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Isolation and Identification of Endophytic Fungi from *Artemisia scoparia* (Asteraceae)

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ABSTRACT: The choice of host plant is of critical importance when working with endophytic fungi. The exploration of endophytic fungi is still an emerging field and all plants seem to harbour fungi with some bioactive content and activities. However, there are certain metabolites that are characteristic of certain biotopes. Thus, a rationale for selecting promising plant sources should be established. Of particular interest are the plants that are used as medicinal plants or plants that populate a unique environment. *Artemisia* is a widely used medicinal plant. In this research work, the endophytic mycota of *Artemisia scoparia* was studied. In order to isolate endophytic fungi, 155 plant segments from 20 samples of *Artemisia scoparia* were collected from its natural habitat in Dachigam National. This habitat is a unique environment and a protected area. Six different fungal isolates were obtained from root, leaf and stem plant parts. Among the identified isolates, the most abundant genera were *Gliocladium solani* followed by *Penicillium melinii* with a colonization frequency of 62 and 37.5% respectively. The objective of this study was to report new data regarding the endophytic fungi found in medicinal plant *Artemisia scoparia*. This systematic investigation revealed that traditional medicinal plants are a rich and reliable source of novel endophytic fungi.

Keywords: Endophytic fungi, Kashmir, Medicinal plant

I. INTRODUCTION

Endophytic organisms are those that live internally in apparently healthy and asymptomatic hosts. Endophytes appear to be ubiquitous; indeed, no study has yet shown the existence of a plant species without endophytes [1]. The term "Endophyte" was introduced by De Bary [2] and was initially applied to any organism found within a plant that causes asymptomatic infections entirely within plant tissues without any symptoms of disease [3]. By definition, an endophytic fungus lives in mycelial form in biological association with living plant at least for some time. Therefore, the minimal requirement before a fungus to be termed as an endophyte should be the demonstration of its hyphae in the living tissue [4]. Endophytic fungi spends the whole or part of its life cycle colonizing inter and/or intra-cellularly inside the healthy tissues of the host plants, typically causing no apparent symptoms of diseases [5]. These are fungal microorganisms which asymptomatically inhabit plant tissues and have been isolated from many species of woody plants and grasses [6,5]. Endophytic fungi are found in all kinds of plants, i.e. trees, grasses, algae and herbaceous plants. Medicinal plants had been used to isolate and characterize directly the bioactive metabolites. However, the discovery of fungal endophytes inside these plants with capacity to produce the same compounds shifted the focus of new drug sources from plants to fungi. Bioactive natural products from endophytic fungi, isolated from different plant species, are attracting considerable attention from natural product chemists and biologists alike which is clearly depicted by the steady increase of publications devoted to this topic during the recent years [1]. The genus *Artemisia* L. (Asteraceae) containing 500 species is the largest genus in the tribe Anthemideae, and one of the largest genera in the family [7]. It is widely distributed mainly across the Northern Hemisphere, being profusely represented in the Old World, with a great centre of diversification in Asia, and also reaching the New World [8]. Several *Artemisia* species have medicinal importance and are used in traditional medicine for the treatment of a variety of diseases and complaints [9]. Genus *Artemisia* is represented by herbs or small shrubs, frequently aromatic [10-11]. *Artemisia scoparia* is a species in this genus, and its English common name is Redstem wormwood.

The plant is anticholesterolemic, antipyretic, antiseptic, cholagogue, diuretic and vasodilator. It has an antibacterial action, inhibiting the growth of *Staphylococcus aureus*, *Streptococci*, *Bacillus dysenteriae*, *B. typhi*, *B. subtilis*, *Pneumococci*, *Corynebacterium diphtheriae*, *Mycobacterium* etc. It is used in the treatment of jaundice, hepatitis and inflammation of the gall bladder. The plant is also used in a mixture with other herbs as a cholagogue [9]. The aerial part of this plant is used in traditional medicine as an antiphlogistic, as a diuretic and for the treatment of hepatitis and urticaria. Phytochemical investigations of the aerial part of *A. scoparia* resulted in the isolation of flavonoids, coumarins, and essential oils [12]. Dachigam National Park, located 22 km from Srinagar, is situated at altitudes ranging from 5500 ft to 14000 ft. and is the last home of the rare Hangul or Kashmir stag. A former game preserve of the erstwhile Maharaja of Jammu and Kashmir, the park has been a protected area since 1910. Located among Himalayan Mountains, the park is spread over an area of 141 sq km. The word 'Dachigam' literally means 'ten villages' because of the fact that as many as 10 villages were relocated out of the forest as part of the efforts to create the game preserve and catchment area. The park due to the variation in altitudes is demarcated into upper and lower regions. The park's terrain ranges from gentle sloped grasslands to cliffs and sharp rocky outcrops. The park boasts of over 500 species of herbs, 50 species of trees and about 20 species of shrubs. Thus, this work was carried out with an objective of isolating and identifying the endophytic fungi from medicinal plant *Artemisia scoparia* which was collected from its habitat at Dachigam National Park, Kashmir, J & K.

