Research Article

Diversity of Endophytic Fungi From *Tribulus terrestris* L. from Eastern Ghat of India (First Report)

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ABSTRACT

Endophytes are a large and diverse group of fungi that colonize healthy plant tissues without causing any symptoms. Medicinal plants are relatively less attacked by the plant pathogens and pests, therefore endophytic micro-biota can be of great value in protecting plants from pests. As microorganisms play a central role in the regulation of ecosystem processes, and they comprises the vast majority of species on Earth. In the present investigation endophytic fungi were isolated from *Tribulus terrestris* L. from Eastern Ghats and the coast Bay of Bengal for the first time. Fifty-four endophytic fungi with different morphologies were isolated from the leaves, stems and roots of *Tribulus terrestris* L. with colonization rates of 14%, 8.5%, and 4.5%, respectively. Furthermore Biodiversity of endophytic fungi in various segments of the plant were determined by statistical analysis Simpson index (1-D), Shannon-wiener index (Hs) and Species richness (R1) which is found higher in leaves than stem and root.

Keywords: Endophytic fungi, *Tribulus terrestris* L., Colonization rate, isolation rate, Simpson index (1-D), Shannon-wiener index (Hs) and Species richness (R1).

INTRODUCTION

Endophyte constitutes an important component of microbial biodiversity. Endophytes commonly refer to a group of fungi that reside asymptomatically inside the plants and its different parts, demonstrated that fungal endophytes are ubiquitous in plant kingdom and even lichens, with an estimate of at least 1 million species[1, 2]. Diverse fungal community composition and isolation frequencies of endophytes are found in various host plants. The relationship of endophytes with single or multiple plant hosts can be described in terms of host specificity, host selectivity or host preference, host recurrence[3]. The endophytic fungal community confirmed host specificity at species level but this specificity could be influenced by environmental conditions[4], also known as 'spatial heterogeneity' or 'geographic variation'[5, 6]. Differences of endophytic fungal assemblages in different tissue types have been reported in the same plant species, or even in different tissues of an individual plant, which is a reflection of tissue specificity [7].

Medicinal plants provide a unique environment for endophytes and have been recognized as a repository of endophytes with novel metabolites of pharmaceutical importance[8-10]. From the past few decades, there is increase in the number of publications on endophytes associated with medicinal plants because plant generation bioactive natural products have associated endophytes that produce the same natural products such as Taxol, Podophyllotoxin and Camptothecin. In the case of paclitaxel a highly functionalized diterpenoid and anticancer agent that is found in each of the world's yew tree species (*Taxus* spp.). In 1993, a novel paclitaxel-producing fungus, *Taxomyces andreanae*, from the yew *Taxus brevifolia* was isolated and characterized by (Sterile *et al*., 1993)[11]. Camptothecin a topoisomerase I inhibitor producing endophytic fungi, *Xylaria* sp. M20 and *Fusarium solani* from medicinal trees *Campotheca acuminata* and *Nothapodytes foetida* respectively were isolated and characterized by previous reports[12,13].

Criteria or rationale for plant selection

To isolate novel endophytic microorganisms producing novel bioactive products specific rationale for the collection of each plant for endophytic isolation and natural product discovery is used. Strobel and Daisy (2003)[14], proposed 4 specific hypotheses which govern plant selection strategy are as follow:

I. Plants from unique environmental settings, especially with an unusual biology and possessing novel strategies for survival are seriously considered for study.

II. Plants that have an ethno botanical history (used by indigenous people) that are related to the specific uses or applications of interest are selected for study. These plants are chosen either by direct contact with local peoples or *via* local literature. Ultimately, it may be learnt that the healing power of the botanical source, in fact, may have nothing to do with the natural product of plant, but of the endophytes (inhabiting the plant).

III. Plants that are endemic have an unusual longevity, or that have occupied certain ancient land mass, more likely to harbor endophytes with active natural products than other plants.

IV. Plants growing in areas of great biodiversity also have the prospect of housing endophytes with great diversity.

In the present investigation endophytic fungi have been isolated from *Tribulus terrestris* L. The genus *Tribulus*, belonging to family Zygophyllaceae, comprises about 20 species in the world, of which three species, viz. *Tribulus cistoides*, *Tribulus terrestris*, and *Tribulus alatus*, are of common occurrence in India[15]. Among them, *T. terrestris* (TT) is a well-patronized medicinal herb by Ayurvedic seers as well as by modern herbalists[16]. The plant is used individually as a single therapeutic agent or as a prime or subordinate component of many compound formulations and food supplements. It is an annual shrub found in Mediterranean, subtropical, and desert climate regions around the world, viz. Nepal, India, China, southern USA, Mexico, Spain, and Bulgaria[17].

TT is commonly known as *Gokshur* (Sanskrit); puncture vine, land (or small) caltrops (English); *Gokharu* (Hindi); *Bethagokharu* or *Nanagokharu* (Gujarathi); *Nerinjil* (Tamil) ; and *Khar-e-khusak khurd* (Urdu). It is distributed along a wide geographic perimeter. It is found all over India up to 11,000 ft in Kashmir, Ceylon, and all warm regions of both hemispheres. It is a common weed of the pasture lands, road sides, and other waste places, chiefly in hot, dry, and sandy regions including West Rajasthan and Gujarat in India.[18.]

