with primary health care (Bannerman *et al*., 1983). Nevertheless, indiscriminate exploitation of these plant resources has rapidly declined their population making some of them critically endangered. But now it is known that medicinal plants harbour some distinct fungal endophytes that are believed to be associated with the production of pharmaceutical products (Zhang *et al*., 2006). By definition endophytic fungi are microbes that colonize inner plant tissues without causing any disease symptoms. They have been found associated with every plant species investigated so far. It is believed that plants from unique environmental settings, with an ethno botanical history or which are endemic are likely to house novel endophytic microorganisms as well as microorganisms making novel bioactive products (Strobel and Daisy, 2003). Endophyte research expanded in recent years from cataloguing species to examining the nature of the endophyte/plant interaction with particular emphasis on studying endophytes of medicinal plants in order to discover novel compounds. Despite the omnipresence of endophytic fungi in plants, the extent of their contribution to fungal biodiversity still remains unclear. Besides, few studies have been conducted with regards to the diversity and colonization of the host.

Inspite of largest diversity of endophytic species in tropical and subtropical rainforests, their biodiversity in tropical country is still poorly studied. In the recent years, endophytic fungi associated with medicinal plants are of special interest because of the fact that these microbes have produced metabolites similar to or with more bioactivity than their respective hosts. It has also been speculated that the bioactivity of medicinal plant may be due to its associated endophytes.

* *Corresponding Author*
UGC, Post-Doc Fellow Dept. of Botany North Orissa University, Takatpur, Baripada-3; Cell: +91 9040361091.

Similipal Biosphere Reserve (SBR), in the state of Odisha, India has unique environmental settings with several plant resources with medicinal properties. The Biosphere is also an inhabitant of several aboriginal tribes who has been using these plant resources for curing various ailments. The present study was directed to study endophytic fungi associated with two plant species (*Solanum rubrum* and *Morinda pubescence*) which are used as ethnomedicines by the tribal communities of SBR and their evaluation for antimicrobial activity.

MATERIALS AND METHODS

Study area and collection of samples

The study was conducted in Similipal Biosphere Reserve (SBR) situated between 21 28' to 22 08' North latitudes and 86 03' to 86 37' East longitude. SBR is one of the richest biodiversity regions of the country and endow with several medicinal plants. The biosphere is also inhabited by number of aboriginal tribes who have been using these plant resources for treatment of various diseases. Two plant species (*Solanum rubrum* and *Morinda pubescenc*) were selected for the present study based on their ethno-medicinal uses. Plant samples (leaf, stem and fruit) were collected randomly from five healthy individual plants during monsoon season (June-July, 2012). The samples were collected in plastic bags, immediately brought to the laboratory and stored at 4^0C until isolation procedure was accomplished.

Isolation and identification of endophytes

For isolation of endophytic fungi , healthy plant tissues were washed in running tap water and processed as follows: leaf samples and fruits were surface sterilized by sequentially dipping into 70% ethanol (3min), 1% sodium hypochlorite (4 min) and 70% ethanol (1 min) and were rinsed with sterile distil water thoroughly, then allowed to surface dry under sterile conditions. The leaves were punched into circular segments about 0.5 mm diameter by the help of a sterile puncture. Stems tissues were cut into short pieces of 5-6cm long and then surface sterilized sequentially as described above, rinsed thoroughly with sterile distil water and surface dried.

The outer layer was removed and inner tissues were cut into segments of $2mm^2$ size with sterile scalpel. The efficiency of surface sterilization procedure was verified as per the method described by Schultz et al. (1998). All the segments were then placed on three different sterilized fungal media namely Potato dextrose agar (PDA), Mycological agar (MA), Rose Bengal agar (RBA) and Water agar (WA) medium. In each Petri-dish at least 10 segments were placed in equidistant from each other. The plates were then incubated in BOD incubator at 30±1 ^{0}C. The plates were checked every day for two weeks for the growth of endophytic fungi. The hyphal tip of endophytic fungus growing out of the plant tissues were immediately transferred to a sterile PDA slants and maintained at 4°C. The fungal isolates were identified based on their morphological and reproductive characters using the standard identification manuals (Gilman, 1971; Barnett and Hunter, 1996). The fungal cultures that failed to sporulate were categorized as sterile mycelia.

Data analysis

The relative frequency of colonization (% CF) was calculated as the number bark 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 (Tayung and Jha, 2008).

