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Diversity and Molecular Characterization of Endophytic Fungi Associated with Leaves of *Acacia nilotica*

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Fungal endophytes are colonized in different part of the plants and play important role in survival of plants in stressful habitat. In search of potential endophytic fungi to produce bioactive metabolites in this study we investigate the diversity of endophytic fungi associated with leaves of the *Acacia nilotica* plant. Twenty-six endophytic fungi were subjected to morphological and molecular identification with internal transcribes spacer (ITS) region sequenced. All 26 endophytic fungi were divided into nine genera *Chaetomium, Amesia, Ovatospora, Penicillium, Phialemonium, Colletotrichum, Crinipellis, Acrophialophora, Cribbea.* Most of them belonged to the phylum Ascomycota only one belonging to the phylum Basidiomycota. This study shows that Acacia leaves inhabitant by diverse group of endophytic fungi. The biodiversity analysis showed Chaetomium *sp.* Being dominant with the highest colonization frequency (26.9%). One of the *Chaetomium sp.* showed sequence similarity of 93% with the species reported earlier, Further investigations are in needed to harness the bioactive compounds.

Keyword: *Acacia nilotica*, *Chaetomium* sp, Diversity, Endophytic fungi, ITS sequencing, Leaf.

The microorganisms that colonize living plant tissues internally not inducing any overt symptoms or injury to the host plant are endophytes[1]. They are found in the diverse geographical region ranging from alpine plants to tropical plants including an ecologically adapted plants from hydrophytes to xerophytes. It is speculated that some of the tropical woody plants show hyper diversity of endophytic fungi[2]. According to Suryanarayana[3], this diversity could be attributed to the host abundance or endophyte assemblage in a host. The diversity with host, environment, geography, and tissue type enables endophytes to produce a plethora of bioactive secondary metabolites[4]. In order to harness the bioactive compound from this cryptic resource, the study of the diversity of endophytes in different plant hosts from different regions is essential.

The relations of endophytes with plant hosts may be symbiotic to antagonistic[5]. These endophytes influence plants' nutritional level, Survival, and distribution of plants at different stages[6]. Endophytes produce biologically active secondary metabolites[7] such as VOCs (Volatile

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Organic Compounds)[8], antidiabetic, anticancer, and anti-bacterial[9], insecticidal active compound[10]. Endophytes can also produce structurally diverse secondary metabolites[11]. *Aspergillus awamori,* an endophytic fungus was characterized from the *Acacia nilotica* recorded for the synthesis of antidiabetic peptides[12]. The antimalarial bioactive metabolite epoxycytochalasin *H* against Plasmodium falciparum reported from endophytic fungus *Diaporthe miriciae*[13]. Two new antiviral compounds, cytonic acid A ($C_{32}H_{36}O_{10}$) and B ($C_{32}H_{36}O_{10}$) isolated from endophytic fungi Cytonaema *spp* also reported[14.]

Most of the species of *Chaetomium* are members of non-clavicipitaceous endophytes that had gained the attention of investigators owing to the production of bioactive metabolites. Sharma et al.[15] described a new species isolated from *Jatropha podagrica* in India as *C. jatrophae*. *Chaetomium iranianum* and *C. grande* were isolated and characterized from Egypt and Africa by Blanchette et al.[16] and Abdel-Azeem et al.[17] respectively from the host *Teucrium polium.*

Acacia nilotica (L.) Del. belonging to the family Mimosaceae was selected to investigate the diversity of endophytes associated. The plant is distributed over a wide area in tropical and subtropical regions and found naturally growing throughout the Marathwada region. Leaf of *A. nilotica* shows pharmacological potential such as Chemo preventative, antimutagenic, antibacterial, anticancer[18] astringent, anti-microbial, Aphrodisiac, dressing of ulcers[19] anti-inflammatory and treatment against Alzheimer's diseases have been well documented[20]. To study the induced secondary metabolites by these endophytes, it was thought proper to investigate the endophytes first, the aim of this study was to isolate and characterize fungal endophytes associated with leaves using both morphological methods as well as molecular methods based on genomic DNA's ITS (Internal Transcribed Spacer) region.

MATERIALS AND METHODS

Isolation of fungal endophytes

Plant leaflets were collected and cleaned under running tap water to remove soil debris/ dust from the surface and then with ethanol (70%) for surface sterilization for 3 minutes, 1% sodium hypochlorite for 1 minute, followed by 3 rinses in sterile distilled water for 3 minutes each. Sterilized leaflets (0.5–1.0 cm) were plated on PDA Petri plates. The incubation of the plates was done at 28p C temperature for 3–4 weeks and were observed for emergence of colonies. The fungi from these colonies were subcultures to obtain pure isolates.