It is small prostrate, 10-60 cm height, hirsute or silky hairy shrub. Leaves are opposite, often unequal, paripinnate; pinnae from five to eight pairs, elliptical or blong, lanceolate. Flowers are yellow in color. Its carpel fruits are of characteristic, stellate shape, somewhat round-shaped, compressed, five cornered, and covered with princkles of very light yellow color. There are several seeds in each crocus with transverse partitions between them. The seeds are oily in nature. When fresh, the root is slender, fibrous, cylindrical, frequently branched, bearing a number of small rootlets and is of light brown color. Fruits and roots are mainly used as a folk medicine for the treatment of various ailments. Root occurs in pieces, 7-18 cm long and 0.3-0.7 cm in diameter, cylindrical, fibrous, frequently branched, bearing a number of small rootlets, tough, woody, yellow to light brown in color, surface rough due to the presence of small nodules; fracture fibrous; odor aromatic; taste sweetish astringent. The fruits of the herb are known as “Chih-hsing” in China or goat head in USA. The spiky fruit looks like the cloven hoof of a cow and, hence, is known as go-ksura (cow-hoof). Fruits are faint greenish yellow with spines. They are globose, consisting of five, nearly glabrous, muriculate, wedge-shaped, woody cocci, each with two pairs of hard sharp spines, one pair longer than the other. Tips of spines almost meet in pairs together forming pentagonal framework around the fruit. Outer surface of the schizocarp is rough. There are several seeds in each coccus, with transverse partitions between them. Odor of fruits is faintly aromatic and taste is slightly acrid.

Its various parts contain a variety of chemical constituents which are medicinally important, such as flavonoids, flavonol glycosides, steroidal saponins, and alkaloids. *Tribulus terrestris* L. is used in folk medicine as a tonic, aphrodisiac, palliative, astringent, stomachic, antihypertensive, diuretic, lithotriptic, and urinary disinfectant, antiurolithic, immunomodulatory, antidiabetic, absorption enhancing, hypolipidemic, cardiotonic, hepatoprotective, anti-inflammatory, analgesic, antispasmodic, anticancer, antibacterial, anthelmintic, larvicidal, and anticariogenic activities. The dried fruit of the herb is very effective in most of the genitourinary tract disorders. It is a vital constituent of *Gokshuradi Guggul*, a potent Ayurvedic medicine used to support proper functioning of the genitourinary tract and to remove the urinary stones. *Tribulus terrestris* L. has been used for centuries in Ayurveda to treat impotence, venereal diseases, and sexual debility. In Bulgaria, the plant is used as a folk medicine for treating impotence. In addition to all these applications, the Ayurvedic Pharmacopoeia of India attributes cardiotonic properties to the root and fruit. In traditional Chinese medicine, the fruits are used for treatment of eye trouble, edema, abdominal distension, emission, morbid leukorrhea, and sexual dysfunction. *Tribulus terrestris* L. is described as a highly valuable drug in the Shern-Nong Pharmacopoeia (the oldest known pharmacological work in China) in restoring the depressed liver, for treatment of fullness in the chest, mastitis, flatulence, acute conjunctivitis, headache, and vitiligo. In Unani medicine, *Tribulus terrestris* L. is used as diuretic, mild laxative, and general tonic.[19]

MATERIALS AND METHODS

Collection of plant material

Visakhapatnam (Location 17°40'48.32''N, 83°12'5.8''E.) is situated between the Eastern Ghats and coast of Bay of Bengal. The annual mean temperature ranges between 24.7-30.6 °C (76-87 °F), with the maximum in the month of May and the minimum in January; the minimum temperatures range between 20-27 °C (68-81 °F) and the average annual rainfall recorded is 1,118.8 mm. Sample collection was done in January 2016. The plants are located in the Campus of Andhra University. Healthy (showing no visual disease symptom) and mature plant of *viz-Tribulus terrestris* L. were collected from the Campus, Andhra University. Samples were tagged and placed in separate sterile polythene bags, brought to the laboratory and processed within 24 h of collection[20, 21]. Fresh plant materials were used for the isolation work to reduce the chance of contamination.

Isolation of endophytic fungi

The samples were washed thoroughly in running tap water before processing.

Leaf, stem and root samples were surface sterilized by dipping in 70 % ethanol (v/v) for 1min and 3.5 % NaOCl (v/v) for 3min, rinsed thrice with sterile water and dried.

Bits of 1.0 X 1.0 cm size were excised with the help of a sterile blade. Six hundred segments of each part of plant of *Tribulus terrestris* L., representing 200 leaf segments, 200 stem segments and 200 root segments were placed on the water agar (16%) (WA) medium supplemented with Streptomycin (100 mg/l; Sigma, St. Louis, MO, USA) was used for the isolation of endophytic fungi. The Petri dishes were sealed using parafilm and The Petri dishes were incubated at 25°C till the mycelia start growing from the samples[22-25]. After incubation, individual fungal colonies were picked from the edge with a sterile fine tipped needle and transferred onto Potato Dextrose Agar (PDA, HiMedia, India) medium for further identification. All the media and glassware were sterilized by autoclaving at 121°C and 15 lb pressure for 20 min. Media pouring, handling and endophytic fungal isolation were performed in the sterile laminar air flow unit (Klennzaids, Chennai) type 2. The identification was done based on the conidial characteristics. All isolates were maintained in cryovials on PDA layered with 15% glycerol (v/v) at -80 °C in an Ultrafreezer (Cryoscientific Pvt. Ltd., Chennai, India) at the Department of Microbiology, College of Science and Technology, Andhra University, Visakhapatnam, India.