II. METHODOLOGY

A. Collection of Plant samples

Twenty (20) symptoms-less whole plant samples were collected from different regions of Dachigam National Park, J&K. The samples were collected in clean paper bags and brought to laboratory where they were further processed within 24 hours after collection.

B. Isolation of endophytic fungi from Artemisia scoparia

The method most commonly used to detect and quantify endophytic fungi is isolation from surface-sterilised host tissue [13]. Surface-sterilization of plant material usually entails treating the plant material with a strong oxidant or a general disinfectant for a brief period, followed by a sterile rinse to remove residual sterilant [13]. Highly sterile condition was maintained for the isolation of endophytic fungi. The isolation was done according to the method described by [14]. The surface sterilization was done by sodium hypochlorite (NaOCl) and 75% ethanol and by using autoclaved double-distilled water for rinsing. The efficiency of surface sterilization was ascertained for every segment of tissue following the imprint method [15]. The plant parts, namely stem, root, and leaf were then inoculated onto the medium of Potato dextrose agar (PDA) in a petri-dish supplemented with antibiotic Streptomycin sulphate (100µg/ml). Because endophytic fungi are slow to emerge, prolonged incubation is required and thus, plates were incubated for 7days at 25ºC in a growth chamber having humidity control. The plates were sealed properly with parafilm to avoid desiccation of the medium and any contamination during this period. Then isolation of endophytic fungi from these master plates was done by transfer of hyphal tips to fresh potato dextrose agar plates with great precaution to obtain pure cultures for identification.

C. Preservation of endophytic fungi

The purified fungal isolates were transferred separately to PDA slants with proper labelling and kept at 4ºC.

D. Identification of Endophytic Fungi

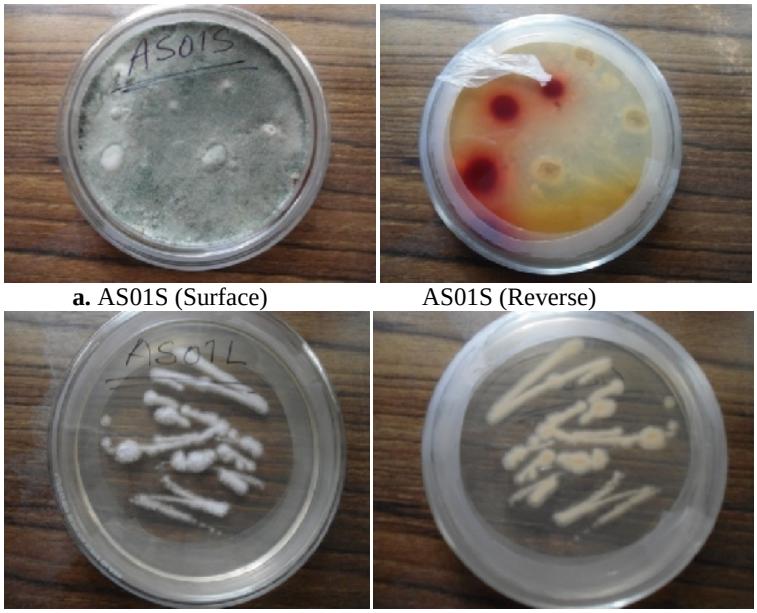
The isolated endophytic fungal strains were grown on specified media for their identification. The fungi were identified on the basis of their morphological and cultural characteristics [16-19]. For characterisation of the morphology of fungal isolates, semi-permanent slides were prepared from cultures by Culture Slide Technique and were stained with Lacto-phenol cotton blue and examined with a bright-field and phase-contrast microscope. Identification was based on morphological characteristics such as growth pattern, hyphae, colour of colony and medium, surface texture, margin character, aerial mycelium, mechanism of spore production and characteristics of the spore [20]. Fungal morphology was characterized by using a semiautomatic image analysis system consisting of an Olympus microscope (Olympus, New Hyde Park, NY, U.S.A.) operated as phase contrast, a charge coupled device (CCD) camera (Sony, Cambridge, U.K.), a PC with a frame-grabber, and the image analysis software (SIS, Olympus, Germany). Sample preparation and measurement was done as described by Papagianni *et al*. [21]. A magnification of 100X was applied for measurement of mycelial particles to estimate the individual mycelia and other micro-morphological features.

