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Diversity and Cold Adaption of Endophytic Fungi from Boerhavia Diffusa L. from Eastern Ghat of India

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Abstract:-The aim of present study was to isolate the endophytic fungi from the medicinal plant Boerhavia diffusa L. During the present investigation, endophytes were isolated from the symptomless leaves, stem and root. A total of 81 strains were recovered from different tissues of Boerhavia diffusa L. This is the first report of isolating the endophytic fungi from Boerhavia diffusa L. from the eastern Ghat. Moreover the richness and diversity of the endophytic fungi were different in different part of plant. Frequency of colonization of endophytic fungi was found higher in the leaf segments, than the stem and root i.e. 17%, 14.5%, 9% respectively.Furthermore Biodiversity of endophytic fungi in various segments of the plant were determined by statistical analysisSimpson index (1-D), Shannon-wiener index (Hs) and Species richness (R1).

Keywords:-Endophytic fungi, Boerhavia diffusa L, Eastern Ghat, CR, IR,Simpson index (1-D), Shannon-wiener index (Hs) and Species richness (R1).

I. INTRODUCTION

Endophytes are the plant-associated microorganisms that live within the living tissues of their host plants without causing any harm to them. Endophytic fungi have been found in healthy tissues of terrestrial plants taxa (Arnold, 2007).Endophytes constitute a remarkably multifarious group of microorganisms ubiquitous in plants and maintain an imperceptible association with their hosts for at least a part of their life cycle. Their enormous biological diversity coupled with their capability to biosynthesize bioactive secondary metabolites has provided the impetus for a number of investigations on endophyte. There is a need to search new ecological niches for potential of natural bioactive agents for different pharmaceutical, agriculture and industrial application; these should be renewable, eco-friendly and easily obtainable natural products discovery in the search for new drugs and is the most potent source for the discovery of novel bioactive compounds. Therefore, a large number of bioactive compounds are isolated from the plants, bacteria, fungi and many other organisms. Endophytic fungi being the most promising of these have been a source of various such bioactive compounds. Many of these compounds are being used for the treatment of a number of diseases.Moreover, certain endophytic fungi are capable of synthesizing the medicinal products produced in plants (Tan and Zou, 2001).Endophytic fungi colonizing the plant tissues usually get nutrition and protection from the host plant. In turn they enhance tolerance of the host plants by producing certain functional metabolites (Redman *et al*, 2002). In the present study we have isolated the endophytic fungi from the Boerhavia diffusa L. Boerhaavia diffusa L.is a perennial creeping weed, prostrate or ascending herb, up to 1m long or more, having spreading branches. The stem is prostrate, woody or succulent, cylindrical, often purplish, hairy, and thickened at the nodes. Leaves are simple, thick, fleshy, and hairy, arranged in unequal pairs, green and glabrous above and usually white underneath. The shape of the leaves varies considerably - ovate-oblong, round, or subcordate at the base and smooth above (Kirtikar, 1956). Flowers are minute and subcapitate. These are hermaphrodite, pedicellate, and white, pink, or pinkish-red in color. The roots are stout and fusiform and woody. This trailing herb is mainly collected after rainy season in India (Ayurvedic Pharmacopeia of India, 2005).

The genus *Boerhaavia* has several species, and is distributed in the tropical, subtropical, and temperate regions of the world (Heywood, 1978). It is found in Australia, Nepal, India, China, Egypt, Pakistan, Sudan, SriLanka, South Africa, USA and in several countries of the Middle East. Out of the 40 species of this genus, 6 species are found in India - B.diffusa, B.chinensis, B.erecta, B.repens,B.rependa, and B.rubicunda. It is found throughout the warmer parts of the country up to an altitude of 2000m in the Himalayan region. It grows well on wastelands and in fields after the rainy season (Chopra, 1969).

- *Boherhavia diffusa*Linn.

Classification
Kingdom: Plantae
Subkingdom: Tracheobionta
Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Caryophyllidae
Order:Caryophyllales
Family: Nyctaginaceae (four o'clock)
Genus: *Boerhavia*
Species*: diffusa Linn.*

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Fig 1: Boerhavia Diffusa L.

