Diversity and enzymatic activity of foliar endophytic fungi isolated from medicinal plants of Indian dry deciduous forest

Prathyusha P.*, Rajitha Sri A. B. and Satya Prasad K.

Mycology and Plant Pathology Laboratory, Department of Botany, Osmania University, Hyderabad, A. P., India

ABSTRACT

Twenty Four foliar endophytic fungal species were isolated from the living asymptomatic leaves of 37 medicinal plants from Bhadrachalam dry deciduous forest along the river Godavari of Khammam district, Andhra Pradesh, India. Morphotypes of mycelia sterilia are predominant among frequent fungal endophytes. Endophytes associated with Andrographis paniculata are more in number while Holorrhina antidysentrica supported minimum endophytic fungi. Species of Acremonium, Cladosporium, Curvularia, Alternaria and Colletotrichum were frequently isolated. Arthrinium phaeospermum is a rare and infrequent endophyte mostly isolated from Andrographis paniculata. Badarisima sojae, Emericella nidulans, Sordaria fimicola, Stachybotrys chartarum, Tretopileus sphaeroporus are the new endophytes reported for the first time. Among the 24 endophytic fungi, majority produced amylase (58%) followed by pectinase (45%) and protease (33%). None of the endophytes exhibited lipolytic or lignolytic activity. The results provide valuable information on endophytic fungal diversity from natural dry deciduous forest flora under the threat of inundation by the ongoing Polavaram irrigation project. Enzymatic ability of the endophytes suggests their potential use as novel agents for new drugs and other industrially important secondary metabolites.

Key words: Bhadrachalam forests, Endophytic fungi, Extracellular enzyme activity, Medicinal plants

INTRODUCTION

Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites of pharmaceutical importance [23, 28, 29,]. Endophytes represent a huge diversity of microbial adaptations that have developed in special and isolated environments. Their diversity and specialized habituation make them an exciting field of study in the search for new medicines or novel drug like molecules [11]. The fungus that lives within photosynthetic plant tissue by forming symbiotic relationship with host is considered an endophytic fungus [22] and it does not cause any harmful effect to the host [19, 21]. Growth of most endophytes depends on readily available compounds as soluble sugars. The ability of endophytic fungi to produce enzymes for degrading cellulose and lignin is a probable strategy that allows some endophytes to decay tissues and persist as saprobes after host senescence [12, 17], to change their mode of life from an endophyte to saprobe or pathogen [24]. Further 60% of enzymes used in industrial processes are produced by few genera of fungi distributed of worldwide [25]. Endophytes are capable of synthesizing bioactive compounds that can be used by plants for defense against pathogens and some of these compounds proved useful for novel drug discovery. These unique compounds represent a huge diversity and offer an enormous potential for exploitation in medicinal, agricultural and industrial uses [7, 8, 30]. In most of the studies on endophytic fungi from tropics, anamorphic fungi were commonly isolated and substantial number of fungi failed to sporulate in culture [6, 18]. The commonly isolated mycelia sterilia have been grouped into morphotypes based on morphological characteristics [10]. Natural habitats are fast degrading and disappearing due to anthropogenic intervention in the name of development. One such habitat in southern India, a deciduous forest of Bhadrachalam is at the verge of disappearance due to the ongoing Polavaram irrigation project that was given national status in recent times. Bhadrachalam forest spread along the river Godavari in the state of Andhra Pradesh, India is a dry deciduous forest with local pockets of moist deciduous forest elements which provides a special environment for endophytic
fungi to diversity. The threat of submergence by the ongoing Polavaram project necessitates the assessment and conservation of plant and microbial diversity. In this paper we report the diversity of fungal endophytes from the medicinal plants collected from Bhadrachalam forest and their enzymatic abilities for prospective use.

MATERIALS AND METHODS

Sources of Endophytes
Endophytic fungi were isolated from healthy medicinal plants of Bhadrachalam forest located along the river Godavari, Khammam dist., Andhra Pradesh, India.

Isolation and culture of endophytic fungi
The leaf samples were collected using the perforated bags to avoid the desiccation of plant material. The samples were rinsed gently in running water to remove dust and debris. After proper washing, leaves were cut into 3-4 mm × 0.5-1 cm pieces under aseptic conditions. Plant material was treated with 75% ethanol for 1 min followed by immersion in sodium hypochlorite and again in 75% ethanol for 30 seconds [3]. They were finally rinsed with de-ionized sterile distilled water and blot dried on sterile tissue paper, sterilized leaves 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.

