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Endophytic fungi play an important role in the metabolism of host plant. *Oroxylum indicum* (L.) Vent. is an endangered medicinal tree. In the present study isolation of endophytic fungi from different parts i.e. leaf, shoot and root of this tree was carried out. Two sp. of *Alternaria* (from shoot of *O. indicum*), *Geotrichum* sp. (from root of *O. indicum*) were identified on the basis of morphological and biochemical studies. Other two fungal isolates were identified by ITS sequencing. DNA barcoding targeting the internal transcribed spacer (ITS) region of the nuclear ribosomal repeat has been regarded as a prerequisite procedure. *Polycephalomyces sinensis* and *Phomopsis phaseoli* (both from leaf of *O. indicum*) were identified by ITS sequencing

Key words: Endophytic fungi, microscopic characters, macroscopic characters, enzyme activity, ITS sequencing

INTRODUCTION

The endophytic fungi play important physiological and ecological roles in their host life. Recent investigations have been intensified by the potentialities of endophytic fungal strains in production of bioactive metabolites and antibiotics. Present work describes the isolation strategy of endophytic fungi from different parts i.e. leaf, shoot and root of *Oroxylum indicum* (L.) Vent. It is an important tree which has become endangered in many parts of India (Dalal and Rai *et al.* 2006).There is no any report regarding isolation of endophytic fungi from this tree. Object of the present research is to isolate and study characters of endophytic fungi from different parts (leaf, shoot and root) of *Oroxylum indicum* (L.) Vent.

The species level identification of sterile and/or arthroconidium-forming filamentous fungi presumed to be ascomycetes based upon morphological or physiological features alone is usually not possible due to the limited amount of hyphal differentiation. Therefore, a reliable molecular approach capable of the unambiguous identification of clinical isolates is needed. One hundred sixtyeight presumptive ascomycetes were screened by sequence analysis of the internal transcribed spacer (ITS) regions in an effort to obtain a species identification.

Sequence based identification is a powerful tool for many fungi. Sequencing of the ribosomal genes has emerged as a useful diagnostic tool for the rapid detection and identification of fungi, regardless of whether morphologically distinct structures are produced (Balajee *et al.* 2009). One of the most common ribosomal targets for sequence identification is the internal transcribed spacer (ITS) region. This region contains two informative regions ITS1 and ITS2 which are located between the 18S and 28S ribosomal subunits and which are separated by the 5.8S ribosomal subunit (Leaw *et al.* 2006). The ITS region can be amplified from a broad spectrum of fungi with primers ITS-1 and ITS-4 and can generally be recovered in a single PCR, since the amplicon is usually <“400 to 700 bp in length (Kim *et al.* 2011).

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MATERIALS AND METHODS

Plant Material Collection

The healthy leaf, shoot and root of medicinally important endangered tree *Oroxylum indicum* (L.) vent. were collected from the reserve forest area of Jabalpur district Madhya Pradesh, India.

Sterilization of Plant Material

Plant parts (leaf, shoot and root of *O. indicum*) were surface sterilized with 0.1% mercuric chloride. A small portion from the middle of the each segment of surface sterilized leaf, shoot and root was cut into pieces (0.5-1cm) aseptically for inoculating into sterilized PDA and SDA media. Before inoculation both media amended with a pinch of streptomycin (to avoid bacterial contamination). After plant material inoculation petriplates were incubated at 28^{0}C in a BOD incubator and monitored every day for the growth of endophytic fungal colonies.

Identification of Endophytic fungus

The colonies of isolated fungus from leaf, shoot and root pieces were studied for their macroscopic features (i.e., color, shape and growth of cultured colonies etc) and microscopic features (i.e., the structure of hyphae, conidia and conidiophores) data observed were then compared with the descriptions of endophytic fungus species in the literature and matches were recorded.

Enzymatic Activity

The endophytic fungal isolates were checked for the production of various hydrolyzing enzymes like amylase on Starch Agar Media (SAM), protease on Gelatin Agar Media (GAM), lipase on Peptone Agar Media (PAM) with Tween 80 and cellulase on Czapekdox Agar Media (CMC).

