

## Endophytic Fungi Occurring in *Ipomoea carnea* Tissues and their Antimicrobial Potentials

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### ABSTRACT

The aim of this work was to study the endophytic fungi associated with the tissues of *Ipomoea carnea*, a common invasive plant of India. A total of 69 isolates belonging to ten taxa comprising 1.45% Zygomycetes, 10.14% Coelomycetes, 62.32% Hypomycetes, 18.84% sterile mycelia and 7.25% unidentified species were obtained. Species of *Curvularia*, *Aspergillus*, *Fusarium*, *Colletotrichum* and sterile fungus were isolated as dominant endophytes. Colonization frequency of *Curvularia* (7.25%) was highest which was isolated from all the tissues. The samples collected during the monsoon harbored more endophytes and showed higher species richness than the samples obtained in summer season. Of the total isolates, 15 isolates (21.74%) displayed antimicrobial activity, inhibiting at least one of the test microorganisms that comprised of pathogenic bacteria (*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, *Pseudomonas fluorescens*, *Shigella dysenteriae*) and fungi (*Trichophyton rubrum*, *Aspergillus fumigatus*, *Trichophyton sp.*). The results provided promising baseline information on the endophytic fungal diversity associated with *I. carnea* tissues and their potential exploitation as antimicrobial agents.

**Key words:** *Ipomoea carnea*, Endophytic fungi, *Curvularia*, antimicrobial activity

### INTRODUCTION

Fungi that colonized inner plants tissues without causing any disease symptoms are called endophytic fungi. Over the years, several plants have been investigated for fungal endophytes, both from the temperate (Bills 1996) and tropical plants host (Suryanarayan et al. 2002, 2003). Studies have shown that fungal endophytes are neither incident residents nor merely latent pathogens of plant hosts but also exert several beneficial effect. Their functional roles may be attributed to protect the host from the insect pests (Azevedo et al. 2000), fungal pathogens (Arnold et al. 2003; Dingle and McGee 2003), or increase the host fitness in harsh environments (Redman et al. 2002). Besides, endophytic fungi have been

recognized as repository of biologically active substances (Tan and Zou 2001).

Strobel and Daisy (2003) proposed the existence of approximately 300,000 vascular plant species in the world, but only a few have been studied for their endophytic microbes. It has been suggested that the plants from unique environmental settings, especially those with an unusual biology and possessing novel strategies for the survival or the plants with medicinal uses should be selected for endophytic study because they are expected to harbor novel endophytes that may produce unique metabolites having diversified applications. Over the years, research priority has been directed to study the endophytic fungi from the medicinal plants due to the fact that these plants shelter microbes that produce bioactive natural products

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and their derivatives with more bioactivity than those of their respective hosts. This gives paucity of work on endophytes of other plant species.

Invasive plants have unique survival features and are less competitive and abundant in their native ranges than in their invaded ranges (Broennimann et al. 2007). It is suggested that associative endophytes of invasive plants could contribute to their greater competitiveness and may also produce novel allelochemicals that might have inhibitory effects (Shipunov et al. 2008). Many invasive species have the natural capability to adapt in wide range of habitats. It is believed that such adaptability might be due to some associated endophytes because recent study indicates that endophytes influence the protection and growth of an invasive plant (Newcombe et al. 2009). However, there is meager information on fungal endophytes of invasive plant species. *Ipomoea carnea*, a native of South America is common invasive plant in all the states of India. The plant has unique survival features, also used as folk medicine for the treatment of skin diseases and leucoderma (Agarwal and Uppadhyay 1979). The aim of the this work, therefore, was to study the endophytic fungi communities associated with *Ipomoea carnea* tissues and their antimicrobial potentials.

## MATERIALS AND METHODS

### Collection of plant materials

*Ipomoea carnea* growing in and around North Orissa University campus (21°93' N and 86°76' E), Orissa, India was identified and two sites were selected. Samplings were done for a period of six months, representing two season, summer (April-June) and monsoon (July-September). The samples were collected from five individual healthy and symptomless plants from each site. The collection of leaves, stems and seeds were made from each selected plant 122 cm above the ground with a help of ethanol-disinfected knife and placed in sterile poly ethylene bags separately. The samples were brought to the laboratory, washed thoroughly in running water and shade dried under fan before isolation procedure was undertaken.

