

Fungal Endophytes in *Aegle marmelos* (L.) *Correa*: Approach for Histological Localization and Enzymes Estimation

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Abstract

In plant-microbe interactions, endophytic fungi are essential because they support the growth, stress tolerance and disease resistance of the host plant. The fungal endophytes of Aegle marmelos (L.) Correa, a medicinal plant with pharmacological qualities, are the subject of this investigation. Seasonal differences in fungal diversity were revealed by isolating endophytes from various plant tissues collected over the course of three seasons. Fungal colonization in plant tissues was shown by histological localization. There were 42 fungal taxa found in all. Several isolates exhibited amylolytic, proteolytic, cellulolytic and lipolytic activities, demonstrating the metabolic capability of endophytes as evidenced by enzymatic processes. The breakdown of macromolecules, fungal colonization and biotechnological applications all depends on these enzymes.

The results demonstrate the metabolic diversity and ecological importance of Aegle marmelos endophytes. Their potential for use in industry, agriculture and pharmaceuticals is highlighted by their capacity to create extracellular enzymes and bioactive chemicals. Plant-endophyte interactions are improved by this work, which also lays the groundwork for future research into endophytes as a source of new bioactive substances and environmentally friendly solutions for a range of sectors.

Keywords: Symbiotic relationship, histological determination, endophytic fungi.

Introduction

A beneficial partnership has been formed through the co-evolution of fungal endophytes and their hosts. Endophytic fungi live inside tissues, beneath the layers of epidermal cells, intercellularly and intra/intracellularly, without infecting their hosts or producing any symptoms of disease¹⁵. Fungal endophytes significantly influence plants by enabling them to adapt to various environments which in turn can enhance the diversity of fungal endophytes²⁴. The close relationship that endophytic microorganisms, especially fungi, have with their host plant has been shown to protect it from diseases and herbivores. All plants in

natural ecosystems have endophytic fungi, which are renowned for their extraordinary capacity to generate novel leads with significant clinical and pharmacological applications^{2,6}.

The demand for medicinal plants has been rising globally as a result of the increased awareness of natural products. Compared to synthetic medications, which are prone to adulteration and adverse effects, they are pharmacologically active, inexpensive and have low toxicity. They also offer a simple solution for a variety of human illnesses¹⁷. Since medicinal plants are regarded as pharmacological agents, they are crucial to the creation of pharmaceuticals. About 80% of people worldwide use herbal and traditional medicine for primary healthcare in underdeveloped countries, according to the WHO⁶. The main benefits of using medicinal plants for a variety of conditions are their safety in addition to their affordability, efficacy and accessibility⁷.

Bael, also known as *Aegle marmelos*, is a resilient subtropical tree that may reach a height of 15 meters despite severe weather. It is the only plant in its genus and has fragrant blooms, alternating leaves with three to five leaflets (Fig. 1c.) and thorny branches. The edible fruits are round, oval, or oblong in shape (Fig. 1b.) and when they ripen, their grey-green shell turns yellowish and they release a fragrant scent. The pulp, which has ten to fifteen seeds covered in a sticky mucilage, is pale orange, sweet and aromatic¹⁴. A variety of this plant's crude extracts have shown a broad range of properties such as anti-inflammatory, antipyretic, analgesic, antioxidant, antibacterial, radioprotective, anticancer, antidiabetic, antihyperlipidemic and antispermato-genic capabilities⁹.

The ripe fruit is a remedy for indigestion, while unripe fruit is used in oil for burning sensations. Various parts of the plant including roots, bark, leaves and seeds, are used in traditional medicine for ailments like fever, eye problems, inflammation and more. The plant also has practical uses, such as its wood for construction and rind for dye and the dried fruit rinds are used as containers. Both Ayurvedic and Homeopathic traditions utilize this plant for a wide range of conditions¹⁰.

The byproduct of microbial cell growth, extracellular enzymes serve a variety of biological and environmental purposes outside the cell. In actuality, endophytic fungi

produce a number of important enzymes including xylanases, proteases, asparaginase, cellulases, pectinases, tyrosinase, gelatinase, chitinase, amylases etc. In order to break down different macromolecules such as proteins, carbohydrates, lignin, organic phosphate and sugar-based polymers into transportable products that may be carried throughout cells and support heterotrophic metabolism, these extracellular enzymes target these molecules^{11–13}. Microbial enzymes are highly biotechnologically significant in molecular biology, pharmaceutical synthesis, textile production, food processing and the production of detergents¹⁴.

Material and Methods

Sample collection: Healthy leaves and stems of *Aegle marmelos* (L.) Correa were collected from Manipal University Jaipur, Rajasthan under aseptic conditions. The samples were chosen at random and sliced with a sterile, sharp blade. Sterilized paper bags were used to carry the materials loosely to the lab. Samples were prepared for histological analysis within two hours of being collected.