Simpson's index of Diversity was calculated using formula: 1–D

$$\text{where } D = \frac{\sum n(n-1)}{N(N-1)}$$

n = the total number of organisms of a particular species

N = the total number of organisms of all species

Shannon-Wiener diversity index was calculated using the following formula:

$$H_s = -\sum_{i=1}^{S} (Pi)\,(\ln Pi),$$

Where

H_s – symbol for the diversity in a sample of S species or kinds

S – the number of species in the sample

Pi – relative abundance of i^{th} species or kinds measures,= n/N

N – total number of individuals of all kinds

n_i– number of individuals of i^{th} species

ln – log to base 2

Fungal cultivation and determination of antimicrobial activity

The endophytic fungi were cultivated in Potato Dextrose Broth (PDB) by inoculating actively growing culture in 250ml Erlenmeyer flasks containing 100ml of the medium. The flasks were incubated at 25^0C with periodic shaking for 15 days. After incubation period cultural broth were filtered for the separation of fungal mycelia and the filtrate were taken for testing their antimicrobial activity. Antimicrobial activity was determined by the agar cup diffusion method against six bacteria (*Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Proteus vulgaris, Klebsiella pneumonia, Shigella flexneri Escherichia coli, Bacillus*) and three pathogenic fungi (*Candida albicans,C. krusei and Trychophyton mentagrophytes*). The test pathogens were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. Over night cultures of bacterial pathogens were swabbed over Muller Hinton agar plates and fungal pathogens on Sabouraud's dextrose agar plates to make a lawn of pathogens. Agar cups were prepared by scooping out the medium with a sterile cork borer (7mm in diameter). The cups were then filled with 100 μ L of the culture filtrate of endophytic fungi and incubated at 37±1°C for 24 h for bacterial and 48 h for fungal pathogens. Antimicrobial activity was determined as growth inhibition of the target organism around agar cup as appearance of clear zones.

RESULTS AND DISCUSSION

Endophytic fungi are known to be ubiquitous and every plant species examined to date have been found colonized with fungal endophytes (Arnold *et al*., 2001). It has been found that a single plant species may harbour hundreds of endophytes and may inhabit all available tissues, including leaves, petioles, stems, twigs, bark, xylem, roots, fruit, flowers, and seeds (Saikkonen *et al*., 1998; Chapela and Boddy, 1988; Fisher *et al*.,1993). In the present study also rich diversity of endophytic fungi were isolated from leaves, barks and fruits tissues of the two plant species. A total of 201 and 257 endophytic isolates were obtained from different tissue fragments of *Solanum rubrum* and *Morinda pubescence* respectively. The endophytic fungal communities of *S. rubrum* comprises of fungi belonging to genera *Aspergillus, Colletotrichum, Curvularia, Penicillium, Trichoderma,* Sterile mycelia and some unidentified species (Table 1). The tissue of *M. pubescence* was found colonized with endophytic fungi of the genera *Aspergillus, Chaetomium, Cladosporium, Curvularia, Nigrospora, Penicillium, Colletotrichum Trichoderma* and Sterile mycelia (Table 2). Both the plant species showed colonization of similar endophytic fungi but species of *Chaetomium, Cladosporium, Nigrospora* and *Torula* were found only in tissues of *M. pubescence*. Among the fungi, genus *Aspergillus* showed highest colonization frequency and consisted of six different species in both the plant tissues. Maximum endophytic isolates were obtained from leaves segments followed by stem and fruit tissues. This finding concurs with earlier reports that colonization of endophytic fungi is more prevalent in leaf than other tissues (Suryanarayan *et al*., 1998; Rajagopal and Suryanarayanan, 2000). Maheswari and Rajagopal (2013) were of the opinion that high colonization of endophytes in leaf tissue may be due to their anatomical structure and supply of nutrient elements on which the endophyte depends. The overall fungal composition of *S. rubrum* consisted of 65% hypomycetes, 10% coelomycetes, 10% sterile fungi and 15% of unidentified species while that of *M. pubescence* comprises of 86% hypomycetes, 8% ascomycetes, 4% coelomycetes, 16% sterile fungi and 4% unidentified species (Table 1 & 2). In both the plants class hypomycetes were dominant over other fungal classes. Many workers have also shown that hyphomycetes dominate the endophytic assemblages both in leaf and bark tissues in several plants species (Mahesh *et al*., 2005; Morakotkarn *et al*., 2006; Maheswari and Rajagopal, 2013). The dominance nature of class hypomycetes may be attributed to their ability to colonize host rapidly by producing abundant asexual spores and most fungi of this class occur as phylloplane flora but they are capable of penetrating the superficial layers of leaf and grow as endophyte, suggesting that phylloplane fungi might have adapted to endophytic mode of life to overcome adverse environmental conditions (Cabral *et al*., 1993).