Identification of endophytic fungi

The identification procedure of endophytic fungi was based on morphology. The isolated species were described according to their macroscopic features (i.e. the color, shape and growth of cultured colonies) as well as microscopic characteristics (i.e. the structure of hyphae, conidia and conidiophores). The microscopic observations were carried out using Zeiss SteREO Discovery.V12, Fluorescence microscope and Compound microscopes. The morphology of fungal culture colony or hyphae and the characteristics of the spore were identified by temporary mounts using lacto phenol cotton blue (LPCB) and viewed under the microscope at 40X. Obtained data were then compared with the descriptions of endophytic fungal species based on the morphological and microscopic features; the isolates were identified by standard mycological manuals[26-29].

Analysis of data

The colonisation rate and isolation rate of endophyte were calculated as the percentage of segments infected by one or more isolate(s).[30-33.]

Total no. of bits/tissues in a sample yielding ≥ 1 isolate

$$\textbf{Colonization rate (CR)} = \frac{\text{Total no. of isolates scored in a given sample}}{\text{Total no. of segments in a sample}} \times 100$$

$$\textbf{Isolation rate (IR)} = \frac{\text{Total no. of isolates scored in a sample}}{\text{Total no. of segments in sample}}$$

Simpson index (D), Shannon–Wiener's diversity (H_S) and Margalef's species richness index (R1) (Shannon CE, Weiner W, 1963; Yuan *et al*., 2010; Maheshwari and Rajagopal, 2013)[34, 35, 33] were used to assess and quantify endophytic fungal diversity in host plants.

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

$$D = \frac{\Sigma n(n-1)}{N(N-1)}$$

Where, n = the total number of organisms of a particular species

N = the total number of organisms of all species.

Shannon-Wiener diversity index (HS) was calculated using the following formula:

$$H_s = -\sum_{i=1}^{S} (Pi)(\ln Pi)$$

Where, Hs-symbol for the diversity in a sample of S species or kinds

S-the number of species in the sample

Pi-relative abundance of i^{th} species or kinds measures= n/N

N-total number of individuals of all kinds

Ni- number of individuals of i^{th} species

ln - log to base 2

Margalef's Species richness R1 was calculated using the following formula

$$R1 = \frac{(S-1)}{\ln(N)}$$

Where, S = total number of species.

N = the total number of isolates of all species.

RESULTS

A total of 54 endophytic isolates were collected from 600 plant tissue samples of leaf (200 segments), stem (200 segments) and root (200 segments) from *Tribulus terrestris* Linn (Zygophyllaceae). 54 endophytic isolates were categorised into 17 taxa, comprising 1 Ascomycetes genera *Chaetomium* sp., 4 Coelomycetes genera *Colletotrichum* sp., *Pestalotiopsis* sp., *Phomopsis* sp. and *Phyllosticta* sp. 5 Hyphomycetes genera *Alternaria* sp., *Aspergillus* sp., *Curvularia* sp., *Fusarium* sp., *Nigrospora*

sp. All the different part of plant tissues were found to harbour various endophytic fungal species with different colonization rate (CR) and isolation rate (IR) (Tables1-2 and Fig.1-2) and the endophytic fungal pictures isolated from three different plants are shown in (Figures 3-19).

Simpson dominance index is comparatively higher in the leaves sharing relatively similar index values 0.933. Shannon–Wiener index indicates that the foliar endophytic diversity is more with index value 2.394 which is due to occurrence of more number of endophytic species than the stem and root (Tables 3).

Table 1: Isolation and colonization rate of endophytic fungi from *Tribulus terrestris* L.

	Leaf	Stem	Root	Total
No. of segments	200	200	200	600
No. of segments yielding endophytic fungi	28	17	09	54
No of isolates	29	19	10	58
Isolation rate	0.145	0.095	0.05	0.09
Colonization rate (%)	14	8.5	4.5	9

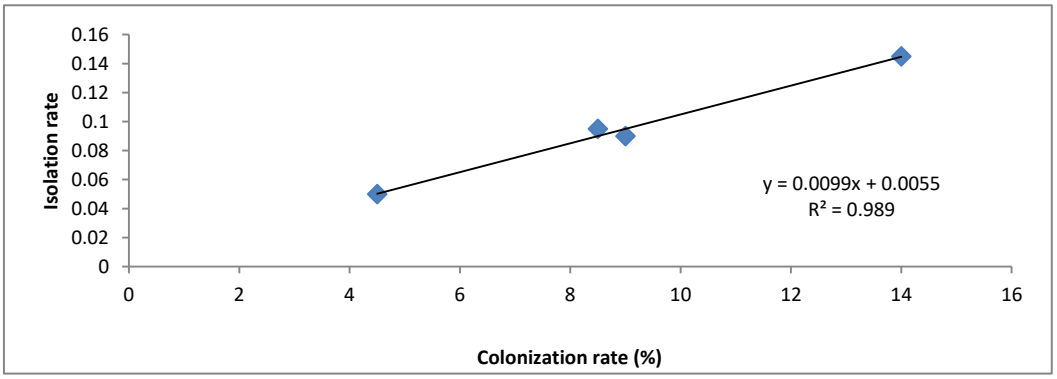

Figure 1: The relationship between colonization rate and isolation rate of Endophytic fungi from *Tribulus terrestris* Linn.