Surface sterilization: Surface sterilization is a critical step to eliminate contamination from surface microbes and only endophytic microorganisms residing within the plant tissue are studied. Plant samples are first washed under running tap water to remove dirt and debris. The tissues then go through a number of chemical sterilization procedures, including a

70% ethanol solution for 1 minute to breakdown surface lipids, a 2% sodium hypochlorite solution for 2 minutes to remove microbiological contamination and a series of rinses in sterile distilled water to get rid of any remaining sterilizing agents. Following sterilization, the tissues are aseptically dried before undergoing additional histological or microbiological analysis^{7,20}.

Histological localization: The process of histologically localizing endophytes entails a number of steps that are intended to identify and visualize their presence within plant tissues: First, plant samples are fixed using a suitable fixative such as formalin-acetic acid-alcohol (FAA) in order to preserve cellular integrity and endophyte structure. The fixed tissues are dehydrated through a tertiary butyl alcohol (TBA) series (table1) substituted by liquid paraffin. Following fixation, the leaf and stem samples were sectioned into small pieces. Finally, thin sections of the embedded tissue, usually 5–10 µm thick, are prepared using a microtome and placed onto glass slides. These sections are then deparaffinized and subjected to lactophenol cotton blue staining. These stains aid in differentiating plant tissues and highlighting the presence of fungal endophytes¹⁷.

Isolation of fungal endophytes: Sample of healthy leaf and stem of *Aegle marmelos* was collected (proceed within 5 hours) from Manipal University Jaipur, Rajasthan in three different seasons.

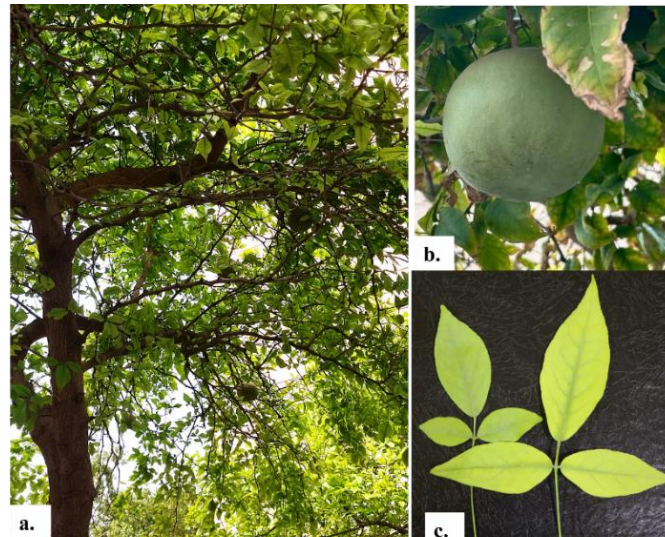


Fig. 1: a. tree, b. fruit and c. compound leaves of *Aegle marmelos* (Bael).

Table 1

The TBA solution's composition and concentration, as well as the processing durations for trees, shrubs and herbs.

S.N.	Conc. Of TBA	Composition	For herb	For shrub	For tree
1.	10%	10ml TBA+45ml Distilled Water+45ml ethyl alcohol	1hour	1hour	2hour
2.	20%	20ml TBA+40ml Distilled Water+40ml ethyl alcohol	4hour	2days	2days
3.	35%	35ml TBA+10ml Distilled Water+55ml ethyl alcohol	30min	1hour	2hour
4.	55%	55ml TBA + 45ml ethyl alcohol	30min	1hour	2hour
5.	75%	75ml TBA+25ml ethyl alcohol	30min	2hour	4hour
6.	100%	100ml TBA	1hour	3hour	4hour

The sample was surface sterilized using 70% ethanol to get rid of surface fats, 2% sodium hypochlorite to get rid of microbiological impurities and many rinses with sterile distilled water to get rid of any remaining chemicals under sterile condition. The sterilized tissues were cut into small segments under sterile conditions and were placed on culture media, such as potato dextrose agar (PDA) supplemented with antibiotics to inhibit bacterial growth. The plates were incubated at an appropriate temperature (typically 25–28°C) for 5–7 days or until fungal growth was observed. Emerging fungal colonies were sub-cultured onto fresh media and were identified based on morphological or molecular characteristics⁸.

Colonization frequency: The calculation of colonization frequency (CF) followed the guidelines provided by Suryanarayanan et al. In brief, the appropriate incubation period was specified for CF counting¹².

$$CF\% = \frac{\text{Number of segments colonized by fungi}}{\text{Total number of segments observed}} \times 100$$

Extracellular Enzymatic estimation: The qualitative production of enzymes (amylase, cellulose, laccase and lipase) by the acquired endophytic isolates was examined using extracellular enzyme assays². The enzymatic activity was determined following the method described by Hankin et al and Sunitha et al.

