ORIGINAL RESEARCH PAPER

Forestry Science

ENDOPHYTIC FUNGUS, ACREMONIUM BORODINENSE, FROM TROPICAL TREE SPECIES, TECTONA GRANDIS LINN.F. MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION

KEY WORDS: Teak, endophyte, Acremonium, Internal Transcribed Spacer, phylogenetic analysis.

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ABSTRACT

Forest trees have symbiotic relationships with endophytic fungi, a prospective microbial resource with the ability to manufacture biologically active substances as secondary metabolites from the host plant, which could provide a number of benefits. In Tamil Nadu, India, a species of Acremonium was discovered during the isolation of endophytic fungi from healthy teak leaf samples. The endophytic fungal isolate's morphological characteristics matched those of Acremonium borodinense, which was validated by genetic analysis. For molecular phylogenetic analysis, the primary amplicon produced by Polymerase Chain Reaction of the Internal Transcribed Spacer (ITS) region was sequenced. According to the current phylogenetic analysis of this ITS sequence, the species of fungus was identified as Acremonium borodinense. In India, the fungus Acremonium borodinense (GenBank Accession number MW882246) is a new record as a teak endophyte.

INTRODUCTION

Plant endophytes are an endosymbiotic microorganism cluster that cause asymptomatic infections in healthy plants and have a symbiotic relationship with the majority of plant families (Sieber et al., 1988). About one million endophytic fungal species alone were reported (Dreyfuss and Chapela, 1994). Biologist from Germany - Anton de Bary invented the name "Endophytes" (de Bary, 1866). Fungal endophytes live inside the living host for the majority of their lives, causing no visible harm or disease signs in the host plant (Bacon and White, 2000) and protecting it from different types of pest species (Carroll, 1991; Azevedo et al., 2000).

Endophytes include a wealth of phytohormones and new bioactive substances with medicinal and industrial use (Joseph and Priya, 2011; Parthasarathi et al., 2012). Surprisingly, microbes associated with plants, rather than host plants themselves, were found to produce secondary metabolites with great therapeutic potential in the current context (Subbulakshmi et al., 2012). An endophytic microorganism Metarhizium anisopliae isolated from Taxus chinensis (Liu et al., 2009), Colletotrichum gloeosporioides, isolated from the leaves of Justicia gendarussa (Gangadevi and Muthumary, 2008), and Colletotrichum gleospoiroides, isolated from the leaves of teak, had the ability to manufacture a large amount of taxol (Senthilkumar et al., 2013). In temperate regions, all woodland plants contain endophytic fungus, with some tree species hosting large number of species and a large complexity of fungal endophyte species in vegetation (Redlin and Carris, 1996).

Tectona grandis Linn.f. (Teak), from the Verbenaceae family, is a tropical tree native to India and harbours a variety of endophytes. Fungal endophytes such as Diaporthe (Phomopsis) spp. Phomopsis sp. (Phomopsis longicolla), Lasiodiplodia theobromae (Singh et al., 2017); Alternaria, Colletotrichum, Nigrospora, Phomopsis and mycelia sterilia in both young and mature leaves and Fusarium, Penicillium, Schizophyllum commune (Chareprasert et al., 2006) only in mature leaves of teak and Phomopsis sp. (Senthilkumar et al., 2014) were reported. Senthilkumar et al., (2014) reported varieties of endophytic fungi in teak leaves of which Botryodiplodia theobromae, Phoma sp. Nigrospora sphaerica and Aspergillus flavus found to possess entomopathogenic significance. Interestingly, in the present study we isolated endophytic fungus Acremonium borodinense a new record from teak leaf. Ito et al., (2000) reported two rare Acremonium sect. Acremonium species, A. borodinense sp. nov. and A. cavaraeanum, from sugarcane rhizosphere soil and a Japanese wooden house outside wall, respectively. The said fungus was not at all reported from India.

Acremonium spp. has a widespread range and can reside in varieties of places including hay, rotting plants, and, in some cases, edible items. Multiple Acremonium endophytes were also documented, including Acremonium sp., an endophyte of Taxus baccata (Strobel et al., 1997), A. zeae, an endophyte of maize kernels (Wicklow et al., 2005), and A. lolii, an endophytic fungus of Lolium perenne L. (Rowan, 1993). (Ulfig et al., 1995, Glenn et al., 1996, Cano et al., 1997, Vidal et al., 1999). Acremonium species are physically very similar to one another and can only be separated by minor changes, making identification difficult. As a result, majority of the etiological agents were only identified as Acremonium spp. (Klimko, 2008; Das et al., 2010; Perdomo et al., 2011), prompting us to conduct molecular and morphological analysis to confirm Acremonium borodinense.

