JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

# ANATOMICAL STUDIES ON THE ASSOCIATION OF ENDOPHYTIC FUNGI AND THEIR ISOLATION FROM *ALANGIUM SALVIIFOLIUM* (L.f.) Wangerin

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https://doi.org/10.15414/jmbfs.3497

ARTICLE INFO

Received 23. 7. 2020
Revised 11. 5. 2021
Accepted 18. 5. 2021
Published 1. 10. 2021

Regular article

OPEN ACCESS

**ABSTRACT**

The present research was carried out to determine the anatomical features of leaves of *Alangium salviifolium* and their endophytic fungal association. *Alangium salviifolium* is an important medicinal plant with various activities reported. It is suggested that the medicinal property of the plant is either because of their endophytes or the endophytes inherit the medicinal properties from their host plant. Hence the detailed study of plant- microbe interaction is important. The anatomical features revealed the fungal mycelium in the ground cells of the abaxial midrib, the adaxial segment of xylem in the midrib. In the T.S of lamina, fungal hyphae are seen in the epidermis and also in the mesophyll tissue beneath the epidermal layer. Three endophytic fungi namely *Aspergillus niger*, *Diaporthe longicolla* and *Schizophyllum commune* were isolated and characterized using their ITS region.

**Keywords:** Endophytic fungi, *Alangium salviifolium*, light microscopy, molecular characterization

## INTRODUCTION

Endophytes live inside the tissues of all plants without any sign of diseases**(Petrini, 1991)**. Endophytes confer greater resistance to host against biotic and abiotic stresses, protects the plant against phytopathogens and produce chemical metabolites similar to that of the host **(Bayat *et al.*, 2009; Gundel *et al.*, 2010)**. Endophytic fungi mainly include Ascomycota, Basidiomycota and Zygomycota**(Tao *et al.*, 2013)**. Microbes are the source of innumerable enzymes and secondary metabolites which have many applications in the biotechnology arena**(Strobel & Daisy 2003)**.
The presence of fungi inside the living leaves is detected mostly by culture-dependent methods. Endophytic fungal hyphae present within the plant tissues are examined directly under light or electron microscope in the direct observation method. By this method even unculturable fungi can also be detected**(Deckert *et al.*, 2001; Lucero *et al.*, 2011)**. Direct observation using anatomical studies for the presence of fungal hyphae inside the leaves have been reported less**(Deckert *et al.*, 2001)**. Hence the current study was developed to locate the endophytic fungi inside the tissues of leaf and to isolate them. The plant chosen is an ethno medicinally important one, *Alangium salviifolium*. Each and every part of this plant is used in Siddha and Ayurveda. The plant belongs to the family Alangiaceae. The common name is sage leaved Alangium. It is a thorny tree and grows up to a height of 5 – 10m. It has antidiabetic**(Kumar *et al.*, 2011; Hepsy Kalrani D *et a.,l* 2012)**, antiulcer **(Sreekanth *et al.*, 2011)**, anti-arthritis**(Jubie *et al.*, 2008)**, anticancer**(Zahan R *et al.*, 2011)**, anti-inflammatory **(Ahad *et al.*, 2012)** properties and so on.

## MATERIALS AND METHODS

### Collection of specimens

The plant material was collected from the Mambakkam forest area, Chennai. The fresh and healthy leaves of the plant were processed in the laboratory after collection. The collected plant was identified as *Alangium salviifolium* by Dr. P. Jayaraman, PARC, Tambaram (Voucher NO. PARC/2019/4044).
The required leaf samples were cut and fixed in FAA (Formalin-5ml + Acetic acid-5ml + 70% Ethyl alcohol-90ml). After 24hrs, graded series of tertiary Butyl alcohol was used for dehydration of specimens as given by Sass, 1940. Paraffin was infiltrated gradually until saturation. The specimens were cast into paraffin blocks.