- a) **Amylolytic activity:** The criterion utilized to assess the capacity to generate amylolytic enzymes was the ability to break down starch. Amylase activity was evaluated by culturing the fungi on glucose yeast extract peptone agar (GYP) medium (1 g glucose, 0.1 g yeast extract, 16 g agar and 1 L distilled water) supplemented with 0.2% soluble starch at pH 6.0. Following incubation, the plates were flooded with a solution of 1% iodine in 2% potassium iodide. Presence of clear zones around the fungal colony indicates the positive results.
- b) **Proteolytic activity:** The proteolytic activity of endophytic fungi was assessed on GYP agar supplemented with 0.4% gelatin, with the pH adjusted to 6.5. The plates were incubated at room temperature for 3–5 days. Enzyme activity was evaluated by measuring the diameters of the clear zones formed around the fungal colonies.
- c) **Laccase activity:** Glucose yeast extract peptone agar (GYP) medium containing 0.05 g of 1-naphthol per liter at pH 6.0 was used. As the fungus grew, the initially colourless medium turned blue, indicating the oxidation of 1-naphthol by laccase.
- d) **Cellulose activity:** Glucose yeast extract peptone agar (GYP) medium containing 0.5% carboxymethyl cellulose was used to assess cellulase activity. After 3–5 days of fungal colony growth, the plates were flooded with 0.2% aqueous Congo red solution and then destained with 1 M NaCl for 15 minutes. The presence of yellow zones around the fungal colonies against the

red background of the medium indicated cellulase activity.

- e) **Lipolytic activity:** The fungi were cultured on peptone agar medium (10 g peptone, 5 g NaCl, 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 16 g agar, 1 L distilled water; pH 6.0) supplemented with 1% Tween 20, which was sterilized separately and added to the medium. After the incubation period, the formation of a visible precipitate around the fungal colony, resulting from calcium salts of lauric acid released by enzymatic activity, indicated positive lipase activity.

Results

Microscopic evaluation of T.S of leaf and stem of *Aegle marmelos*: In microscopical evaluation of T.S of *Aegle marmelos*, we concluded that Lactophenol blue effectively stains endophytes in plant tissues by binding specifically to fungal cell wall components like chitin and glucans, which are absent in plant cells. The cotton blue dye highlights fungal structures such as hyphae and spores, while the lactic acid clears plant tissues, making them translucent and allowing the fungi to stand out. Phenol acts as a preservative and fungicide, preserving fungal morphology during staining. This selective staining of endophytes by lactophenol blue is highlighted by the dark blue staining in the fig. 2.

Histological localization of endophytic fungi on the stem and leaf of *Aegle marmelos*: Histological evidence of fungal formations in plant tissues demonstrates their unique colonization styles. In fig. 3 the mesophyll region of a leaf in the first two panels (A and B) displays mycelium and spores, suggesting fungal invasion in the tissue layers and intercellular gaps. In the leaf tissue, the mycelial networks and fungal spores seem to be well-integrated, indicating a symbiotic or active interaction. The focus moves to the stem tissue in the lower panels (C and D), where endophytic fungi are observed living inside or in between the outer layer's cells. In the context of the plant's structural framework, these pictures demonstrate the tight relationship between fungi and plant tissues, highlighting their endophytic and possibly mutualistic interactions.

Isolation of fungal endophytes: Total 42 fungal cultures were isolated from different plant tissues (petiole, leaf and stem) of *Aegle marmelos* across three seasons at Manipal University Jaipur, Rajasthan and are represented in table 2. During the winter season, characterized by moderate temperatures (17.74°C) and humidity (64.15%), nine fungal taxa were isolated, highlighting the diversity of fungal endophytes adapted to colder conditions. In the summer season, with high temperatures (36.88°C) and low humidity (42.99%), nine heat-tolerant fungal taxa were identified, indicating a shift in fungal communities adapted to arid conditions. The rainy season, with moderate temperatures (29.3°C) and high humidity (69.97%), yielded the highest diversity, with 24 fungal taxa isolated. This demonstrates that the rainy season provides an ideal environment for a

wide range of fungal endophytes due to favorable moisture and temperature conditions. In figures 5 and 6, we have

displayed a few morphological characteristics of these 42 cultures.