Morphological identification

Morphological Identification of the isolated endophytic fungi was done based on colony characters comprising of colour of colony, shape of colony, size of colony, and hyphal characters by using the identification key and microscopical observation of slides stained by cotton blue-stained slides.

Molecular characterization of endophyte

The nuclear DNA extraction was carried out using a spin column kit (Hi-Media, India). The amplification of Internal Transcribed Spacer rRNA gene (600 bp)[21] of the fungi was carried out using GeneAmp™ PCR System 9700. Exonuclease I - Shrimp Alkaline Phosphatase (Exo-SAP)[22] treatment was used for further purification. The purified products of PCR were sequenced in ABI 3500xl genetic analyzer (Life Technologies, USA). The resulting Sequences were then analysed using the Basic Local Alignment Search Tool (BLAST), and the most related sequences were retrieved from GenBank NCBI[23]. The program compared nucleotide sequences to sequences from the relationship databases and calculated the matches' statistical significance, the result of which are given.

Phylogenetic tree

The relationship among the isolates was inferred by the Maximum Likelihood method and Tamura 3-parameter model[24]. The consensus tree was generated from 500 replicates[25] with bootstrapping from the taxa analyzed[25]. The branches with less than 50% bootstrap replicates were merged. In the bootstrap test (500 replicates), the percentage of replication trees in which the related taxa clustered together is shown next to the branches[26]. The Neighbor-Joining method was applied to a matrix of pairwise distances calculated using the Tamura 3 parameter model to produce the initial tree(s) for the heuristic search. There were 07 nucleotide sequences in this study. 1st+2nd+3rd+Noncoding codon positions were included. In the end, the dataset contained

601 positions. MEGA X was used to run the evolutionary analyses.[27]

Statistical analysis

Hata and Futai's formula was used to calculate the colonisation frequency (percent CF) of endophytic fungi[23].

$$\text{Percent Colonization Frequency (\%CF)} = (N_{col}/N_t)100$$

Where,

N_{col} = the number of plant tissue segments colonised by each fungus

N_t = the total number of plant tissue segments examined.

RESULTS

Isolation of endophytic fungi

Endophytic fungi were isolated using potato dextrose agar (PDA) media. From 50 leaflets of *A. nilotica*, a total of 26 endophytic fungal species were isolated.

Morphological identification

The isolate AN2 exhibited morphologically as dense colony, with aerial habit and white coloured colony on PDA plate (Fig.1). Further, observation of the fungus through microscope showed the occurrence of hair-like, brown coloured appendages (setae) on their surface (Fig. 2). The morphological character of the isolated fungus was similar with the morphological structures of *Chaetomium pachypodioides*. AN17 isolate shows light brown and reverse yellowish-brown coloured (Fig 1) and microscopic observation shows the presence of hair-like, dark coloured appendages

