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Diversity of Endophytic Fungi Associated with *Tephrosia Purpurae* from Eastern Ghats and the Coast of Bay of Bengal, India

[1]Kamana Sahani, [2]K. Sunanda Kumari, [3]P. Raja Ramesh,
Corresponding Author: - [4]K.P.J.Hemalatha
[1,2,3,4] Department of Microbiology, Andhra University, Visakhapatnam-530003, India.

Abstract:-Endophytic fungi are eukaryotic organisms that live inside plant tissues with symbiotic association and they have been recognized as a valuable source of novel bioactive metabolites, antibiotics, anti-cancerous compounds, antioxidants, enzymes, vitamins, and pigments. In the present investigation endophytic fungi were isolated from *Tephrosia purpurae* from Eastern Ghats and near the Bay of Bengal for the first time. Seventy two endophytic fungi with different morphologies were isolated from the leaves, stems and roots of *Tephrosia purpurea* with colonization rates of 16.5%, 12.5%, and 7%, 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).

Keywords:-Endophytic fungi, *Tephrosia Purpurae*, Simpson Index (1-D), Shannon-Wiener Index (Hs) and Species Richness (R1).

I. INTRODUCTION

Endophytic fungi represent a group of fungi growing inside the plants generally causing no disease symptoms, the degree of interaction of which with its host varies from mutualism to parasitism. The word *Endophyte* (Gr. Endon, within; phyton, plant) was first coined by De Bary, to refer any organism occurring within plants (De Bary, 1866). Currently, endophytic fungi are of biotechnological interest due to their potential use as biological control agents and as sources of novel, biologically active secondary metabolites. Many of the bioactive agents that are produced by plants (e.g., taxol) can also be produced by endophytic fungi (Stierle *et al*., 1993). Endophytic fungi yield a broad variety of substances, including antioxidants, novel antibiotics, and antimicrobial, immunosuppressant and antiparasitic compounds, and thus are rich sources of biologically active metabolites that have been widely exploited in medicine, agriculture, and industry (Strobel and Daisy 2003; Aly *et al.,* 2011) Here, we isolated endophytic fungi from the plant *Tephrosia purpurea.*

Tephrosia purpurea is a small shrub that grows up to 1.5 meters tall. It has bi-pinnate leaves with 7 to 15 leaflets, the terminal leaflet being solitary. The leaflets are 10 to 32 mm long and 5 to 11 mm wide. The peas like flowers are white to purple and arranged in inflorescences that are up to 25 cm long. The individual flowers have corolla parts that are between 2 to 3 mm long. The pods are straight and somewhat up curved at the terminal end and may range from 20 to 45 mm in length and 3 to 5 mm breadth. When dry, the pods split along two valves to reveal 2 to 9 black rectangular seeds 2.5 to 5 mm long and 1.8 to 3 mm wide (Rao *et al.,* 1984 and Change *et al.,* 1997).

Tephrosia purpurea is a common wasteland weed species of flowering plant belongs to the pea family that has a pantropical distribution. In many parts it is under cultivation as green manure crop. *T. purpurea* is widely distributed throughout the world. It is the native plant of Africa, Southeast Asia to Australia, Western part of Pacific, China, Sri Lanka, Nepal and India. In India, It is found in the areas of Andhra Pradesh, Haryana, Rajasthan, and Tamil Naidu (Orwa *et al.,* 2009).

II. CLASSIFICATION

Kingdom: Plantae
Subkingdom: Tracheobionta
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Rosidae
Order: Fabales
Family: Fabaceae
Genus: *Tephrosia*
Species: *purpurea*

Fig.1: *Tephrosia Purpurae*

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T. purpurea is widely distributed in tropical, sub-tropical and arid regions. This perennial herb is an ingredient in traditional herbal formulations for hepatitis (Dalwadi *et al*., 2014) and exhibits several hepatoprotective activities, including antimicrobial (Kumar *et al.,* 2007) wound-healing (Lodhi *et al.,* 2006) antiulcer (Deshpande *et al*., 2003), immunomodulatory (Damre *et al*., 2003) and anticancer (Kishore *et al*., 2011) activities.