Colonizing Frequency: The colonizing frequency of each endophytic fungus was calculated as according to Suryanarayanan *et al*. (2003):

$$CF(\%)=\frac{Number\ of\ plant\ segments\ colonised\ by\ a\ single\ fungi}{Total\ number\ of\ plant\ segments\ observed}\times 100$$

III. RESULTS

This was the first research regarding the isolation and identification of endophytic fungi of *A. scoparia* species in Kashmir valley. A total of 155 plant segments; including 40 root parts, 50 leaf parts and 65 stem parts were investigated for this purpose. A total of six (6) endophytic fungi were isolated from 20 samples of *A. scoparia* plants, collected from different locations of the National Park. Two endophytic fungi were isolated each from the root parts, leaf parts and stem parts. All endophytic fungi could be cultivated on artificial media and maintained as a pure culture. They exhibited characteristic colony and microscopic morphology that could be used to differentiate them. Most of them belonged to ascomycetes and also fungi imperfecti. Some results of characterisation of colony and microscopic morphological study are shown in Figures 1 and 2. All isolates were identified as belonging to 5 genera, namely *Trichoderma* sp., *Gliocladium* sp., *Aspergillus* sp., *Penicillium* sp., *Alternaria* sp. (Table 1).

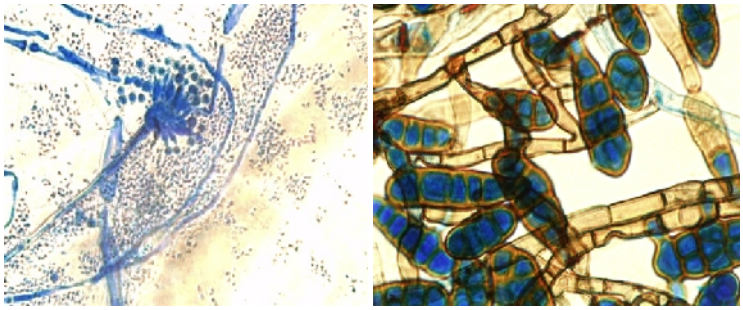


a. AS01S (Surface) AS01S (Reverse)

b. AS01L (Surface) AS01L (Reverse)

Fig. 1. Isolated Endophytic Fungal cultures on PDA.

a. Trichoderma virens b. Gliocladium solani



a. *Aspergillus* spp. **b.** *Alternaria* spp.

Fig. 2. Photographs of microscopic observation of fungi

The identification was confirmed based on morphological characters in *in vitro* culture by Agharkar Research Institute, Pune – a National Facility of fungal culture collection of India (Tables 2 & 3). Isolation rate, a measure of the richness of fungal endophytes colonizing the different parts of the selected medicinal plants calculated as the number of isolates obtained from tissue segments, divided by the total number of segments and expressed as percentages showed that the leaves of *A. scoparia* were very rich with an isolation rate of 0.04% (Table 4).

The colonizing frequency of *Gliocladium solani* was highest, recorded to be 62% which was isolated from leaf parts of the plant (Table 2). The relative frequency (Table 5) used to represent the fungal diversity was also calculated for each fungal endophyte.

Table 1: Colonizing frequency of endophytic fungi in different parts of *A. Scoparia.*

Fungi	Isolates Number	% Frequency of Colonization		
		Root	Leaf	Stem
Penicillium melinii	15	37.50	-	-
Alternariaalternata	9	22.50	-	-
Gliocladiumsolani	31	-	62	-
Trichoderma longibrachiatum	20	-	40	-
T. virens	12	-	-	18.46
Aspergillusversicolor	23	-	-	35.38

Table 2: Morphological features of different fungal endophytes on Czapek Yeast Agar

Species Name	Colony colour	Reverse colour	Colony surface morphology	Colony margin	Diameter (mm)
P. melinii	Dark greyish-green, Olive-green, Dark green; White margin	Orange, Orange-brown; Grey-green, Grey-brown, Yellow-beige margin	Cottony growth	Round	33-57
A.versicolor	White to creamish with reddish exudates	Reddish	Velvety	Round	19.3-20
A.alternata	Olivaceous green with white border	Cream	Cottony with concentric rings	Round	40-50

Table 3: Morphological features of different fungal endophytes on Potato Dextrose Agar.

