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Fungal endophytes associated with actinorhizal plant species, *Elaeagnus latifolia* (*Elaeagnaceae*) and evaluating their antagonistic potential against grey blight disease in tea [causal agent: *Pestalotiopsis theae*] in North-East India

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Abstract

The current investigation explores the antagonistic potential of endophytic fungal diversity associated with *Elaeagnus latifolia* L., an actinorhizal plant species of North-east India against *Pestalotiopsis theae*, the causal agent of grey blight disease in tea. A total of 17 endophytic fungal isolates belonging to 14 different families and three phyla were isolated from various parts of the target plant species. The fungal isolates were characterized using a culture-based approach and microscopic tools and techniques, with particular emphasis on colony shape, size, spore formation characteristics, and fruiting body features. *Nigrospora* sp. showed the highest species density (0.5) amongst all of the fungal isolates. The highest isolation frequency (67%) was observed for *Fusarium* sp., *Nigrospora* sp., *Penicillium chrysogenum* and *Rhizopus* sp. A higher percentage of fungal isolates were obtained from the roots and stems (47% each) compared to leaves (29%). The maximum species richness and diversity indices were observed in stems (15.0), while the minimum values were found in leaves (9.0). The stem had the highest Shannon and Simpson diversity indices (2.02 and 0.860, respectively), followed by the root (1.979 and 0.847, respectively) and the leaves with the lowest values being 1.494 and 0.75, respectively. Among the fungal isolates tested for plant growth-promoting traits, such as phosphate solubilization, starch hydrolysis and efficiency in zinc solubilization, the isolate EF09 showed positive responses for all the tested parameters. The isolate EF09 showed maximum antifungal potential (up to 87.1%) against the significant tea pathogen, *P. theae*, in poisoned food technique *in vitro*. As there exists limited research related to hitherto-unexplored fungal endophytes associated with endemic plant species like *E. latifolia*, the current investigation holds sufficient promises in the isolation of novel

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bioactive molecules from these microbial isolates that might be useful in agriculture to develop sustainable strategies in plant protection in tea.

Keywords – Bioactive molecules – Culture-based approach – Endophytic fungal diversity – Plant growth-promoting traits – Plant protection – Species richness and diversity indices.

Introduction

Endophytes are microorganisms inhabiting healthy plant tissues for all or part of their life cycle without causing apparent detrimental symptoms to the host (Rani et al. 2022). Endophytism is, thus, a symptomless association of beneficial microbes within the plant endosphere (internal plant tissues harbouring a diverse microbiome) (Bhattacharyya et al. 2017, Solanki et al. 2023). Microorganisms known as endophytic fungi invade intracellular and/or extracellular parts within the plant tissues (leaf, seed, stem, trunk, roots, fruits, and flowers) without affecting the host plant integrity. It is anticipated that almost all terrestrial plants are colonized by one or more endophytic fungal species (Dos Reis et al. 2022), which have been isolated from varied plant species across diverse climates and topographies. Plants colonized by endophytic fungi are known to grow faster than non-colonized ones (Baron & Rigobelo 2021), suggesting a long-standing, close, and mutually beneficial plant-microbe interaction (Orozco-Mosqueda & Santoyo 2021). Zhang et al. (2024) reported that endophytic fungi harboured in medicinal plants substantially prepare the host to adapt to extreme environments, including abiotic stress-related conditions. According to the researchers, the fungal communities harboured inside the healthy plant tissues play influential roles in enhancing the absorption of major soil nutrients (Bhattacharyya & Jha 2015), thereby assisting in the maintenance of biogeochemical cycles (Bhattacharyya et al. 2012, Sarkar et al. 2021, Fite et al. 2023) for better soil health and crop prosperity.