Amylolytic Activity

Amylase activity was assessed by growing the fungi on Starch Agar Media (SAM), (beef extract - 0.9g, peptone 1.5 g, agar 6 g, distilled water 400 ml) with 6 g soluble starch, pH 6.0. After incubation of inoculated fungus for five days on 28^{0}C in incubator, the plates were flooded with 1% iodine. The measurement of clear zone surrounding fungal colony was the estimation of production capacity of amylase enzyme by testing fungus.

Cellulytic Activity

Cellulytic activity was assessed by growing the fungi on Czapekdox Agar Media (CMC), ($NaNO_3$-1g, K_2HPO_4- 0.5g, $MgSO_4$.KCl- 0.25g, $FeSO_4$ - 0.005 g,CMC- 5 g,Agar- 10g,distilled water-500ml) pH 6.0. After incubation, degradation of the cellulose was seen as clear zone around the colonies. The plate was then flooded with Congo red (Titen Biotech limited) for 15 minutes with NaCl (5.84 in 50ml. distilled water) for 10 minutes. Formation of clear zone around the fungal colony is the indication of positive cellulytic activity of isolated fungus.

Lipolytic Activity

For lipase activity, the fungi were grown on Peptone Agar medium (Peptone - 5 g, NaCl - 0.5 g, $CaCl_2.H_2O$-0.01 g, Agar- 8 g,distilled water- 500 ml, pH 6.0) supplemented Tween 80 separately sterilized and added 1% to the medium. At the end of the incubation period, a visible precipitate around the colony due to the formation of calcium salts of the lauric acid liberated by the enzyme indicated positive lipase activity.

Proteolytic Activity

Protease activity was assessed by growing the fungi on Gelatin Agar Media (Beef extract - 0.9g, Peptone - 2g,Gelatin - 48g,NaCl - 2g, Agar - 6g, distilled water -400ml) at pH 6.0. After incubation for five days on 28^{0}C in incubator, the plate was then flooded with saturated aqueous tricarboxylic acetic acid (TCA), which resulted in the formation of a precipitate. This made the agar opaque and develops the clear zone around the fungal colony. Protease activity of isolated fungus was estimated by measuring this clear zone.

Identification of fungal strains

The identification of isolates was carried out at the sequencing facility of Microbial Culture Collection (MCC), NCCS, Pune (www.nccs.res.in/mcc). At the facility, genomic DNA was isolated by the standard phenol/chloroform extraction method (Sambrook *et al.* 2012), followed by PCR amplification of the ITS regions using universal primers ITS1 [5'-TCC GTA GGT GAA CCT GCG G -3'] and ITS4 [5'-TCC TCC GCT TAT TGA TAT GC -3']. The amplified ITS PCR product was purified by PEG-NaCl pre-

cipitation and directly sequenced on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA) as per manufacturer's instructions. Essentially, sequencing was carried out from both ends so that each position was read at least twice. Assembly was carried out using Lasergene package followed by EzFungi search (Kim *et al.* 2012) for identification.

RESULTS AND DISCUSSION

The isolates used in the study were identified as probable or presumptive ascomycetes on the basis of their macroscopic and microscopic, and physiological features. Although a limited number of features of filamentous ascomycetes are not diagnostic, they are suggestive for placement of the isolates in the phylum Ascomycota. Growth is typically rapid except in OL/1, often up the side of the tube or plate; colony colors and back ground are different i.e. off white (OL/1) (Fig.1), green (OL/2) (Fig.3), black (OS/1) (Fig.5), grey (OS/2) (Fig.7) with dark brown background, creamish with pink back ground (OR) (Fig. 9) on PDA and SDA (Table 1).

