Antimicrobial potentials of endophytic fungi inhabiting three Ethno-medicinal plants of Similipal Biosphere Reserve, India

J Mohanta, K Tayung, U Mohapatra

Introduction

The search for new antibiotics to overcome the growing human problems of drugs resistance in microorganisms and appearance of new diseases has been rapidly increasing around the world. Realizing the capability of microorganisms to produce diverse bioactive molecules and the existence of unexplored microbial diversity, research is underway to isolate and screen microbes of diverse habitat and unique environment for discovery of novel metabolites. One such unexplored and less studied microorganism is the endophytic fungi, which are defined as those microbes that colonize healthy tissues of plants, at least for a part of their life cycle, without causing apparent disease symptoms in their host (Petrini, 1991; Wilson, 1995). Different works carried out so far regarding the role of endophytes in host plants indicate that they can stimulate plant growth, increase disease resistance, improve plant's ability to withstand environmental stresses and recycle nutrient (Sturz and Nowak, 2000). Besides these, endophytes are also recognized as rich sources of bioactive metabolites of multifold importance (Tan and Zou, 2001; Strobel and Daisy, 2003).

In developing countries, the indigenous communities have been using medicinal plants in different ways for the treatment of various diseases, which in turn has resulted in scientific discoveries, with a wealth of literature on plant extracts and their biological activities. However, recently it has been reported that fungal endophytes residing within these plants could also produce metabolites similar to or with more activity than that of their respective hosts (Strobel, 2002). Therefore, it is believed that search for novel compounds should be directed towards plants that commonly serve indigenous populations for medicinal purposes and plants growing in unique environmental setting or interesting endemic locations as they are expected to harbor novel endophytes that may produce unique metabolites with diversified applications (Strobel and Daisy, 2003). With this knowledge, the present investigation was carried out to study fungal endophytes associated with three medicinal plants used as ethno medicine by the tribal communities of Similipal Biosphere Reserve, India and to evaluate these endophytes for antimicrobial activity against some human pathogens.
MATERIALS AND METHODS

LOCATION AND STUDY AREA

Plant materials were collected from Similipal Biosphere Reserve located at 21°16' to 22°08' N latitude and 86°4' to 86°37' E longitude. Similipal Biosphere Reserve is a rich biodiversity hotspot of eastern India representing a great aesthetic treasure as well as a grand repository of biological wealth. Samples were collected during February-March 2007 at an altitude of 80 – 869 m above Mean Sea Level (MSL). The mean temperature during the study period was 21±2 °C. The plant species chosen for the present study were Andrographis paniculata, Acorus calamus and Drynaria quercifolia.

COLLECTION OF PLANT PARTS

Four plants of each species were selected and 8 samples (rhizome and stem) from each plant were randomly cut off with an ethanol-disinfected sickle and placed separately in sterile polythene bags to avoid moisture loss. The materials were transported to laboratory within 12h and stored at 4°C until isolation procedures were completed.

ISOLATION OF ENDOPHYTIC FUNGI

The collected samples were washed thoroughly with sterile distilled water and air dried before they are processed. The materials were then surface sterilized by immersing them sequentially in 70% ethanol for 3min and 0.5% NaOCl for 1min and rinsed thoroughly with sterile distilled water. The excess water was dried under laminar airflow chamber. Then, with a sterile scalpel, outer tissues were removed and the inner tissues of 0.5cm size were carefully dissected and placed on petri-plates containing different mycological media. The media were supplemented with streptomycin sulphate (100mg/L) to suppress bacterial growth. The plates were then incubated at 25±2 °C until fungal growth appeared (Figure 1). The plant segments were observed once a day for the growth of endophytic fungi. Hypal tips growing out the plated segments were immediately transferred into PDA slant and maintained at 4 °C. The fungal isolates were identified based on their morphological and reproductive characters using standard identification manuals (Barnett and Hunter, 1972; Subramanian, 1971). The fungal cultures that failed to sporulate were categorized as sterile mycelia. All the isolates are maintained in Potato dextrose agar slant in the department of Botany, North Orissa University.