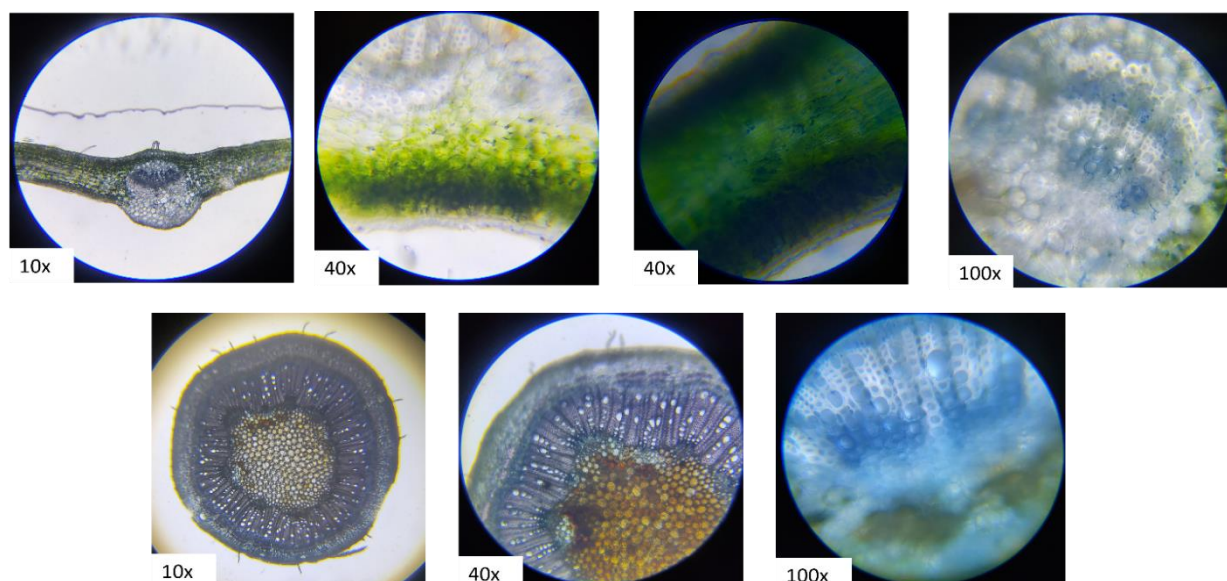


Fig. 2: Microscopic assessment of T.S. of *Aegle marmelos* leaves and stems at several magnification levels

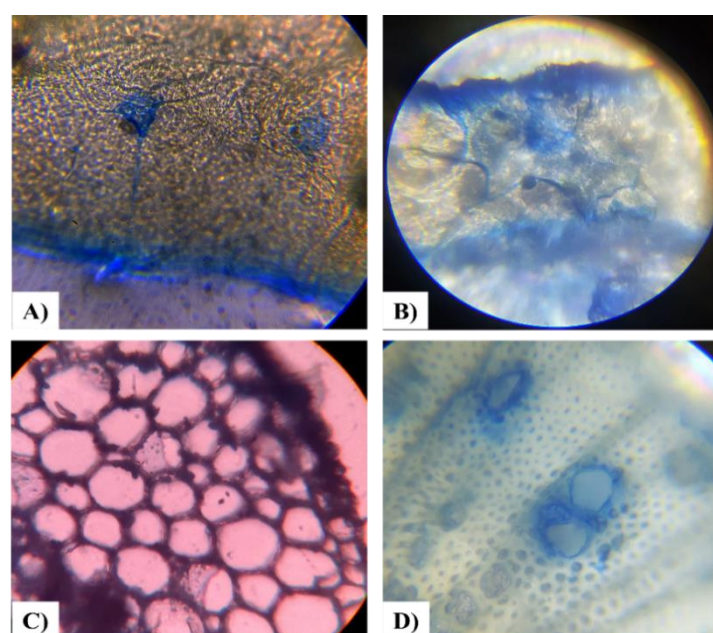


Fig. 3: Evidence of fungal structures in plant tissues by histology: A-B) Mycelium and spores in the leaf mesophyll; C-D) Endophytic fungi that live inside the outer layer of stem cells.

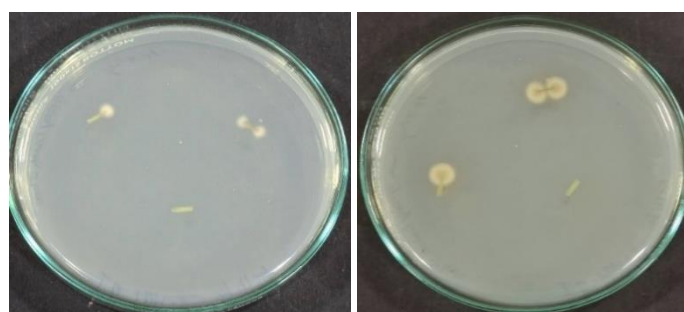


Fig. 4: Early-stage growth of fungal endophytes on PDA agar plates, showing initial hyphal development from inoculated plant tissue fragments.