The most important plant constituents are alkaloids, terpenes, sterols, glycosides, saponin, gums, fatty acids, lactones, coumarins, carbohydrates, waxes, amino acids, proteins, Tephrosin, diguelin, isotephrosin, rotenone, tannins, phytosterols, glycosides, purpurin, isolonchocarpin etc. which are medicinally important. It is used in the treatment of diuretic, enriches the blood, useful in bronchitis, wounds, boils, pimples, liver and spleen diseases, asthma, inflammation, antiulcer, hepatoprotective, used in poisoning due to snakebite, useful in enlargement of spleen, antidiarrhoel, also used in tympanitis and dyspepsia. It is also used in poisoning against rat bite. It is also used in diseases of lungs and chest, tonic to intestine, improves the appetite, treatment of piles, syphilis, and gonorrhoea. Blood purifier, antihelmintic, digestible, antipyretic, alexeteric, cures diseases of liver, spleen, heart, blood, cures tremors, ulcers, leprosy, asthma, bronchitis, piles.

III. MATERIALS AND METHODS

A. Collection of Plant Material

Visakhapatnam (Location 17.7041N, 83.2977E.) is situated between the Eastern Ghats and the 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. The plants are located in the Campus of Andhra University. Healthy (showing no visual disease symptom) and mature plant of *Tephrosia purpurae* were collected from the Campus, Andhra University. Sample collection was done in winter season (January 2016). Samples were tagged and placed in separate sterile polythene bags, brought to the laboratory and processed within 24 h of collection (Fisher and Petrini, 1987; Suryanarayanan *et al*., 1998). Fresh plant materials were used for the isolation work to reduce the chance of contamination.