FUNGAL CULTIVATION AND EXTRACTION OF METABOLITES

The fungal endophytes were cultivated on Potato dextrose broth (Himedia) by placing agar blocks of actively growing pure culture (3mm in diameter) in 250ml Erlenmeyer flasks containing 100ml of the medium. The flasks were incubated at 25±1 °C for 3 weeks with periodical shaking at 150 rpm. After the incubation period, the cultures were taken out and filtered through sterile cheesecloth to remove the mycelia mats. The fungal metabolites were extracted by solvent extraction procedure where ethyl acetate was used as an organic solvent. Equal volume of the filtrate and ethyl acetate was taken in a separating funnel and was shaken vigorously for 10 min. The solution was then allowed to stand, where the cell mass got separated and the solvent so obtained was collected. Ethyl acetate was evaporated and the resultant compound was dried in vacuum evaporator using MgSO₄ to yield the crude extracts. The crude extracts were then dissolved in Dimethyl sulphoxide (DMSO) for antimicrobial bioassay (Figure 2).

EVALUATION OF ANTIMICROBIAL ACTIVITY

Altogether eight common human pathogens were used to evaluate the antimicrobial activity of endophytic crude extracts. The test pathogens include three Gram-positive bacteria i.e Staphylococcus epidermidis (Se), Bacillus subtilis (Bs), Staphylococcus aureus (Sa), three Gram-negative bacteria i.e Klebsiella pneumoniae (Kp), Shigella flexneri (Sf), Escherichia coli (Ec) and two fungal pathogens i.e Candida albicans (Ca) and Candida tropicalis (Ct). All the test pathogens were obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India.

For antimicrobial evaluation, agar cup disc diffusion method was followed (Grammer, 1976). Nutrient agar plates were inoculated with overnight culture of each bacterial suspension. Similarly for the fungal pathogens, Sabouraud’s agar plates were inoculated with each fungal suspension. The plates with the inoculated organisms were evenly spread out with sterile cotton swabs. Agar cups were prepared by scooping out the media with a sterile cork borer (7mm in diameter). The cups were then filled with 100µL of the crude extract that was already dissolved in DMSO. The plates were then incubated at 35±1 °C for 24 h and the zone of inhibition was recorded and compared with the control (i.e a cup filled with DMSO solution only).

DETERMINATION OF MINIMUM INHIBITORY
CONCENTRATION

Minimum inhibitory concentration was determined only for the crude metabolites produced by the Fusarium sp. Micro broth dilution assay technique was followed for the purpose. The assay was performed in sterile 96-well plates. Overnight culture of the each test organisms (approx. $10^4 - 10^5$ CFU) were seeded into the wells and the crude metabolites was tested at concentration from 1000 µg to 75 µg/ml. Three wells were inoculated for a given concentration. The plates were incubated for 24 hr at 35±1 °C. MIC was determined as the least concentration of the crude metabolites that inhibited the growth of the test organisms.

RESULTS AND DISCUSSION

Three potential medicinal plants used by the tribal communities of Similipal Biosphere Reserve were selected for endophytic studies (Table 1).

Figure 1

Table 1: Medicinal plants selected for endophytic studies

<table>
<thead>
<tr>
<th>Plant name</th>
<th>Family</th>
<th>Local name</th>
<th>Medicinal properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acorus paniculata</td>
<td>Acoraceae</td>
<td>Nervendra</td>
<td>Used in treatment of advance stage of dysentery, dyspepsia, toxic and stimulant</td>
</tr>
<tr>
<td>Acorus calamus</td>
<td>Acoraceae</td>
<td>Paysamka</td>
<td>Used for treatment of dyspepsia, flatulence, and loss of appetite. Administered during cough, fever and cold. Also used as antispasmodic and tonic.</td>
</tr>
<tr>
<td>Dryneria quercifolia</td>
<td>Drynariaceae</td>
<td>Ghari punaki</td>
<td>Used as astringent, treatment of dyspepsia and fever, dried rhizome used as anthelmintic.</td>
</tr>
</tbody>
</table>

All the plant species were found colonized with endophytic fungi. The endophytes were isolated using three different mycological media namely potato dextrose agar (PDA), malt extract agar (MEA) and water agar (WA). In many instances these media were commonly used for isolation of endophytes (Mahesh et al., 2005; Tejesvi et al., 2005). Maximum endophytes were obtained in potato dextrose agar medium and minimum in water agar media (Table 2).