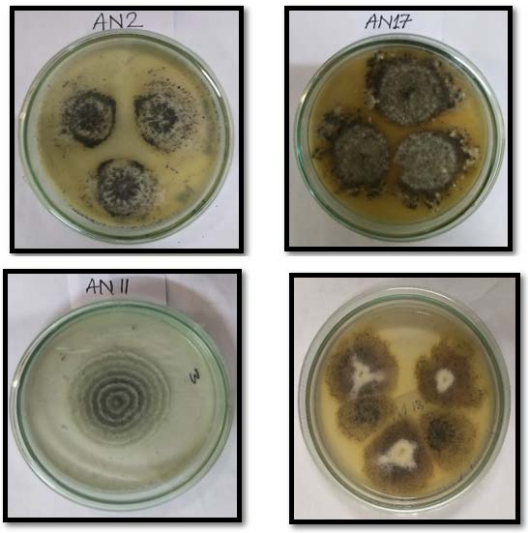

Fig. 1. Endophytic fungi grow in PDA medium

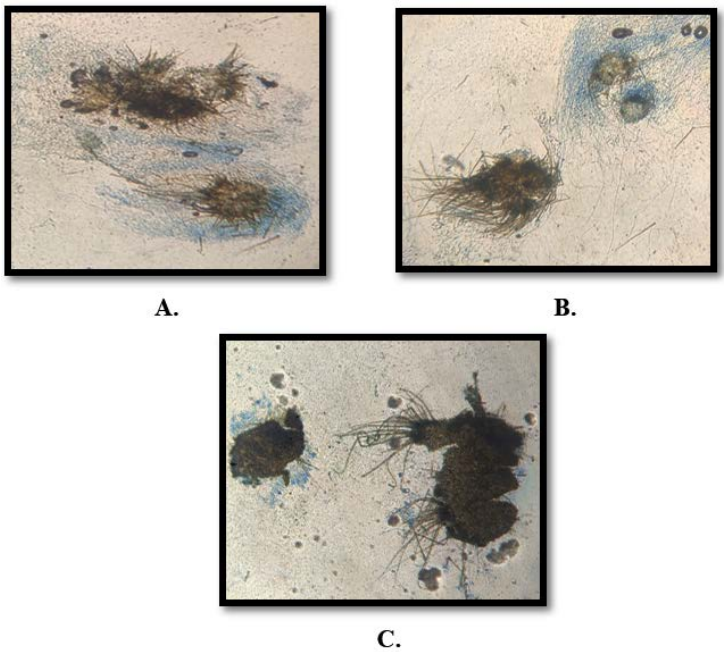

Fig. 2. Microscopic images of endophytic fungi stained with Cotton blue stain. Images represent A. AN 2, B. AN 17, C. AN 11

(setae) and lateral hairs like structures matching with the morphological features of *Chaetomium Microthecia* (Fig 2).

Molecular characterization of endophytes

The ITS1–5.8S–ITS2 sequences of the most similar endophytic fungal isolates were retrieved from GenBank (NCBI), and phylogenetic tree (Fig 3) was created. The sequences showed different taxonomic affinities between the endophytic isolates extracted from GenBank. These sequences were submitted to GenBank, NCBI, with accession numbers listed in Table 1.

Table 1. NCBI- Accession number, %Sequence similarity and %Query Cover

Endophytic Isolates (GenBank Acc. no. of the ITS sequence)	Query coverage	% sequence similarity	Organism with the highest sequence identity, GenBank Acc. No.
AN2 (MW238764)	99%	96%	*Chaetomium pachypodioides*
AN11 (MW239168)	100%	93%	*Chaetomium globisporum*
AN14 (MW239165)	100%	100%	*Chaetomium globosum*
AN17 (MW242834)	100%	100%	*Chaetomium microthecia*
AN18 (MW242837)	100%	100%	*Chaetomium pseudoglobosum*
AN21 (MW248483)	100%	93%	*Chaetomium globisporum*
AN25 (MW242840)	100%	93%	*Chaetomium globisporum*

Table 2. % Colonization frequency of endophytic fungi isolated from *Acacia nilotica*

Sr. No	Endophytic fungi	No.of Isolates	% CF
1	Chaetomium sp.	07	26.9
2	Amesia sp.	05	19.2
3	Ovatospora sp.	03	11.5
4	Penicillium sp.	02	7.6
5	Phialemonium sp.	02	7.6
6	Colletotrichum sp.	01	3.8
7	Crinipellis sp.	01	3.8
8	Acrophialophora sp.	01	3.8
9	Cribbea sp.	01	3.8

Statistical analysis

The biodiversity analysis exhibited that the fungal colonization by these endophytes was significant and diverse in leaf. Graphical representation is done by using R-studio software. Chaetomium *sp.* showed the highest colonization (26.9%) frequency, (Table 2 and Fig 4)