Due to their immense potential in conferring plants with the ability to adapt to diverse stress and produce industrially and pharmaceutically active compounds, this group of fungi has become an important component of modern human civilization (Tiwari & Bae 2022). Interest has, therefore, been exaggerated in the production of bioactive compounds from endophytic fungi and their potential to produce pharmacologically active substances with unique health benefits against multiple human ailments, including cancers, diabetes, and neurological disorders. Additionally, microbial cell factories (MCFs), including fungi, are researched for plant growth-promoting (PGP) potential, production of toxins and enzymes, and insecticidal and immunosuppressive properties (Sarma et al. 2021, Omomowo et al. 2023). Waqar et al. (2024) suggest that plants can derive indirect advantages from endophytic fungi through various means such as competing for space or nutrients, mycoparasitism, and activating defence mechanisms. These defence mechanisms involve the production of secondary metabolites and the induction of systemic resistance (ISR) against a diverse array of plant pests and phytopathogens (Bhattacharyya et al. 2023a).

Investigations on endophytic microbe-mediated agricultural development (Burragoni & Jeon 2021) show substantial positive findings in the sustainable improvement of PGP potential and prominent microbial antagonism against phytopathogens. Endophytic fungi, including *Aspergillus flavus, A. niger, Fusarium oxysporum, Penicillium* spp., and *Rhizopus stolonifer* isolated from diverse plants, were reported for their abilities to produce different PGP phytohormones (Waqas et al. 2012, Poveda et al. 2021) and mineral solubilizing activities, including the solubilizations of phosphate, zinc and potassium (Bhattacharyya & Jha 2012). It is, thus, noteworthy to mention that due to their ubiquitous distribution in plant tissues, a vast majority of plants are recognized as prime repositories of fungal endophytes with novel metabolites of pharmaceutical and agricultural importance (Gauda et al. 2016). It has recently been reported that even though most endophytes are host-specific, certain groups of endophytic fungi possess a greater occurrence in some plants, indicating the host-preference nature of this unique microbiome (Manganyi & Ateba 2020).

North-east India serves as the biogeographical gateway for greater India (Bhattacharyya & Jha 2014) and is recognized as a biodiversity hotspot, renowned for its potential genetic resources, globally. *Elaeagnus latifolia*, belonging to the family *Elaeagnaceae*, is locally known as *Soh-shang*

in the Khasi hills of Meghalaya and *Mirika Tenga* in Assam (Bhattacharyya & Bhattacharyya 2021). It is a large evergreen spreading woody shrub with rusty-shiny scales that often become thorny. The flowers are usually hermaphrodite and are pollinated by bees. Indigenous people have found many uses for *Soh-shang* fruits, besides enjoying them as fresh fruit (Seal 2011). Fruits are eaten raw and could be utilized for making jam, jelly and refreshing drinks. The fruit is considered to be a very rich source of vitamins, essential fatty acids, minerals, and other bioactive compounds. However, very few attempts have been made to enumerate the endophytic fungal assemblages associated with this economically important actinorhizal plant species of Northeast India, which gradually become endemic with restricted geographic distributions and habitat destruction. Being a natural host of beneficial microbes like endophytes, endomycorrhizae, and microfungal species, including nitrogen-fixing bacteria like *Frankia* sp. and *Pseudomonas* sp., this plant harbours potential uses for soil restoration activities as well as habitat reconstruction for development of ecological succession (Bhattacharyya et al. 2023b). Considering these points, the present investigation has been designed to isolate the hitherto-unexplored endophytic fungal diversity associated with *Elaeagnus latifolia*, which could be of key importance in developing sustainability in plant protection, drug discovery and other agriculture and industry-related components.