Figure 2

Table 2: Isolation of endophytes from three medicinal plants on various media

<table>
<thead>
<tr>
<th>Host</th>
<th>Part used</th>
<th>Media used</th>
<th>No. of colonies recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acorus paniculata</td>
<td>Stem</td>
<td>PDA</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MEA</td>
<td>06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WA</td>
<td>01</td>
</tr>
<tr>
<td>Acorus calamus</td>
<td>Rhizome</td>
<td>PDA</td>
<td>09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MEA</td>
<td>06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WA</td>
<td>02</td>
</tr>
<tr>
<td>Dryneria quercifolia</td>
<td>Rhizome</td>
<td>PDA</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MEA</td>
<td>09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WA</td>
<td>02</td>
</tr>
</tbody>
</table>

Media: PDA-Potato dextrose agar, MEA-Malt extract agar, WA-Water agar
*Colonies recovered 50 segments.

Altogether 60 fungal endophytes belonging to 14 genera were isolated from the three medicinal plants (Table 3). Out of which, 31 endophytes (51.66%) were obtained as filamentous forms and 29 of them (48.33%) as yeast colonies. Colonization of endophytes was found to be variable in the three medicinal plants. In Acorus calamus, yeast colonies were obtained in highest numbers and filamentous forms were isolated in lowest numbers. In Andrographis paniculata, filamentous forms were isolated in highest numbers but yeast colonies were obtained in lowest numbers. However, in Dryneria quercifolia, colonization of yeast and filamentous forms were more or less same in numbers. Despite the broad occurrence of endophytes in plant organs, many endophytic fungi appear specialized to particular host tissues (Arnold et al., 2000). Furthermore, fungal endophytes are reported to be host specific at the same time several species can also be isolated from different host (Suryanarayanan et al., 2002). Such host specific endophytes have also been observed in our present study in the three medicinal plants.
Species of Curvularia were dominant and were isolated only from Andrographis paniculata whereas colonization and dominant nature of Fusarium was observed in Acorus calamus. Similarly, species of Penicillium and Alternaria were dominant and host specific to Drynaria quercifolia. Although the role of endophytes on host plants is less studied, different functions have been described. When beneficial, such association can stimulate plant growth, increase disease resistance and improve the plant’s ability to withstand environmental stresses (Sturz and Nowak, 2000). It had been discussed that species of Curvularia as endophytes help plants to withstand high constant soil temperatures of 50°C and intermittent temperatures as high as 65°C (Johri, 2006). In our present investigation also, we have isolated species of Curvularia as a host specific endophytes to Andrographis paniculata. Since this plant has been observed to survive in high temperature during summer and in other adverse environmental condition, we therefore speculate that, such beneficial association might have been developing between the fungus and the host, which needs thorough investigation.

Fungal endophytes have been recognized as repository of novel secondary metabolites for potential therapeutic use (Tan and Zou, 2001). Further, Daisy and Strobel (2003) necessitated that medicinal and endemic plants should use for endophytic studies as they are expected to harbor rare and interesting endophytes with novel bioactive metabolites. This has lead to the discovery of several bioactive compounds from fungal endophytes and wealth of literature on antimicrobial activity of endophytic fungi isolated from medicinal plants (Raviraja et al., 2006; Li et al., 2006; Tayung and Jha, 2006). In our present study also, we have demonstrated crude metabolites extracts of fungal endophytes isolated from the three medicinal plants showed considerable antimicrobial activity against a panel of human pathogenic microorganisms. Out of the 31 fungal endophytes isolated as filamentous forms, 13 isolates (41.9%) could display antimicrobial activity inhibiting at least one of the test pathogens (Table 4). Among the potent strains, 19.3% displayed both antibacterial (Gram-positive & Gram-negative) and antifungal activity inhibiting at least one of the test pathogens. However, only 6.4% of the strains showed antimicrobial activity against all the test pathogens. Among the potent strains, crude metabolite of an endophytic fungus identified as Fusarium sp. displayed significant antimicrobial activity against entire test pathogens. The extract was significantly effective against both Gram-positive and Gram-negative bacteria (Figure 3 & 4) and moderately effective against the fungal pathogens. This showed the broad-spectrum nature of the metabolite.
Figure 5
Figure 2: Crude metabolites extract of some endophytic fungi and metabolite extracted from sp. (Fm) dissolve in DMSO.

