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Characterization of endophytic fungi isolated from *Anaphalis contorta* (D. Don) Hook. f., a medicinal plant from Manipur

N. KISTU SINGH* AND M. SHYAMKESHO SINGH

Department of Botany, Manipur University, Canchipur-795003, Imphal, Manipur, India

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Endophytic fungi constitute an important part of microbial diversity. Endophytic fungi have unique genetic and biological systems to produce many bioactive compounds. In the present work, endophytic fungi were isolated from *Anaphalis contorta*, a medicinal plant locally used by the people of Manipur. Major plant portions viz., leaf, stem, root and inflorescence were used for isolation. A total of 40 different endophytic fungi were isolated of which 36 isolates produced spores which belongs to Sordariomycetes, Dothideomycetes, Eurotiomycetes and Mucoromycetes and 4 isolates were sterile. Identification was based on morphological characteristics. All the isolated fungi were tested for antagonistic activity and grouped under 5 antagonistic classes; antagonistic inhibition percentage were calculated for 11 isolates against pathogenic strains of *Curvularia lunata*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Aspegillus niger* and *Aspergillus flavus*. The isolates were also tested for the production of 5 extracellular enzymes. Number of isolates producing protease, lipase, amylase, cellulase and laccase were 35, 37, 39, 39, and 28 respectively. Phosphate solubilisation, ammonia production and HCN production were assessed qualitatively for plant growth promotion abilities. The present study reveals that endophytic fungi are abundantly harboured in all parts of the plant. The antagonistic activity suggests an important source of antimicrobial compounds as well as effective biocontrol agent. The production of extracellular enzymes and plant growth promotion activities shows a high potential for clinical microbiology, production industry and agriculture.

Key words: Endophytic fungi, bioactive compounds, *Anaphalis contorta*, antagonistic activity, extracellular enzymes.

INTRODUCTION

The term 'endophytic fungi' refers to group of fungi which lives within photosynthetic plant tissue by forming symbiotic relationship with host and has no harmful effect to the host plant (Rodriguez *et al*., 2009; Saithong *et al*., 2010; Shekhawat *et al*., 2010). They inhabit plant hosts for all or part of their life cycle. They colonized the internal plant tissues beneath the epidermal cell layers and lives within the intercellular spaces (Strobel, 2003). These endophytic fungi produce essential secondary metabolites to enhance the production of plant secondary metabolites which are a source of very important chemical constituents in plant defence as well as pharmaceutical industry (Arnold *et al*., 2003).

Many medicinal plants were screened for the presence of endophytic fungi from various parts for the purpose of bioprospecting (Khan *et al*., 2010; Naik *et al*., 2008; Strobel, 2002). Endophytic fungi protect their host against insects, pests, pathogens and even from herbivores (Mainowski & Belesky, 2006). These group of fungi were drawing a great attention after the discovery of fungi *Taxus brevifolia*, which produces the anti-cancer drug 'taxol' (Pandi *et al*., 2011). Plants growing in tropical or semitropical areas host a greater diversity of endophytes than those growing in dry or cold areas (Banerjee, 2011). In addition, fungal endophytes have been recognized as a repository of novel compounds of immense value in agriculture, industry and medicine (Tan & Zou, 2001; Strobel & Daisy, 2003; Kumar & Hyde, 2004). Medicinal plants and their endophytes are important resources for discovery of natural products (Schulz *et al*., 2002). *Anaphalis contorta* (D.Don) Hook.f. is a small annual herb of Asteraceae family having high therapeutic value and uses in the treatment of high blood pressure,

*Corresponding author: nkistusingh@gmail.com

intestinal disorder, cuts and injuries, etc. (Joshi, 2011; Khare, 2008).

Nowadays biological control is considered as safe option for plant disease management and sustainable agriculture (Dawson *et al*., 2002). Many endophytes have been evaluated for their biocontrol potential against various plant pathogens (Arnold *et al*., 2003; Holmes *et al*., 2004; Ting *et al*., 2010). Endophytic fungi produces extracellular enzyme as a resistance mechanism against pathogenic invasion and to obtain nutrition from host (Sunitha *et al*., 2013). Further, 60% of enzymes used in industrial processes is produced by few genera of fungi distributed worldwide (Suryanarayanan *et al*., 2003). Several endophytes have been examined for their ability to produce growth promoting metabolites similar to that produced by their host plants, but in higher quantities (Zhao *et al*., 2010). Recently, it has been reported that endophytic fungi can produce phytohormones, particularly gibberellins (GAs), that enhance crop growth and alleviate the harmful effects of abiotic stresses (Khan *et al*., 2011). In the present study endophytic fungi were isolated from leaf, stem, root and inflorescence of *Anaphalis contorta* collected from Imphal-West district of Manipur and assessed their diversity, antifungal activities, enzyme assay and plant growth promotion activities. This is the first report on endophytic fungi documented from this medicinal plant.

MATERIALS AND METHODS

Collection of Plant sample

Healthy and symptomless plants were collected from Thangmeiband, Imphal-West district of Manipur. The collection was done during the month of March. Different plant parts such as leaf, stem, root, and inflorescence were used for isolation. The plants were brought to the laboratory in sterilized bags and isolation was done within a few hours of sample collection.

Surface sterilization and Isolation

Endophyte isolation was performed following methods given by Hallmann *et al*. (2006) with few modifications. The plant samples were thoroughly washed in running tap water for about 30 minutes to remove all the surface adherents and soil particles before undergoing surface sterilization. Surface sterilization had been performed by transferring the plant parts through a series of sterilizing agents. First, the plant parts were washed in sterilized distilled water twice and then sequentially submerged to 70% ethanol for 2 minutes, 4% sodium hypochlorite (NaOCl) solution for 3 minutes, 70% ethanol for 30 seconds and again washed in sterilized distilled water thrice for 1 minute each to remove the chemicals from the plant materials. The plant materials were blot dried in the filter paper and cuts at a size of 0.5 cm to 1 cm and were aseptically transferred to PDA plates supplemented with Streptomycin sulphate ($500 mgL^{-1}$) to reduce bacterial growth. The plates were incubated at 28±1°C for 5 to 7 days and regularly check for fungal growth. The fungal hyphal tips that emerged from the plant bits were transferred to fresh PDA plates without antibiotics to make pure culture and incubated. The endophytic fungi were then stored to PDA slants at 4°C for further studies and were sub-cultured from time to time.

Identification

Colony morphology such as colour, texture, growth rate and fruiting bodies were used as important identifying characters. Microscopic observations were made by mounting 10-12 days old fungal cultures on lactophenol cotton blue stain at 25x and 40x magnification. Morphological identification was made by referring to taxonomic keys, monographs and papers relating to fungi identification (Barnett & Hunter, 1998; St-Germain & Summerbell, 2011; Watanabe, 2002). All the endophytic fungal isolates were deposited and acquired accession number from NFCCI, Agharkar Institute, Pune.

Data analysis

Data obtained from the isolation of endophytic fungi were used to calculate Colonizing Frequency Percentage, Endophytic Infection Rate (Suryanarayanan *et al*., 2003) and Relative Percentage Occurrence of different groups of endophytic fungi (Kumar & Hyde, 2004).

