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Comparative analysis of diversity and distribution of Fungal endophytes in different parts of *Aegle marmelos* L.

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Abstract

An endophyte is a microorganism that resides internally in a plant without causing harm to the host plant. In most cases, endophytes are transmitted through seeds, promoting seed germination as well as the growth of newly developing host plants. Endophytes increase the nutrient input and stress tolerance capacity of growing young plants. Endophytes, which include bacteria and fungi, grow inside plants either within cells or between cells. Endophytic fungi can have symbiotic, mutualistic, or antagonistic interactions with their host plants. Endophytic fungi produces bioactive compounds with anti-tumor, antifungal, and antibacterial properties. These substances may also be toxic to the host plant due to the presence of some enzymes and taxol-like substances. However, they become an excellent source of drugs and show potential applications in the food, cosmetic, and medicine industries. *Aegle marmelos* is commonly used in Ayurvedic and Unani medicines to treat health issues such as diarrhoea and dysentery. More than 20 endophytic fungi have been recorded from *Aegle marmelos*.

Key words : Endophytes, Bioactive compounds, Fungi, *Aegle marmelos*, Secondary metabolites

B*ael* is a holy as well as medicinal plant in India. It is found in Bengal, the Central and South Indian parts, and the Himalayan regions. Sri Lanka, Burma, Thailand, Nepal, Pakistan, etc. are other nearby parts of our country where they grow abundantly. Bael leaves are also used in the worship of Lord Shiva. So, the trees are cultivated near the temple area. *Bael* fruit has many essential vitamins like vitamin C, vitamin A, niacin, riboflavin, and some other important minerals like P and Ca, etc.. *Bael* is rich in essential phytochemicals such as limonene, linalool, citrella, citral, caryophyllene oxide, humulene oxide, and marmeline.

Fungi work as reservoirs for many important bioactive compounds as well as secondary metabolites. Due to their diverse and ubiquitous nature, endophytic fungi are considered superior creatures[3]. There is a mutualistic interaction between endophytes and the host. Here, the host provides shelter and nutrition, while endophytes provide chemical protection[4,17]. The word "endophyte" was first explained by de Bary in 1866. According to him, endophytes are microbes that live in plant tissue at a certain point in their life cycle. Endophytes forms their colony in fruits, seeds, leaves, bark, and flowers of the host plant, stimulate the growth, disease-resistant capacity, and stress tolerance power of the host[18,19]. Mostly, they enter through roots, leaves, flowers, stems, and seed cotyledons into the host plant[5]. Several studies are available to show the formation of antimicrobial compounds after the host-endophytic fungi interaction[2,13]. There was a huge amount of bactericidal, fungicidal, and cytotoxic secondary metabolites formed by endophytic fungi, which were isolated from many medicinal plants. Production of phytohormones and enhancement in the availability of potassium, zinc, and phosphate-like elements are also good gifts of endophytic fungi to host plants[14,15]. Secondary metabolites are known as a source of medicinal products. Many Indian plant scientists studied the presence of endophytic fungi in different medicinal plants like *Bauhinia phoenicea, Adhatoda zeylanica, Clerodendron serratum, and Terminalia arjuna*[11,12]. The aim and objective of this study are the isolation and identification of endophytic fungi from the *Aegle marmelos* tree.

a) *Collection of samples :*

Healthy and mature plant materials like stem, bark, and leaves were collected from the *Aegle marmelos* plants found in the R.B.S. College campus. Bael plants grow abundantly in the entire area of UP and Uttarakhand. It can be easily seen in the college as well as nearby the college. Bark samples were collected at 145 cm above ground level and 1-2 cm in depth with the help of a sterile machete. Small discs of approximately 0.5 cm in diameter were prepared with the help of a sterilized pinch cutter. Now, a total of 15 samples were prepared, 5 of each type (bark, leaves, and stem).