### Sectioning

The paraffin embedded specimens were sectioned (10-12 µm thickness) using Rotary Microtome. Dewaxing of the sections was by the customary procedure **(Johansen, 1940)**. The sections were stained with toluidine blue, a polychromatic stain, as per the method published by **O'Brieu et al., (1964)**. The cellulose walls were stained pink colour, lignified cells and protein bodies as blue and suberin as dark green *etc*. Safranin and fast green were also used wherever necessary.
The temporary preparations were mounted in glycerin and observed under microscopy. Nikon labphoto 2 microscopic unit was used to take photographs of different magnifications. Anatomical features were described as given in the standard Anatomy books **(Esau, 1964)**.

### Isolation of endophytes

The fresh and healthy leaves of plant were processed in laboratory after collection. The collected leaves were surface sterilized and cut it into small pieces of 1 cm which was placed on the Potato Dextrose Agar medium for isolation of endophytic fungi. Streptomycin was added to the medium to suppress bacterial contamination. The cultures were incubated for 3 weeks at 28°C in laboratory condition. After incubation period, colonies were isolated, subcultured and identified.

### Molecular characterization

Fungal DNA was isolated using the NucleoSpin® Plant II Kit (Macherey-Nagel). Primers ITS-1F (TCCGTAGGTGAACCTGCGG) and ITS-4R (TCCTCCGCTTATTGATATGC) were used to amplify the fungal DNA. The quality of DNA was checked using agarose gel electrophoresis. The PCR mixture (20µl total volume) contained 10.8 µl Milli Q water, 2 µl each of dNTP mix, Taq buffer and both the forward and reverse primers, 1 µl DNA template and 0.2 µl of Taq DNA Polymerase. Thirty five cycles were run each with denaturation step at 94ºC, annealing at 50ºC and extension step at 72ºC. After 35th cycle, Final extension @ 72ºC for 7 minutes was carried out. The PCR products were electrophoresed in agarose gel and sequenced in applied biosystems 3500 genetic analyzer and the sequences were submitted to GenBank. The sequences were compared using the NCBI BLAST program. Phylogenetic relationship was established by the neighbor joining method in MEGA software.

## RESULTS AND DISCUSSION

The leaf sections showed the presence of endophytic fungi. In cross sectional view the leaf exhibits thick midrib and thin smooth lamina. The midrib has a wide semicircular abaxial arc, vascular strand and somewhat smaller adaxial flat segment of vascular strand (Figure 1.1 & 2). The ground tissue of the abaxial midrib has a thin arc of fungal invested region. This region is thin and darkly stained. Some of the ground cells of the abaxial midrib also possess fungal mycelium (Figure 1.2). There is no fungal mycelium in the phloem cells or xylem cells (Figure. 2).