Colonizing frequency (%) of an endophyte species was calculated as per the following equation: Colonizing frequency (%) $= \frac{\text{Number of segments colonized by a single fungus}}{\text{Total number of segments observed}} \times 100$

Endophytic infection rate (%) was calculated as per the following equation:Endophytic infection rate (%) $= \frac{\text{Number of infected segments}}{\text{Total number of segments observed}} \times 100$

Relative percentage occurrence of each group is calculated as per the following equation: Relative percentage occurrence (RPO) $= \frac{\text{Density of colonization of one group}}{\text{Total density of colonization}} \times 100$

Diversity indices

Species diversity were calculated in terms of dominance, richness and evenness.

Simpson's dominance index (D), Simpson's diversity index (1-D),Species richness (S), Shannon–Wiener index (H) and Evenness(E) index were used to calculate the diversity (Jena & Tayung, 2013; Sharma *et al*., 2018; Verma *et al*., 2007).

Simpson's dominance index was calculated using the formula: $D = \frac{\sum n(n-1)}{N(N-1)}$; where, n= total number of organisms of a particular species; N= total number of organisms of all species

Simpson's diversity index was calculated using the formula: $1 - D = 1 - \frac{\sum n(n-1)}{N(N-1)}$

Species richness index was calculated by the total number of species found in each part: S = Number of species;

Shannon-Wiener index was calculated using the formula: H = -" (pi logpi); where, pi = Number of individuals of species i/ Total number of samples

Evenness was calculated using the formula: H/H_{max} ; where, H= Shannon-wiener index; H_{max} = ln (N); N= Number of species

Antagonistic activities

The endophytic fungal isolates were screened for antagonistic activity by in-vitro dual culture technique against a pathogenic strain of *Curvularia lunata*. Five days old 9 mm mycelial discs of both the pathogen and the fungal isolates were placed 6 cm apart on opposite sides of a 90 mm petridish, containing PDA medium. After 10 days of inoculation their degree of antagonism were scored on a scale of 1-5 (Bell *et al*., 1982). Class 1 = isolate completely overgrew the pathogen and covered the entire medium surface, class 2 = isolate overgrew at least two-thirds of the medium surface, class 3 = isolate and the pathogen colonized approximately one-half of the medium surface and neither organism appeared to dominate the other, class 4 = the pathogen colonized at least two-thirds of the medium surface and appeared to withstand encroachment by the isolate, and class 5 = the pathogen completely overgrew the isolate and occupied the entire medium surface.

Some of the isolates that showed good antagonistic activities were selected and calculated their Inhibition percentage (I%) against three fungal plant pathogens (*Curvularia lunata, Fusarium oxysporum* and *Rhizoctonia solani*) and two fungal human pathogens (*Aspergillus niger* and *Aspergillus flavus*) according to the formula (Hajieghrari *et al*., 2008): I% = [(r1–r2)/r1] × 100; r1 = mycelial radial growth of the pathogen on control plate, r2 = mycelial radial growth of the pathogen on dual culture plate. I% was calculated after 7 days of incubation on PDA medium at 28±1°C. All the assay was replicated three times.

Qualitative extracellular enzyme activities

Qualitative production of 5 extracellular enzymes, viz. protease, lipase, amylase, cellulase (Rajput *et al*., 2016) and laccase (Yadav *et al*., 2015) were assessed by the digestion of suspended or dissolved substrate in solid agar medium after incubation for 5-7 days at 28°C.

Protease activity

Protease assay was performed by growing the endophytic fungi on glucose yeast peptone agar (GYPA) media amended with 1% skim milk and the pH was adjusted to 6.5. After 5 days of incubation the clear zone appeared around the fungal colony indicated the presence of protease enzyme

Lipase activity

The fungi were grown on the peptone agar (PA) medium at pH 6 supplemented with Tween 20. A clear zone around the colony indicated the presence of lipase enzyme.

Amylase activity

Amylase activity was assessed by growing the fungi on glucose yeast extract peptone (GYP) agar at pH 6 containing 1% soluble starch. After 5 days incubation, the plates with fungal colony were flooded with a solution of 1% iodine and 2% potassium iodide. The appearance of clear zone surrounding the colony was considered positive for amylase enzyme.

Cellulase activity

To glucose yeast peptone (GYP) agar media, CMC (carboxy methyl cellulose) was added which acted as a substrate and endophytic fungi were inoculated. The plates were incubated at 28°C for 5 days. The plates were then flooded with 1% Congo red dye solution for 20 minutes and destained with NaCl solution for 15 minutes. Appearance of the light yellow area around the fungal colonies indicated the presence of cellulase enzyme.

Laccase activity

Glucose yeast peptone (GYP) agar medium amended with 1-napthol, 0.005% at pH 6 was prepared and fungi were inoculated and incubated for 6-8 days. On oxidation of 1-napthol by laccase, the medium change from clear to blue colour.

Plant Growth Promotion activities

Phosphate solubilisation assay

Fungal isolates were evaluated for their ability to solubilize inorganic phosphate in Pikovskaya's agar medium containing calcium phosphate. The petriplates were inoculated withisolated endophytes and incubated at 27°C for seven days. The presence of clear zone around the fungal colonies indicates the solubilisation of phosphate (Ripa *et al*., 2019).

Ammonia production assay

Fungal endophytes were tested for the production of ammonia in peptone water. Freshly grown fungal cultures were inoculated in 10 ml peptone water in each tube separately and incubated for 48-72 hrs at 27°C. Nessler's regent (0.5ml) was added in each tube. Development of brown to yellow colour indicates a positive test for ammonia production (Mahfooz *et al*., 2017).

HCN production assay

The isolates were inoculated in test tubes containing Bennett agar (1 g of yeast extract; 1 g of beef extract; 2 g of casein enzyme hydrolysate; 10 g of dextrose and 18 g of agar) amended with glycine (4.4 g l^{-1}). Whatman filter paper No. 1 flooded with the solution containing 0.5% picric acid in 2% sodium carbonate for a minute were then inserted inside the test tubes without touching the medium. The tubes were sealed with parafilm and incubated at 28 ± 1°C. Development of brown to red colour after incubation of 10 days indicated HCN production (Potshangbam *et al*., 2017).

RESULTS

Isolation of endophytic fungi

In the present study, 280 plant segments of *Anaphalis contorta* (70 leaf, 70 stem, 70 root and 70 inflorescence) were taken and processed for the isolation of endophytic fungi. A total of 40 different fungi were isolated (Fig.1).

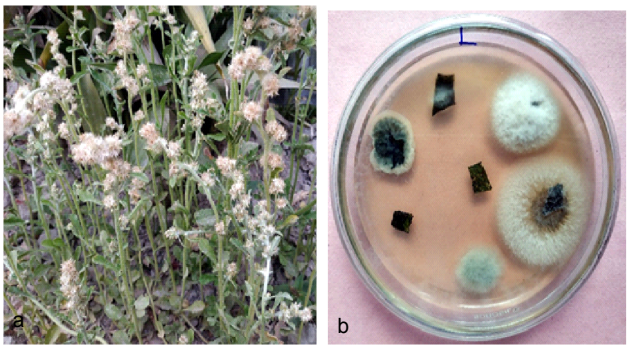

Fig 1: Photographs showing (a) Host plant; (b) Emergence of fungal hyphae from plant segments after inoculation on PDA plate.