Fungi growing out of the plant explants were subcultured on separate PDA slants and stored at 4°C for further identification. The fungi were identified based on the cultural characteristics and direct microscopic observations of the fruiting bodies and spores of fungi using standard manuals [5, 2]. Non sporulating strains were induced for sporulation by culturing them on malt extract agar (MEA) and water agar (WA). Those cultures which failed to sporulate were grouped under mycelia sterilia.

Calculation of colonization frequency and Relative frequency
Colonization frequency (%) of an endophyte species was equal to the number of segments colonized by a single endophyte divided by the total number of segments observed ×100 [26].

\[
\text{Colonization frequency} = \frac{\text{Number of segments colonized by fungi}}{\text{Total number of segments observed}} \times 100
\]

Relative frequency (RF) of isolation, used to represent fungal density, was calculated as the number of isolates of a species divided by the total number of isolates, and expressed also as a percentage [8, 20].

\[
\text{Relative frequency} = \frac{\text{Number of isolates of a species}}{\text{Total number of isolates}} \times 100
\]

Extracellular enzyme assay
Production of different extracellular enzymes such as amylase, cellulase, protease, laccase, lipase [1], pectinase [24] were assessed based on the digestion of the dissolved or suspended substrate in agar medium after for 3-5 days of incubation at room temperature.

Amylase
The activity of amylase was determined by inoculating selected isolates in glucose yeast extract peptone (GYP) agar medium with 2% soluble starch. After 3-5 days incubation time, the fully formed cultures were flooded with 1% iodine in 2% potassium iodide. The clear zone in the form of halos was visualized around the fungal colony (Fig 4).

Cellulase
For celluloletic activity, the isolates were grown on yeast extract peptone agar medium amended with 0.5% Na-carboxy methyl cellulose. After incubation, the plates were flooded with 0.1% Congo red and destained with 1M sodium chloride for 15 min. A clear halo around the colony indicates the cellulase activity (Fig 5).

Laccase
The laccase activity was observed by growing the isolates on GYP agar medium and 1-naphthol, 0.005% was added along with the medium. As a result of oxidation of 1-naphthol by laccase enzyme produced by endophytic fungi, a change in colour to blue takes place.
Lipase
The lipase activity was assessed by growing the fungi on peptone agar medium amended with sterile Tween 20. After 4-5 days of incubation clear halos appear around the colony indicating the lipase activity.

Pectinase
Pectinolytic activity was tested by growing the fungi in pectin agar medium. After 4-5 days of the incubation period, the plates were flooded with 1% aqueous solution of hexadecyl tri methyl ammonium bromide. A clear zone formed around the fungal colony indicated pectinolytic activity (Fig 6).

Protease
The proteolytic activity was determined by growing the isolates on GYP agar medium supplemented with sterilized 0.4% gelatin. After 4-5 days of the incubation, the culture was flooded with saturated aqueous ammonium sulphate. The clear zone around the colony indicates the hydrolysis of gelatin in media and the unhydrolysed gelatin is precipitated by ammonium sulphate (Fig 7).

RESULTS AND DISCUSSION
A total of 335 endophytic fungal strains isolated from 37 medicinal plants from a dry deciduous forest of Bhadrachalam, Khammam district of Andhra Pradesh, India were identified into 24 taxa based on morphological characters. The endophytes which failed to sporulate were commonly isolated from majority of plants and named as morphotypes under mycelia sterilia (Table 1). These morphotypes are predominant among frequent fungal endophytes with colonization frequency of 15% and relative frequency of 19% (Fig 1, Fig 2). *Andrographis paniculata* has the highest number of isolates while *Holorrhina antidysentrica* recorded minimum endophytes with only two isolates. Species of *Acremonium*, *Cladosporium*, *Curvularia*, *Alternaria* and *Colletotrichum* were the frequent endophytes along with many known but less frequently isolated fungi in this study. These results are in conformity with the earlier findings from tropical endophytic fungi [4, 9, 16]. *Arthrinium phaeospermum* is a rare and infrequent endophyte predominantly isolated from *Andrographis*. Five fungi, viz., Badarisima sojae, *Emericella nidulans*, *Sordaria fimicola*, *Stachybotrys chartarum* and *Tretopileus sphaerophorus* are reported as new endophytes for the first time.

Each of the 37 medicinal plants harboured at least one, often many endophytic fungi with high endophytic diversity in *Andrographis paniculata*, *Terminalia bellerica* and *Wrightia tinctoria* (Fig 3).