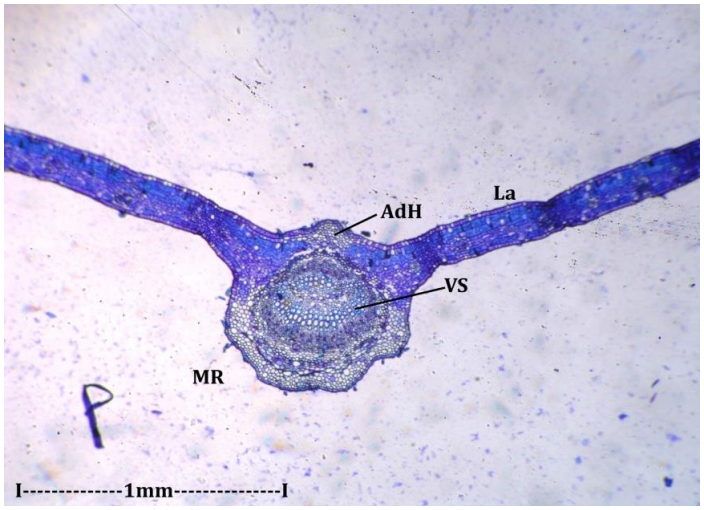


**Figure 1.1** T.S of leaf through midrib

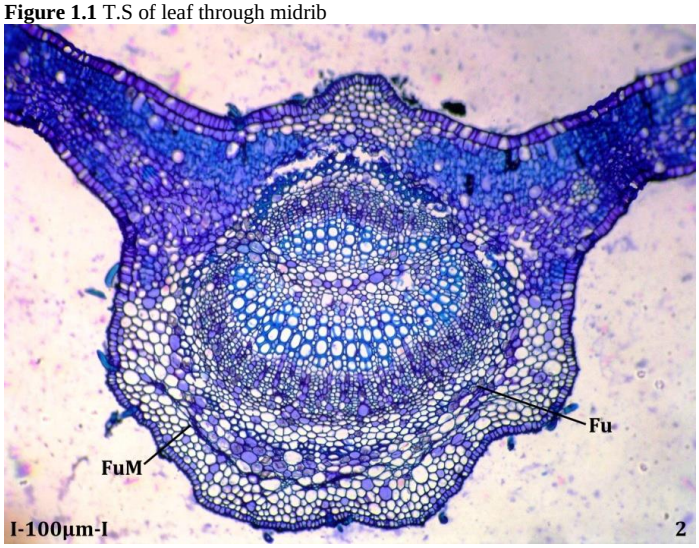


**Figure 1.2** T.S of midrib showing fungal mycelium

**Legends** : AdH – Adaxial Humb, FuM – Fungal mycelium , La – Lamina, MR – Midrib, VS – Vascular strand

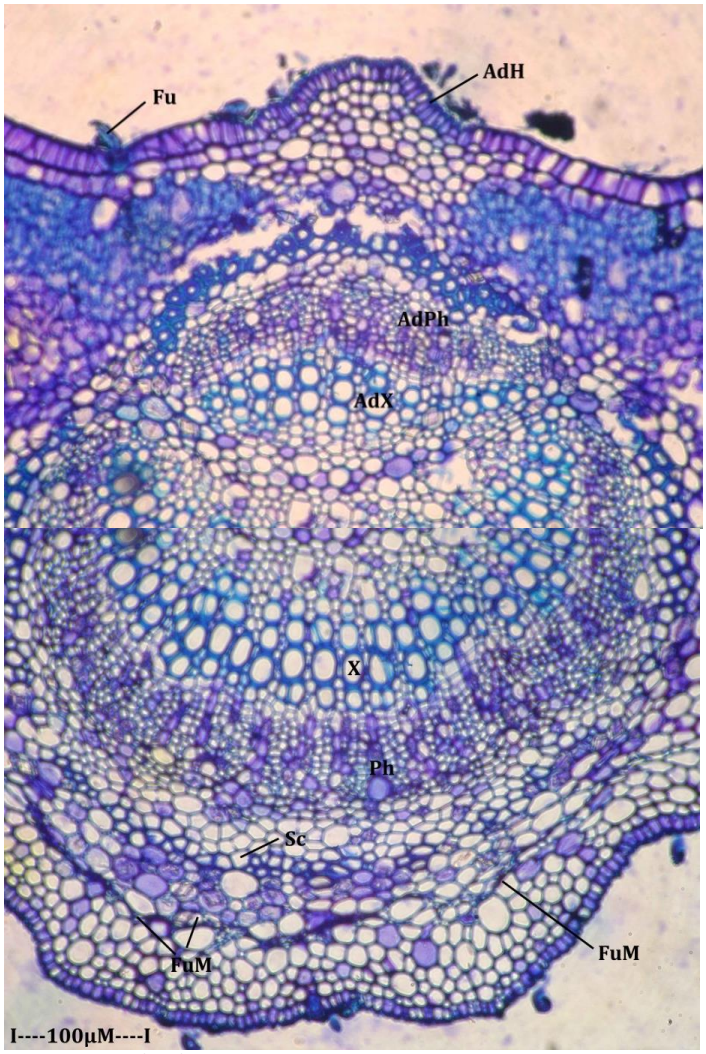


**Figure 2** T.S of leaf midrib – enlarged

**Legends** :AdH – Adaxial Humb, AdPh – Adaxial Phloem, AdX – Adaxial Xylem, FuM – Fungal mycelium, Ph – Phloem Sc – Sclerenchyma , X-Xylem

Around the wide circular, vessel elements of adaxial segment of xylem, there are dark, thick layers of fungal mycelium. It seems that fungal mycelium has penetrated the xylem zone through the adaxial part of phloem rays (Figure. 3.1). In the marginal part of the leaf, the fungal mycelium is seen on the surface of the epidermal cells. The mycelium has not yet penetrated the mesophyll tissue (Figure. 3.2).