Identification of endophytic fungi

The isolated endophytes were identified based on their morphology and group under 16 genera, 13 families and 4 classes (Dothideomycetes, Mucoromycetes, Eurotiomycetes, Sordaromycetes) along with 4 morphotypes of Sterile mycelia. *Penicillium*, *Trichoderma*, *Fusarium*, *Colletotrichum* and *Cladosporium* were the most frequently isolated genera (Table 1, Fig.2)).

Table 1: Identification of endophytic fungi isolated from *Anaphalis contorta*

Isolate code	Endophytic fungi	NFCCI Accession No.	Family	Order
F5c32	*Alternaria alternate*	NFCCI 4795	Pleosporaceae	Dothideomycetes
L6f13	*Alternaria* sp.	NFCCI 4724	Pleosporaceae	Dothideomycetes
S1a7	*Arthrinium* sp. 1	NFCCI 4726	Apiosporaceae	Sordariomycetes
L1g1	*Arthrinium* sp. 2	NFCCI 4769	Apiosporaceae	Sordariomycetes
F3a10	*Chaetomium* sp.	NFCCI 4797	Chaetomiaceae	Sordariomycetes
S3a5	*Cladosporium* sp. 1	NFCCI 4765	Cladosporiaceae	Dothideomycetes
S5b17	*Cladosporium* sp. 2	NFCCI 4764	Cladosporiaceae	Dothideomycetes
F2a9	*Cladosporium* sp. 3	NFCCI 4727	Cladosporiaceae	Dothideomycetes
S7a25	*Cladosporium* sp. 4	NFCCI 4732	Cladosporiaceae	Dothideomycetes
S6a18	*Cladosporium* sp. 5	NFCCI 4760	Cladosporiaceae	Dothideomycetes
F1a8	*Cladosporium* sp. 6	NFCCI 4733	Cladosporiaceae	Dothideomycetes
S5c7	*Cladosporium* sp. 7	NFCCI 4734	Cladosporiaceae	Dothideomycetes
S2b4	*Cladosporium* sp. 8	NFCCI 4758	Cladosporiaceae	Dothideomycetes
L2f2	*Colletotrichum* sp. 1	NFCCI 4728	Glomerellaceae	Sordariomycetes
L3a3	*Colletotrichum* sp. 2	NFCCI 4729	Glomerellaceae	Sordariomycetes
L1d1	*Colletotrichum* sp. 3	NFCCI 4761	Glomerellaceae	Sordariomycetes
L3i3	*Curvularia* sp.	NFCCI 4766	Pleosporaceae	Dothideomycetes
F5d32	*Epicoccum nigrum*	*	Pleosporaceae	Dothideomycetes
R6b15	*Fusarium* sp. 1	NFCCI 4767	Nectriaceae	Sordariomycetes
R8b21	*Fusarium* sp. 2	NFCCI 4730	Nectriaceae	Sordariomycetes
R7a20	*Fusarium* sp. 3	NFCCI 4759	Nectriaceae	Sordariomycetes
L6UN	*Gliocladium* sp.	NFCCI 4768	Hypocreaceae	Sordariomycetes
R1a4	*Mucor* sp.	NFCCI 4771	Mucoraceae	Zygomycetes
R9d28	*Myrothecium indicum*	NFCCI 4798	Stachybotryaceae	Sordariomycetes
S8a26	*Neocosmospora solani*	*	Nectriaceae	Sordariomycetes
F6a19	*Penicillium* sp. 1	NFCCI 4770	Aspergillaceae	Eurotiomycetes
L7c23	*Penicillium* sp. 2	NFCCI 4735	Aspergillaceae	Eurotiomycetes
F11c38	*Penicillium* sp. 3	NFCCI 4763	Aspergillaceae	Eurotiomycetes
R12a31	*Penicillium* sp. 4	NFCCI 4793	Aspergillaceae	Eurotiomycetes
R10a29	*Phoma* sp. 1	NFCCI 4794	Didymellaceae	Dothideomycetes
R10c29	*Phoma* sp. 2	NFCCI 4796	Didymellaceae	Dothideomycetes
L2d2	*Trichoderma* sp. 1	NFCCI 4725	Hypocreaceae	Sordariomycetes
L2e2	*Trichoderma* sp. 2	NFCCI 4723	Hypocreaceae	Sordariomycetes
F4a22	*Trichoderma* sp. 3	NFCCI 4763	Hypocreaceae	Sordariomycetes
S9a27	*Trichoderma* sp. 4	NFCCI 4762	Hypocreaceae	Sordariomycetes
F5b32	*Verticillium* sp.	*	Plectosphaerellaceae	Sordariomycetes
L5f12	Sterile mycelia Morphotype 1	**		
L5h12	Sterile mycelia Morphotype 2	**		
L7b23	Sterile mycelia Morphotype 3	**		
L7g23	Sterile mycelia Morphotype 4	**		

* Applied for accession no.

** No accession no. for sterile form

Data analysis

The Colonization Frequency Percentage (CF%) vary with plant parts. Both leaf and stem parts was shown maximum colonization by *Colletotrichum* sp.2 (15.71%, 11.43% respectively), root by *Penicillium* sp.2 (12.86%) and inflorescence by *Trichoderma* sp.3 (5.71%) (Table 2, Fig.3).

The endophytic fungi were harboured mostly in leaves with Endophytic Infection Rate Percentage (EIR%) of 92.86% and least is found in inflorescence with 41.43% (Table 3). Some endophytic fungi are found to occur in almost all parts and some show tissue specificity and confined to only certain plant part.

The Relative Percentage Occurrence (RPO) of endophytic fungi in *Anaphalis contorta* is dominated by Sordariomycetes (49.47%) followed by Dothidiomycetes (21.58%), Sterile mycelia (14.21%), Euromycetes (11.58%) and Mucoromycetes (3.16%) (Fig.4).

Diversity indices

Simpson's dominance index is shown maximum in root (0.117); leaf, stem and inflorescence are comparatively lower. Simpson's diversity index were high in inflorescence (0.916), leaf (0.915), stem (0.911) and low in root (0.883). Shannon-Wiener index was highest in leaf (2.622) and lowest in root