Table 2: Diversity of endophytic fungi isolated from leaf, stem and root of *Tribulus terrestris* L.

Class	Endophytic fungi	Leaf	CR (%)	Stem	CR (%)	Root	CR (%)
Ascomycetes	***Chaetomium sp.2***	03	1.5	-	-	-	-
Coelomycetes	***Colletotrichum* sp.**	02	1	02	1	-	-
	***Pestalotiopsis* sp.**	02	1	-	-	-	-
	***Phomopsis* sp.1**	03	1.5	01	0.5	-	-
	***Phomopsis* sp.2**	02	1	03	1.5	-	-
	Phyllosticta sp.	02	1	-	-	-	-
Hyphomycetes	***Alternaria sp.1***	-	-	02	1		
	Alternaria sp.2	03	1.5	01	0.5	-	-
	***Aspergillus* sp.1**	**05**	**2.5**	-	-	-	-
	***Aspergillus* sp.2**	02	1	**04**	**2**	01	0.5
	***Aspergillus* sp.3**	-	-	-	-	02	1
	***Curvularia* sp.1**	01	0.5	-	-	-	-
	***Curvularia* sp.2**	-	-	01	0.5	-	-
	***Curvularia* sp.3**	01	0.5	02	1	-	-
	Fusarium sp.1	-	-	-	-	02	1
	Fusarium sp.2	-	-	01	0.5	01	0.5
	Nigrospora sp.	02	1	-	-	**03**	**1.5**
Total		**28**		**17**		**9**	

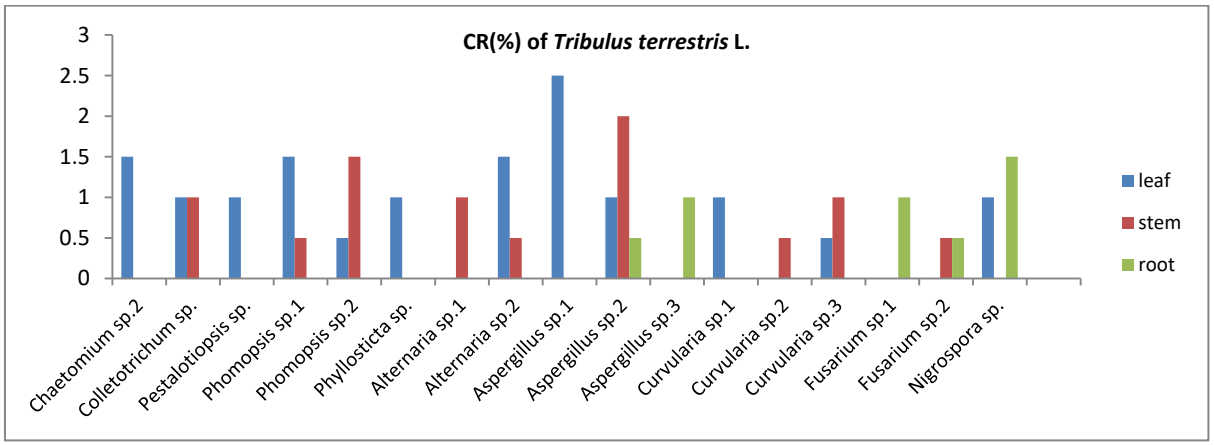

Figure 2: Colonization rate of different endophytic fungi from *Tribulus terrestris* Linn.

Table 3: Dominance and richness of species diversity of endophytic assemblages in different tissues of *Tribulus terrestris Linn.*

Tissue	Total no. of taxa	Total no. of isolate	Simpson index(1-D)	Shannon-wiener index (Hs)	Species richness (R1)
Leaf	12	28	0.933	2.394	3.301
Stem	09	17	0.911	2.068	2.823
Root	05	09	0.861	1.522	1.820

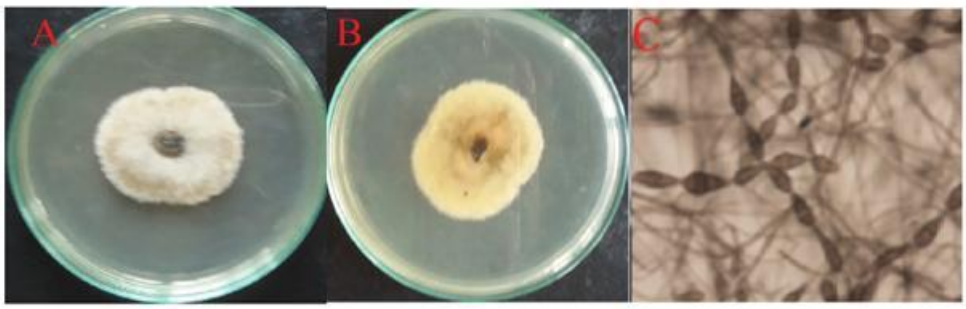

Figure 3: *Alternaria* sp.1. A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image