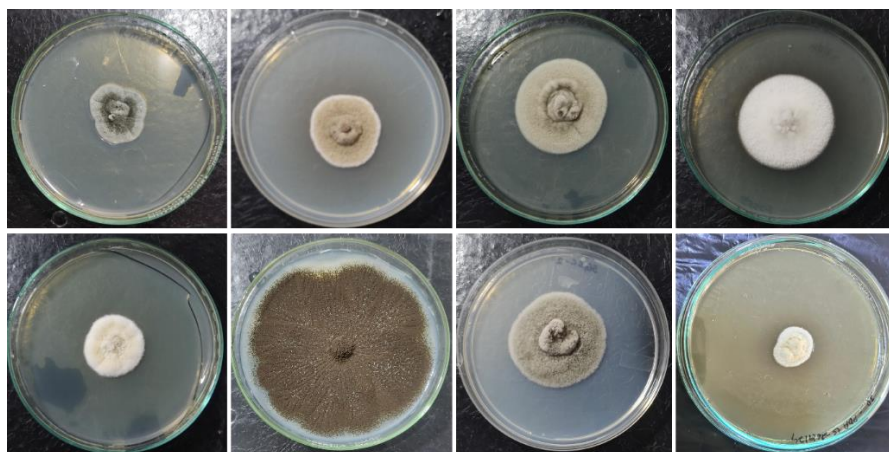


Fig. 5: Variety in morphology of endophytic fungi that are derived from leaves and stems of *A. marmelos*.

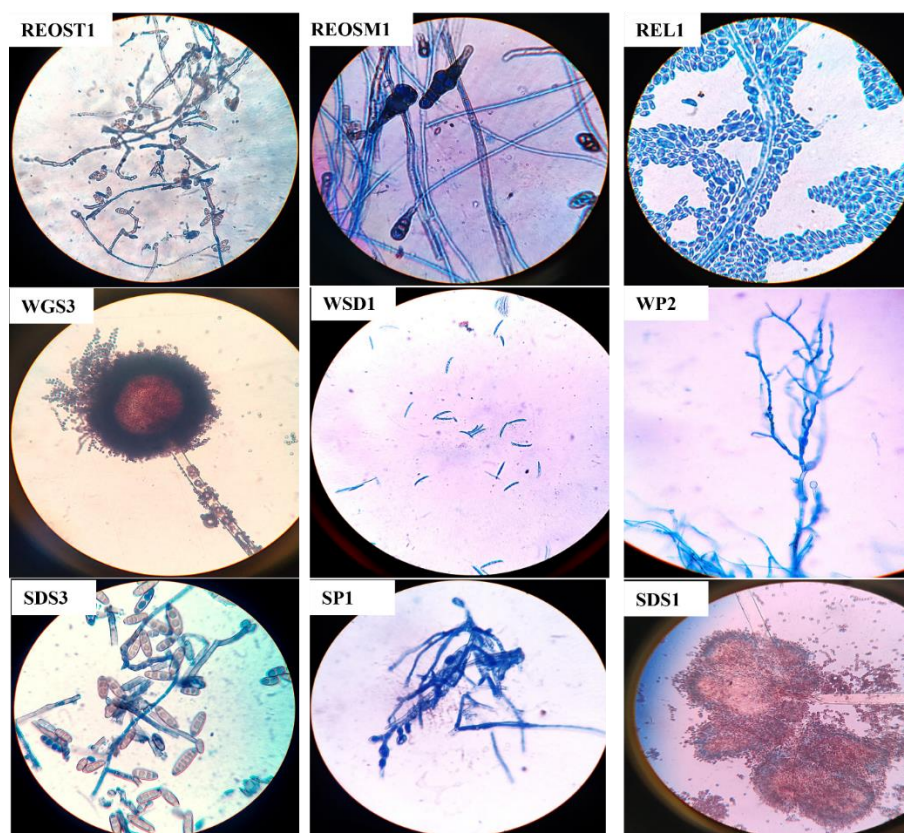


Fig. 6: The diversity of endophytic fungal species linked to the plant is highlighted by the microscopic observation of stained endophytic fungi isolated from *Aegle marmelos*, which reveals unique morphological characteristics such as spore structures, hyphal arrangements and conidia

Extracellular Enzymatic estimation: The enzymatic activity profiles of various fungal cultures tested for amylolytic, proteolytic, laccase, cellulase and lipolytic activities. Table 3 provides a comparative summary of positive and negative results for these enzyme types across different fungal isolates, highlighting the metabolic diversity among the cultures. Fig. 6 showcases representative enzymatic activity assays, with visible zones of substrate degradation or colour changes indicating positive enzymatic activity. These results underline the potential of these fungi for biotechnological applications based on their enzymatic capabilities.