Furthermore, as grey blight disease in tea (causal agent: *Pestalotiopsis theae*) is creating enormous devastation in the northeastern tea industry, the adoption of sustainable solutions against the serious tea pathogen seems urgent. The most prevalent symptoms of *Pestalotiopsis theae* in tea leaves are light to dark brown disease patches with a greyish center on the upper surface and concentric zonation that extends from the centre almost to the very edge of the field (Borah et al. 2024). Management of grey blight pathogenesis in tea using chemical fungicides is a hasty approach utilized by small and marginal farmers across the globe, as it could inhibit the growth of the phytopathogen with minimal effort. Nevertheless, the excessive and thoughtless use of chemical fungicides has elevated the risk of environmental pollutants in soil and environment and the creation of maximum residue limits (MRLs) (Bhattacharyya et al. 2018, Sarmah et al. 2020, Bhattacharyya et al. 2024a) due to the increased incidence of free and reactive chemical substances. Agrochemicals, including chemical fungicides, have been proven to deteriorate the quality of soil, destabilize the dynamics of beneficial microbial populations (Bhattacharyya et al. 2024b; Bhattacharyya et al. 2024c), and hinder the activities of important soil enzymes. Bhattacharyya et al. (2023b) and Mahanta et al. (2023) have shown a relationship between the agrochemical side effects and decreased soil fertility and toxicity development. This investigation, thus, aims to achieve sustainability in plant protection in tea by screening endophytic strains from *E. latifolia* against *P. theae*, the causal agent of grey blight pathogenesis in tea. Isolation and screening of endophytic microbes against significant tea pathogens, including *P. theae*, *Sphereostilbe repens* (causal agent of violet root rot disease) and *Fusarium solani* (causal agent of Fusarium die-back disease), have been performed by Bora & Barthakur (2013). The researchers used a culture-dependent approach to grow and characterize the endophytic microbial isolates based on culture characteristics and colony morphology, and measurement of spores and fruiting bodies using microscopical tools and techniques. The ability of endophytes to produce secondary metabolites with novel structures creates the potential for using such microbial biopesticides in plantation crops like tea. According to tea experts, since the majority of chemical pesticides and antibiotics are currently prohibited for use in tea (Borah et al. 2024), the current approach offers enormous potential for the development of effective antagonistic molecules and products from isolated endophytic microbiomes that might be useful in developing integrated disease management (IDM) programs against grey blight pathogenesis under field evaluation. Naturally occurring endophytic strains inhabiting *E. latifolia* may inhibit the growth of target phytopathogens through hyperparasitic/antagonistic activities. The establishment of large numbers of metabolically active populations of endophytic microbes seems essential for rapid success in developing climate-smart tea cultivation. Determination of colonization frequency and diversity indices of endophytic microbes associated with the endemic plant species *E. latifolia* with putative endophytism is also studied in the current investigation, which holds significant perspectives in agriculture and forestry.

Materials & Methods

Collection of the plant specimen and identification

A field survey was carried out at three distinct locations in North-east India: Jorhat, Sivasagar, and Meghalaya (Fig.1). Jorhat is a biodiverse zone in Assam with a geographical area of 2859.3 sq. km, located between 26.46 °N latitude and 96.16 °E longitude. Sivasagar is located at 26.45 °N latitude and 95.25 °E longitude, while Meghalaya is located at 25.65 °N latitude and 91.88 °E longitude (Fig. 1). The purpose of the survey was to collect the target plant species, *Elaeagnus latifolia* (Fig. 2), which is known for its abilities to form symbiotic relationships with multiparous beneficial microbiotas. The collected plant specimens were carefully pressed, dried, and mounted on herbarium sheets for further study and reference. The plant specimen was identified as *Elaeagnus latifolia* with the assistance of senior scientists from the Botanical Survey of India (BSI), Shillong, Meghalaya, India.