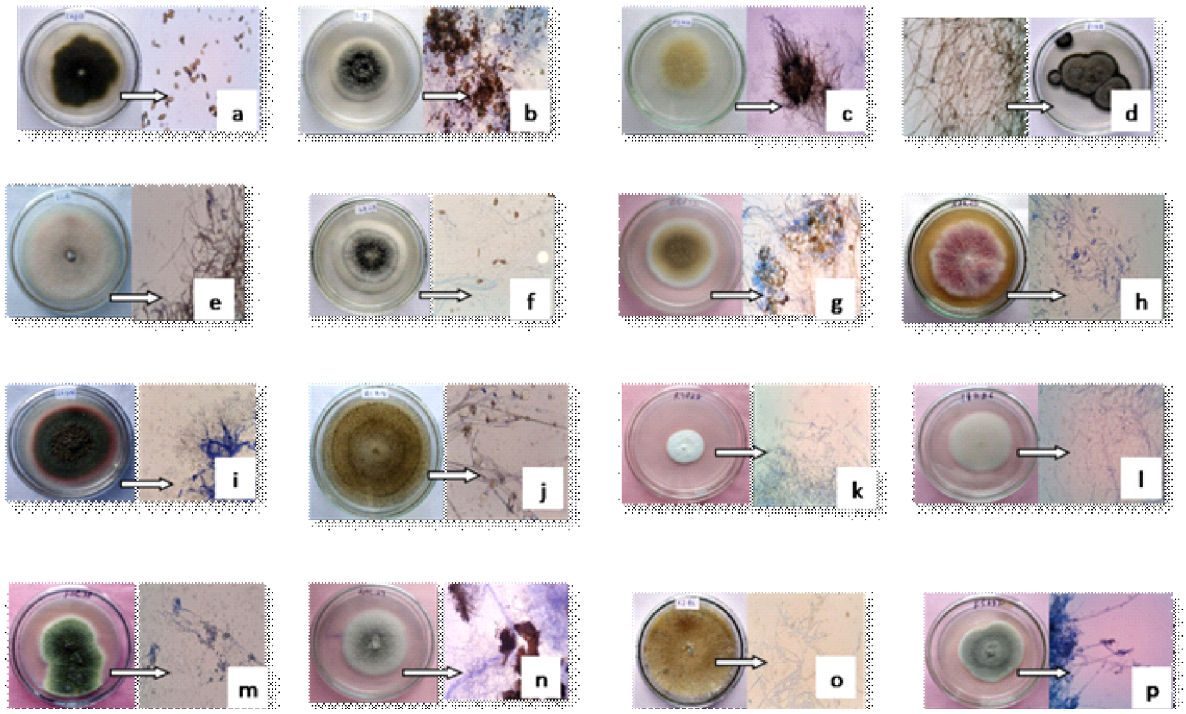

Fig.2 Morphological identification of 16 genera of endophytic fungi isolated from *Anaphalis contorta*
a. . *Alternaria* sp; b. *Arthrinium* sp. 2; c.*Chaetomium* sp.;d. *Cladosporium* sp.6; e.*Colletotrichum* sp.3; f. *Curvularia* sp.;g.*Epicoccum nigrum*; h.*Fusarium* sp.3; i. *Gliocladium* sp.j. *Mucor* sp.; k. *Myrothecium indicum*; l.*Neocosmospora solani*; m. *Penicillium* sp.3; n. *Phoma* sp.1; o. *Trichoderma* sp.1; p. *Verticillium* sp.

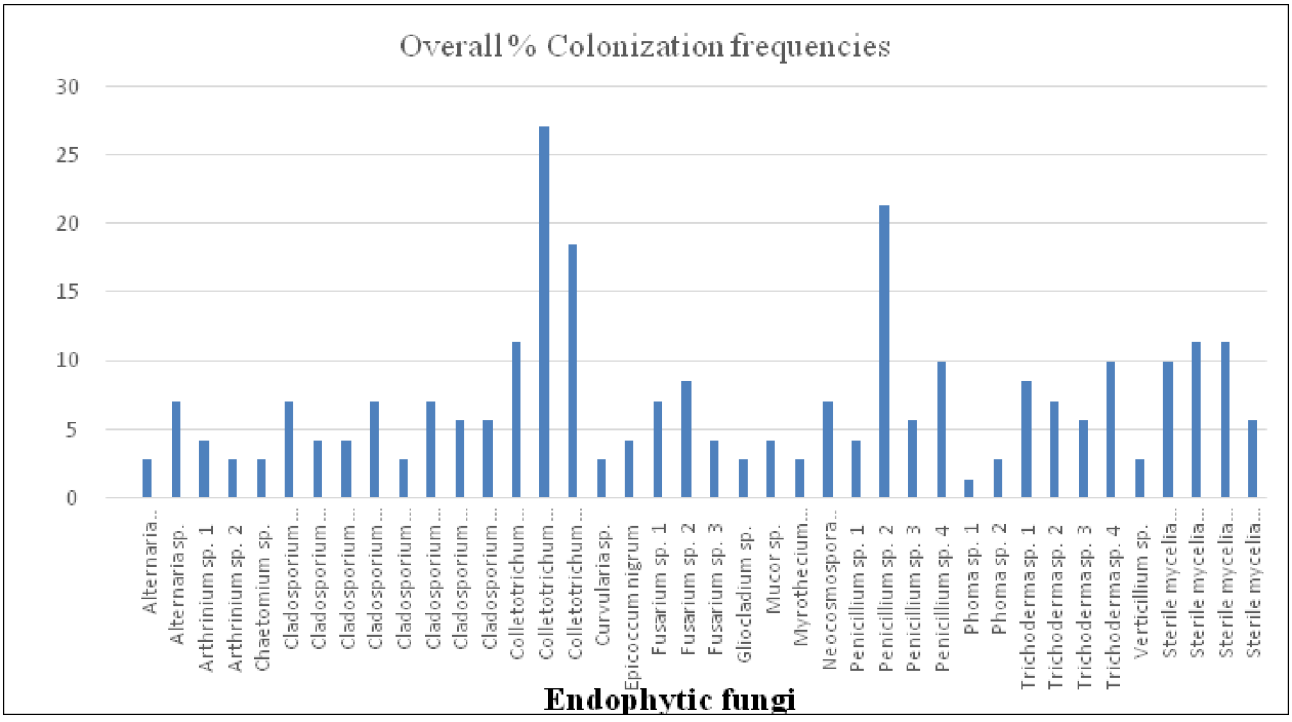

Fig 3: Percentage Colonization frequency of endophytic fungi isolated from *Anaphalis contorta*.

Table 2: Percentage Colonization Frequency of endophytic fungi isolated from *Anaphalis contorta*

Endophytic fungi	% Colonization frequencies			
	Leaf	Stem	Root	Inflorescence
Alternaria alternate	0.00	0.00	0.00	2.86
Alternaria sp.	5.71	0.00	0.00	1.43
Arthrinium sp. 1	0.00	4.29	0.00	0.00
Arthrinium sp. 2	2.86	0.00	0.00	0.00
Chaetomium sp.	0.00	0.00	0.00	2.86
Cladosporium sp. 1	0.00	7.14	0.00	0.00
Cladosporium sp. 2	0.00	4.29	0.00	0.00
Cladosporium sp. 3	0.00	0.00	0.00	4.29
Cladosporium sp. 4	2.86	4.29	0.00	0.00
Cladosporium sp. 5	0.00	2.86	0.00	0.00
Cladosporium sp. 6	0.00	4.29	0.00	2.86
Cladosporium sp. 7	0.00	5.71	0.00	0.00
Cladosporium sp. 8	1.43	4.29	0.00	0.00
Colletotrichum sp. 1	11.43	0.00	0.00	0.00
Colletotrichum sp. 2	15.71	11.43	0.00	0.00
Colletotrichum sp. 3	10.00	7.14	1.43	0.00
Curvularia sp.	2.86	0.00	0.00	0.00
Epicoccum nigrum	0.00	0.00	0.00	4.29
Fusarium sp. 1	0.00	0.00	7.14	0.00
Fusarium sp. 2	0.00	0.00	5.71	2.86
Fusarium sp. 3	0.00	0.00	4.29	0.00
Gliocladium sp.	2.86	0.00	0.00	0.00
Mucor sp.	0.00	0.00	0.00	4.29
Myrothecium indicum	0.00	0.00	2.86	0.00
Neocosmospora solani	0.00	5.71	1.43	0.00
Penicillium sp. 1	0.00	0.00	0.00	4.29
Penicillium sp. 2	4.29	0.00	12.86	0.00
Penicillium sp. 3	1.43	0.00	4.29	0.00
Penicillium sp. 4	0.00	0.00	4.29	5.71
Phoma sp. 1	0.00	0.00	1.43	0.00
Phoma sp. 2	0.00	0.00	2.86	0.00
Trichoderma sp. 1	7.14	0.00	0.00	1.43
Trichoderma sp. 2	5.71	0.00	0.00	1.43
Trichoderma sp. 3	0.00	0.00	0.00	5.71
Trichoderma sp. 4	0.00	10.00	0.00	0.00
Verticillium sp.	0.00	0.00	0.00	2.86
Sterile mycelia Morphotype 1	10.00	0.00	0.00	0.00
Sterile mycelia Morphotype 2	11.43	0.00	0.00	0.00
Sterile mycelia Morphotype 3	2.86	7.14	1.43	0.00
Sterile mycelia Morphotype 4	5.71	0.00	0.00	0.00