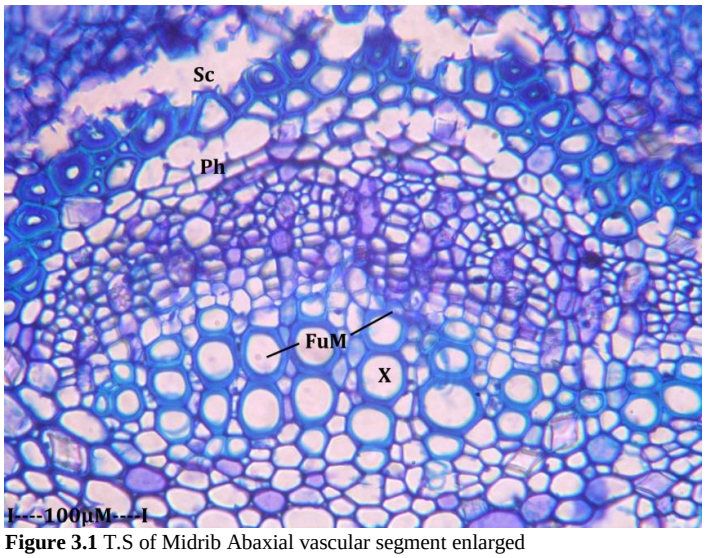


**Figure 3.1** T.S of Midrib Abaxial vascular segment enlarged

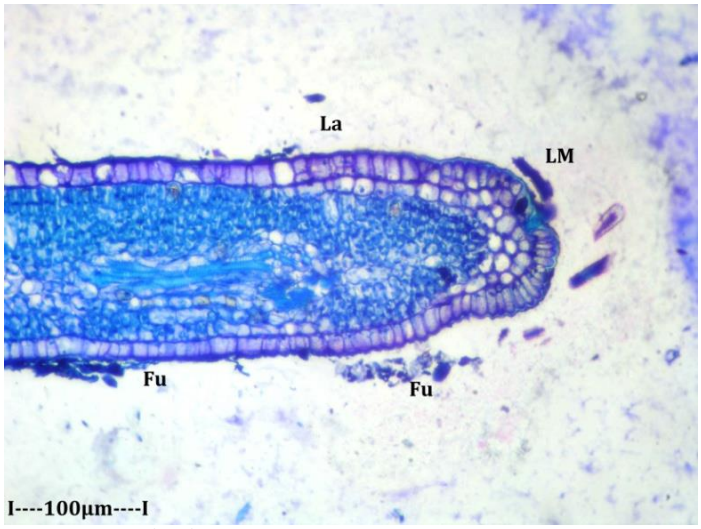


**Figure 3.2** T.S of leaf margin
**Legends** : FuM – Fungal mycelium, Ph – Phloem, Sc – Sclerenchyma ,, X- Xylem La – Lamina , LM – Leaf Margin

Numerous prismatic crystals are seen along with sclerenchyma bundle sheath of the vascular bundle (Figure. 4.1 &2) in the ground tissue of midrib. It seems that the crystal boundary forms a barrier for the entry of fungal mycelium. When the sections are highly magnified a thick intercellular growth of the fungus is seen in the phloem tissue. The fungal filaments penetrate in between the phloem elements and forms dark thick irregular masses in the phloem region (Figure 5.1). When T.S of lamina was examined under the microscope dark mycelium growth is evident in the mesophyll tissue beneath the epidermal layer (Figure 5.2).