b) *Isolation and Culture of Endophytic Fungi:*

Surface sterilization of plant material is crucial for the accurate study of endophytes. Many bacteria and fungi contaminate the surface of the sample. For this purpose, all the samples were sterilized using protocol of Verma *et al*.,[21]. Samples were washed with running tap water to remove any extra debris and dust that was present on them. Next, the samples were treated with 70% ethyl alcohol (5 sec.) and then 4% sodium hypochlorite (90 sec.). Then, all samples were rinsed with distilled water. The surfaces of all the samples were now free from epiphytes. Extra moisture was removed by sterile blotting paper. The outer bark was removed with a sterile blade, and the inner cortex was cut into 1.0 cm×1.0 cm. pieces. Small dishes of leaves of 5 mm size were prepared after sterilization. All the prepared samples were put in Petri dishes containing sterilized PDA. To examine the surface sterilization, 0.1 ml of sterile water was poured into the sterilized PDA in the Petri dishes and rolled the surface of the sterilized

plant sample material was on the sterilized Petri dishes.

Many culture media are available for endophytic fungi, in which PDA is frequently used. 500 grams of peeled potatoes boiled in 500 ml of water were filtered to obtain starch extract. Agar powder was boiled with 500 ml of water and dextrose in a separate beaker and cooked to obtain a custard-like consistency. Furthermore, both mixtures were mixed with each other to make the volume 1 litre. The culture medium must be slightly acidic, and the pH range must remain between 5.8 and 6.0. The use of antibiotics is necessary to inhibit the growth of endophytic bacteria. Broad-spectrum antibiotics streptomycin (250 mg/L) were used. PDA media was sterilized in an autoclave at 121 °C and 15 lb pressure for 15 minutes.

Paraffin Petri dishes were incubated at 28±2°C in a dark/light cycle for 10–20 days. Pure colonies were obtained by subculturing and streaking. Stocks were stored at -20 °C. After 3 days, tissues were seen as filamentous growth. After 2 to 3 days, it was been subcultured on fresh PDA media. Isolation of endophytic fungal strains was done with the help of the streak method. Five-day-old cultures were streaked on the center of the PDA-containing plate and incubated for 3 days at 28 °C. After isolation of the endophytic fungus, identification was done at the genus level by observing the presence of septate or aseptate mycelium, spore mass colour, fruiting body, sporophyte, the shape of conidia, colony color, spore chain morphology, presence and type of pigments in the colony, etc. Fungal components were put on sterile slides, colored with cotton blue and lactophenol, and mounted with glycerin. All the slides were examined with the help of light microscopy under the power of 400X and 1000X. Subterranean mycelium, aerial hyphae, shape of asexual or sexual reproductive bodies.

c) *Biochemical characterization of endophytes:*

Catalase test : The following tests were performed to determine the qualitative catalase activity. A loop of 18 to 24 hour old isolated culture was smeared on a clean, dry glass slide, and a drop of 3% H_2O_2 mixed with a sterile toothpick was used to evaluate the development of oxygen via effervescences, indicating good catalase activity[7].

Citrate test : The fungal isolates were qualitatively evaluated for citrate as an energy source using Simmons' citrate agar with a Bromothymol blue indicator. The isolates were selected from the core of a colony and streaked onto slanted media in test tubes using a back-and-forth motion, incubated aerobically at 35-37°C, and monitored for a colour shift from green to blue along the slant for citrate utilization[9].

Cellulase Test : For detection of cellulase activity, bacterial isolates were individually inoculated on Carboxymethy-lcellulose (CMC) agar plates and incubated for 48 h at 28 ± 2 °C. After incubation, the plates were flooded with 0.1% Congo red for 20 min and then de-stained with 1 M NaCl for 15 min. The formation of a clear zone indicated positive for cellulase activity[10].

Xylanase Test : For xylanase activity, the isolates were inoculated on xylan agar

medium containing birch wood xylan (0.5%). After four days of incubation at 28 ± 2 ºC, the xylanolytic activity of the endophytic isolates was determined by flooding the plates with 1.0% Congo red for 15 min, followed by de-staining with 1 M NaCl[6]. Development of clear zones around the colonies was considered positive for xylanase production.

Protease Test : Protease production was assessed by inoculating the cultures on skim milk agar plate and incubating at 28 ± 2ºC for 2 days. Formation of zone of hydrolysis around the bacterial colony indicates positive result for protease production[1].

Urease Test : urease activity was observed by Christensen's urea agar according to the method described by Kurtzman *et al.,*[8].