Table 3: Endophytic Infection Rate % of different plant parts of *Anaphalis contorta*

Plant parts	Endophytic Infection Rate %
Leaf	92.86
Stem	70.00
Root	57.14
Flower	41.43

(2.329). Species richness is maximum in leaf (17). Evenness is highest in stem (0.972) and lowest in root (0.908)(Table 4) .

Dual-culture antagonistic activity

Preliminary dual-culture assay of the isolates against *Curvularia lunata* revealed that 23 isolates (5 Class I, 4 Class II & 14 Class III) out of total 40 isolates possessed good antagonistic characteristics (Fig.5). Further evaluating Inhibition percentage (I%) of 11 endophytic fungal isolates against pathogenic strains of *Curvularia lunata*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Aspergillus niger* and *Aspergillus flavus*, it was found that maximum inhibition for *C. lunata* was shown by *Trichoderma* sp.4 (100±0.000%) and minimum by Sterile mycelia Morphotype 2 (49.39±0.075%); maximum inhibition of *F. oxysporum* was shown by *Mucor* sp. (71.48±0.095%) and minimum by *Penicillium* sp.2 (30.00±0.028%); maximum inhibition for *R. solani* was shown by *Trichoderma* sp.2 (75.56±0.044%) and minimum by Sterile mycelia Morphotype 2 (40.89±0.120%); maximum inhibition of *A. niger* was shown by *Trichoderma* sp.2 (92.65±0.074%) and minimum by *Mucor* sp. (58.58±0.017%); and

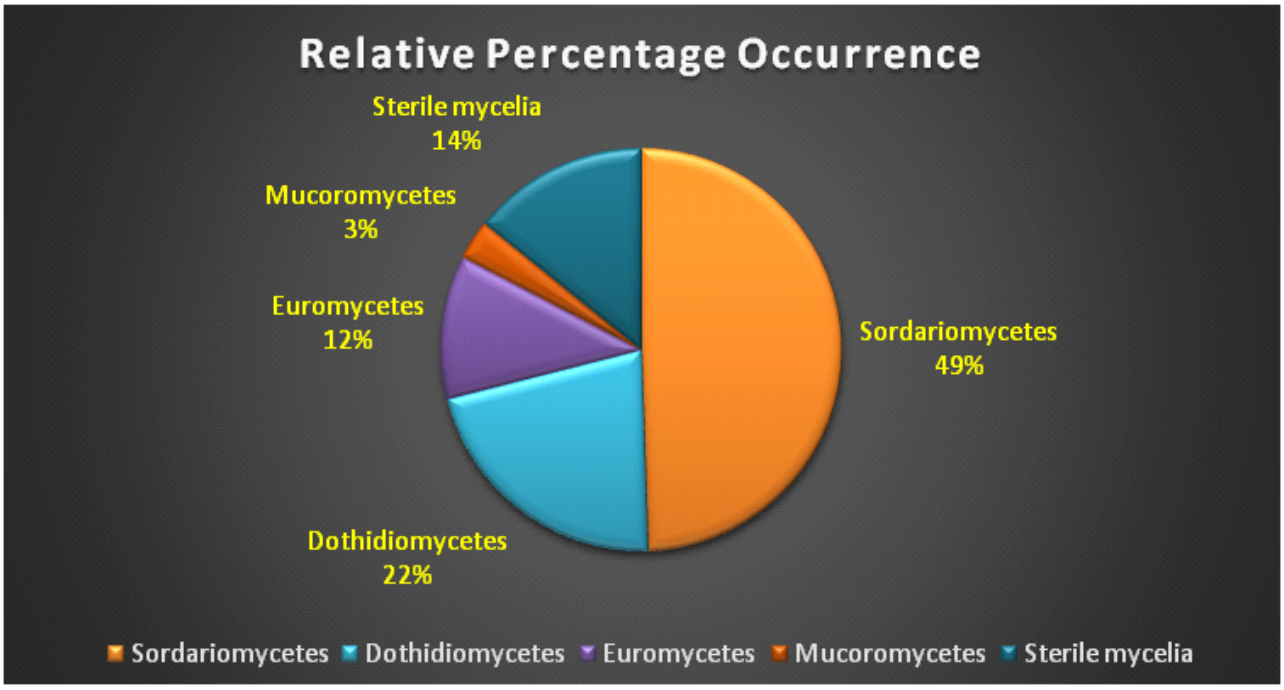

Fig 4: Relative Percentage Occurrence of the endophytic fungal groups isolated from *Anaphalis contorta*.

Table 4: Diversity indices of endophytic fungi associated with leaf, stem, root, and inflorescence of *Anaphalis contorta*

Diversity indices	Leaf	Stem	Root	Inflorescence
Simpson's dominance index (D)	0.085	0.089	0.117	0.084
Simpson's diversity index (1-D)	0.915	0.911	0.883	0.916
Species richness (S)	17	13	13	14
Shannon-Wiener index (H)	2.622	2.492	2.329	2.551
Evenness (E)	0.925	0.972	0.908	0.967