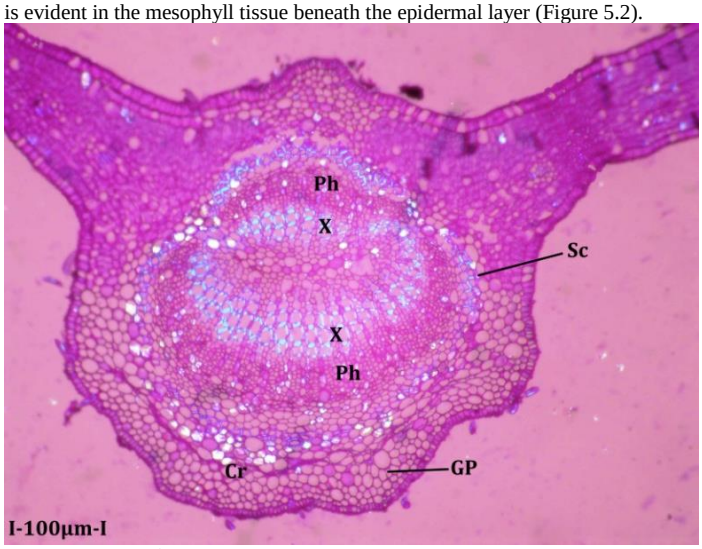


**Figure 4.1** T.S of midrib showing crystal distribution

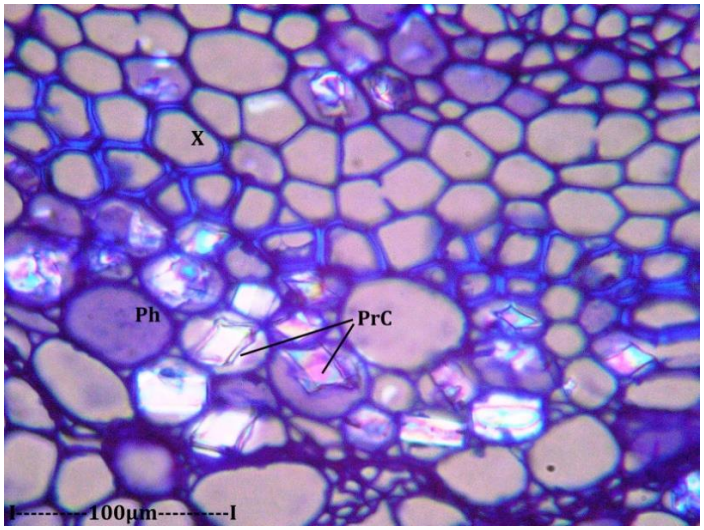


**Figure 4.2** Prismatic crystal in phloem parenchyma
**Legends** : Cr –Crystal, GP – Ground Parenchyma, PrC – Prismatic Crystal, Ph – Phloem, Sc- Sclerenchyma, X- Xylem