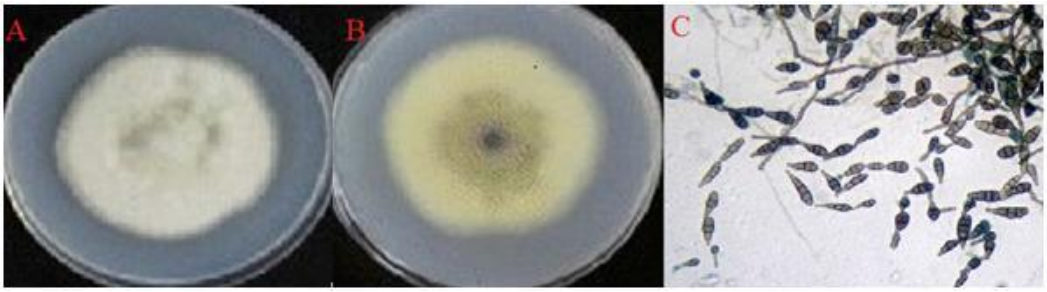

Figure 4: *Alternaria* sp.2. A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image

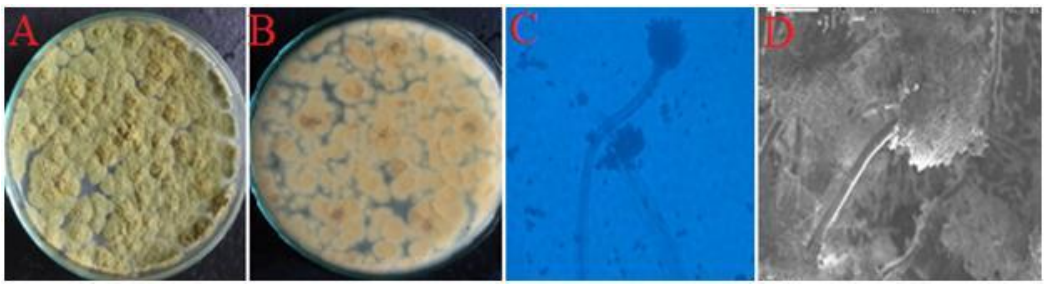

Figure 5: *Aspergillus* sp.1 A. Front view in PDA, B. Reverse view in PDA, Microscopic image D. SEM image

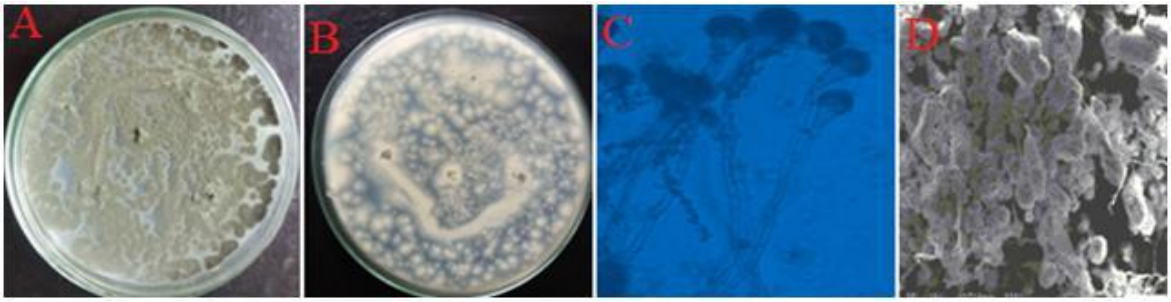

Figure 6: *Aspergillus* sp. 2. A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image D. SEM image

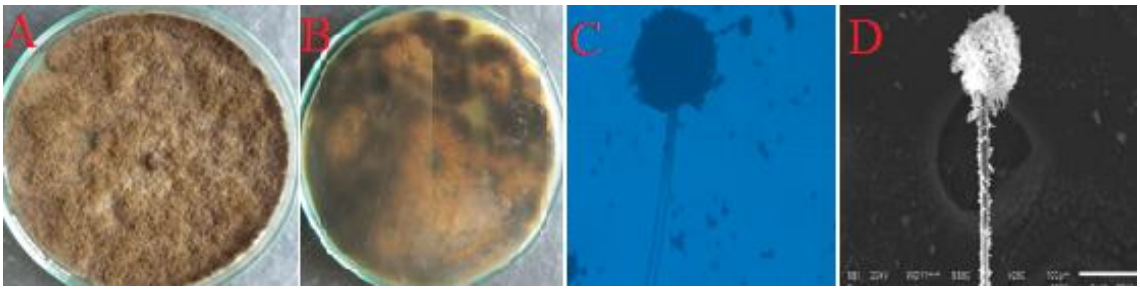

Figure 7: *Aspergillus* sp. 3. A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image D. SEM image