Diversity and species richness of endophytic fungi were studied in different tissues of *S. rubrum* and *M. pubescence*. The result indicated that both the plant species were rich in endophytic fungi. The Shannon-Wiener index in leaf and stem tissue of *S. rubrum* were 2.872 and 2.808 respectively. The number of isolates was more in leaf tissue than bark and fruit tissues but comparatively lesser in species richness and the same was true for *M. pubescence* (Table 3). This showed that bark tissues were richer in species diversity than leaf and fruit tissues. Similarly, the Simpson diversity index was also almost same for all the tissues in both the plant species.

The values of diversity indexes thus suggested that the endophytic colonization in the tissues of both plants were even in nature indicating uniform occurrence of various species. More number of endophytic isolates in leaf tissue may be due to that fact that sampling were done in wet season and in many instances leaves sampled during the wet season harboured more endophytes than those screened during the dry season (Rodrigues, 1994; Wilson and Caroll, 1994). The rich diversity of *Aspergillus* species as endophytes in different tissues of both the plants may be due germination of more number of spores of this fungi due to favourable environmental condition such as humidity and precipitation which are normally high during monsoon season. Seasonal variation plays a major role in endophyte harvesting where environmental conditions pave the way for the symbiotic microbes to survive and explore; precipitation may be one of the major factors that influences the infection of endophytes. It has been reported that precipitation may influence the infection of endophytic fungi as well (Sahashi *et al*., 2000).

The endophytic fungi were evaluated for their antimicrobial activity against some clinically significant human pathogens. The result indicated that all the isolates showed varying degree of antimicrobial activity against the test pathogens. 80% of the endophytic isolates of *S. rubrum* showed antibacterial activity against Gram positive bacteria and 85% of isolate showed activity against Gram negative bacteria inhibiting at least one test pathogens. Again 20% isolates could inhibit both the Gram positive and 10% of the isolates showed inhibition against all the tested Gram negative bacteria. 35% of the isolates displayed antifungal activity against all the test fungal pathogens and one isolate showed considerable antimicrobial activity against all the test pathogens (Table 4). Similarly, the endophytic isolates of *M. pubescence* showed 72% and 92% antibacterial activity against Gram positive and Gram negative bacteria respectively inhibiting at least one of the test pathogens. Out of the total isolates 5 isolates (24%) showed antibacterial activity inhibiting both the Gram positive bacteria and 7 isolates (28%) showed antifungal activity against all the test fungal pathogens (Table 5).

It is known that the endophytic fungi existing in the plant are potential sources of antimicrobial substances and this has also been demonstrated in earlier studies (Tayung and Jha, 2006; Mohanta *et al*., 2008; Padhi and Tayung, 2012). However, this is perhaps the first report of associated endophytic fungi and their antimicrobial potentials from *S. rubrum* and *M. pubescence* which are used as ethno-medicines by certain aboriginal tribes of Similipal Biosphere Reserve, India. The study thus agrees with the assumption that endophytic microbes associated with medicinal plants could be potential sources of bioactive metabolites for therapeutic applications. The result also indicated that some

endophytic isolates showed considerable antifungal activity against the three fungal pathogens (*Candida albicans, Candida krusei and Trychophyton mentagrophytes*). Such finding in the present scenario is quite significant because systemic infections caused by fungi especially in patients with impaired host defence mechanisms, have become increasingly serious. Various antifungal agents have been explored, but the control of many of the fungal diseases has not yet been achieved. Currently we are working on some potent endophytic isolates from characterization and structure elucidation of the bioactive metabolites.

Table. 1: Occurrence of endophytic fungi in different tissues of *Solanum rubrum*.