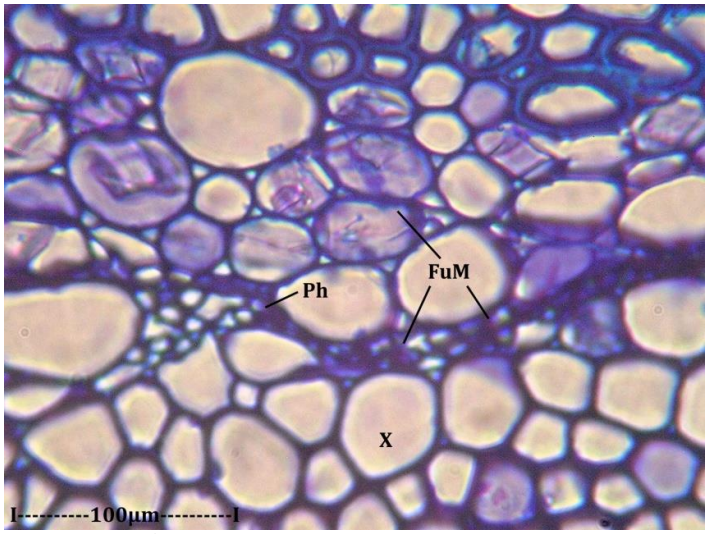


**Figure 5.1** T.S of midrib showing phloem and xylem elements

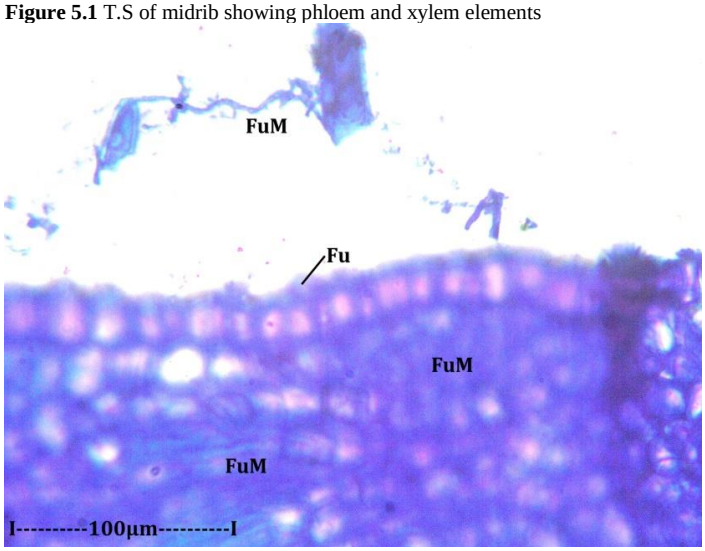


**Figure 5.2** A portion of leaf T.S showing epidermal layer with fungal infection
**Legends** : Fu – Fungus FuM – Fungal mycelium Ph – Phloem X- xylem

In some of the sections, fungal mycelium is seen spreading over the surface of the epidermal cells. They are also seen penetrated into the epidermis, inner cells of the palisade tissue. The mycelium is thin wiry and non-septate (Figure 6.1 &2).

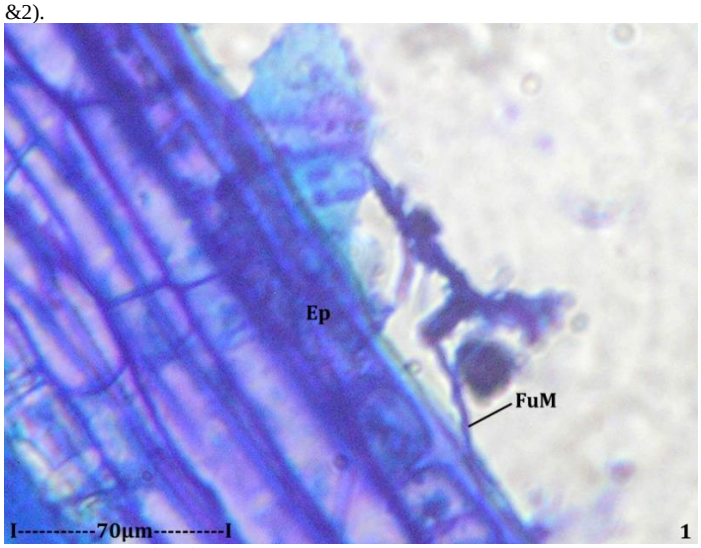


**Figure 6.1** T.S of leaf – Epidermal cells showing

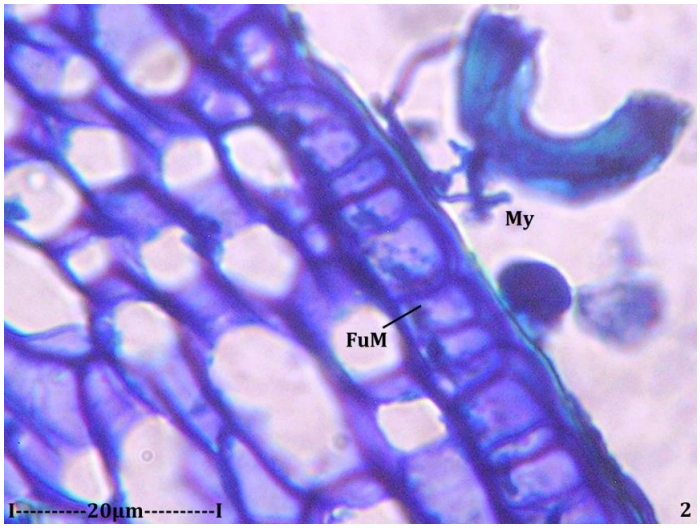


**Figure 6.2** Fungus mycelium seen on the epidermis and fungal mycelium penetrating to mesophyll tissues
**Legends** : Ep – Epidermis FuM – Fungal mycelium