Microscopically, sterile ascomycetes may display hyphae only or hyphae with conidia. Out of all isolated fungi, only OL/1 was not capable to produce conidia on its aseptate hyphae (Fig.2). Remaining ascomycetes do, however, produce conidia in culture. Most are arthroconidia, in long terminal chain as seen in OL/2 (Fig.4) or barrel shaped (6-12 x 3-6 µm in size), hyline and terminal arthoconidia developed by separation on maturation of enlarge and dense apical cell from septate and branched hyphae in OR. Presence of two hyline spots on both corners was the special features of arthoconidia in OR (Fig.10).

In OS/1 septate, highly branched, simple and brown colored hyphae with short conidiophores were visible. Conidiophores bear club shaped, septate, dark brown colored conidia of size 50-80 x 7-18µm.Conidial wall with small rounded warts creating a slightly rough surface in conidia (Fig. 6). Conidium of OS/2 was similar in structure with conidia of OS/1. Color of conidia was pale brown and size was 20-200 x 7-18µm. Conidia of OS/1has a long chain on other end (Fig. 8).

All isolated ascomycetes fungi were capable to produce amylase enzyme with different capacity. Enzyme amylase produced by OL/1, OL/2, OS/1, OS/2 and OR (Fig. 11-15), enzyme cellulose produced by OS/1, OS/2 and OL/1 (Fig.16-18), enzyme Protease produced by OR and OL/2 (Fig. 19-20) and enzyme lipase produced by OL/1, OL/2, OS/2 and OR (Fig.21-24) (Table 2). On the basis of typical structure of spores *Alternaria* species (OS/1 and OS/2) and *Geotrichum* sp. (OR) were identified.

The identification reports for OL/1 and OL/2 were generated using EzFungi (*http://www.ezbiocloud.net/ezfungi)* Database for OL/1 and OL/2. The confidence in identification is limited by both the availability and the extent of homology shown by the ~700 bp sequence of sample with its closest neighbor in the database. OL/1 was identified as *Phomopsis phaseoli (*Accession No. EU196746) with 97.53% (12/485) pair wise similarity and OL/2 was identified as *Polycephalomyces sinensis*(Accession No. AY857543) with 99.61% (2/501) pair wise similarity and deposited in Microbial Culture Collection (MCC), NCCS, Pune.

Sequence Text (in FASTA format)

(OL/1)

C G T A G G G T G A A C C T G C G G A G G G A T C A T T G C T G G A A C G C G C C T C G G C G C A C C C A G A A A C C C T T T G T G A A C T T A T A C C T A c C T G T T G C C T C G G C G C A G G C C G G C C T T T G T C A A A G A A G G C C C C C T G G G A C A G G G A G C A G C C C G C C G G C G G C C A A C T A A A C T C T T G T T T C T A T A G T G A A T C T C T G A G T G A A A A C A T A A A T G A A T C A A A A C T T T C A A C A A C G G A T C T C T T G G T T C T G G C A T C G A T G A A G A A C G C A G C G A A T G C G A T A A G T A A T G T G A A T T G C A G A A T T C A G T G A A T C A T C G A A T C T T T G A A C G C A C A T T G C G C C C T C T G G T A T T C C G G A G G G C A T G C C T G T T C G A G C G T C A T T T C A A C C C T C A A G C C T G G C T T G G G A T G G G G C A C T G C T C T C T C G C G G G A G C A G G C C C T G A A A T C T A G T G G C G A G C T C G C C A G G A C C C C G A G C G T A G T A G T T A C A T C T C G C T C T G G A A G G C C C T G G C G G T G C C C T G C C G T T A A A C C C C C A A C T C T G A A A A T T G A C C T C G G

(OL/2)