### Isolation of endophytes

For the isolation of endophytes, samples (stems, leaves and seeds) were surface sterilized by immersing them sequentially in 70% ethanol for 3min and 0.5% sodium hypochloride (NaOCl) for 1min and rinsed thoroughly with sterile distilled water (Bills, 1996). The excess water was dried under laminar airflow chamber. For stem samples, outer barks were removed and inner tissues of 1 x 0.5 cm (approx.) segments were carefully dissected out with a sterile scalpel. The seeds were dissected in two equal halves after removing the seeds coat and leaves were cut into 0.5 cm<sup>2</sup> sizes (approx.) without midrib under aseptic conditions. In each Petri dish, 5-6 segments were placed on Potato Dextrose Agar medium (PDA), supplemented with penicillin-G (60mg L<sup>-1</sup>) and streptomycin sulphate (80 mg L<sup>-1</sup>) to inhibit the bacterial contaminant. For each sample, 200 segments were plated and the plates were incubated at room temperature (30° C approx.) for 4-6 weeks in dark. The plated segments were observed once a day for the growth of endophytic fungi. Hyphal tips growing out the plated segments were immediately transferred into PDA slant, purified and maintained at 4°C. The efficiency of surface sterilization procedure was tested as per the method described by Schultz et al. (1998). The fungal isolates were identified based on their morphological and reproductive characters using the standard identification manuals (Barnett and Hunter 1998; Subramanian 1971). The fungal cultures that failed to sporulate were categorized as sterile mycelia. All the isolates were maintained in Potato dextrose agar slant.

### Data analysis

The relative frequency of colonization (% CF) was calculated as the number of segments colonized by a specific fungus divided by the total number of segments plated X 100 and dominant endophytes were calculated as percentage colony frequency divided by the sum of percentage of colony frequency of all endophytes X 100.

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

$$\frac{\sum n(n-1)}{N(N-1)}$$

Where D = -----  
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:

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

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

$S$  – the number of species in the sample

$P_i$  – relative abundance of  $i^{\text{th}}$  species or kinds measures,  $= n_i/N$

$N$  – total number of individuals of all kinds

$n_i$  – number of individuals of  $i^{\text{th}}$  species

$\ln$  – log to base 2

### Fungal cultivation and metabolites extraction

The fungal endophytes were cultivated on Potato dextrose broth by placing the agar blocks of pure culture (3mm in diameter) of actively growing culture in 250ml Erlenmeyer flasks containing 100ml of the medium. The flasks were incubated in BOD shaking incubator for three weeks at  $27 \pm 1^\circ\text{C}$  with periodic shaking at 150 rpm. The culture was filtered through sterile cheese cloth to remove the mycelial mats. The liquid broth was extracted with equal volume of ethyl acetate in a separating funnel by vigorous shaking for 10 min. The cell mass was separated and the from the filtrate, ethyl acetate was evaporated. The resultant compound was dried with  $\text{MgSO}_4$  and concentrated to yield the crude extract. The crude extract was then dissolved in Dimethyl sulphoxide (DMSO) for antimicrobial bioassay.

### Determination of Antimicrobial activity

The antimicrobial activity of the crude metabolites was determined by the agar cup diffusion method against six bacteria (*Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi*, *Pseudomonas fluorescens*, *Shigella dysenteriae*) and three fungi (*Trichophyton rubrum*, *Aspergillus fumigatus*, *Trichophyton* sp.) as test pathogens. These test microbes were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India. 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 medium with a sterile cork borer (7mm in diameter). The cups were then filled with 100 $\mu\text{L}$  of the crude extract dissolved in DMSO and incubated at  $35 \pm 1^\circ\text{C}$  for 24 h for bacterial and 48 h for fungal pathogens. The plates were observed for zone of inhibition and compared with the control (i.e., a cup filled with DMSO solution only). Minimum inhibitory concentration (MIC) was determined only for the bacterial pathogens (*B. subtilis* and *E. coli*) by microbroth dilution assay on 96-wells microplates according to the National Committee for Clinical Laboratory Standard (NCCLS, 2003). All the determinations were conducted in duplicate.

### Estimation of crude metabolites

The  $\lambda$ -max in ethyl acetate of the crude metabolites of endophytic fungi was determined by using a UV spectrophotometer (SPECORD 210). A standard curve of the bioactive metabolites was calibrated and the concentrations of the bioactive metabolites produced were determined by comparing the optical density at 230 nm.