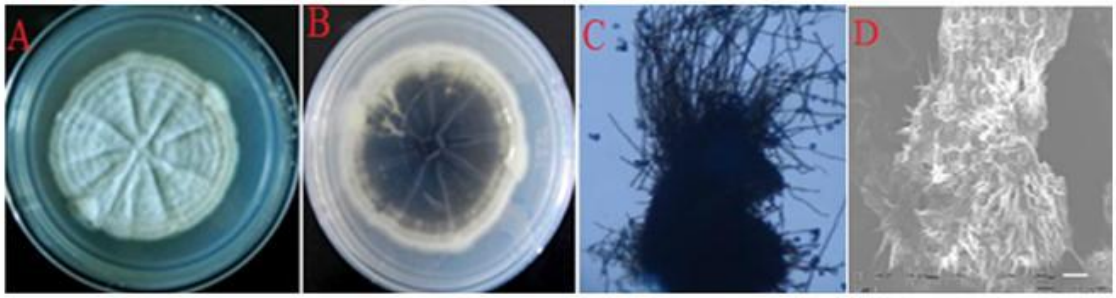

Figure 8: *Chaetomium* sp.1 A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image, D. SEM image

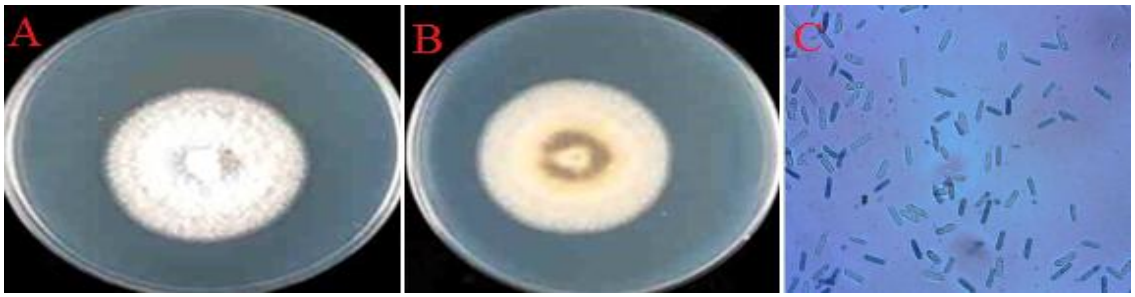

Figure 9: *Colletotrichum* sp. A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image

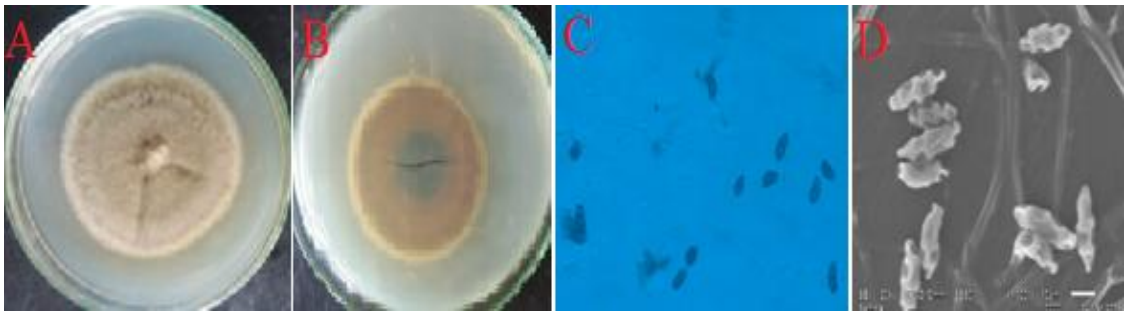

Figure 10: *Curvularia* sp.1 A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image, D. SEM image

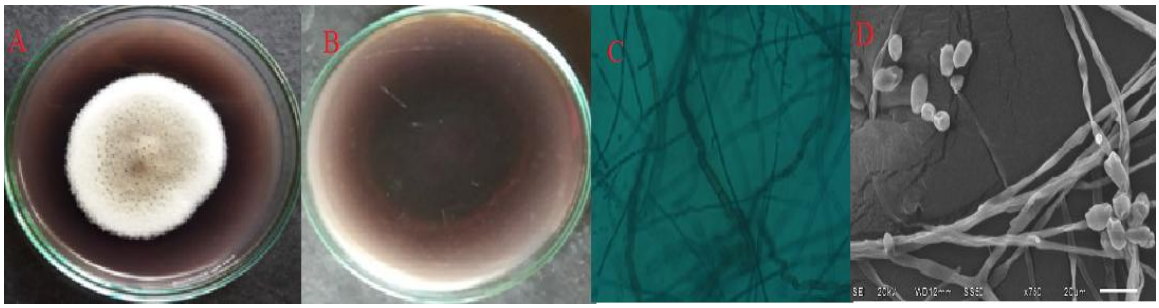

Figure 11: *Curvularia* sp.2, A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image, D. SEM image