C T T C C G T A G G G T G A C C T G C G G A A

Table 1 : Macroscopic and microscopic characters of isolated fungi

Macroscopic Characters					
Characters	*Phomopsis phaseoli* (OL/1)	*Polycephalomyces sinesis* (OL/2)	*Alternaria* sp. (OS/1)	*Alternaria* sp. (OS/2)	*Geotrichum* (OR)
Type of Colony	Fluffy, slow growing, smooth	Colonies fast growing , flat, powdery or velvety in texture	Thick, fast growing	Thick, Fast growing	Smooth, slimy,white fast-growing
Zone area of colony (after 5 days)	2.6 cm	3.2 cm	3.2 cm	2.3 cm	2.7cm
Color of colony	Off white	Green	Black	Dark grey	Creamish
Area background	Light brown	Green, yellow brown	Dark brown	Brown	Pink
Microscopic characters					
Hyphae	Aseptate	Multiseptate	Highly septate	Branched chains	Septate
Type of Mycelium	Branched	Branched into chains	Branched	Branched chains	Branched into chains
Type of spores	Not found	Arthoconidia	Short conidiophores club shaped with pointed apical end	Club shaped with pointed long apical end	Barrel-shaped arthroconidia, conidiophores absent.
Size of Spores	Not found	5-12 microns	18-83 x 7-18 microns	20-200 x 7-18 microns	6-12 x 3-6 microns
Color of Spores	Not found	Light green	Dark brown	Pale brown	Pink

Table 2 : Comparative enzymatic activity of isolated fungi

Name of the isolated strain	Amylase		Protease		Cellulase		Lipase	
	Zone	Zone size (cm)	Zone	Zone size (cm)	Zone	Zone size (cm)	Zone	Zone size (cm)
Phomopsis phaseoli (OL/1)	+	0.17	_		+	0.16	+	1.9
Polycephalomyces sinensis (OL/2)	+	4.06	+	6.55	_		+	1.2
Alternaria (OS/1)	+	1.82	_		+	0.82	+	1.3
Alternaria (OS/2)	+	1.3	_		+	0.41	_	
Geotrichum sp.	+	12.3	+	1.7.	_		+	1.8

G G A T C A T T A C C G A G T G A G G G T C C C A C G A G G C C C A A C C T C C C A TCCGTGTTGAACTACACCTGTTGCTTCGGC G G G C C C G C C G T G G T T C A C G C C C G G C C G C C G G G G G C C T T G T G C C C C G G G C C C G C G C C C G C G A A G A C C C C T C G A A C G C T G C C C T G A A G G T T G C C G T C T G A G TATAAAATCAATCATTAAAACTTTCAACAACGGATC TCTTGGTTCCGGCATCGATGAAAACGCA GCGAAATGCGATAAGTAATGTGAATTGCAGA ATTCCGTGAATCATCGAATCTTTGAACGCA C A T T G C G C C C C C T G G C A T T C C G G G G G G C A T G C C T G T C C G A G CGTCATTGCTAACCCTCCACCCGGCTG GTGTGTTGGGTCGGCGTCCCCCCCG G G G G A C G G G C C C G A A A G G C AGCGGCGGCGCCGCGTCCG ATCC TCGAG CGCATGG GGCTTTGTCACGCG CTCTGG TAGGGT CGGCCGGCT GGCCACCAG CGACC TCAC GGT CACCT ATTTTTTCT CTTAGGT TGACCT CGGATC AGGTA GGGATA CCCGCT GAACTT AAGCATATC

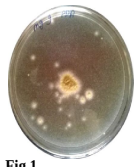


Fig 1


Fig 2


Fig. 3

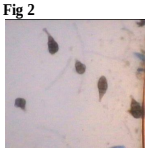


Fig. 4


Fig. 5

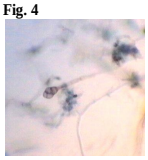


Fig.6


Fig. 7

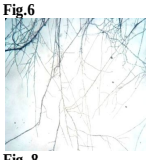


Fig. 8

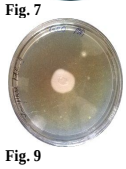


Fig. 9

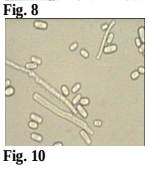


Fig. 10

Photographs showing cultures of fungi isolated from different organs of *Oroxylum indicum*

Fig.1: Petriplate culture of OL/1, **Fig.: 2:** Microscopic view of OL/1, **Fig.3:** Petriplate culture of OL/2, **Fig. 4:** Microscopic view of OL/2, **Fig.5:** Petriplatecuture of OS/1, **Fig.6:** Microscopic view of OS/1, **Fig.7:** Petriplate culture of OS/2, **Fig.8:** Microscopic view of OS/1, **Fig.9:** Petriplate culture of OR, **Fig.10:** Microscopic view of OR