Morphological characterization is very important, as species names are needed in plant pathology, quarantine and most applied industrial aspects. The classification of endophytes is founded on morphological similarities (Crous et al., 2007; Zhang et al., 2008). Morphological identifiers are used to identify various fungi, particularly reproductive structures. However, numerous fungi do not have these structures in artificial cultures, making classification and identification more challenging (Barseghyan and Wasser, 2010). However, when recognising closely related or physically similar endophytes, extreme caution should be exercised because some of the fungi's morphological characteristics are medium-dependent (Zhang et al., 2008; Hyde and Soytong, 2007). Morphological characters alone is not always conclusive, so taxonomists use modern techniques like molecular analysis based on DNA sequences, which is recognised as an efficient method for revealing genetic relationships between strains and could be used to definitively identify and evaluate isolates at any taxonomic rank (Burns et al., 1991). As a result, studying endophyte communities is critical for getting the most out of this valuable resource.

2. MATERIALS AND METHODS

2.1. Collection of tree leaves samples

The endophyte fungi were isolated from Tectona grandis leaves. Samples were collected from a healthy T. grandis tree free of insect and disease infestation in Somandurai, Pollachi, Tamil Nadu, between 10°25'56.6" N latitude and 76°59'15.6" E longitude, properly labelled, and transported to Coimbatore where they were processed within 2 days after sampling.

2.2. Isolation of fungal endophytes

Leaf samples that were not damaged were chosen and properly cleaned under running tap water before being surface sterilised and dried. 1 cm^2 leaf segments were cut from the midrib portion of each leaf and the surface sterilised by soaking in 70% ethanol for a minute, then 4 percent sodium hypochlorite (v/v) for 2 minutes, followed by three 1-minute rinses in sterile water (Suryanarayanan et al., 1998). To suppress bacterial development, sterilised leaf segments (@ 2/plate) were plated in each petri dish containing potato dextrose agar supplemented with the antibiotic streptomycin. The dishes were incubated for 21 days at 26°C. The plates were checked for spore formation on a regular basis. Forming colonies were then injected aseptically onto fresh PDA plates to achieve pure cultures. PDA slant tubes were used to keep isolate cultures pure. The morphological and molecular characteristics of individual mycelial growth were studied.

2.3. Characterization of fungal isolates morphologically

The pure fungal isolates were divided into groups based on their physical characteristics (colour, texture), as well as their pace of growth. Lacto phenol cotton blue stain was used for microscopic study of fungal mycelium and spores, which were viewed under a light microscope (model Nikon optiphot-2) with a Nikon camera. In the culture, shape, size, texture, spore production, growth pattern, colony colour, and structure of conidial heads and conidia were all observed in the culture. Ito et al. (2000) used important features from established technical guides to help identify the fungal isolates.

2.4. Molecular characterization of fungal isolate

2.4.1. Extraction of genomic DNA and PCR amplification

The genomic DNA was isolated from the mycelial mat of morphologically recognised Acremonium borodinense using a slightly modified version of Nicholson's (2001) method. The universal primers ITS1 (5' TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCG CTTATTGAATATGC 3') were used to amplify the internal transcribed spacer (ITS) region (White et al., 1990). When resolved on agarose, a single distinct PCR amplicon band of about 700 bp was seen. To remove impurities, the PCR amplicon was purified. On an ABI 3730xl Genetic Analyzer, forward and reverse DNA sequencing reactions of PCR amplicons were performed with ITS1 and ITS4 primers using the BDT v3.1 Cycle sequencing kit.

2.4.2. Sequence analysis

At Eurofins Genomics India PVT Ltd in Bangalore, the amplified product was sequenced using the Sanger dideoxy technique. By using BLAST search analysis, the acquired ITS region sequence was compared to those in the GenBank. The first ten sequences were chosen and aligned using the multiple alignment software application, CLUSTAL W, based on their maximum identity score. The MEGA software version 7.0 was used to produce the distance matrix and build the phylogenetic tree. Kumar et al. (2006; Kumar et al., 2007). An accession number was obtained after submitting the nucleotide sequence to NCBI.

3. RESULTS AND DISCUSSION

Endophytic fungi colonise forest trees in large numbers; their colonisation is widespread, and all temperate forest trees host them. In twigs of Carpinus caroliniana and needles of Abies alba (Bills and Polishook, 1991; Sieber-Canavesi and Sieber 1987, 1993), more than 120 species were found; four different species were isolated from a single Douglas fir needle, several different genotypes of Lophodermium piceae colonised single Norway spruce needles (Muller et al., 2001), and Acremonium sclerotigenum (Prathyusha et al., 2015). Through morphological and molecular analysis, an endophytic fungus was isolated from a teak leaf and identified as Acremonium borodinense. The majority of Acremonium species are saprophytic, meaning they can be found in dead plant tissues, soil, and as endophytes in many plant species (de Almeida et al., 2011; Selim et al., 2011). Ito et al., (2000) described the isolation of a unique Acremonium species, A. borodinense, from sugarcane rhizosphere soil in Japan. According to classical mycology, most endophytic fungi, both cultivable and non-cultivable, is recognised according to phenotypical observations of spore, spore manner, mycelium peridium shape etc. (Barseghyan and Wasser, 2010). Because the genus Acremonium is morphologically simple in terms of taxonomy, species categorization is difficult. Morphological and molecular approaches were used to identify the fungus isolate.