The studies on the localization of endophytic fungi have been reported less. Direct observation studies help us to understand the interaction between the host and its endophyte, mode of infection and the extent of their association. The usage of various stains like lactophenol cotton blue, LOH-aniline blue , Pianese IIIB Stain etc were also made by different authors to study the anatomical association of endophytic fungi with their hosts. **Reyna *et al.*, (2012)** detected the endophytic fungus *Undifilum oxytropis* within the petioles and leaves of *Oxytropis sericea*. Electron microscopy and confocal microscopy were employed to detect the endophytic fungus inside the tissues. **Johnston *et al.*, (2006)** employed microscopic techniques and labeled with fluorescent dye that detect the (1/3)-β-D-glucan within fungal cell. Insitu hybridization technique was employed by **Pirttilä *et al.*, (2005)** to study the distribution of endophytes in scots pine. Endophytic fungi were found to be localized in the stem and leaf tissue of *Stevia rebaudiana* and was observed with the help of lactophenol cotton blue by **Kumari & Chandra, (2013)**. Similar results have been reported for the localization of endophytic fungi in the leaf of *Catharanthus roseus* **(Lakra *et al.*, 2013)** and *Tinospora cordifolia* **(Mishra Y *et al.*, 2015)**.
The plant tissues specifically leaves were the repository for the endophytic fungi as per earlier reports. The endophytic fungi were cultured on PDA and after 3 weeks of incubation they were subcultured for pure culture. Molecular identification of all 3 fungi has been successfully carried out for the identification of endophytic fungi. DNA was isolated from fungal mycelium and sequenced with ITS1 and ITS 4 primers. The NCBI BLAST search for similarity revealed the closest match to *Aspergillus niger* (MT090011), *Diaporthe longicolla* (MN173138) and *Schizophyllum commune* (MH857808) respectively. The sequences were submitted in GenBank and provided with accession number (Table 1). Phylogenetic tree were constructed (Figure 7-9). Two of the three isolates belong to Ascomycetes whereas *Schizophyllum commune* belongs to Basidiomycetes. This corroborates with the findings of earlier researchers who reported the dominance of Ascomycetes over Basidiomycetes in the endophytic fungal community **(Porras-Alfaro & Bayman, 2011; Wehner *et al.*, 2014)**.

**Table 1** Taxonomic affiliation of endophytic fungi isolated from *Alangium salviifolium* and their GenBank accession numbers

| Isolate Codes | Accession Numbers | The Closet GenBank Taxa | Similarity % |
|---|---|---|---|
| **EF1** | MN821058 | *Aspergillus niger* ( MT090011) | 100 |
| **EF2** | MN765101 | *Diaporthe longicolla* ( MN173138) | 99.65 |
| **EF4** | MN821480 | *Schizophyllum commune* ( MH857808 ) | 99.53 |