Discussion

These findings are consistent with earlier research showing that humid circumstances provide ideal growing habitats for endophyte variety²⁷. According to White et al²⁸, histological localization demonstrated that endophytes were present in both intracellular and intercellular areas of plant tissues, highlighting their potential function in boosting plant resistance to pathogens and environmental challenges. Additionally, the isolated endophytes' enzymatic activities such as the synthesis of cellulase, amylase and protease indicate that they play a part in macromolecular breakdown, promoting the cycling of nutrients within plant tissues.

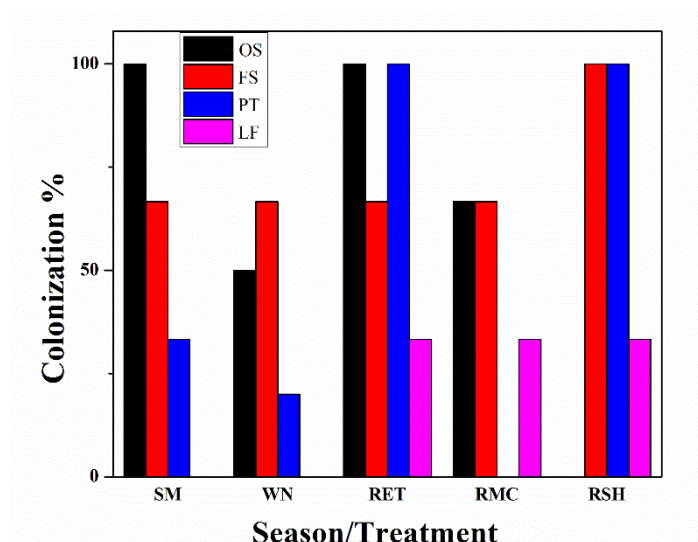


Fig. 7: The frequency of fungal colonization varies with the season, peaking during the rainy season, suggesting ideal conditions for endophyte growth. The chemical treatments given to plant samples during the various seasons are represented on the x-axis (SM: Summer, WN: Winter, RET: Rainy Season with Ethanol Treatment, RMC: Rainy Season with Mercuric Chloride Treatment, RSH: Rainy Season with Sodium Hypochlorite Treatment) and the percentage of colonization frequency is displayed on the y-axis. The graph illustrates the impact of both seasonal fluctuations and chemical treatments on fungal colonization and it is presented according to colonization frequency data.

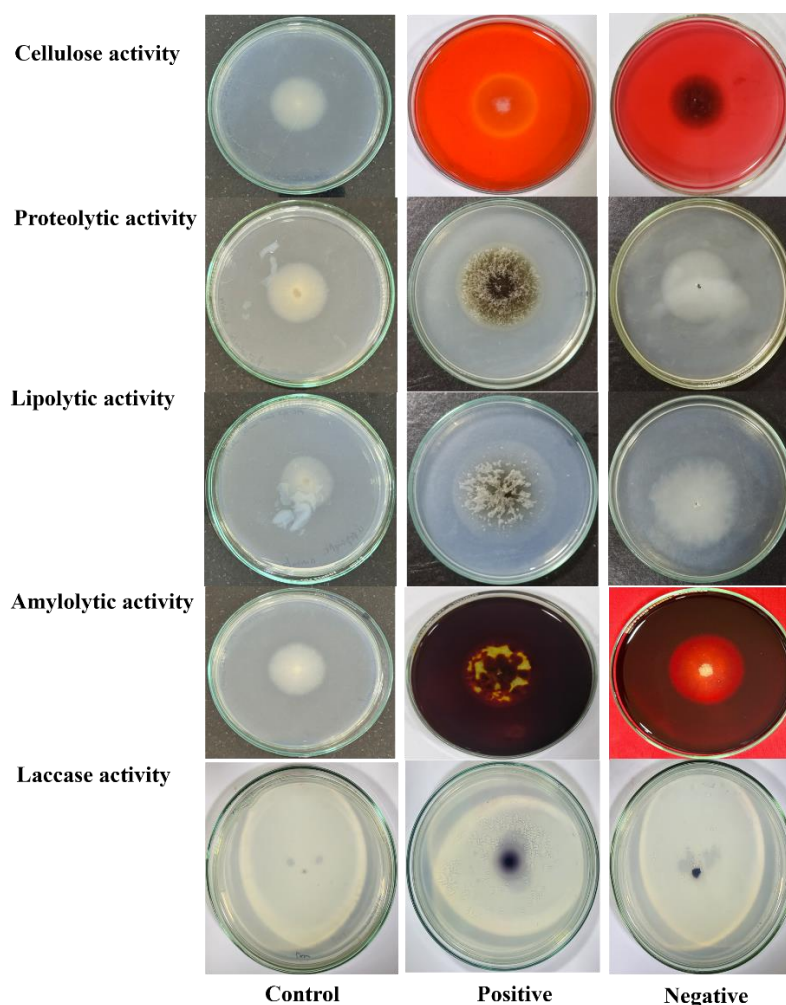


Fig. 8: Enzymatic activity assays of fungal cultures, showing differences in the activities of cellulase, lipase, laccase, amylolytic and proteolytic enzymes on their respective substrates