The study revealed the presence of various endophytic fungi in different parts of *Aegle marmelos*. The discussion explores the significance of these findings in the context of plant-microbe interactions.

After the observation, it was found that most members of ascomycetes as well as deuteromycetes developed on culture medium. Figure 1. represents the PDA used to culture endophytic fungi in sterilized bark, leaves, and stem pieces of *Aegle marmelos*.

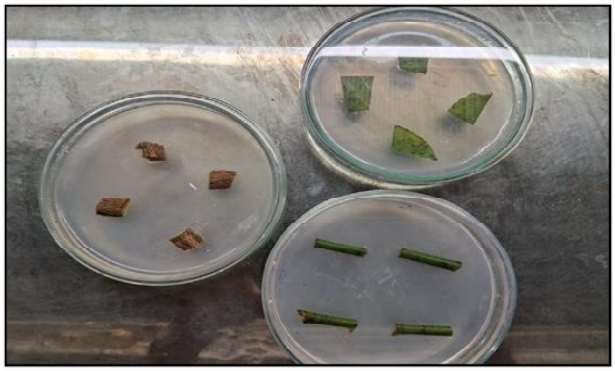

Fig. 01. Sterilized bark, leaves and stem pieces of Aegle marmelos on PDA to culture endophytic fungi.

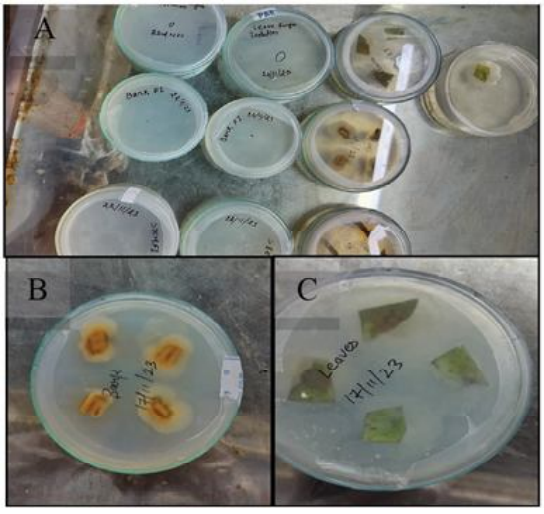

Fig. 02. Different Stages of colony development of Endophytic fungi of *Aegle marmelos*

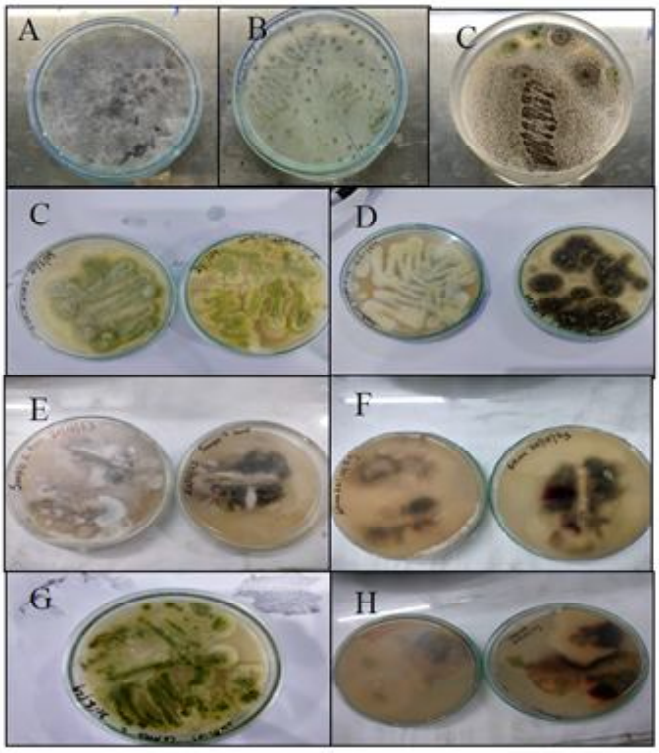

Fig. 03 Full Growth of Streaking Isolate during Subculture

Aseptate and septate both types of mycelium are seen in Fig. 04 A and 04 B.