## RESULTS

A total of 69 isolates belonging to ten taxa including three isolates of sterile mycelia and two unidentified species were obtained from the leaves, barks and seeds of *Ipomoea carnea* (Table 1). Of the total species isolated, 1.45% was Zygomycetes, 10.14% Coelomycetes, 62.32% Hypomycetes, 18.84% sterile mycelia and 7.25% unidentified species. Among the endophytes, species of *Curvularia*, *Aspergillus*, *Fusarium*, *Colletotrichum* and sterile mycelia were dominant. The colonization frequency of *Curvularia* (7.25%) was highest and was isolated from all the tissues. Maximum endophytes were recovered from the seeds during the monsoon season, while in summer, more endophytes were isolated from the barks with colonization frequency of 8.5 and 7.5%, respectively. However, less numbers of endophytes were isolated from the barks during the monsoon with colonization frequency of 4.0% and least endophytes were obtained from the seeds during the summer with colonization frequency of 3.0% (Table 1).

**Table 1** - Occurrence of endophytic fungi in different tissues of *Ipomea carnea* in two different seasons.

Endophytic fungi	Colonization frequency (% CF)						Total	Frequency of dominant endophytes
	Summer			Monsoon				
	Stem	Total	Seed	Stem	Leaf	Seed		
<i>Acremonium</i> sp.	--	--	--	--	1.0	0.5	03	4.35
<i>Aspergillus</i> sp.	--	1.5	--	--	1.0	--	05	7.25
<i>Aspergillus flavus</i>	--	--	--	1.0	--	--	02	2.90
<i>Aspergillus fumigatus</i>	--	--	--	0.5	--	1.0	03	4.35
<i>Cladosporium</i> sp.	--	--	--	--	--	0.5	01	1.45
<i>Colletotrichum</i> sp. 1	--	2.0	--	--	--	--	04	5.80
<i>Colletotrichum</i> sp. 2	--	--	1.5	--	--	--	03	4.35
<i>Curvularia</i> sp. 1	--	3.5	--	--	--	1.5	10	14.50
<i>Curvularia</i> sp. 2	1.5	--	--	--	1.0	0.5	06	8.70
<i>Fusarium</i> sp. 1	--	--	1.5	--	--	--	03	4.35
<i>Fusarium</i> sp. 2	--	--	--	--	--	1.0	02	2.90
Sterile fungus sp. 1	4.0	--	--	--	--	--	08	11.60
Sterile fungus sp. 2	1.0	--	--	--	--	--	02	2.90
Sterile fungus sp. 3	--	--	--	--	1.0	0.5	03	4.35
<i>Rhizopus</i> sp.	--	--	--	--	--	0.5	01	1.45
<i>Trichoderma</i> sp.	1.0	--	--	--	--	--	02	2.90
<i>Trichoderma viridae</i>	--	--	--	1.5	--	--	03	4.35
<i>Trichoderma harzianum</i>	--	--	--	1.0	--	0.5	03	4.35
Unidentified sp. 1	--	--	--	--	--	2.0	04	5.80
Unidentified sp. 2	--	--	--	--	0.5	--	01	1.45
N <sup>o</sup> . of isolates recovered	15	14	06	08	09	17	69	--
*Colonization frequency	7.5	7.0	3.0	4.0	4.5	8.5	--	--

Among the isolates, *Acremonium*, *Cladosporium* and *Rhizopus* were represented by the single species and were isolated only during the monsoon season. More isolates of sterile mycelia were obtained from the barks during the summer season. Species of *Fusarium* were isolated only from the seeds in both the seasons. High colonization of *Collectotrichum* was observed from the leaves and bark samples collected during the summer. In general, more endophytes were isolated during the monsoon than in the summer

season. The Shannon-Wiener and Simpson diversity indices for fungi were highest in the seeds obtained during the monsoon season but were lower in the leaves sample collected during the summer (Table 2). It indicated higher number of isolates but low in species richness. However, monsoon isolates showed higher species richness, although the number of isolates was less than that of summer. In general, the samples obtained during the monsoon showed higher species richness than from those obtained in the summer.