Table 2
Seasonal Isolation of Fungal Taxa from *Aegle marmelos* Tissues

S.N.	NAME	Reverse	Front	Morphology	Plant Tissue	Season	Environmental Conditions
1.	RELM-1	BLACK	BROWN	Septate Hyphae	Petiole Leaf Stem	Rainy	Temperature:29.3°c Humidity:69.97 %
2.	REL-1	BROWNISH PEACH	BROWNISH PEACH	Spores			
3.	REYSM-1	BROWN	WHITE	Hyphae			
4.	REYSM-2	BROWNISH RED	WHITISH YELLOW	Hyphae			
5.	REYSM-3	BLACK AND BROWN	GREENISH BLACK	Spores			
6.	REYSM-1 OC	BROWN	WHITISH ORANGE	Conidial Hyphae			
7.	REOST-1	BLACK	BLACK	Spores			
8.	REOST-2	BLACK	BROWN	Spores			
9.	REOSM-3	BLACK	BLACK	Spores			
10.	REP-1	PALE YELLOW	BROWN	Conidial Hyphae			
11.	RMCYSL-1	YELLOW	WHITE	Hyphae			
12.	RMCYSL-2	BROWN	PALE YELLOW	Conidial Hyphae			
13.	RMCYSL-3	BROWN	WHITE	Hyphae			
14.	RMCYST-1	BLACK	GREENISH BLACK	Hyphae			
15.	RMCOSM-3	BLACK	BLACK	Spores			
16.	RMCOST-1	ORANGE	ORANGE	Spores			
17.	RMCOST-2	BLACK	OFF WHITE	Conidial Hyphae			
18.	RSHYM-1	DARK BROWN	DARK BROWN	Sporulated			
19.	RSHYM-2	GREENISH	ORANGE	Hyphae			
20.	RSHYM-3	LIGHT BROWN	WHITE	Conidial Hyphae			
21.	RSHYM-4	BROWNISH ORANGE	BROWNISH ORANGE	Conidial Hyphae			
22.	RSHYT-1	BROWN	WHITE	Conidial Hyphae			
23.	RSHYT-2	BROWISH PEACH	BROWISH PEACH	Spores			
24.	RSHYT-3	BROWN	GREY	Conidial Hyphae			
25.	RSHOS-1	BROWN	WHITISH ORANGE	Conidial Hyphae			
26.	RSHOS-2	BLACK	BLACK	Spores			
27.	RSHOS-3	BROWN	WHITE	Hyphae			
28.	RSHP-1	LIGHT BROWN	OFF WHITE	Hyphae			
29.	RSHP-2	BROWNISH RED	WHITISH GREY	Hyphae			
30.	WYS1	BROWN	LIGHT BROWN VELVET	Conidial Hyphae	Petiole Leaf Stem	Winter	Temperature:17.74°c Humidity:64.15%
31.	WYS2	BLACKISH GREY	COTTONY GREY	Hyphae			
32.	WYS3	BROWN	COTTONY OFF WHITE	Conidial Hyphae			

33.	WYS4	BROWN	WHITE COTTONY	Hyphae			
34.	WOS1	WHITE	BLACK	Spores			
35.	WOS2	WHITE	ORANGE	Spores			
36.	WP3	GREY	GREY WITH WHITE BOUNDARY COTTONY	Conidial Hyphae			
37.	WP4	BLACKISH GREY	GREY	Hyphae			
38.	WP1	GREY	GREY	Conidial Hyphae			
39.	WP2	BLACKISH GREY	WHITE COTTONY	Septate Hyphae			
40.	SYS1	LIGHT BROWN	OFFWHITE	Hyphae	Petiole Leaf Stem	Summer	Temperature:36.88°C Humidity:42.99%
41.	SYS2 (1)	BROWN	WHITE	Hyphae			
42.	SYS2(2)	BLACK	WHITISH GREY	Branched Hyphae			
43.	SYS3	BROWN	WHITE	Hyphae			
44.	SYS4	BROWN	WHITE	Hyphae			
45.	SP1	GREYISH BLACK	GREYISH BLACK	Septate Hyphae			
46.	SOS1	BLACK	BLACK	Spores			
47.	SOS2(1)	BROWN	BLACK	Spores			
48.	SOS2(2)	BLACK	BLACK	Spores			
49.	SOS2(3)	BLACK	BROWN	Spores			
50.	SOS2(4)	BLACK	BROWN	Spores			
51.	SOS2(5)	BLACK	BROWN	Spores			