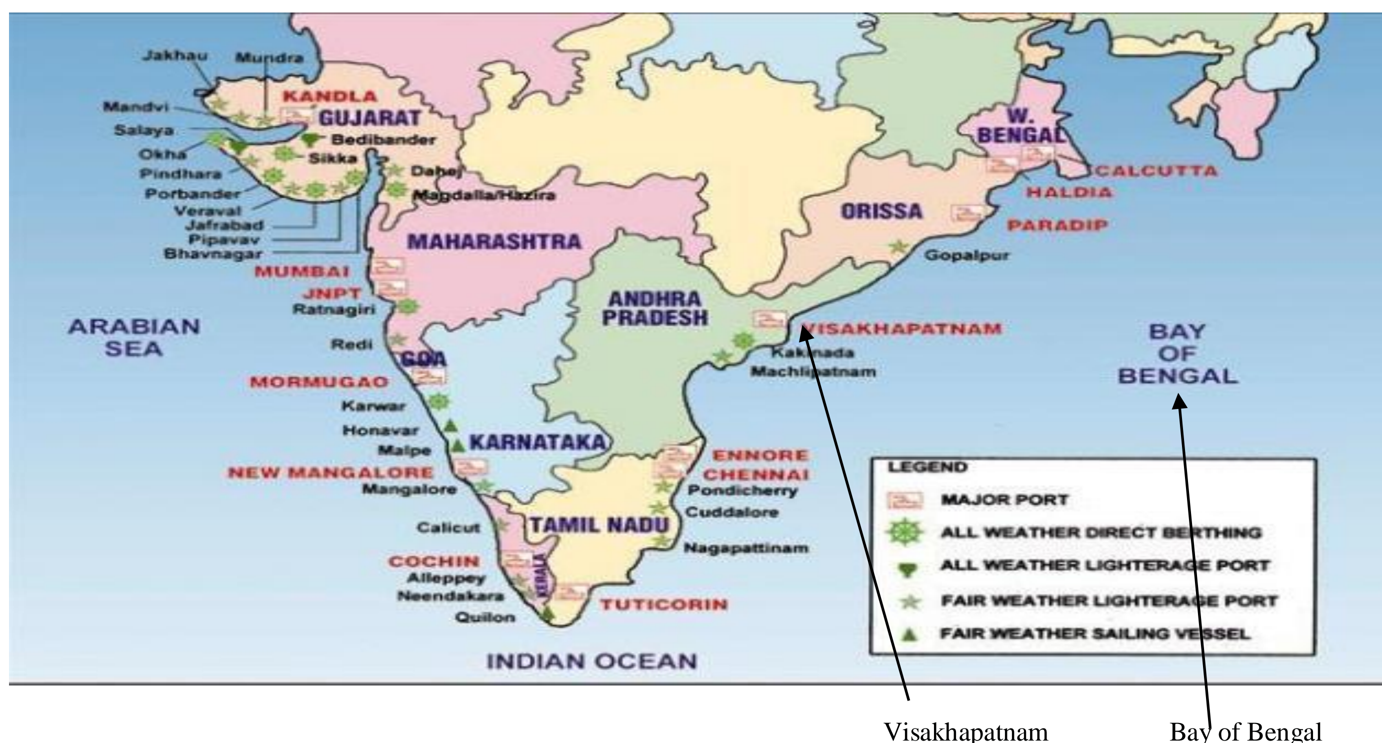

Fig.2: Visakhapatnam and Bay of Bengal

B. Glassware, Chemicals and Media

The glassware used were made up of borosilicate glass obtained from M/s Borosil India Limited. The chemicals used were of analytical grade obtained from M/s Himedia Laboratories Pvt. Limited Mumbai, India & M/s Sigma-Aldrich Pvt. Limited USA. The Media used for the experiments were obtained from M/s Himedia Laboratories Pvt. Limited Mumbai, India.

C. 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 one min and 3.5 % NaOCl (v/v) for three min, 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 *Tephrosia purpurae* plant 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) which 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 (Schulz *et al.,* 1993; Strobel *et al.,* 2003; Huang *et al*., 2008 and Wang *et al.,* 2012). 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.

D. 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 (Ellis, 1993a, 1993b; 1971c; Barnett and hunter, 1998; Gilman, 1971).

E. 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) (Petrini and Fisher, 1988; Hata and Futai, 1995; Photita *et al*., 2001; Maheshwari and Rajagopal, 2013).
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) 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 (H_S) 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 ith species or kinds measures= n/N
N-total number of individuals of all kinds
N_i- number of individuals of ith 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.

IV. RESULTS

A total of seventy two endophytic isolates were collected from 600 plant tissue samples of leaf (200 segments), stem (200 segments) and root (200 segments) from *Tephrosia purpurae* (Fabaceae). 72 endophytic isolates were categorised into 16 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 parts of plant tissues were found to harbour various endophytic fungal species with different colonization rate (CR) and isolation rate (IR) (Tables 1-2). Figures 3 shows the correlation between isolation rate and colonization rate of endophytic fungi isolated from *Tephrosia purpurae* plant and Figure 4 shows the colonization rate of all the endophytes isolated from different parts of *Tephrosia purpurae* plant.

Simpson dominance index is comparatively higher in the leaves sharing relatively similar index values 0.968. Shannon-Wiener index indicates that the foliar endophytic diversity is more with index value 2.519 which is due to occurrence of more number of endophytic species than the stem and root (Tables 3).