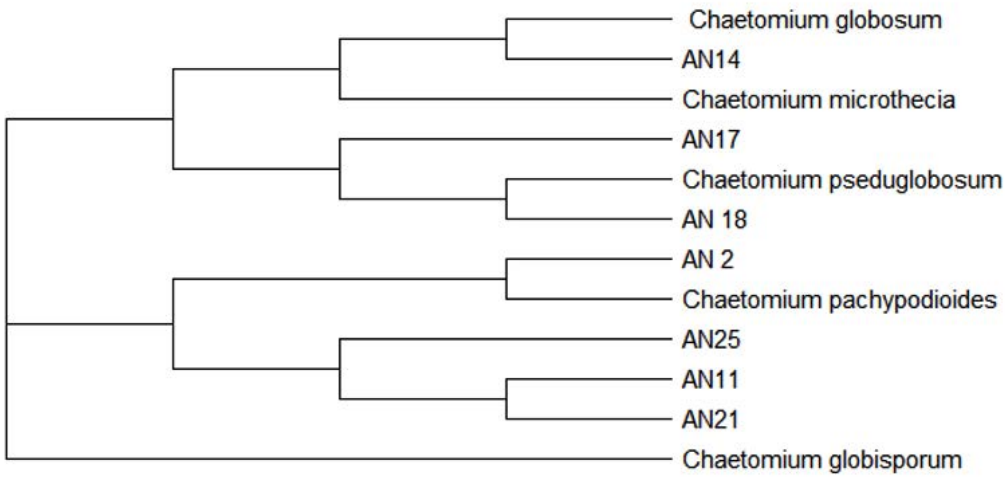

Fig. 3. Phylogenetic tree of endophytic fungi isolated from *Acacia nilotica*

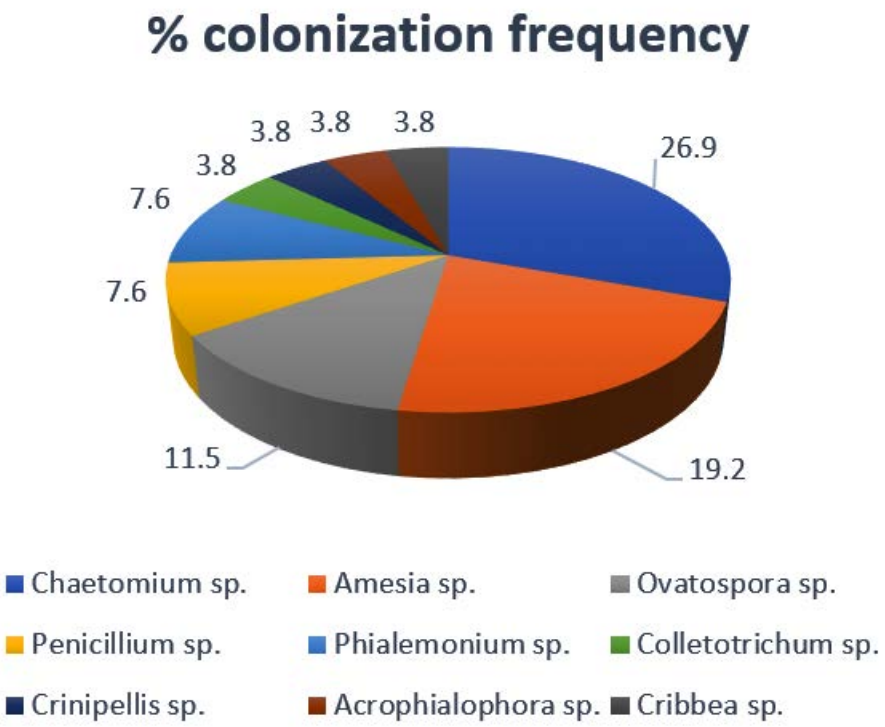

Fig. 4. % Colonization frequency of endophytic fungi isolated from *Acacia nilotica*

DISCUSSION

The alarming increase in microbial resistance has led the researchers to find out the novel sources of antimicrobial agents to control the drug resistant pathogens. Keeping this in mind, endophytic fungi were isolated from leaflet of the *A. nilotica*, followed by the proper surface sterilization. A total of 26 endophytic fungal species were isolated from 50 leaflets. Potato dextrose agar (PDA) media was used to isolate endophytic fungi. Out of total isolates of endophytic fungi (n=26) isolated from the host, most of them belonged to Phylum Ascomycota followed by one species of Crinipellis belonging to phylum Basidiomycota. Ascomycota phylum includes endophytic fungi from classes such as Sordariomycetes, Eurotimycetes, and Incertaesedis. The ITS1–5.8S–ITS2 sequences of all nearest neighbours of the endophytic fungal isolates were retrieved from GenBank (NCBI) and phylogenetic tree was constructed. The sequences studied showed diverse taxonomic affinities in the isolates. The same were submitted to NCBI and obtained accession numbers. Five isolates AN23 (*Crinipellis tabtim*), AN11(*Chaetomium globisporum*), *AN7* (*Phialemonium obovatum*), AN4 (*Cribbea turbinispora), AN3* (*Penicillium capsulatum),* showed a sequence similarity of less than 95 % with known organisms in the GenBank. These endophytes could likely be from novel fungal lineages. The biodiversity analysis showed that colonization of endophytic fungi was more significant and more diverse in leaf. Chaetomium *sp.* Showed the highest colonization (26.9%) frequency, while *Colletotrichum sp., Crinipellis sp., Acrophialophora sp., Cribbea sp.* showed the least colonization frequency (3.8%).

CONCLUSION

From the study it is concluded that endophytic fungi show hyper diversity in tropical plants[2]. Out of 26 isolates maximum endophytes belonging to Ascomycota phylum[28]. Endophytic fungi belong to *Chaetomium sp.* were the most abundant fungi occurring in the leaf. With hyper

diversity of species and ability to inhabit different environments, Chaetomium sp. might acquire biosynthetic gene clusters, which enables to produce various secondary metabolites for the adaptation of different ecological environments. Chaetomium sp. can be used for futuristic bioactive molecule production exhibiting significant cytotoxic, apoptotic and antioxidant potential[29]. Fungal endophyte, *C. globosum* produced chrysin as an alternative resource [30]. It is reported that about more than 200 compounds with potential bioactive potential have been isolated from Chaetomium sp[31]. Recently Chaetomium sp. have taken much attention to be used to manage different economically important diseases. C. globosum has been identified as a potential antagonist of Cochliobolus sativu[32].

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Conflict of interest

We declare no conflict of interest in the manuscript.

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