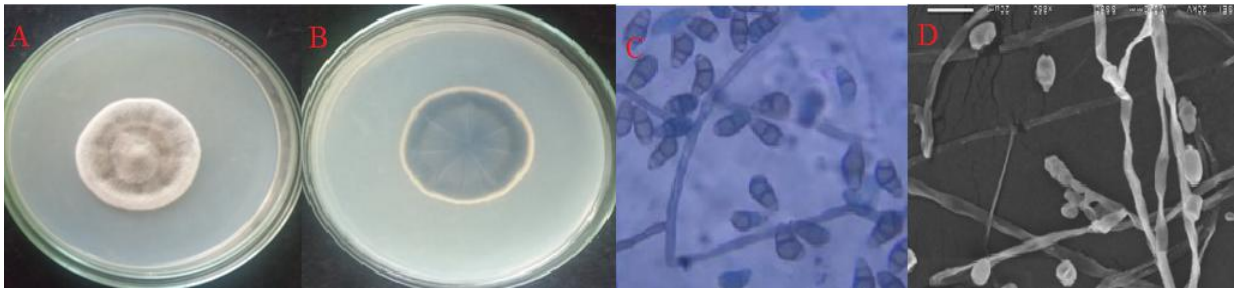

Figure 12: *Curvularia* sp. 3, A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image, D. SEM image.

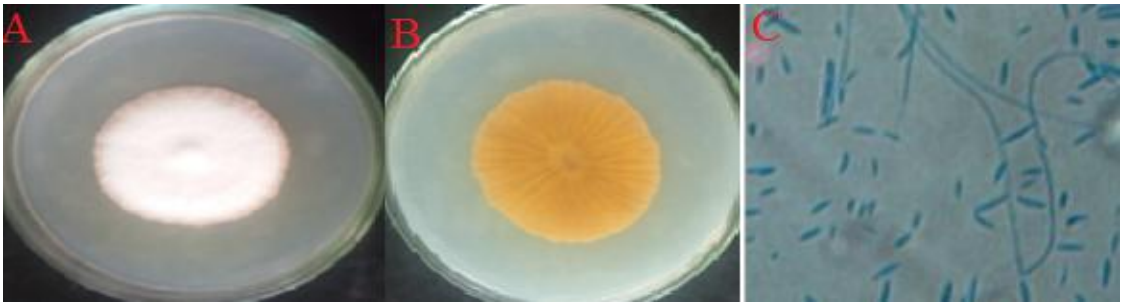

Figure 13: *Fusarium* sp.1 A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image

Figure 14: *Fusarium* sp. 2 A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image

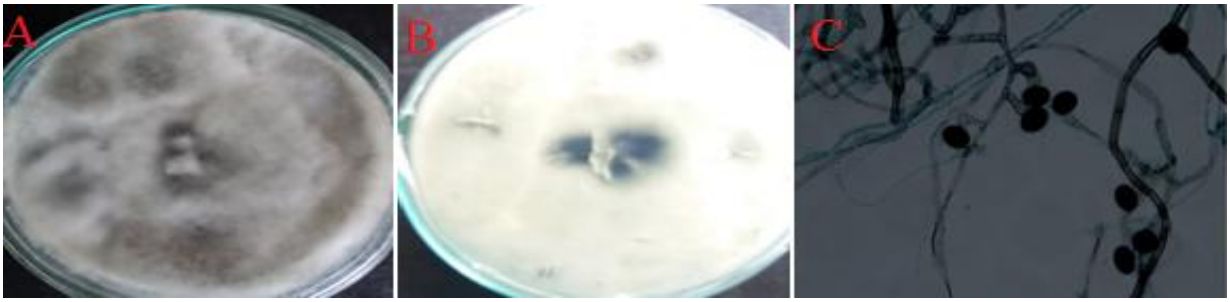

Figure 15: *Nigrospora* sp. A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image

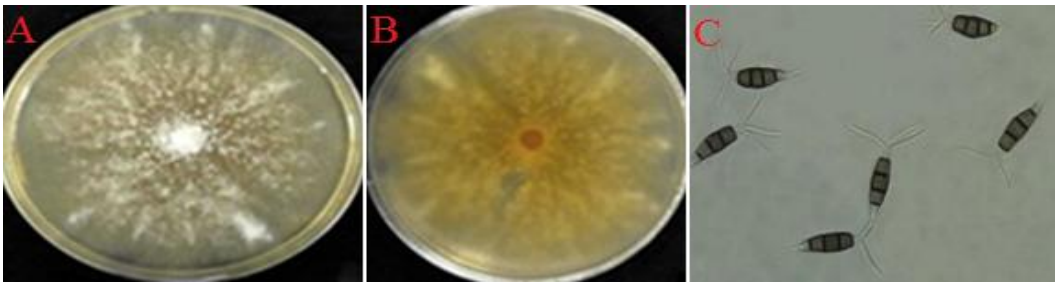

Figure 16: *Pestalotiopsis* sp. A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image