	Leaf	Stem	Root	Total
No. of segments	200	200	200	600
No. of segments yielding endophytic fungi	33	25	14	72
No of isolates	34	26	16	76
Isolation rate	0.17	0.13	0.08	0.12
Colonization rate (%)	16.5	12.5	7	12

Table 1: Isolation and Colonization Rate of Endophytic Fungi from *Tephrosia Purpurae*

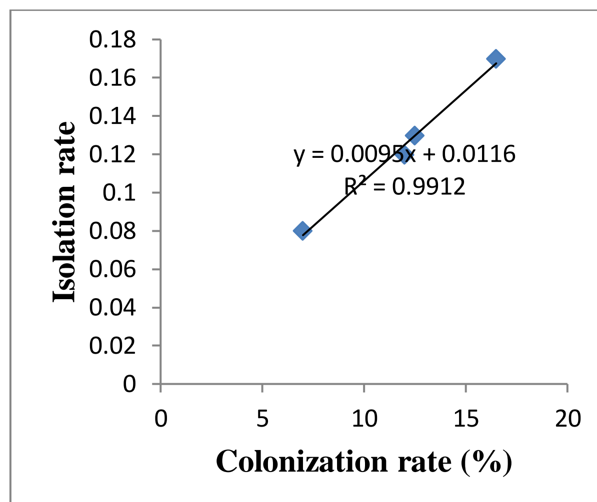

Fig. 3: The Relationship between Colonization Rate and Isolation Rate of Endophytic Fungi From *Tephrosia Purpurae*.

Class	Endophytic fungi	Leaf	CR (%)	Stem	CR (%)	Root	CR (%)
Ascomycetes	***Chaetomium sp.1***	**04**	**2**	03	1.5	-	-
	Chaetomium sp.2	03	1.5	-	-	-	-
Coelomycetes	***Colletotrichum* sp.**	03	1.5	02	1	-	-
	***Pestalotiopsis* sp.**	02	1	02	1	-	-
	***Phomopsis* sp.1**	03	1.5	01	0.5	-	-
	***Phomopsis* sp.2**	02	1	03	1.5	-	-
	Phyllosticta sp.	03	1.5	-	-	-	-
Hyphomycetes	***Alternaria sp.1***	02	1	04	2	03	1.5
	Alternaria sp.2	02	1	01	0.5	-	-
	***Aspergillus* sp.1**	03	1.5	-	-	-	-
	***Aspergillus* sp.2**	01	0.5	01	0.5	02	1
	***Aspergillus* sp.3**	-	-	-	-	01	0.5
	***Curvularia* sp.3**	02	1	**05**	**2.5**	02	1
	Fusarium sp.1	-	-	-	-	**05**	**2.5**
	Fusarium sp.2	-	-	03	1.5	-	-
	Nigrospora sp.	03	1.5	-	-	01	0.5
Total		**33**		**25**		**14**	

Table 2: Diversity of Endophytic Fungi Isolated From Leaf, Stem and Root of Tephrosia Purpurae

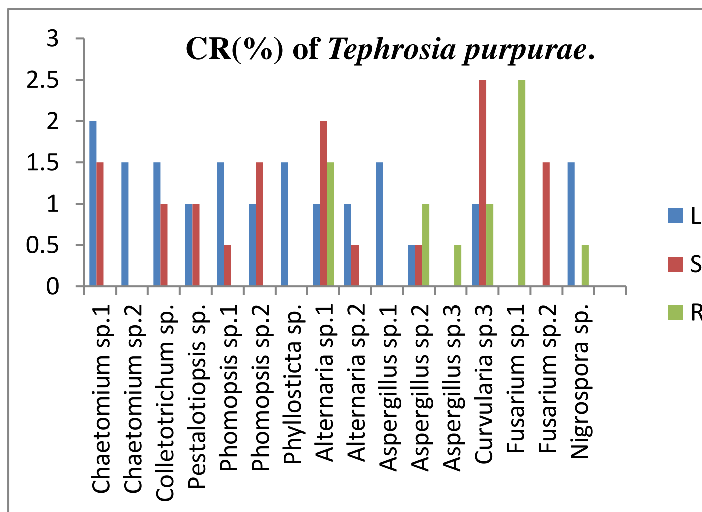

Fig.4: Colonization Rate of Different Endophytic Fungi from Tephrosia Purpurae

Tissue	Total no. of taxa	Total no. of isolate	Simpson index(1-D)	Shannon -wiener index (Hs)	Species richness (R1)
Leaf	13	33	0.968	2.519	3.431
Stem	10	25	0.91	2.168	2.796
Root	06	14	0.835	1.630	1.894