Endophytic fungi	Colonization frequency (CF%)				Frequency of dominant endophytes
	Leaf	Stem	Fruit	Total	
Hypomycetes					
Aspergillus niger	4.5	3.5	1.5	19	9.45
Aspergillus fumigatus	5.0	2.5	3.5	22	10.94
Aspergillus versicular	2.5	1.5	1.0	10	4.97
Aspergillus sydowi	3.5	3.0	2.0	15	7.46
Aspergillus fonsecaeus	2.5	1.5	1.5	11	5.47
Aspergillus terricola	2.0	1.0	--	06	2.98
Curvularia lunata	2.0	2.0	0.5	09	4.47
Curvularia geniculata	3.5	2.5	--	12	5.97
Penicillium purpurogenum	1.0	1.5	0.5	06	2.98
Penicillium lanosum	2.5	2.0	--	09	4.47
Penicillium oxalicum	4.0	1.5	1.0	13	6.46
Trichoderma viridae	3.0	1.5	1.0	11	5.47
Trichoderma lignorum	2.5	2.5	--	10	4.97
Coelomycetes					
Colletotrichum sp.1	3.5	1.5	--	10	4.97
Colletotrichum sp.2	1.5	--	1.5	06	2.98
Mycelia Sterilia					
Morphotype sp.1	2.0	1.5	2.5	12	5.97
Morphotype sp.2	2.5	1.0	--	07	3.48
Unidentified					
Unidentified sp.1	2.0	0.5	--	05	2.48
Unidentified sp.2	--	1.5	--	03	1.49
Unidentified sp.3	1.5	--	--	03	1.49
No of isolates recovered	103	65	33	201	--
Colonization frequency	51.5	32.5	16.5	--	--

Table. 2: Occurrence of endophytic fungi in different tissues of *Morinda pubesence*.

Endophytic fungi	Colonization frequency (CF%)				Frequency of dominant endophytes
	Leaf	Stem	Fruit	Total	
Hypomycetes					
Aspergillus clavatus	3.5	2.5	2.0	17	6.61
Aspergillus fumigatus	4.5	3.0	1.5	18	7.00
Aspergillus versicular	2.5	2.0	2.0	13	5.05
Aspergillus sydowi	3.0	1.5	1.5	12	4.66
Aspergillus flavus	5.0	3.5	2.5	20	7.78
Asperigillus sp.	1.5	1.0	--	05	1.94
Curvularia lunata	3.0	2.0	1.0	12	4.66
Curvularia interseminata	1.5	2.0	--	07	2.72
Curvularia subulata	2.0	1.0	--	06	2.33
Penicillium purpurogenum	4.0	2.5	1.5	16	6.22
Penicillium albidum	3.5	2.0	--	11	4.28
Trichoderma koningi	2.0	2.5	2.0	13	5.05
Trichoderma sp.	--	--	1.5	03	1.16
Cladosporium herbarum	2.5	2.0	0.5	10	3.89
Nigrospora zimmermann	2.5	1.5	1.5	11	4.28
Nigrospora sphaerica	3.5	1.0	--	09	3.50
Torula sp.	1.0	--	--	02	0.77
Ascomycetes					
Chaetomium dolichotrichum	1.5	2.5	--	08	3.11
Chaetomium globosum	3.5	1.5	1.5	13	5.05
Coelomycetes					
Colletotrichum sp.	4.0	1.5	--	11	4.28
Mycelia Sterilia					
Morphotype sp.1	3.0	2.5	1.0	13	5.05
Morphotype sp.2	1.5	1.5	--	06	2.33
Morphotype sp.3	--	2.0	0.5	05	1.94
Morphotype sp.4	2.5	1.5	--	08	3.11
Unidentified					
Unidentified sp.	2.0	1.5	--	07	2.72
No of isolates recovered	127	89	41	257	--
Colonization frequency	63.5	44.5	20.5	--	--

Table. 3: Species richness and diversity of endophytic fungi in two ethno-medicinal plants.

Plant name	Plant part	Total isolates	Species richness	Diversity indices	
				Shannon-Wiener	Simpson
S rubrum	Leaf	103	19	2.872	0.950
	Stem	65	18	2.808	0.950
	Fruit	33	11	2.246	0.907
M pubesence	Leaf	127	23	2.787	0.957
	Stem	89	23	2.997	0.962
	Fruit	41	14	2.561	0.941