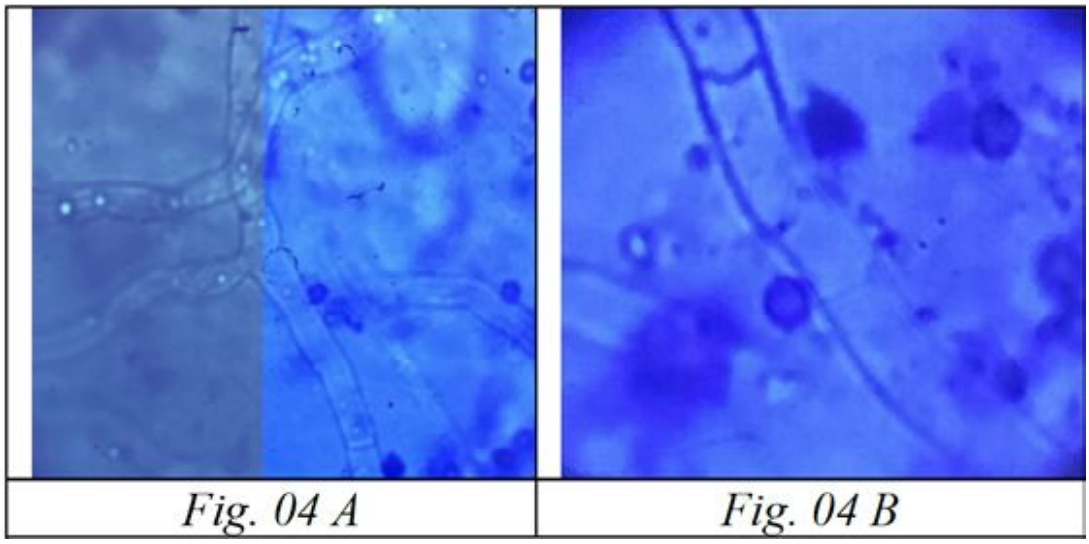

Fig. 04 Microscopic Observation of Mycelium of Endophytic fungi. After Staining with Cotton Blue and Lactophenol.

Here, the final stage of subculture was also seen. After that, different slides with the help of cotton blue and lactophenol from randomly selected parts of the colony of an isolated endophytic fungus were prepared. Well-developed septate and aseptate mycelium are seen here during the study (Fig. 04A, 04B).

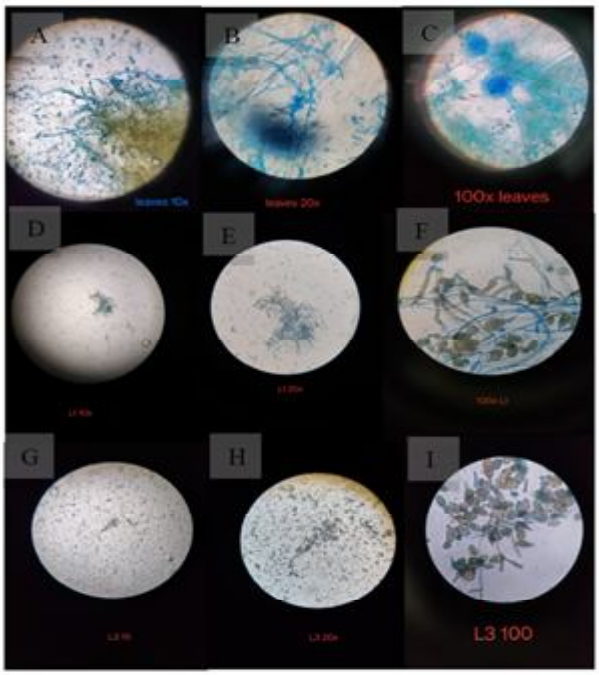

Fig.05 Endophytic fungal growth in Leaves

Different developmental stages of endophytic fungi of stems, bark, and leaves are seen in Figs. 05, 06, and 07. During the study of the endophytes of leaves, the spores, sporangia, and mycelium of *Aspergillus* sps. are clearly seen in Fig. 05 C, while *Curvularia* sps. and *Alternaria are* in Figs. 5F and 5I, respectively.