Figure 6
Figure 3: Antibacterial activity of the crude metabolite extract of sp. (Fm) against

Figure 7
Figure 4: Antibacterial activity of the crude metabolite extract of sp. (Fm) against

Figure 8
Table 4: Antimicrobial activity of the metabolites obtained from potent Endophytic strains

<table>
<thead>
<tr>
<th>Endophytic fung</th>
<th>Sa</th>
<th>Se</th>
<th>Sa</th>
<th>Ee</th>
<th>St</th>
<th>Re</th>
<th>Ca</th>
<th>Ox</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curvularia fumata</td>
<td>---</td>
<td>---</td>
<td>+</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Curvularia sp.</td>
<td>---</td>
<td>---</td>
<td>+</td>
<td>---</td>
<td>---</td>
<td>+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Drechslera sp.</td>
<td>+</td>
<td>---</td>
<td>---</td>
<td>+</td>
<td>---</td>
<td>+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Sterile mycelia 1</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Fusarium sp.</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Fusarium moniliforme</td>
<td>++</td>
<td>++</td>
<td>---</td>
<td>+</td>
<td>---</td>
<td>+</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Monographium sp.</td>
<td>+</td>
<td>---</td>
<td>---</td>
<td>+</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Acremonium sp.</td>
<td>+</td>
<td>---</td>
<td>---</td>
<td>+</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Penicillium sp.</td>
<td>+</td>
<td>---</td>
<td>---</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Sterile mycelia 2</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Alternaria alternata</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>---</td>
<td>---</td>
<td>+</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Trichoderma sp.</td>
<td>++</td>
<td>++</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>+</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Sterile mycelia 3</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>ND</td>
</tr>
</tbody>
</table>

Sa - Staphylococcus aureus; Se - Bacillus subtilis; Sa - Staphylococcus aureus; Es - Escherichia coli; St - Shigella flexneri; Ec - Escherichia coli; Ca - Candida albicans; Ox - candida tropicalis

--- indicate no activity; ** indicate partial inhibition; ND - not determined
+ indicate zone diameter < 10mm; ++ indicate zone diameter of 10-15mm;
+++ indicate zone diameter > 15mm

The metabolite was very effective and showed highest zone of inhibition against Shigella flexneri and less effective with lowest zone against Staphylococcus aureus. The MIC of the metabolite ranges from 76.6 µg/ml to 376.6 µg/ml with
The fungus was isolated from rhizome of Acorus calamus, which is a plant of ethno medicinal importance. Fusarium as endophyte has been reported from several plant species (Mahesh et al., 2005; Nalini et al., 2005) and its antimicrobial potential have been studied and reported (Wang et al., 2007). However, to our knowledge this is the first report of Fusarium as endophyte and its antimicrobial activity from Acorus calamus. It has been estimated by the World Health Organization (WHO) that approximately 80% of the world’s population from developing countries rely mainly on traditional medicines (mostly derived from plants) for their primary health care. And at least 119 chemical compounds, derived from 90 plant species, are important drugs currently in use in one or more countries (Balick et al., 1996). However, due to over exploitation of these genetic resources and other biotic interferences, many plants used as medicines have become critically endangered or are in verge of extinction. Since, it is believed that these plant species may harbor quite distinct and potential fungal endophytes that might produce novel metabolites with multifold applications, research priority should be directed to study them because disappearance of any of these plant species will also disappears the entire suite of associated endophytes. The study of plant-associated endophytes could therefore provide the best possible way of acquiring novel metabolites. The present study thus, reinforced the assumption that endophytes of ethno medicinal plants could be a promising source of antimicrobial substances. We are currently working on to characterize the active metabolites of some potent strains.

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