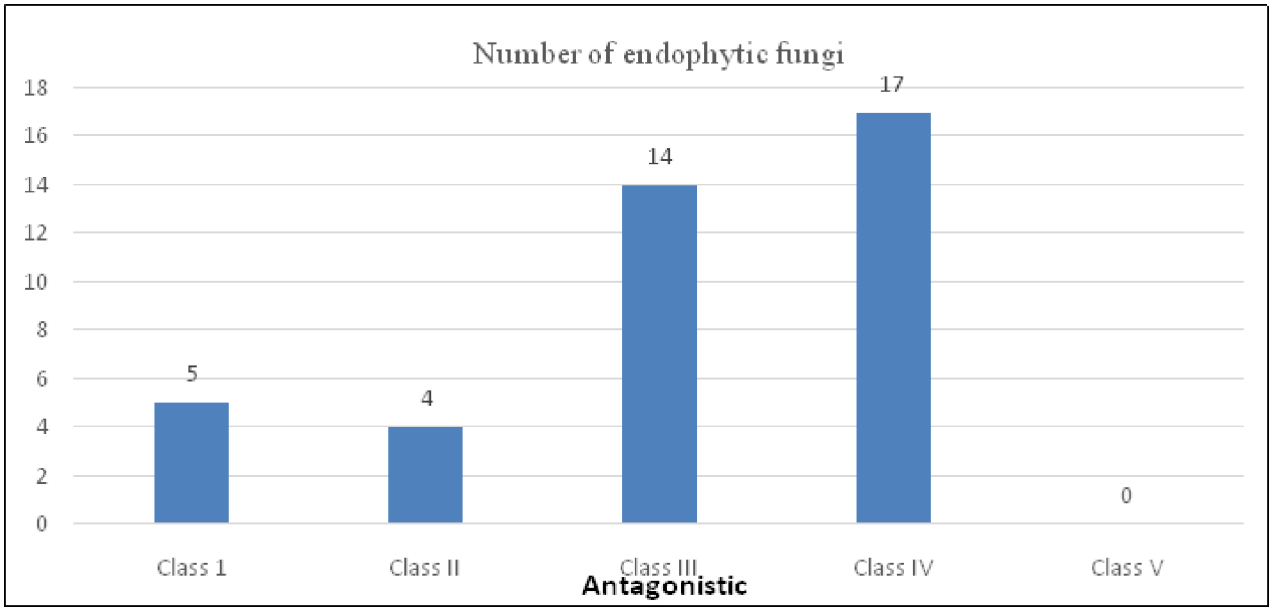

Fig.5 Antagonistic classes shown by the isolated endophytic fungi against *Curvularia lunata* by dual culture method.

Table 5: Evaluation of Inhibition percentage (I %) of endophytic fungi against 5 fungal pathogens

Endophytic fungi	Pathogenic fungi				
	Curvularia lunata	*Fusarium oxysporum*	*Rhizoctonia solani*	*Aspergillus niger*	*Aspergillus flavus*
Mucor sp.	77.52±0.066	71.48±0.095	51.11±0.054	58.58±0.017	44.09±0.141
Trichoderma sp. 2	78.79±0.059	51.85±0.052	75.56±0.044	92.65±0.074	61.29±0.206
Fusarium sp. 1	50.18±0.045	33.33±0.029	48.89±0.110	76.47±0.039	40.32±0.249
Colletotrichum sp. 3	50.00±0.130	34.44±0.029	45.11±0.057	63.98±0.055	11.29±0.449
Arthrinium sp. 1	71.21±0.222	41.48±0.018	44.67±0.099	63.48±0.018	5.38±0.898
Trichoderma sp. 3	75.15±0.061	59.63±0.076	48.44±0.055	63.48±0.072	56.45±0.218
Fusarium sp. 2	58.48±0.179	44.81±0.018	45.11±0.057	63.72±0.018	6.45±0.635
Penicillium sp. 2	79.52±0.132	30.00±0.028	70±0.080	66.91±0.034	66.67±0.113
Trichoderma sp. 4	100±0.000	70.37±0.025	62±0.085	76.96±0.062	52.69±0.315
Trichoderma sp. 1	77.27±0.103	54.07±0.072	43.78±0.058	76.47±0.039	61.83±0.117
Sterile mycelia morphotype 2	49.39±0.075	33.70±0.044	40.89±0.120	80.15±0.042	39.78±0.299

Data are means of percentage of growth inhibition (triplicate) ± standard deviation (SD)

maximum inhibition of *A. flavus* was shown by *Penicillium* sp.2 (66.67±0.113%) and minimum by *Arthrinium* sp.1 (5.38±0.898%). The genus *Trichoderma* have shown strong antagonist activity (Table 5, Fig.6).

Extracellular enzyme activity

Most of the isolated endophytic fungi produced industrially important enzymes. Protease production activity on agar medium were shown by 35 isolates, lipase activity by 37 isolates, amylase by 39 isolates, cellulase by 39 isolates and laccase by 28 isolates (Table 6).

Plant growth promotion activity

Out of the 40 isolates screened for plant growth promotion abilities, only 2 isolates show phosphate utilization activity; 37 isolates produced ammonia and 20 isolates produced HCN (Table 7).

DISCUSSION

Endophytic fungi constitute the most unexplored and diverse group of organisms (Khan *et al*., 2007). They reside asymptomatically in internal tissues of all higher plants and are store house of biologically active compounds (Selim *et al*., 2012). However only a few plants have been studied for their endophytic diversity and their potential to produce bioactive compounds (Strobel and Daisy, 2003). Endophytes exist in a complex interaction with their host plants and isolating them from their native habitat might affect their metabolic capabilities. Plants havingethnobotanical importance will provide the best opportunities to isolate endophytic fungi (Prabukumar *et al*., 2015). As tropical and subtropical climates harbour most of the world's plant diversity, endophytic diversity in this climatic zone is also higher (Rajendran, 2016).

The surface sterilization of plant tissues is the most crucial step in the isolation process and aims to eliminate the external microorganisms, maintaining only the internal endophytic communities of the host plant. It has been established that pathogenic-endophytic lifestyles are interchangeable. So, a fungus that is pathogenic in one ecological niche can be endophyte in another ecosystem (Chowdhary & Kaushik, 2015). The application of the fresh leaves of *A. contorta* as an antiseptic to cuts/wounds could be attributed to their antimicrobial activity (Joshi, 2011).

From 280 segments of plant material a total of 40 different isolates were obtained. As different endophytic fungi were found to occur in all plant parts, the host plant is found to be rich in diversity. The infection rate was maximum in leaf segments (81.40%) and minimum in inflorescence (28.57%), this might be due to presence of more photosynthetic tissues in leaves. The dominant

Table 6: Qualitative extracellular enzyme activity of endophytic fungi on petriplate