Table 3

Enzymatic Activity Profile of Five Important Enzyme Types in Various Fungal Cultures

S.N.	Fungal culture	Amylolytic	Proteolytic	Laccase	Cellulose	Lipolytic
1.	WP1	+	+	+	+	+
2.	WP2	+	+	-	+	+
3.	WP3	+	-	+	+	+
4.	WP4	+	+	+	+	+
5.	WYS1	-	-	-	-	-
6.	WYS2	+	+	+	+	+
7.	WYS3	+	+	-	+	+
8.	WYS4	+	+	+	+	+
9.	WOS1	+	+	-	-	+
10.	WOS2	-	+	-	+	+
11.	SYS3	+	-	-	+	-
12.	SYS2(2)	+	+	-	+	+
13.	SYS4	+	+	-	+	+
14.	SYS2(1)	+	-	-	+	-
15.	SOS1	-	+	-	+	+
16.	SOS2(1)	+	+	+	-	+
17.	SOS2(2)	+	+	+	-	+
18.	SOS2(3)	+	+	+	-	+
19.	SP1	+	-	+	+	+
20.	REYSM-3	+	+	+	-	+
21.	REOST-1	-	+	-	+	+
22.	REOST-2	+	+	+	-	+
23.	RSHLL1	-	-	+	+	-

24.	RSHYT1	-	+	-	-	-
25.	RSHYM1	+	-	-	--	+
26.	RM COSM	-	+	-	-	-
27.	RM CYSL1	-	+	-	+	-
28.	RM CYS2(F)	+	+	-	-	+

Similar research on microbial enzyme production has shown that such enzymatic capabilities are not only essential for plant-endophyte interactions but also have potential for industrial uses^{4,9}. Amylolytic enzymes were successfully produced, purified and immobilized in another study, which also showed how stable and effective they were in hydrolyzing polysaccharides. This demonstrated the potential of enzymatic applications, in particular, immobilized enzymes, in promoting environmentally friendly methods of producing bioethanol²⁴. Through the process of photosynthesis, plants are the primary source of the cellulose pool in the biosphere. Consequently, it is the primary component of plant biomass, with lignin and hemicellulose coming in second and third^{18,22}.

The use of cellulases in biomass hydrolysis and biofuel production is presently the focus of several projects funded by various organizations worldwide¹. *Trichoderma sp.*, *Geocladium sp.*, *Chaetomium sp.* and *Penicillium sp.* are among the many cellulolytic fungi that are recognized to be important in agriculture because they improve seed germination, accelerate plant growth and flowering, strengthen root systems and boost crop yields^{27,28}. These results offer a foundation for investigating endophytes as a source of bioactive substances for long-term biotechnological fixes.

Conclusion

This study emphasizes how important endophytic fungi are to the ecological and metabolic processes of the medicinally important plant *Aegle marmelos* (L.) Correa. A thorough examination showed that the plant's leaves, stems and petioles were colonized by a varied collection of fungal endophytes. The diversity and frequency of endophyte colonization were greatly impacted by seasonal fluctuations, with the rainy season producing the greatest number of taxa. This implies that fungal communities are significantly shaped by environmental factors like temperature and humidity.

The spatial distribution of fungal endophytes within plant tissues was elucidated through the use of lactophenol cotton blue staining in histological localization procedures. The intimate symbiotic or mutualistic relationship between *Aegle marmelos* and its endophytes is highlighted by the observation of mycelium and spores in the mesophyll and stem layers. The plant's adaptability, resilience and therapeutic potential are probably influenced by this relationship. Significant metabolic capacities such as amylolytic, proteolytic, cellulolytic and lipolytic activities, were discovered by enzymatic profiling of the isolated endophytes.

These enzymes, which are necessary for the breakdown of macromolecules, demonstrate the potential of these fungi in a range of industrial settings including bio-remediation, food processing and pharmaceuticals. Furthermore, endophytes' capacity to generate extracellular enzymes emphasizes their function in promoting connections between plants and fungus and presents a promising direction for biotechnological research.

The study concludes by highlighting the fungal endophytes' ecological significance and biotechnology potential in *Aegle marmelos*. The results offer a solid basis for further investigation into using endophytes as a source of new bioactive substances and long-term fixes for industrial and therapeutic uses. Further investigation into these microbial populations may lead to novel developments in biotechnology, medicine and agriculture.

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