Table.3: Dominance and Richness of Species Diversity of Endophytic Assemblages in Different Tissues of *Tephrosia Purpurae*

V. DISCUSSION

Endophytes have been reported from all major groups of plants including algae (Hawksworth, 1988; Zuccaro *et al*., 2008; Suryanarayanan *et al*., 2010), lichens (Petrini *et al*., 1990; Suryanarayanan *et al*., 2005), mosses (Petrini, 1986; Schulz *et al*., 1993), bryophytes (Davis *et al*., 2003), ferns (Petrini, 1986; Petrini *et al*., 1992), conifers (Carroll *et al*., 1977; Carroll and Carroll 1978; Petrini and Muller, 1979; Petrini and Carroll, 1981; Petrini, 1986; Giordano *et al*., 2009) and angiosperms (Taylor *et al*., 1999; Arnold *et al*., 2000). Endophytes occur in wide range of habitats, such as coastal mangroves (Kumaresan and Suryanarayanan 2001), temperate evergreen forests (Espinosa-Garcia and Langenheim, 1990), xeric regions (Suryanarayanan *et al*., 2003) and tropical forests (Dreyfuss and Petrini, 1984; Laessoe and Lodge, 1994; Rodrigues and Samuels, 1990; Rodrigues *et al*., 1993; Rodrigues, 1994; Arnold *et al*., 2000; Raviraja, 2005).

Tropical and subtropical climates harbor most of the world's plant diversity, so endophytic diversity in this climatic zone is also higher as almost all vascular plant species examined to date are found to possess endophytic bacteria and fungi (Firakova *et al*., 2007). Tropical and sub tropical medicinal trees have been studied for their associated fungal endophytes. (Suryanarayanan and Rajagopal, 2000; Tejesvi *et al*., 2005; Mahesh *et al*., 2005; Suryanarayanan *et al*., 2009). Most undescribed fungal diversity lies within the tropical plant associated fungi, yet the diversity and ecological role of endophytes in tropical angiosperms are almost entirely unexplored (Hawksworth, 1993; Rodrigues and Petrini, 1997; Azevedo *et al*., 2000). Several medicinal herbs and shrubs have been intensively screened for endophytic fungi, by (Schulz *et al*., 1993; Li *et al*.,2001; Strobel, 2002; Raviraja, 2005; Krishnamurthy *et al*.,2008; Sun *et al*.,2008; Sowparthani and Rajagopal, 2011; Orlandelli *et al*.,2012; Gautam *et al*., 2013).

In the present investigation endophytic fungal colonization and isolation rates were comparatively more in leaf than stem and root tissues of three plants which are found to be within the range (2-100%) as many host plants studies in tropical regions.(Kumar and Hyde, 2004; Raviraja, 2005; Huang *et al*., 2008; Sun *et al*., 2008; Xing *et al*., 2010; Chaeprasert *et al*., 2010; Thalavaipandian *et al*., 2011; Siqueira *et al*., 2011 and Suwannarach *et al*., 2011). The composition of the endophytic fungal community differs between root, stem, leaf of *Tephrosia purpurae* (Fabaceae) which is relatively similar to the studies of Ze-Ping Luo *et al*., (2015).

In *Tephrosia purpurae* (Fabaceae) there is significant difference in overall Simpson, Shannon diversity indices values as mentioned in (Tables 3). But Simpson and Shannon diversity indices comparatively higher for leaves due to high species richness value as reported by Suwannarach *et al*., (2011). A comparative analysis of species richness and diversity of the endophytic fungal community associated with *Tephrosia purpurae* (Fabaceae) is consistent with the other studies of endophytes isolated from different tissues from medicinal plants. (Raviraja, 2005; Chareprasert *et al*., 2006; Huang *et al*., 2008)

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