Chemically the plant contains some bioactive compounds such as flavonoids, alkaloids, steroids, triterpenoids, lipids, lignins, carbohydrates, proteins, and glycoproteins which are medicinally important. It is used for the treatment of various disease like Abdominal Pain, Anemia, Ascites, Asthma, Calculi, Cancer (abdominal), Cataract, Cholera, Cough, Debility, Dropsy, Dyspepsia, Edema, Fever, Gonorrhea, Guinea Worms, Hemorrhages (childbirth) Hemorrhages (thoracic) Hemorrhoids, Inflammation (internal), Jaundice, Liver, Heart, Menstrual, Ophthalmic, Renal & Urinary Disorders, Rheumatism, Snakebite, Spleen (enlarged), Weakness, Albuminuria, BeriBeri, Blenorrhagia, Chologogue, Cystitis, Hepatitis, Hydropsy, Nephritis, Sclerosis (Liver), Sterility, Yaws, Erysipelas, Joint Pain, Lumbago, Nephritis, Urticaria, Abscess, Boil, Convulsions, Epilepsy.(Santhosha*et al.*, 2011).

II. 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. Sample collection was done in January 2016. Healthy and mature plants of *Boherhavia diffusa* 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 24h 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.

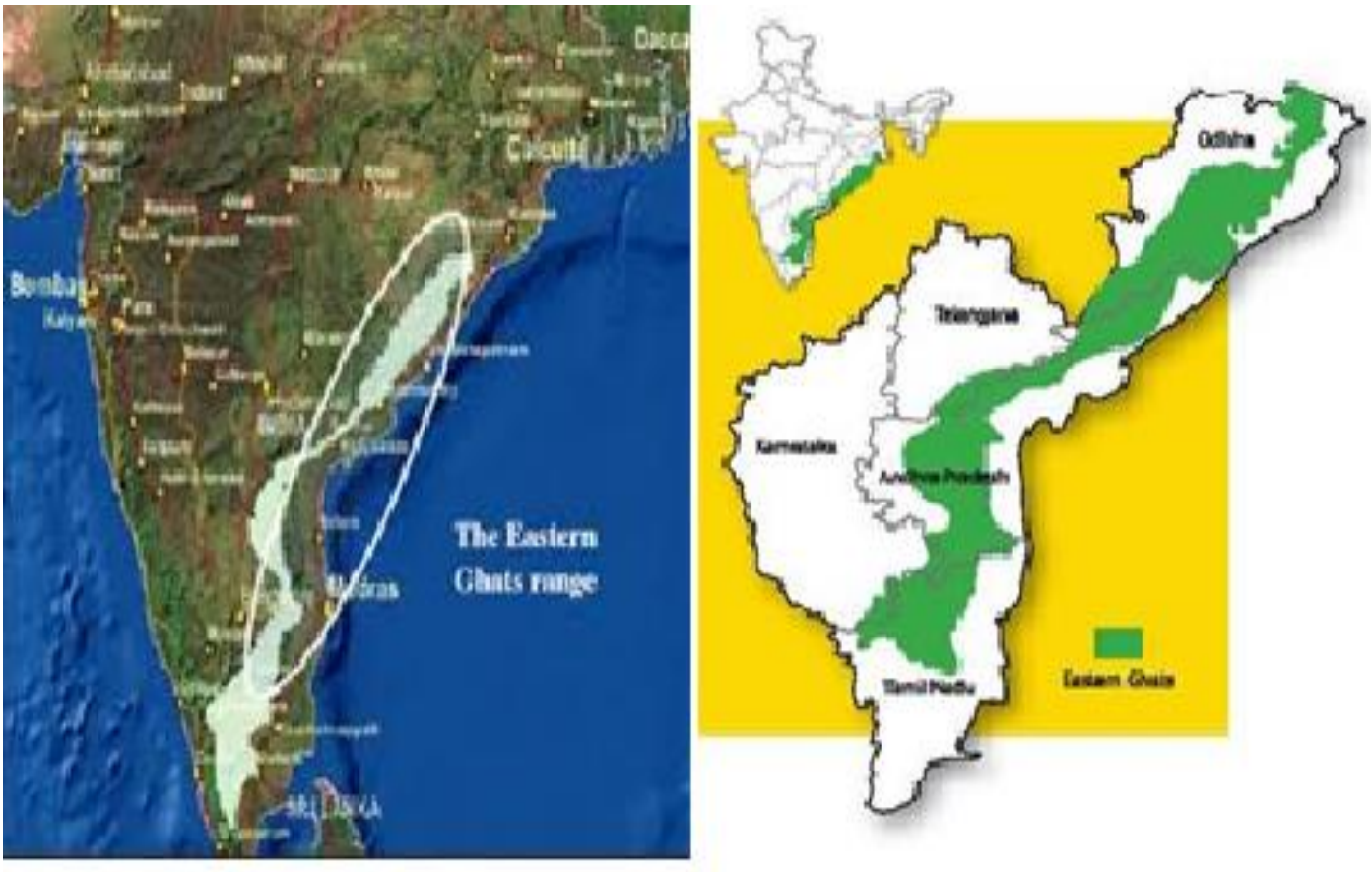

Fig 2: Figures Showing the Eastern Ghats of India

B. 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 partof the plant of*Boerhavia diffusa* L. representing 200 leaf segments, 200 stem segments and 200rootwere placed on the water agar (16%) (WA) medium supplemented with Streptomycin (100 mg/l; Sigma, St. Louis, MO, USA).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 isolates were maintained in cryovials on PDA layered with 15% glycerol (v/v) at -80 °C in an Ultrafreezer (CryoscientificPvt. Ltd., Chennai, India) at the Department of Microbiology, College of Science and Technology, Andhra University, Visakhapatnam, India.

C. 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).

D. 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

Colonization rate (CR) = Total no. of isolates scored in a given sample X 100
Total no. of segments in a sample

Isolation rate (IR) = Total no. of isolates scored in a sample