Isolate code	Endophytic fungi	Enzyme activities				
		Protease	Lipase	Amylase	Cellulase	Laccase
F5c32	*Alternaria alternate*	+	+	+	+	+
L6f13	*Alternaria* sp.	+	+	+	+	+
S1a7	*Arthrinium* sp. 1	+	+	+	+	-
L1g1	*Arthrinium* sp. 2	+	+	+	+	-
F3a10	*Chaetomium* sp.	-	+	+	+	+
S3a5	*Cladosporium* sp. 1	+	+	+	+	+
S5b17	*Cladosporium* sp. 2	+	+	+	+	+
F2a9	*Cladosporium* sp. 3	+	+	+	-	+
S7a25	*Cladosporium* sp. 4	+	+	+	+	+
S6a18	*Cladosporium* sp. 5	+	+	+	+	-
F1a8	*Cladosporium* sp. 6	+	+	+	+	+
S5c7	*Cladosporium* sp. 7	+	+	+	+	+
S2b4	*Cladosporium* sp. 8	+	+	+	+	+
L2f2	*Colletotrichum* sp. 1	+	+	-	+	+
L3a3	*Colletotrichum* sp. 2	+	+	+	+	+
L1d1	*Colletotrichum* sp. 3	+	+	+	+	+
L3i3	*Curvularia* sp.	+	+	+	+	+
F5d32	*Epicoccum nigrum*	+	+	+	+	+
R6b15	*Fusarium* sp. 1	+	+	+	+	+
R8b21	*Fusarium* sp. 2	-	+	+	+	+
R7a20	*Fusarium* sp. 3	+	+	+	+	+
L6UN	*Gliocladium* sp.	+	+	+	+	-
R1a4	*Mucor* sp.	+	+	+	+	-
R9d28	*Myrothecium indicum*	-	+	+	+	-
S8a26	*Neocosmospora solani*	+	+	+	+	+
F6a19	*Penicillium* sp. 1	+	+	+	+	+
L7c23	*Penicillium* sp. 2	+	-	+	+	-
F11c38	*Penicillium* sp. 3	+	-	+	+	-
R12a31	*Penicillium* sp. 4	+	+	+	+	-
R10a29	*Phoma* sp. 1	+	+	+	+	-
R10c29	*Phoma* sp. 2	+	+	+	+	+
L2d2	*Trichoderma* sp. 1	-	-	+	+	-
L2e2	*Trichoderma* sp. 2	+	+	+	+	+
F4a22	*Trichoderma* sp. 3	+	+	+	+	+
S9a27	*Trichoderma* sp. 4	-	+	+	+	-
F5b32	*Verticillium* sp.	+	+	+	+	+
L5f12	Sterile mycelia Morphotype 1	+	+	+	+	+
L5h12	Sterile mycelia Morphotype 2	+	+	+	+	+
L7b23	Sterile mycelia Morphotype 3	+	+	+	+	+
L7g23	Sterile mycelia Morphotype 4	+	+	+	+	+

+ indicates presence of enzyme; - indicates absence of enzyme

endophytic fungi of the host plant was found to be *Colletotrichum* sp. 2 having overall CF% of 27.14%. Several species of *Colletotrichum* causes various leaf spot disease, fruit rot disease and anthracnose disease in many plants but it shows beneficial effect in this host plant. None of the *Colletotrichum* spp., *Fusarium* spp., and *Trichoderma* spp. were found in inflorescence, stem and root segments respectively. *Mucor* sp. was confined to root; *Curvularia* sp. and sterile form were found only in leaf. Only two fungi were common in leaf, stem and root tissues, i.e. *Colletotrichum* sp. 3 and Sterile mycelia Morphotype 3. This provides a clear evidence for tissue specificity of endophytic fungi. Few fungal taxa that had been less frequently isolated were *Curvularia*, *Arthrinium*, *Verticillium*, *Epicoccum*, *Phoma*. All the fungal endophytes reported from *A. contorta* in the present study belong to Ascomycota except *Mucor* sp. (Zygomycota). Ascomycota is the largest and one of the most diverse phylum of fungi (Schoch *et al*., 2009).

Simpson's diversity index value range between 0 and 1, where an increase in index value equates to an increase in species diversity. Highest value of Simpson's diversity was found in inflorescence tissue (0.916). Leaf segments show higher values in both Species richness and Shannon-Wiener index, so we can conclude that leaf tissues have higher endophytic diversity than other plant tissues. In our study 23 isolates show prominant

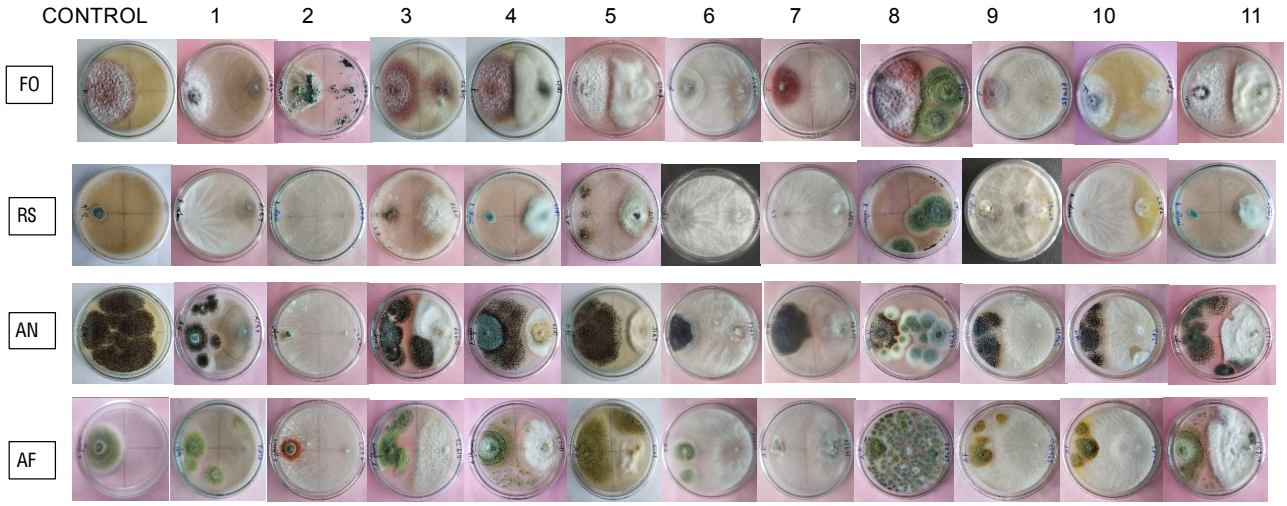

Fig 6: Antagonistic activity of eleven fungal endophytes (1-11) against *Curvularia lunata* (CL), *Fusarium oxysporum* (FO), *Rhizoctonia solani* (RI), *Aspergillus niger* (AN) and *Aspergillus flavus* (AF) using petriplate dual culture method.
Fungal pathogen inoculated on left side; Endophytic fungi inoculated on right side
Endophytic fungi (1=*Mucor* sp.; 2=*Trichoderma* sp.2; 3=*Fusarium* sp.1; 4=*Colletotrichum* sp.3; 5=*Arthrinium* sp.1; 6=*Trichoderma* sp.3; 7=*Fusarium* sp.2; 8=*Penicillium* sp.2; 9=*Trichoderma* sp.4; 10=*Trichoderma* sp.1; 11=Sterile mycelia Morphotype 2).

antagonistic activity. Three fungal plant pathogens, *Curvularia lunata*, *Fusarium oxysporum* and *Rhizoctonia solani* were collected from Central Agricultural University, Imphal and two fungal human pathogen *Aspergillus niger* and *Aspergillus flavus* were collected from Jawaharlal Nehru Institute of Medical Sciences, Imphal. The antagonistic inhibition percentage of eleven endophytic fungi were higher against *Curvularia lunata* and *Aspergillus niger*. The genus *Trichoderma* was found to be the most important biocontrol agent. The biocontrol potential of *Trichoderma* species had already been well documented (Amin & Razdan, 2010; Herrera-Estrella and Chet, 2003; Schuster & Schmoll, 2010). Their antagonistic activity was due to the production of anti-fungal compounds and lytic enzymes. The plant pathogens caused serious loss on vegetables and cereals and human pathogens caused allergic reactions, lung infections, and infections in other organs. It can be concluded that fungal endophytes harboured inside *Anaphalis contorta* hold great promise not only as biocontrol agents but also as sustainable resource of novel antifungal secondary metabolites. No record of fungal disease of *A. contorta* has been recorded. This suggests that either plant's defence system is quite strong or its endophytes manage to control the infection of plant pathogen.