![Fig 1: Colonization frequencies of different endophytic taxa isolated from 37 medicinal plants of Bhadrachalam forest. “Others” include rare and infrequent endophytic fungal species](image-url)
Fig 2: Relative frequencies of different endophytic taxa isolated from 37 medicinal plants of Bhadrachalam forest. “Others” include rare and infrequent endophytic fungal species.

Fig 3. Endophytic fungal diversity of medicinal plants from Bhadrachalam forest, Andhra Pradesh, India.
Fig 4. Amylase activity on Starch medium

Fig 5. Cellulase activity on GYP medium

Fig 6. Pectinase activity on Pectin Agar medium
Table 2: Extra cellular enzyme activity of endophytic fungi from medicinal plants collected from Bhadrachalam forests

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Endophytic fungi</th>
<th>Amylase</th>
<th>Protease</th>
<th>Cellulose</th>
<th>Pectinase</th>
<th>Laccase</th>
<th>Lipase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acremonium sp.</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td></td>
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</tr>
<tr>
<td>2</td>
<td>Alternaria alternata</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Arthrinium phaeospermum</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>++</td>
<td></td>
<td></td>
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<tr>
<td>4</td>
<td>Aspergillus ochraceus</td>
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<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Chaetomium sp.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Cladosporium cladosporioides</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Colletotrichum dematium</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Colletotrichum gloeosporioides</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Curvularia sp.</td>
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<td>+</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>10</td>
<td>Drechslera sp.</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
</tr>
<tr>
<td>11</td>
<td>Emericella nidulans</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>12</td>
<td>Fusarium solani</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>13</td>
<td>Glomerella cingulata</td>
<td>-</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>14</td>
<td>Khuskia oryzae</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>15</td>
<td>Leptosphaeria chartarum</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>16</td>
<td>Nigrospora sphaerica</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>-</td>
</tr>
<tr>
<td>17</td>
<td>Penicillium frequentans</td>
<td>+</td>
<td>+++</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>18</td>
<td>Pestalotiopsis microspora</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
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<tr>
<td>19</td>
<td>Pestalotiopsis mangiferae</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>Pestalotiopsis glandicola</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>Phomopsis sp.</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>22</td>
<td>Sordaria fimicola</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>Stachybotrys chartarum</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>24</td>
<td>Tetrapleura sphacelopora</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

- = No production; + = Weak production; ++ = Medium production; +++ = High production

Extracellular enzymes offer an added advantage to the endophyte in utilizing various cell wall constituents during senescent stages and in decomposition of dead leaves. Majority of the endophytes, secreted amylase (58%) followed by pectinase (45%), protease (33%) and no one is lipolytic or lignolytic (Table 2). Sordaria fimicola produced amylase alone with high activity. Many endophytes A. phaeospermum, A. ochraceus, C. dematium and P. frequentans were positive for protease activity and this trait is of clinical importance. The endophytic isolates from Oscimum species have been reported to produce proteases and implicated in clinical applications in the treatment of diabetes [15]. However, no protease activity present in endophytes [14]. Cellulase production was relatively low in Pestalotiopsis mangiferae, P. glandicola, Penicillium frequentans, Drechslera sp., Cladosporium cladosporioides, Acremonium sp. and Alternaria alternata while other fungi are non-producers of cellulase. No laccase and lipase activity were evident in all the 24 endophytic fungi. Pestalotiopsis microspora and P. mangiferae were good source of pectinase followed by A. phaeospermum, Glomerella cingulata and Stachybotrys chartarum. None of the fungi showed laccase activity. The endophytic nature of these fungi might be the reason for the lack of laccase activity, since an active enzyme might cause damage to host plant [13]. Many fungal endophytes produced a diversity of enzymes in this study may serve as a good source for industrial applications as fungal enzymes are more stable than other enzymes.

Diverse foliar endophytic fungal species inhabiting the medicinal plants form important components of microbial diversity and serve as a resource for chemical compounds of medicinal and industrial importance. They play a beneficial role in the physiology of host plants and their enzyme production ability might influence litter.
decomposition and carbon and nutrient cycling [30]. The ability of extracellular enzymes by the endophytes suggests their potential to access detrital C, N and P nutrient uptake [16]. Several of the common and some of the rare and new endophytic fungi reported in this paper may be explored for the secondary metabolites of importance in pharma and agri-industries. Since Bhadrachalam dry deciduous forest is at the verge of inundation by the Polavaram project, the endophytic fungi isolated from medicinal plants of the area not only enrich the information on fungal diversity but helps to conserve the rare and novel fungal endophytes for future exploitation.

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