Endophytic fungi colonize a plant without causing visible disease symptoms. Endophytes of medicinal plants have the capacity to synthesize same or similar active substances with their hosts, are common in plants (Lin *et al.* 2010), and have been found to be ubiquitous within all examined plants. Previous studies have found that some endophytic fungi


Fig.11

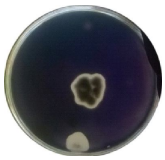


Fig.12

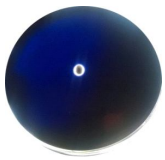


Fig.13


Fig.14

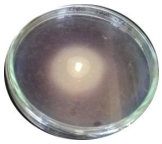


Fig.15

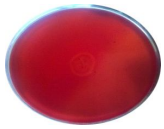
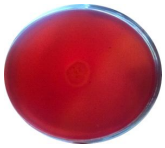


Fig.16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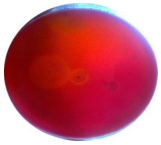
Fig.18

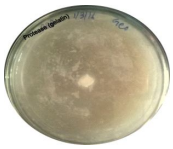
Fig.19

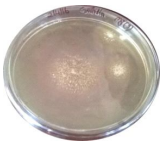
Fig.20

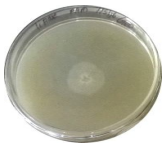
Fig.21


Fig.22


Fig.23


Fig.24

Photographs showing enzyme activity of isolated fungi from *Oroxylum indicum*

Fig.11: Amylase activity of OS/1, **Fig.12:** Amylase activity of OS/2, **Fig.13:** Amylase activity of OL/1, **Fig.14:** Amylase activity of OL/2, **Fig.15:** Amylase activity of OR, **Fig.16:** Cellulase activity of OS/2, **Fig.17:** Cellulase activity of OL/1, **Fig.18:** Cellulase activity of OS/1, **Fig.19:** Protease activity of OR, **Fig. 20:** Protease activity of OL/2, **Fig.21:** Lipase activity of OR, **Fig.22:** Lipase activity of OS/1, **Fig.23:** Lipase activity of OL/1, **Fig.24:** Lipase activity of OL/2.

have roles within the plant in relation to growth (Doty, 2011), enhanced stress resistance (Ownley *et al.* 2010), degradation of pollutants (Sun *et al.* 2011) and the production of bioactive substances in the host. In medicinal plants, some endophytic fungi have been found to produce secondary metabolites that have medicinal value.

In the present work, five endophytic fungi were isolated from different organs of *Oroxylum indicum*. Two fungi (*Phomopsis phaseoli* and *Polycephalomyces sinensis*) were isolated from leaf, two fungi (two sp. of *Alternaria*) from shoot and one fungus (*Geotrichum* sp.) from root of *Oroxylum indicum* were isolated and worked out.

OL/1 is identified as *Phomopsis phaseoli*, member of ascomycota known as the plant pathogen, infecting soybean, sweet potato and peanut and known as the imperfect fungi. It is rarely found to cause human diseases. In literature, presence of two spores is reported (Eugenio *et al.* 2010) (i) α spores- hyaline, fusiform, straight, aseptate single-celled, clear and oval to fusoid and (ii) β spores- hyaline, filiform, straight or more often hamate, aseptate very rare with oily drops, single celled, clear, long and thin with a characteristic curve or bend.

In present work, only sterile hyphae were detected and fungus was identified on the basis of ITS sequencing.

OL/2 is identified as *Polycephalomyces sinensis (Ophiocardyceps sinensis*), member of *ascomycota* that parasitizes larvae of host moths and produces a fruiting body valued as an herbal remedy found in mountain regions of India, Nepal and Tibet (Dong *et al*. 2012). It is known in english colloquially as caterpillar fungus and mistakenly referred to as vegetable caterpillar. There are many colloquial names for this fungus due to its historical significance in traditional Chinese medicine (Shrestha *et al.* 2010).