**Table 2** - Species richness and diversity of endophytic fungi in tissues of *Ipomoea carnea*.

Plant Part	Season	Total isolates	Species richness	Diversity indices	
				Shannon	Simpson
Stem	Summer	15	04	0.524	0.686
	Monsoon	08	04	1.321	0.821
Leaf	Summer	14	03	0.341	0.670
	Monsoon	09	05	1.581	0.889
Seed	Summer	06	02	0.693	0.600
	Monsoon	17	10	1.894	0.923

Of the total isolates, 15 strains (21.74%) showed activity against the tested microorganisms inhibiting either bacterial or fungal pathogens. Almost all the isolates exhibited antibacterial activity inhibiting at least one of the test bacterial pathogens but 46.6% of the isolates displayed both antibacterial and antifungal activity (Table 3). Out of the total isolates displaying antimicrobial activity, two isolates inhibited all the test

pathogens. Among the bacterial pathogens, *E. coli* was found to be most susceptible to all the crude extracts. Almost all the fungal pathogens showed resistant to the crude extracts, except the metabolites of *Curvularia* which exhibited antifungal activity against all the tested fungal pathogens, i.e., *Trichophyton rubrum*, *Aspergillus fumigatus* and *Trichophyton* sp.

**Table 3-** Antimicrobial activity of metabolites of some endophytic fungi

Endophytic fungi	Zone of inhibition (mm)								
	Sa	Bs	Ec	St	Pf	Sd	Tr	Af	Tp
<i>Aspergillus</i> sp	+	--	+	+	--	+	--	--	--
<i>Collectotrichum</i> sp. 1	+	+	+	+	+	++	--	--	--
<i>Collectotrichum</i> sp. 2	--	+	++	--	+	+	--	--	--
<i>Cladosporium</i> sp.	+	+	+	+	--	+	+	--	--
<i>Curvularia</i> sp. 1	+	+	++	+	+	+	+	+	+
<i>Curvularia</i> sp.2	+	++	++	++	++	++	+	+	+
<i>Mycelia sterilia</i> sp. 1	+	+	+	++	+	+	--	--	--
<i>Mycelia sterilia</i> sp. 2	--	++	++	--	+	+	--	--	--
<i>Mycelia sterilia</i> sp.3	--	--	+	--	+	++	+	--	--
<i>Fusarium</i> sp. 1	++	+++	+++	++	++	++	--	--	--
<i>Fusarium</i> sp. 2	+	+	++	++	+	++	+	--	--
<i>Trichoderma</i> sp	--	--	+	--	+	+	--	--	--
<i>Trichoderma viridae</i>	+	--	+	--	--	+	--	--	--
Unidentified sp. 1	++	+++	+++	++	+++	+++	++	--	--
Unidentified sp. 2	--	+	+	--	+	--	--	+	--
Negative control	--	--	--	--	--	--	--	--	--

Negative control: dimethyl sulphoxide (the medium to dissolve crude metabolites) 100  $\mu$ l

Sa- *Staphylococcus aureus*; Bs- *Bacillus subtilis*; Ec- *Escherichia coli*; St- *Salmonella typhi* ; Pf- *Pseudomonas fluorescens*; Sd- *Shigella dysenteriae*; Tr- *Trichophyton rubrum* ; Af- *Aspergillus fumigatus*; Tp- *Trichophyton* sp.

--: no antimicrobial activity; +: the inhibition zone is less than 10 mm;

++: the inhibition zone is from 10 mm to 20 mm; +++: the inhibition zone is above 20 mm

Considerable antimicrobial activity was observed in three endophytic fungi, *Curvularia* sp. 2, *Fusarium* sp. 1 and an unidentified strain. The metabolites of these three endophytic fungi were studied for their minimum inhibition concentration (MIC) considering one Gram positive and Gram negative bacteria, *B. subtilis* and *E. coli*, respectively. The crude extract of *Curvularia* sp. 2

showed highest MIC value of 500 $\mu$ g ml<sup>-1</sup> against *B. subtilis* while that of the unidentified strain showed lowest MIC of 62.5 $\mu$ g ml<sup>-1</sup> against *E. coli*. All the three crude extracts exhibited lower MIC values against *E. coli* indicating higher efficacy of these metabolites in inhibiting this test bacterium. The zone of inhibition and MIC values are presented in Table 4.

**Table 4-** Inhibition zone and MIC values of some potent endophytic fungi

Endophytic fungi	Zone of inhibition (mm)		Minimum inhibitory concentration ( $\mu$ g/ml)	
	<i>B. subtilis</i>	<i>E. coli</i>	<i>B. subtilis</i>	<i>E. coli</i>
Unidentified sp.1	19.0 $\pm$ 1.4	29.5 $\pm$ 2.12	250	62.5
<i>Fusarium</i> sp. 1	20.5 $\pm$ 0.70	24.0 $\pm$ 1.41	250	125
<i>Curvularia</i> sp. 2	16.5 $\pm$ 0.70	19.7 $\pm$ 2.12	500	250

Values are means of three replicates

$\pm$  Standard Deviation

The crude metabolites of these fungi were also determined for their  $\lambda$ -max by calibrating the concentration of the metabolites dissolved in ethyl acetate using UV spectrophotometer at 230 nm. Three different standard curves were obtained corresponding to their optical density. The  $\lambda$ -max (peak wavelength) were 1.346, 1.503 and 1.093 for *Curvularia* sp. 2, *Fusarium* sp. 1 and an unidentified strain, respectively.