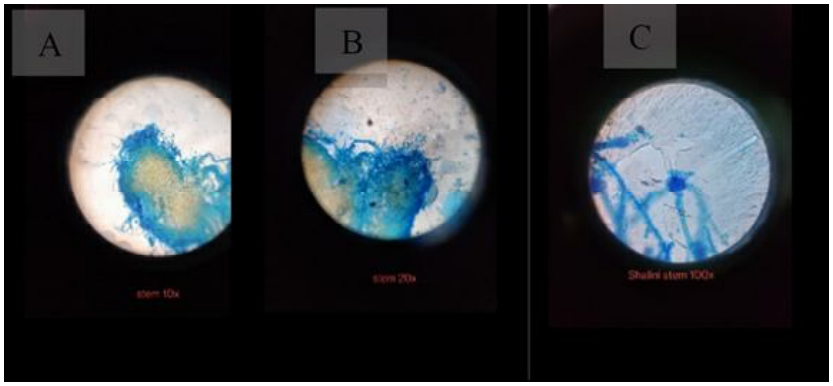

Fig.06 Endophytic fungal growth on stem

Only *Aspergillus* sp. is seen in stem Fig. 06 C. However, in the bark, three types of endophytic fungi were visible, which were *Rhizopus*, *Fusarium,* and *Aspergillus*. The method used for the isolation and identification of all fungi is very old and traditional. Here, mycelium was stained first and then studied. Five types of fungal genera were identified from different parts of *Aegle marmelos* during our study. *Aspergillus* sp. was present in all samples of leaves, stems, and bark, while *Rhizopus* sp. and *Fusarium* were found only in bark. *Curvularia* and *Alternaria* were restricted in their leaves.

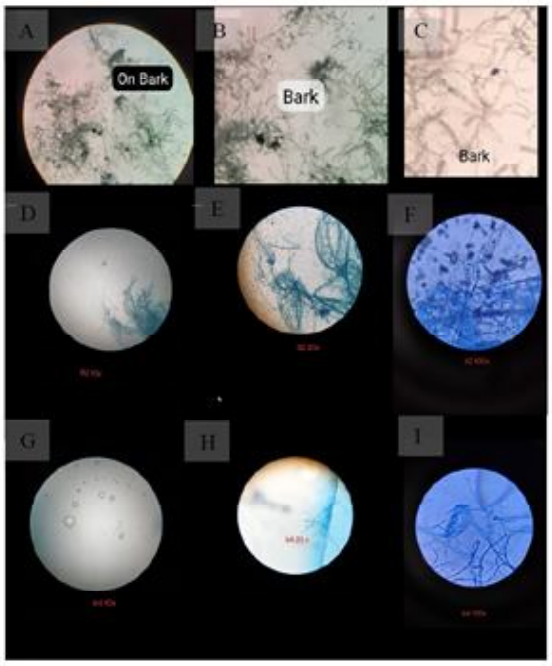

Fig. 07. Endophytic fungal growth in bark

The methods employed for isolating and identifying fungi were based on traditional practices. Mycelium was inter or intracellular. Five types of the fungal genus were identified from the different parts of *the Aegle marmelos* plant during our study. *Aspergillus* sps are present in leaves, stems, and bark, while *Rhizopus* sps and *Fusarium* sps are found only in the bark. *Curvularia* and *Alternaria* remain restricted in their leaves. (See table 01).

Table-01. The Distribution of different types of Endophytic Fungi

S.N.	Name of Fungi	Part of plants		
		Leaves	Stems	Bark
1.	*Aspergillus* sps	Positive	Positive	Positive
2.	*Rhizopus* sps	Negative	Negative	Positive
3.	*Curvularia* sps	Positive	Negative	Negative
4.	*Alternaria* sps	Positive	Negative	Negative
5.	*Fusarium* sps	Negative	Negative	Positive

Biochemical Characterization of endophytes : This section outlines the biochemical tests conducted, including the enzymes tested and the carbohydrates identified in the endophytic fungi.

Endophytes secrete a range of extracellular enzymes, including amylase, protease, pectinase, and dextrase. But here, no such type of enzyme was seen during biochemical characterization.