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 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.

III. RESULTS

81endophytic isolates werecategorised into 15 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 plants tissues i.e. root, stem, leaf were found to harbour various endophytic fungal species with different colonization rate (CR) and isolation rate (IR) (Tables1-2). Figure 3 represents the correlation between colonization rate and isolation rate and figure 4 represents the colonization rate of different endophytic fungi in the different tissue of *Boerhavia diffusa* L.

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Simpson dominance index is comparatively higher in the leaves sharing relativelysimilar index values 0.937. Shannon-Wiener index indicates that the foliar endophytic diversity is more with indexvalue2.542 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	34	29	18	81
No of isolates	35	30	19	84
Isolation rate	0.175	0.15	0.095	0.14
Colonization rate	17	14.5	9	13.5

Table1: Isolation and Colonization Rate of Endophytic Fungi From *Boerhavia Diffusa* L.

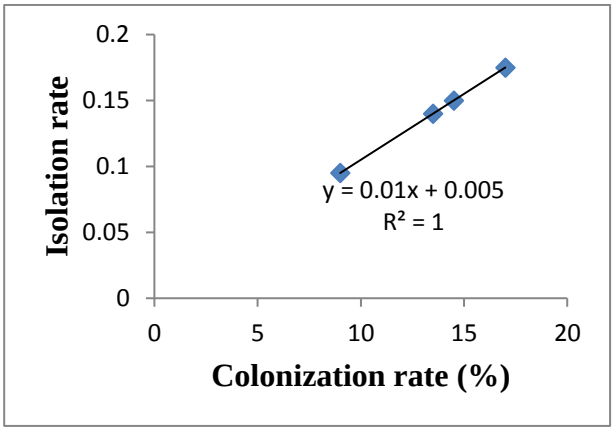

Fig 3: The Relationship Between Colonization Rate and Isolation Rate of Endophytic Fungi from *Boerhavia Diffusa* Linn

Class	Endophytic fungi	Leaf	CR (%)	Stem	CR (%)	Root	CR (%)
Ascomycetes	***Chaetomium sp.2***	02	1	01	0.5	01	0.5
Coelomycetes	***Colletotrichum* sp.**	03	1.5	-	-	-	-
	***Pestalotiopsis* sp.**	02	1	01	0.5	-	-
	***Phomopsis* sp.1**	01	0.5	02	1	-	-
	***Phomopsis* sp.2**	02	1	01	0.5	-	-
	Phyllosticta sp.	03	1.5	-	-	-	-
Hyphomycetes	***Alternaria sp.1***	01	0.5	-	-	-	-
	Alternaria sp.2	03	1.5	01	0.5	-	-
	***Aspergillus* sp.1**	**05**	**2.5**	02	1	01	0.5
	***Aspergillus* sp.2**	03	1.5	**07**	**3.5**	03	1.5
	Aspergillus* sp.*3	01	0.5	02	1	**04**	**2**
	Curvularia* sp.*3	**05**	**2.5**	04	2	03	1.5
	Fusarium sp.1	01	0.5	04	2	03	1.5
	Fusarium sp.2	01	0.5	01	0.5	01	0.5
	Nigrospora sp.	01	0.5	03	1.5	02	1
Total		**34**		**29**		**18**	

Table 2: Diversity of endophytic fungi Isolated from leaf, stem and root of Boerhavia diffusa Linn.