3.1. Morphological identification of A. borodinense

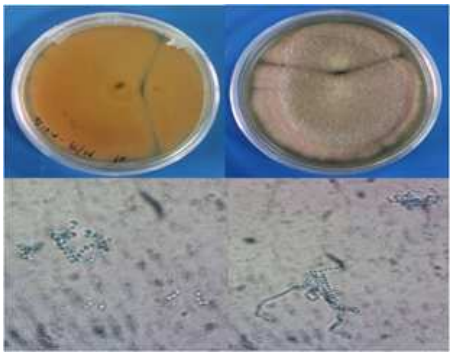

Fig 1. Colony and spore morphology of A. borodinense fungal endophytic isolate from teak leaf

The fundamental goal of morphological identification is to classify fungal isolates based on morphological similarities. Endophyte used in this study was initially white, but as the incubation period progressed, it turned pale yellow with greyish colony appearances (Fig. 1). Conidia heads have two forms of Phialidic conidia: ellipsoidal rough-walled and cylindrical smooth-walled (Gams, 1971; Ito et al., 2000). A. borodinense, on the other hand, differs from A. dimorphosporum in that it grows quicker at 25 °C (27–29 mm in diameter after 10 days) and can grow at 37 °C. Furthermore, its cylindrical conidia are slightly curved and smaller (4.5–5.5 m long), while its ellipsoidal and rough-walled conidia are larger (4.2–5.5 3–4 m). The said morphological features are in agreement with the morphological characters of Acremonium borodinense described in the earlier report of Ito et al., (2000) which was further identified through molecular studies. Senthilkumar et al., (2014) reported 18 dominant fungal endophytes viz., Botryodiplodia theobromae, Phoma eupyrena, Colletotrichum gleosporiies. Phomopsis sp. Fusarium sp, Phoma sp, Phomopsis longicola, Nigrospora sphaerica, Aspergillus flavus, Aspergillus fumigates, Phyllostica sp, Alternaria sp. Xylaria sp. Aspergillus niger, Chloridium sp. Clasporium cladosporioids, Fusarium clamydosporium and Pestalotiopsis in leaves of teak collected from different locations in Tamil Nadu.

3.2. Teak leaf endophytic fungal isolate of A. borodinense: Molecular characterization

In recent investigations, molecular methods have been utilised to accurately determine endophytic fungus (Promputtha et al., 2005; Sette et al., 2006; Morakotkarn et al., 2007). The study of endophytic fungi at the molecular level is

critical for the conservation and exploitation of fungal resources in plants. In some cases, it aids in the identification of genus and species, as well as within species (Schoch et al. 2012). In present study, amplification of the fragment of ITS region of A. borodinense fungal endophyte through PCR resulted in a single discrete PCR amplicon band of size 700bp (Fig 2).

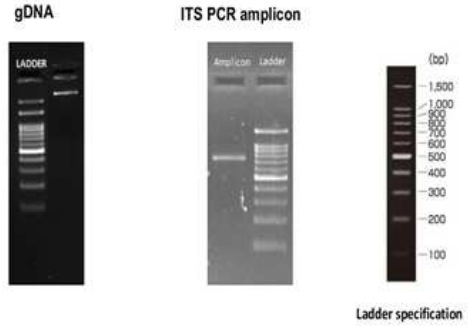

Fig: 2. PCR amplification of ITS region of the endophytic fungal isolate A. borodinense of teak

The ITS is widely used as molecular identifier that has been frequently used to differentiate different fungal species (Schoch et al., 2012). The ITS region of the fungal endophyte A. borodinense and its related species were analyzed to identify their phylogenetic connection. Singh et al. (2017) recently explored the use of ITS sequences to authenticate morphological determination of T. grandis fungal endophytes assemblage in three tissue components, namely the bark, leaf, and stem. The forward and reverse sequencing data were combined to create a consensus sequence for the PCR amplicon. The fungal endophyte Acremonium borodinense was identified by its 18S rDNA nucleotide sequences. The sequences of A. borodinense were found to be 93.41 percent (NCBI sequence ID MH424154.1) and 92.25 percent (NCBI sequence ID HE608635.1) comparable in the NCBI GenBank database. Several studies have studied the molecular identification of fungal genus to species and within species using ITS sequencing followed by bioinformatics analysis proved to be very effective (Hogberg and Land, 2004). The first fifteen sequences (Table 1) were chosen based on their maximum identity score and aligned using Clustal W, a multiple alignment software application.