## DISCUSSION

Endophytic fungi have been found associated with every plant species investigated so far from tropical and temperate hosts, yet they are poorly investigated because of their cryptic and ephemeral nature (Rodrigues and Petrini 1997; Strobel 2002). There are few reports on the endophytic fungi of invasive plant species, despite their abundance in certain invasive hosts (Shipunov et al. 2008). In the present study, rich endophytic fungal diversity was obtained comprising Zygomycetes, Coelomycetes, Hypomycetes, sterile mycelia and unidentified genera from the tissues of *Ipomoea carnea*, a common invasive species. Among the endophytes, class Hyphomycetes was dominant. Such dominance of Hyphomycetes as endophytes has also been reported from several plants such as *Azadirachta indica* and *Terminalia indica* (Mahesh et al. 2004; Tejesvi et al. 2005), indicating their ubiquity among the plant kingdom. Environmental conditions may also play an important role in assemblages and diversity of endophytic fungi. It is generally believed that plants growing in lush tropical rainforests, where competition for light and nutrients might be severe, could be most likely to host the greatest number of endophytes than the temperate parts of the world. The dominant endophytic isolates were species of *Curvularia*, *Aspergillus*, *Fusarium* and *Colletotrichum*. These fungi are also found commonly as the plant pathogens and they might have evolved to endophytic lifestyle due to loss of virulence (Freeman and Rodrigues 1993). This can be exemplified from the fact that species of *Fusarium* is regarded as destructive plant pathogens with important economic impacts and also cause severe human infections (Guarro and Gene 1995). But, at present, they have been isolated as endophytes from many plant species

with some of them displaying biological activity (Chakravarthi et al. 2008; Kour et al. 2008; Deng et al. 2009). Among the dominant endophytes, the colonization frequency of *Curvularia* was highest indicating their systemic colonization in the host tissues. Such systemic colonization of *Curvularia* has also been reported from *Dichantheium lanuginosum*, a plant growing in geothermal sites of Lassen Volcanic National Park (LVNP) and Yellowstone National Park (YNP), where average annual temperature varied between 20 and 50°C. Artificially inoculated plants with the spores of this *Curvularia* sp. could resist constant soil temperatures of 50°C for three days and intermittent temperatures as high as 65°C for ten days (Redman et al. 2002). It could also be speculated that the colonization of *Curvularia* in the tissues of *I. carnea* might have similar role, as this plant grew in extreme environments.

The isolation of *Fusarium* from the seeds and *Collectotrichum* from the leaves and stems samples indicated tissue specificity of these fungi. Similar, many earlier workers have also demonstrated tissue and host specificity of endophytes (Cannon and Simons 2002). In the present study, more endophytic isolates were obtained during the monsoon season. It also showed higher species richness than the summer isolates. This could be due to higher precipitation rates which might have favored spore germination in fungi indicating horizontal transfer of the endophytes in the host tissues. A strong correlation has been observed between the endophyte infection levels and cumulative precipitation (Wilson 2000). In many instances, leaves sampled during the wet season harbored more endophytes than those screened during the dry season (Rodrigues 1994; Suryanarayanan et al. 1998). It is known that the endophytic fungi existing in the plant are potential sources of antimicrobial substances (Strobel 2003) and this has also been demonstrated in earlier studies (Tayung and Jha, 2006; Mohanta et al. 2008). The indication of three different peaks ( $\lambda$ -max) in the crude metabolites of the endophytes suggested the presence of active substances, which could be purified for the antimicrobial agents. Endophytic fungi with antimicrobial activity have been reported mostly from the medicinal plants. However, invasive plant species still remain largely unexplored and unstudied. Considering their unusual biology and adaptability in adverse

environmental conditions, invasive species might harbor interesting endophytic microbes with multiple applications. The present study on endophytes of *I. carnea* with antimicrobial activity could be an endeavor in this direction.

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