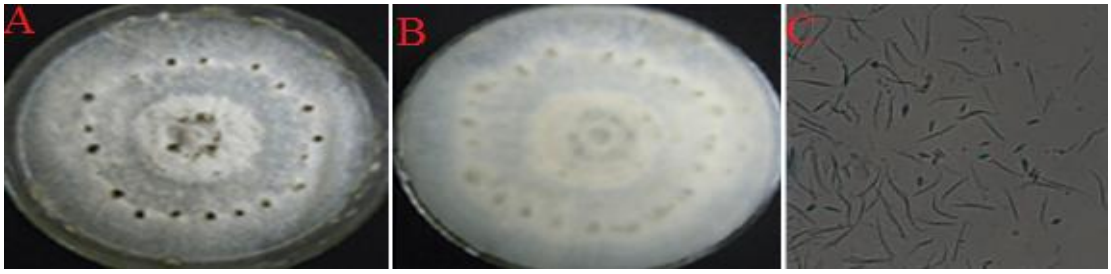

Figure 17: *Phomopsis* sp. 1. A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image

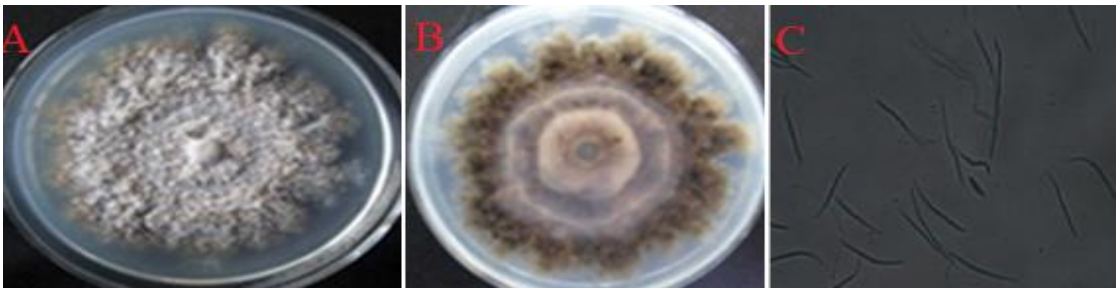

Figure 18: *Phomopsis* sp. 2. A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image

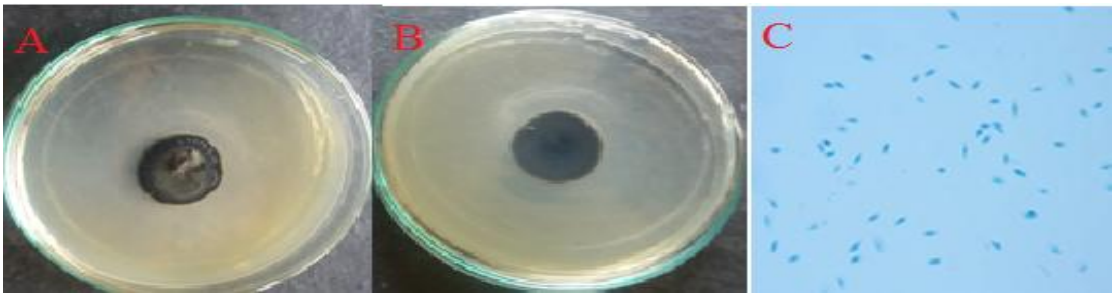

Figure 19: *Phyllosticta* sp. A. Front view in PDA, B. Reverse view in PDA, C. Microscopic image

DISCUSSION

In the present study significant diversity and distribution were observed for the foliar endophytes in terms of isolation rate which is in agreement with a greater number of isolates which were isolated from leaf samples showing range between 50% to 95% occurrence in the tropical region, documented from the studies of recent studies [36-49].

Suryanarayanan and Thennarasan, (2004)[50] reported the temporal variation in foliar endophyte assemblages of *Plumeria rubra*. A temporal relationship with reference to endophyte colonization in host was observed such as, ascomycetes and their anamorphic states invariably constitute the endophyte populations of leaves [51, 52]. This correlation was appearing in the present study due to the occurrence and distribution of species of ascomycetes *Aspergillus and Chaetomium* were dominated in the leaves of species *Tribulus terrestris* Linn (Zygophyllaceae).

Hyphomycetes, a class of Deuteromycotina, ranked first among the endophytic fungal community obtained in the present investigation. *Aspergillus* sp., *Fusarium* sp., *Curvularia* sp. *Nigrospora* sp., all were isolated from all the plant tissues and this may be a fine example of host and tissue specificity from the present investigation. Hyphomycetous fungi are common endophytes among plants inhabiting temperate, tropical and rainforest vegetation[53, 54, 42-46].

Leaf harbored a greater number of endophytic fungi with high diversity than stem as indicated by previous reports [55-57, 36-38, 42-46] (Table 1-2). This may be attributed to the morphological and anatomical features of the leaf tissue, due to the large surface area exposed to the outer environment and the presence of stomata providing passage to the entry of fungal mycelia. This may also be one of the reasons for endophytes of leaf which had greater colonization frequency than that of stem and root.

CONCLUSION

Hence from the present study we can conclude that medicinal plant can be repository of endophytic fungi. Almost all plants are known to harbor endophytes. The choice of the plant to be used for exploring endophytes for bio actives is important. Therefore, medicinal plants which are known to be used since centuries as an alternative source of medicine are a valuable source for bioprospecting endophytes. Endophytes therefore, represent a chemical reservoir for new compounds such as, anticancer, immunomodulatory, antioxidant, antiparasitic, antiviral, antitubercular, insecticidal etc. for use in the pharmaceutical and agrochemical industries

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