Table 1. The best BLAST match sequences of A. borodinense isolated from teak leaves

S.No	Phylum	Closest NCBI match	Total Score	Query Cover	E value	GenBank Accession	Per. Identity
1	Ascomycota	*Acremonium pinkertoniae*	628	100%	9.00E-176	NR159611.1	93.88 %
2	Ascomycota	Acremonium sp	628	100%	6.00E-176	MF063330.1	93.88 %
3	Ascomycota	Hypocreales sp.	628	100%	9.00E-176	KF428417.1	93.88 %
4	Ascomycota	Phomopsis sp.	628	100%	9.00E-176	KF848941.1	93.88 %
5	Ascomycota	*Acremonium pinkertoniae*	628	100%	9.00E-176	MW857184.1	93.88 %
6	Ascomycota	Peniophora sp.	628	100%	6.00E-176	HQ607873.1	93.88 %
7	Ascomycota	Acremonium sp.	628	100%	6.00E-176	HQ607846.1	93.88 %
8	Ascomycota	Beauveria sp.	628	100%	6.00E-176	HQ607818.1	93.88 %
9	Ascomycota	Ophiocordyceps sp.	621	99%	1.00E-173	MT349946.1	93.82 %
10	Ascomycota	*Acremonium borodinense*	617	100%	2.00E-172	MH424154.1	93.41 %
11	Ascomycota	Acremonium sp.	612	97%	9.00E-171	LC317407.1	93.75 %
12	Ascomycota	Beauveria sp.	586	99%	5.00E-163	GQ169468.1	92.22 %
13	Ascomycota	Clavicipitaceae sp.	562	96%	9.00E-156	DQ778912.1	92.16 %
14	Ascomycota	Clavicipitaceae sp.	556	96%	4.00E-154	DQ778911.1	91.91 %
15	Ascomycota	*Acremonium borodinense*	551	93%	2.00E-152	HE608635.1	92.25 %

3.3. Phylogenetic tree analysis of the endophytic fungal isolate A. borodinense of teak leaf

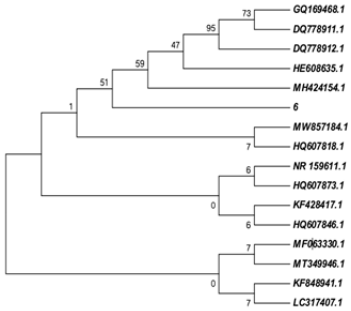

Fig: 3. From neighbor-joining analysis of 18S rRNA internal transcribed spacer data sets, phylogenetic trees of the endophytic isolate Acremonium borodinense and related species were produced

The phylogenetic tree is used to determine the evolutionary relationships of organisms that have common ancestors. The evolutionary distances were checked using the Maximum Composite Likelihood technique. The evolutionary history was deduced using the Kimura 2-parameter model and the Maximum Likelihood technique (Kimura, 1980). The evolutionary history of the taxa studied is represented by a bootstrap consensus tree generated from 1000 replicates (Felsenstein, 1985). Branches that relate to partitions that are replicated in less than 50% of the time are collapsed. Phylogenetic tree was constructed using Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances calculated using the MCL method. The sequence of Acremonium borodinense was clustered with the best matched 15 nucleotide sequences in the current study using phylogenetic analysis (Fig.3). 1st+2nd+3rd+Noncoding codon locations were included. Gaps and missing data were removed from all positions. The gene sequence of the endophytic fungal endophyte was closely related to Acremonium borodinense GenBank accession No MH42154.1 with a bootstrap value of 59 percent, followed by Acremonium borodinense GenBank accession No HE608635.1 with a bootstrap value of 47 percent. Khan et al. (2021) reported the endophytic association of Acremonium with Lilium davidii for the first time, and he discovered that the 544 bp long ITS rDNA gene sequence of Lilium davidii bulb showed a 100 percent similarity with Acremonium sp. strain HL3-2 (KT192220.1), and he submitted the ITS rDNA gene sequence to GenBank. The visual identification of the fungal endophyte isolated from the leaf of teak as Acremonium borodinense was also verified by the molecular analysis. Teak endophytic fungal isolate gene sequences matched and grouped with Acremonium borodinense. The obtained accession no. for the ITS sequence from GenBank was Mw882246.

4.0. CONCLUSION

Tree endophytes are mostly harmless colonizers without which the plants will not survive many environmental stresses. Interestingly, we have isolated endophytic fungus Acremonium borodinense from the leaf of teak. The morphological and molecular studies confirmed the fungal isolate as Acremonium borodinense which was reported as a first record as an endophyte of teak. Since endophytes and

their secondary metabolites have the biological activity as of its host species, the isolated endophyte can be exploited for its pharmacological significance as of teak its host plant.

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