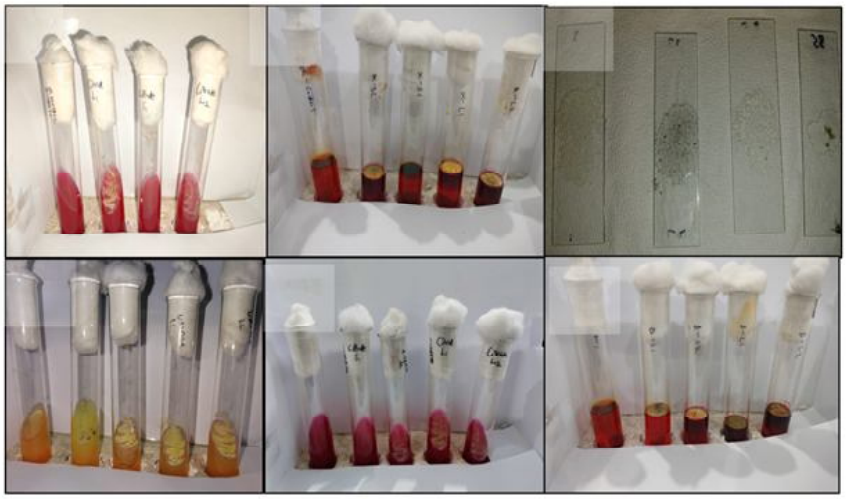

Fig. 08. Biochemical Characterization

Generally, members of Ascomycotina as well as Deutromycotina developed their colony in culture. We identified the following genera under light microscopy.

Table-02. Biochemical Characterization

	S1	S2	L1	L2
Catalase Test	Negative	Negative	Negative	Negative
Citrate Test	Negative	Negative	Negative	Negative
Cellulase Test	Positive	Positive	Positive	Positive
Protease Test	Positive	Positive	Positive	Positive
Xylanase Test	Positive	Positive	Positive	Positive
Urease Test	Negative	Negative	Negative	Negative

1. *Aspergillus* sps.*:* The colonies of Aspergillus sps. appeared greenish-black and powdery on PDA. Initially, the colony growth was white, which later changed colour to black with powdery conidia development. The colony edges turned pale yellow and fissured. Smooth conidia form on the conidiophore, protruding from a septate hypha. The conidial heads were radial, and the conidiophores were dark and smooth, with numerous round-shaped conidia at the top.

2. *Rhizopus* sps.: The colonies of *Rhizopus* sps. appeared dark grey-brown on P.D.A. The mycelium was branched and cottony, with black globular outgrowths. Black sporangia were observed at the tip of the unbranched sporangiophore, with rounded and numerous spores present.

3. *Curvularia* sps.: Woolly, olive green to brownish black colonies were seen on P.D.A. medium. Conidiophores were erect, straight, and large in size, with transverse septa.

4. *Alternaria* sps.: A yellowish brown to black colony was seen on P.D.A. media. Larger, multicellular conidia with horizontal and vertical septa were seen during microscopy.

5. *Fusarium* sps: A pale yellowish, fast-growing colony develops on PDA. Slimy dots were visible in culture due to the formation of sporodochia. Intercellular mycelium is visible.

The study found various endophytic fungi in *Aegle marmelos*, with most members of ascomycetes and deuteromycetes developing on culture medium. The method used for isolating and identifying fungi was traditional, with mycelium stained first and then studied. Five types of fungal genera were identified from different parts of the plant, with *Aspergillus* sp. present in all samples of leaves, stems, and bark, while *Rhizopus* sp. and *Fusarium* were found only in the bark. *Curvularia* and *Alternaria* were restricted in their leaves. The study highlights the importance of understanding plant-microbe interactions in fungi growth.

This biochemical characterization of endophytic fungi revealed that they secrete a range of extracellular enzymes, including amylase, protease, pectinase, and cellulase. However, no enzymes were found during the characterization, only three types of carbohydrates—maltose, dextrose, and xylose. The fungi were found to be beneficial to both plants and humans due to their production of bioactive compounds, phytohormones, antibacterials, and antivirals. These compounds are beneficial for agricultural and industrial purposes, attracting the attention of pharmacologists, mycologists, and biotechnologists. Endophytes are beneficial to both plants and humans due to their potential as sustainable sources in various fields of human interest. The study highlights the importance of understanding the biological properties of endophytic fungi in the development of sustainable agriculture and environmental solutions.

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