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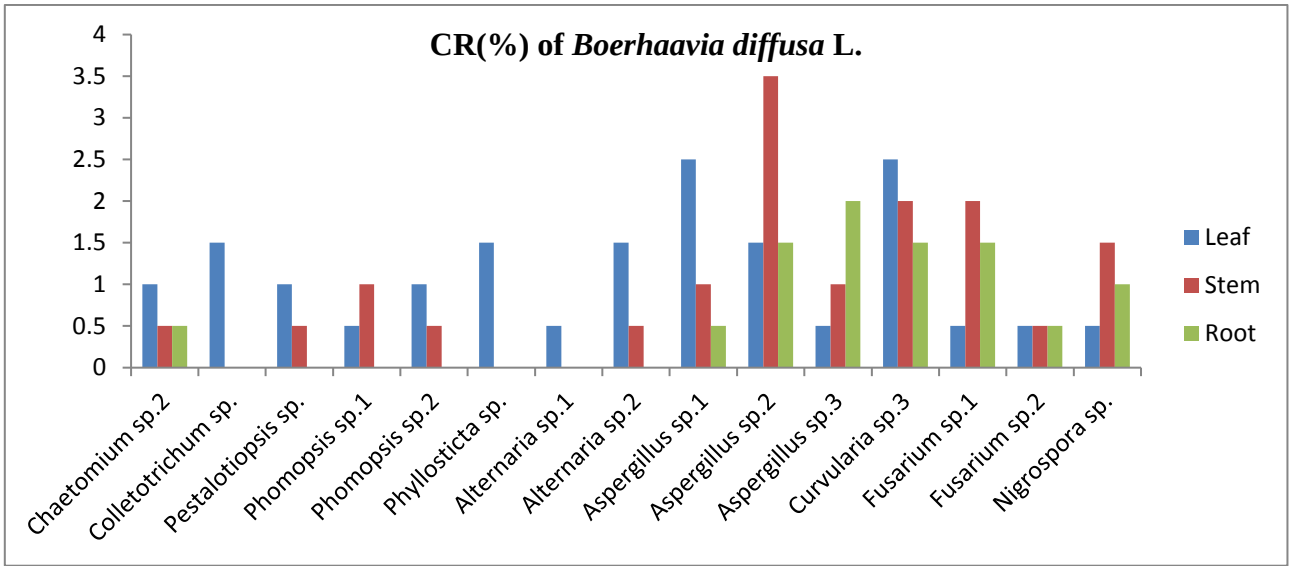

Fig 4: Colonization Rate of Different endophytic fungi from Boerhavia diffusa Linn

Tissue	Total no. of taxa	Total no. of isolate	Simpson index(1-D)	Shannon-wiener index (Hs)	Species richness (R1)
Leaf	15	34	0.937	2.542	3.970
Stem	12	29	0.903	2.258	3.266
Root	08	18	0.895	1.955	2.421

Table 3: Dominance and Richness of Species Diversity of Endophytic Assemblages in Different Tissues of Boerhavia Diffusa Linn.

IV. DISCUSSION

In the present investigation endophytic fungal colonization and isolation rates were comparatively more in leaf than stem and root tissues 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 variation in colonization rates from tropical palm endophytes was reported as between 0.0 to 98.0% (Frohlich *et al*., 2000; Taylor *et al*., 1999 and Lumyong *et al*., 2009). In addition, the colonization rate in studies on banana endophytes was reported to range from 29.6-67% (Photita *et al*., 2001). The colonization rate from *Amomumsiamense* reported by (Bussaban *et al*., 2001) varied from 70-83% and overall foliar colonization rate of *Centella asiatica* collected in Madagascar was 78% (Rakotoniriana *etal*., 2008).

Colonization rate between the range 50% to 99% for leaf tissues obtained from different plant communities, occupying tropical regions was reported by(Arnold *et al*., 2001; Cannon and Simmons, 2002; Raviraja, 2005; Arnold and Lutzoni, 2007; Krishnamurthy *et al*.,2008; Chaeprasert *et al*.,2010; Hilarino *et al*., 2011; Gond *et al*.,2012; Maheswari and Rajagopal, 2013 and Gautam, 2013) which is very much closer to the colonization rate obtained for the leaf tissues of *Boerhavia diffusa* L. in present study(Table1-2).

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 (Lodge *et al*.,1996; Raviraja, 2005; Chareprasert *et al*.,2006; Krishnamurthy *et al*.,2008; Vega *et al*.,2010; Gazis and Chaverri 2010; Kharwar *et al*.,2010; Chaeprasert *et al*.,2010; Thalavaipandian *et al*., 2011; Kharwar *et al*.,2011; Siqueira *et al*.,2011; Kharwar *etal*.,2012; He *et al*.,2012 and Gond *et al*.,2012).

In*Boerhavia diffusa* L. (Nyctaginaceae) 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 *Boerhavia diffusa* L. (Nyctaginaceae) 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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