In present work clusters of long chain of small conidia is observed. They were very dense and present in large numbers. *Polycephalomyces sinensis*is identified on the basis of ITS sequencing.

Alternaria, a member of ascomycetes is known as pathogenic fungi. At least 20% of agricultural spoilage is caused by *Alternaria* species. They are known as major plant pathogens. Not all *Alternaria* species are pests and pathogens; some have shown promise as biocontrol agentsagainst invasive plant species. Some species have also been reported as endophytic microorganisms with highly bioactive metabolites (Kelman *et al.* 2015).

The antimicrobial potential of endophytic fungi *Alternaria* isolated from the leaf tissues of *Tectonagrandis* against *Staphylococcus aureus, Candida albicans* (Phongpaichit *et al.* 2006) was studied. Fermentation cultures of *Alternaria sp.* show inhibitory activity against both gram positive and gram negative bacteria and also against *Candida albicans* (Raviraja *et al.* 2006).

Despite the pathogenic nature of the fungus, it has been utilized for management of several weeds, including *Sesbania exaltata* a noxious weed of soybean (Boyette *et al.* 2007). *Alternaria destruens* has been reported to have herbicidal activity against Dodder, a parasitic weed and it works well in combination with other herbicides as well. It has been reported as an endophyte from *Artimisia* sp. (Huang *et al.* 2009).

In literature club shaped spores are present as single or form long chains on septate hyphae. Thick colonies (black and grey) are reported (Nowicki *et al.* 2012) in some *Alternaria* sp. In present work *Alternaria* sp. is identified on the basis of similarity of spore structure as described in literature. But the isolated spores of *Alternaria* fungi are showing different characters so they are identified as two different sp. of *Alternaria.*

Geotrichum sp. is a plant pathogen, which causes sour rot of citrus fruits, tomatoes, carrot and some vegetables (Thornton *et al.* 2010). It is also used widely in the production of certain dairy products including rind cheeses such as Camebert, Reblochan and others. The fungus can also be found in a Scandinavian like product known as villi where, it is responsible for the products velvety texture (Etienne *et al.* 2008).

In present work OR is showing cylindrical arthoconidia, flat, white, dry, fast-growing hyphae and absence of conidiophores. Similar characteristics are also observed in earlier work (Watanabe *et al.* 2010). So *Geotrichum* sp. is identified on the basis of growth pattern of mycelium and structure of spores.

The above isolated fungal strains are capable for production of different enzymes so they may prove beneficial in industrial applications. The knowledge of endophytic fungus from a medicinal plant is useful to understand the metabolite production strategy of that particular organ of plant.

Sequence-based identification is a powerful tool for many fungi. ITS region displays enough sequence variability to allow the identification of many fungi to the species level. Comparison of the top hits from the Gene Bank database for the ITS and D1/D2 regions showed a number of isolates that returned the same species name for both the ITS region and the D1/D2 region (Anna *et al.* 2009).The ITS sequence lengths obtained from Gene Bank ranged from 95% to 99% compared to the complete ITS sequences that we derived by sequencing with primers ITS-1 and ITS-4. In the BLASTn results for each isolate yielded ascomycetes identification to the species level with 97.53% (12/485) for OL/1 as *Phomopsis phaseoli and* 99.61% (2/ 501) for OL/2 identified as *Polycephalomyces sinensis* of the isolates.

The above isolated fungal strains are capable for production of different enzymes so they may prove beneficial in industrial applications. The knowledge of endophytic fungus from a medicinal plant is useful to understand the metabolite production strategy of that particular organ of plant.

Three parts (leaf, shoot and root) of *Oroxylum indicum* (L.)Vent. were taken for endophytic fungi isolation. The isolated fungi were characterized and identified as *Polycephalomyces sinensis* (from leaf), *Phomopsis phaseoli* (from leaf), two species of *Alternaria* (from shoot) and *Geotrichum* sp. (from root) family all from ascomycetes.

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