Table. 4: Antimicrobial activity of endophytic fungal isolates of *S. rubrum.*

Endophytic fungi	MTCC 424	MTCC 426	MTCC 736	MTCC 737	MTCC 1457	MTCC 3384	MTCC 9215	MTCC 227	MTCC 8476
Aspergillus niger	--	--	+	+	--	+	--	+	--
Aspergillus fumigatus	--	+	--	+	--	+	+	+	--
Aspergillus versicular	--	++	--	+	--	--	--	--	--
Aspergillus sydowi	+	--	--	+	--	--	+	+	--
Aspergillus fonsecaeus	--	--	--	--	++	+	+	++	--
Aspergillus terricola	--	--	--	--	--	--	--	--	+
Colletotrichum sp.1	--	--	--	+	+	--	+	+	--
Colletotrichum sp.2	+	+	--	--	+	--	+	+	+
Curvularia lunata	--	+	+	--	--	+	+	+	--
Curvularia geniculata	+	+	--	++	+	+	+	++	++
Penicillium purpurogenum	--	+	+	--	--	--	--	++	--
Penicillium lanosum	--	--	+	+	--	--	+	+	+
Penicillium oxalicum	--	+	+	+	--	--	+	++	+
Morphotype sp.1	--	++	+	+++	+++	++	++	+	++
Morphotype sp.2	++	+++	++	++	+++	+++	+++	++	+++
Trichoderma viridae	--	+	+	--	+	+	+	--	--
Trichoderma lignorum	--	--	--	++	--	--	--	--	--
Unidentified sp.1	--	--	--	+	+	--	--	--	--
Unidentified sp.2	--	+	--	--	--	--	--	--	+
Unidentified sp.3	--	+	+	--	--	--	+	+	+

Inhibition expressed by the diameter of inhibition zones: --, no inhibition; +, < 10 mm; ++, 10–15 mm; +++, >15 mm.

MTCC 424- *Pseudomonas aeruginosa*; MTCC 426- *Proteus vulgaris*; MTCC 736- *Bacillus subtilis*; MTCC 737- *Staphylococcus aureus*; MTCC 1457-*Shigella flexneri*; MTCC 3384-*Klebsiella pneumonia*; MTCC 9215-*Candida krusie*; MTCC227-*Candida albicans*; MTCC 8476-*Trichophyton mentagrophytes.*

Table . 5: Antimicrobial activity of endophytic fungal isolates of *M. pubescence.*

Endophytic fungi	MTCC 424	MTCC 426	MTCC 736	MTCC 737	MTCC 1457	MTCC 3384	MTCC 9215	MTCC 227	MTCC 8476
Aspergillus clavatus	--	--	+	+	--	--	--	+	+
Aspergillus fumigatus	--	+	--	--	+	--	--	+	+
Aspergillus versicular	--	--	--	--	+	--	+	--	--
Aspergillus sydowi	+	--	+	--	--	--	--	--	--
Aspergillus flavus	--	+	+	+	+	--	--	+	+
Aspergillus sp.	--	--	--	+	+	+	--	--	+
Chaetomium dolichotrichum	--	+	+	--	+	++	--	+	--
Chaetomium globosum	--	--	--	--	+	++	--	+	--
Cladosporium herbarum	--	--	--	+	+	--	--	--	--
Colletotrichum sp.	--	+	++	++	++	+	++	++	+
Curvularia lunata	+	+	--	--	+++	--	+++	++	++
Curvularia interseminata	--	--	--	+	+	++	--	+	++
Curvularia subulata	--	+	+	--	++	--	--	--	--
Nigrospora zimmermann	+	+	--	--	--	--	--	+	--
Nigrospora sphaerica	+	--	--	+	--	--	+	+	+
Penicillium purpurogenum	--	--	++	--	++	++	+	+	++
Penicillium albidum	--	+	+	--	--	+	++	++	+
Morphotype sp.1	+	--	+	++	++	--	+	--	--
Morphotype sp.2	+	--	--	--	+++	+	--	--	--
Morphotype sp.3	--	--	++	++	+	+	+++	++	+
Morphotype sp.4	--	+	+	--	--	--	--	--	--
Trichoderma koningi	--	--	+	--	+	+	+	+	+
Trichoderma sp .	--	--	--	+	+	++	+	+	+
Torula sp.	--	--	--	--	+	+	+	++	--
Unidentified sp.	--	+	--	+	--	--	--	--	--

Inhibition expressed by the diameter of inhibition zones: --, no inhibition; +, < 10 mm; ++, 10–15 mm; +++, >15 mm.

MTCC 424- *Pseudomonas aeruginosa*; MTCC 426- *Proteus vulgaris*; MTCC 736- *Bacillus subtilis*; MTCC 737- *Staphylococcus aureus*; MTCC 1457-*Shigella flexneri*; MTCC 3384-*Klebsiella pneumonia*; MTCC 9215-*Candida krusie*; MTCC227-*Candida albicans*; MTCC 8476-*Trichophyton mentagrophytes*

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