**Legend:** EF1 - *Aspergillus niger*, EF2 - *Diaporthe longicolla*, EF3 - *Schizophyllum commune*

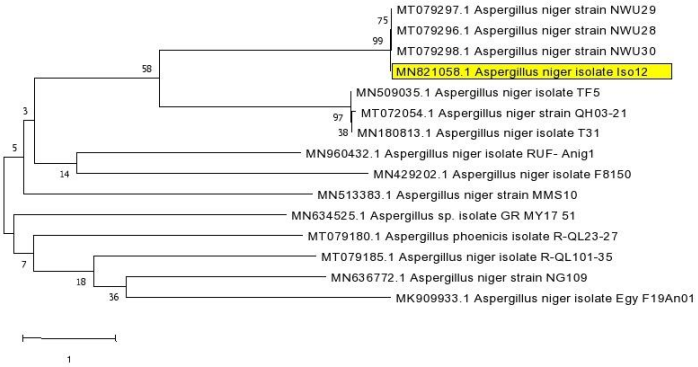


**Figure 7** Phylogenentic tree of ITS gene sequences of *Aspergillus niger*

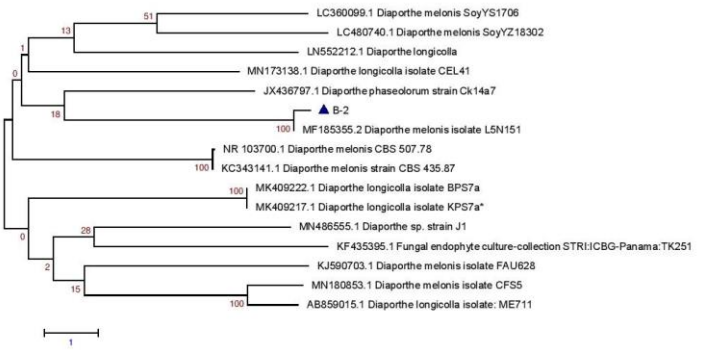


**Figure 8** Phylogenentic tree of ITS gene sequences *Diaporthe longicolla*

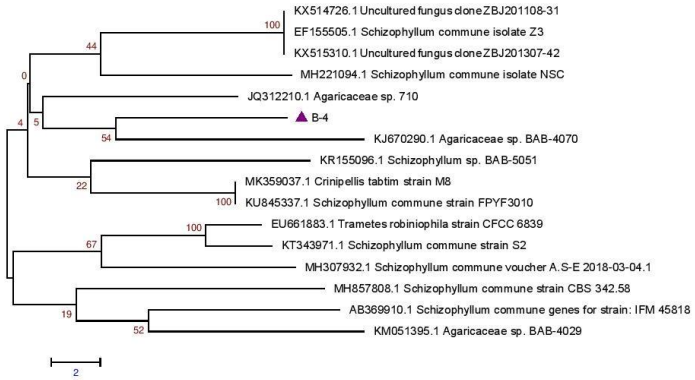


**Figure 9** Phylogenentic tree of ITS gene sequences *Schizophyllum commune*

Many studies were documented for the isolation of endophytic fungi from various medicinal plants since fungal endophytes are known to possess novel secondary metabolites that have pharmaceutical value. The endophytes isolated from this study have been reported in several other studies from various plants. *Aspergillus niger* is one of the commonly reported endophyte since it is cosmopolitan in nature. *Aspergillus niger* was isolated as an endophytic fungus from many plants such as *Withania somnifera*, *Cannabis sativa*, *Achilea millefolium etc*.,**(Tenguria & Khan, 2015, Lubna *et al.*, 2018, Satari AH *et al.*, 2018)**. Although Diaporthe sps. are mostly saprobe or pathogen, reports were also available for the isolation of those as endophytes**(Alberto *et al*, 2016, Agusta *et al* in 2006)**. Secondary metabolites of the endophytic fungus *Diaporthe longicolla* isolated from *Pogostemon cablin* were reported to possess invitro cytotoxic effects**( Wang *et al.*, 2016)**. *Schizophyllum commune* has been reported as an endophyte by **Gorky & Jenifer (2016)** from *Tectonia grandis*,**(Orlandelli *et al.*, 2012)** from *Piper hispidum* and many others. **(Supaphon *et al.*, 2014)** isolated *Schizophyllum commune* from sea grass *Enhalus acoroides* and evaluated its antimicrobial activity.

## CONCLUSION

The localization of endophytic fungi associated with *Alangium salviifolium* was reported for the first time in this study. Endophytes are a promising tool for the development of antibiotic resistant drugs and to protect the plant from

phytopathogens. Hence further detailed study of these endophytic fungi associated with this plant is currently being undertaken which would give us insight into their various bioactivities.

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