Endophytic fungi from *A. contorta* produced protease, lipase, amylase, cellulase and laccase enzyme by agar-plate technique. Enzyme production facilitates the penetration of endophytes in the host plant and is also involved in competition with other pathogenic microorganism (Fouda *et al*., 2015; Ribeiro *et al*., 2011). Their enzyme production ability might influence litter decomposition, nutrient cycling and nutrient uptake by host (Pragathi *et al*., 2013) Protease has diverse application in wide variety of industries like biscuit manufacturing, brewing industries, photographic industries. Proteases are also used in clinical applications especially in treatments like Diabetes. As protease is found to be biotechnologically important enzyme, 35 isolates of endophytic fungi can be industrially exploited from the host plant. The use of skim-milk agar plates was found to be an easy and rapid technique to screen for qualitative protease production. Lipases are a class of enzymes, which catalyze the hydrolysis of long chain triglycerides. Microbial lipases are produced by fungal, yeast, and bacterial species. Industrial lipases application covers various industries such as oleo-chemicals, detergents, polymers, food processing, pharmaceutical, waste, cosmetics and biodiesel (Guerrand, 2017). Amylase enzyme have a wide variety of industrial applications, such as in the food, fermentation, textile, paper, detergent, pharmaceutical, and sugar industries. Amylases are derived from several sources, including plants, animals, and microorganisms. Enzymes from microbial sources are generally more suited to

Table 7: Qualitative extracellular enzyme activity of endophytic fungi on petriplate

Isolate code	Endophytic fungi	Plant growth promotion activities		
		Phosphate solubilisation	Ammonia production	HCN production
F5c32	*Alternaria alternate*	-	+	-
L6f13	*Alternaria* sp.	-	+	+
S1a7	*Arthrinium* sp. 1	-	-	-
L1g1	*Arthrinium* sp. 2	-	+	-
F3a10	*Chaetomium* sp.	-	+	-
S3a5	*Cladosporium* sp. 1	-	+	-
S5b17	*Cladosporium* sp. 2	-	+	-
F2a9	*Cladosporium* sp. 3	-	+	-
S7a25	*Cladosporium* sp. 4	-	+	+
S6a18	*Cladosporium* sp. 5	-	+	+
F1a8	*Cladosporium* sp. 6	-	+	-
S5c7	*Cladosporium* sp. 7	-	+	-
S2b4	*Cladosporium* sp. 8	-	+	+
L2f2	*Colletotrichum* sp. 1	-	+	+
L3a3	*Colletotrichum* sp. 2	-	+	+
L1d1	*Colletotrichum* sp. 3	-	+	-
L3i3	*Curvularia* sp.	-	+	-
F5d32	*Epicoccum nigrum*	-	+	-
R6b15	*Fusarium* sp. 1	-	-	+
R8b21	*Fusarium* sp. 2	-	+	+
R7a20	*Fusarium* sp. 3	-	+	-
L6UN	*Gliocladium* sp.	-	+	+
R1a4	*Mucor* sp.	-	+	-
R9d28	*Myrothecium indicum*	-	+	-
S8a26	*Neocosmospora solani*	+	+	-
F6a19	*Penicillium* sp. 1	-	+	+
L7c23	*Penicillium* sp. 2	-	+	+
F11c38	*Penicillium* sp. 3	-	+	+
R12a31	*Penicillium* sp. 4	-	+	-
R10a29	*Phoma* sp. 1	-	+	+
R10c29	*Phoma* sp. 2	-	+	+
L2d2	*Trichoderma* sp. 1	-	+	-
L2e2	*Trichoderma* sp. 2	-	+	+
F4a22	*Trichoderma* sp. 3	-	-	+
S9a27	*Trichoderma* sp. 4	-	+	+
F5b32	*Verticillium* sp.	+	+	+
L5f12	Sterile mycelia Morphotype 1	-	+	+
L5h12	Sterile mycelia Morphotype 2	-	+	-
L7b23	Sterile mycelia Morphotype 3	-	+	+
L7g23	Sterile mycelia Morphotype 4	-	+	-

+ indicates presence of enzyme; - indicates absence of enzyme

industrial demands, being cheaper to produce, controllable, and reliable. Thirty-nine isolates produce amylase enzyme. Cellulase is the third most industrially significant enzyme worldwide, due to their applications in cotton processing, paper recycling, juice extraction, enzymatic detergents, and animal food additives (Sajith *et al*., 2016). Laccase enzyme can oxidize phenolic and non-phenolic aromatic compounds, which increases interest in various industrial applications, including food, pulping, textile, wastewater treatment, and bioremediation (Couto and Toca-Herrera, 2007). Laccase can also be used in analytical applications including biosensors, enzymatic, and immunochemical assays (Madhavi and Lele, 2009). Each isolate was able to produce one or more extracellular enzymes. Twenty-four isolates were able to produce all five enzymes. The potential endophytic fungi are being investigated for quantitatively extracellular enzyme production in liquid media.

Promotion of plant growth is the most important characteristic of fungal symbiosis (Hassan, 2017). Microbial phosphate solubilization is one of the fundamental processes that contributes to the plant growth. Phosphate solubilising microorganisms render insoluble phosphate into soluble form

through processes such as acidification, chelation, and exchange reactions in the soil environment by the secretion of organic acids which is mediated through lowering of pH (Vassilev *et al*., 2006). Phosphorus deficiencies are wide spread on soil throughout the world and one of the limiting factors for crop productivity. Phosphorus fertilizers represent major cost for agricultural production. Solubilization of insoluble phosphorus by microorganisms was reported by Pikovskaya (Sharma *et al*., 2013). Almost all the isolates produce ammonia. Ammonia is the source of Nitrogen in plants. However, at higher pH a larger proportion of the ammonium becomes toxic unionized ammonia. Ammonia is very toxic because it is lipid soluble and raises intracellular pH, thus inhibiting protein synthesis and enzyme activity (Doyle & Butler, 1990). Nitrogen is vital to plant growth, being required in the synthesis of chlorophyll, proteins, enzymes, DNA and RNA. Ammonia production can help satisfy the nitrogen demand of the host plant and in excess reduces the colonization of plants by pathogens. HCN production has been postulated to play an important role in the biological control of pathogens (Reeth *et al*., 2014). HCN is a volatile product that exhibits antifungal action (Ngoma *et al*., 2013).

There is a need of further in-depth studies of these isolated endophytes. By growing these endophytes on large scale, modifying culture conditions and supplying some stimulants might help in getting better production of particular bioactive and antimicrobial compounds of pharmaceutical importance. Another factor that encourages endophyte study is the importance of the organic approach in agricultural food crops which will replace the chemicals that have direct or indirect impact on the environment and human health. Therefore, the used of microbial resources as biocontrol agents for controlling diseases, growth promotion and increased yield are an alternative need. It can be concluded that results obtained from the present study encourages us to further investigate on the selected fungal endophytes in order to develop a strong biological agent which have wide applicability to various field and also towards organic food crop production.

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