Species Name	Colony colour	Reverse colour	Colony surface morphology	Colony margin	Diameter (mm)
G. solani	White at first then became salmon pink	Reddish-orange	Cottony	Floccose, wavy	25-35
T. longibrachiatum	White latere became yellowish-green to deep-green	Green	Downy	Concentric rings with wavy margin	50-60

Table 4: Isolation Rate.

Plant species	Site of Isolation	Isolation Rate (%)
Artemisia scoparia	Roots	0.02
	Leaves	0.04
	Stems	0.03

Table 5: Relative Frequency of Isolation.

Plant species	Endophytic Fungi	Relative Frequency of Isolation (%)
A. scoparia	*P. melinii*	62.5
	A. alternata	37.5
	G. solani	60.7
	T. longibrachiatum	39.2
	T. virens	34.3
	A. versicolor	65.7

IV. DISCUSSION

Medicinal plants are considered as a repository of "endophytic micro-organisms" living in their internal tissues. The quest for identifying novel bio-actives from the endophytic fungi has resulted in the sampling of host plants such as herbs, shrubs, tree species, and vines in unique places of ecological adaptations around the world. Such niches harbour great species diversity, un-intervened by human activities [22-23].

Efforts in this direction to sample plants located in such habitats around the world with potential ethno-medicinal values have resulted in the isolation of fungal endophytes, unique to a particular plant species with distinct bioactivity. Thus, in view of its habitat in a protected area of a National Park, *Artemisia scoparia* was taken for this study after thorough perusal of literature on this plant. All isolates from this plant were identified as belonging to 5 genera, namely *Trichoderma* sp., *Gliocladium* sp., *Aspergillus* sp., *Penicillium* sp., *Alternaria* spp. (Table 1).

Among the Asteraceae family only few plant species of the genus *Artemisia* have been screened for the evaluation of endophytic fungi. In a study, conducted by Huang *et al*. [24]. the taxonomic identities and phylogenetic relationships of fungal endophytes isolated from three plant species of *Artemisia*; namely *A. capillaris*, *A. indica*, and *A. lactiflora*, using a combination of morphological and molecular approaches were investigated. The results suggested that some of the isolated endophytes exhibited host and tissue specificity. Recently many studies have been conducted about the endophytic fungal biodiversity, taxonomy, reproduction, host ecology and their effects on the host plants [25-26,14]. In another study, a total of 108 isolates of endophytic fungi were isolated from the medicinal plant *Artemisia argyi* [27]. A work was initiated to study the endophytic fungal population in *Withania somnifera* (L.) Dunal., a commonly used medicinal plant. A total of 643 segments (202 leaf, 391 stem, and 50 root samples) from 20 different plants were screened for their endophytic mycoflora. Thirty-three fungal strains of 24 species were isolated on the basis of morphology; four belonged to the class ascomycetes and 20 to class deuteromycetes. The highest species richness as well as frequency of colonization was in stem; almost all the fungi were found to be organ-specific [28]. 473 segments from 7 plants of *Avicennia marina* collected from different locations of Karankadu mangrove forest were processed for the presence of endophytic fungi. A total 10 fungal species were isolated. Among the endophytic flora, *Phoma* was the most prominent genus. Overall colonization frequency from stem was 8.85% [29]. Gond and his co-workers [30] isolated endophytic fungi from *Nyctanthes arbor-tristis* and also evaluated their antimicrobial activity. A total of 19 endophytic fungi were isolated from 400 segments of healthy leaf and stem tissues of the plant. Eighteen endophytic fungi were obtained from leaf, while only ten from stem. A total of 3634 endophytic fungal isolates were recovered from 4800 leaf, stem and bark segments of 10 medicinal plants of Western Ghats, India, during monsoon, winter and summer seasons. These isolates belonged to coelomycetes (26.35%), hyphomycetes (21.76%), Xylariaceae (0.6%) and mycelia sterilia (3.55%). Colonization frequency of endophytic fungi varied significantly between seasons. The fungal community from leaves was most diverse followed by stem and bark tissues [31]. The colonizing frequency of *Gliocladium solani* isolated from *Artemisia scoparia* was highest, recorded to be 62% which was isolated from leaf parts of the plant (Table 1).

CONCLUSIONS

Endophytic fungi from *Artemisia scoparia*is a rich source of many fungal endophytes which could be later used for the evaluation of their bioactive properties. The bioactivity if any found in the fungal endophytic metabolites could later be correlated to the folk claimed medicinal potential of this medicinal plant.

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