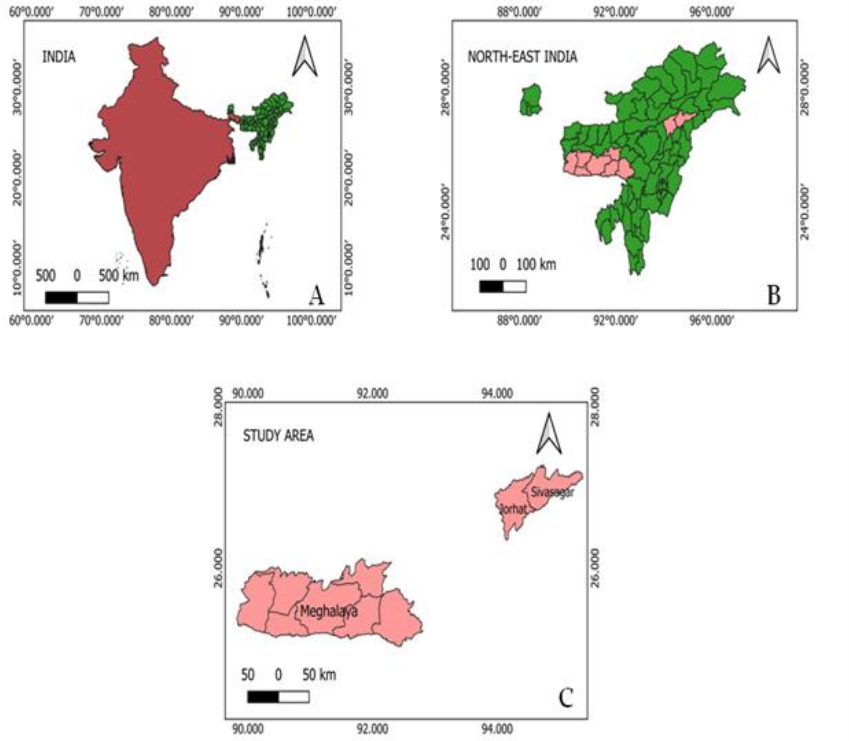


Fig. 1 – Map showing the sampling locations. A India map highlighting the north-eastern region (green in colour). B Map showing the North-East India indicating survey locations (light pink in colour). C Locations of field survey (Jorhat district, Sivasagar district and states of Meghalaya).

Isolation of endophytic fungi

Pre-sterilized plastic bags were used for the collection of plant samples. Excess moisture was removed during the sampling. The leaf, root and stem samples were stored at 4 °C for further microbial characterization and analysis. Asymptomatic healthy plant materials were thoroughly washed in tap water, followed by a three-step surface sterilization technique using standard protocol (Coombs & Franco 2003). For this, the selected plant segments were immersed in 99% ethanol for 60 seconds, followed by exposure to 3.125% sodium hypochlorite (NaOCl) solution for 6 minutes. The segments were finally treated with 90% ethanol for 30 seconds. Sterilized distilled water (SDW) was used to rinse the surface sterilized segments. The segments were then allowed to dry under sterile conditions. Two different protocols were used for the isolation of endophytic strains from the target plant species. In one method, leaves, stems and roots were cut to uniform sizes (5 mm) and plated on endophytic specific low nutrient isolation media like synthetic low

nutrient agar (SNA) and Carnation leaf agar (CLA) (Arnold & Herre 2003) supplemented with streptomycin (100 mg/L) to suppress the bacterial growth while the other protocol follows plating of ground plant samples on SNA media. The ground plant sample was mixed with 100 ml SDW to get the homogenous suspension and plated accordingly using the serial dilution plate (SDP) technique. Culture plates were carefully sealed with parafilm and incubated at 30 ±1°C in a Biochemical Oxygen Demand (BOD) incubator for 5–seven days. Regular monitoring of culture plates is required to measure the biological growth of the endophytic fungal colonies. Fungi grown in the samples were transferred onto fresh potato dextrose agar (PDA) plates and respective broth cultures (potato broth medium) for isolation and characterization of pure colonies.

Fig. 2 – Photographs showing the natural habitat of the target plant, and field survey for sample collection for enumeration of endophytic fungal assemblances. A Natural habitat of the plant specimen. B Sample collection. C Field survey and enumeration of endophytic fungal assemblances in *E. latifolia*.

Identification of endophytic fungal isolates

Laboratory microbial accession numbers were given for data recording purposes to each fungal isolate recovered in the current investigation. Microfungal species details like their area and substrate of isolation, colour, smell, nature of hyphae and spores and other key morphological features like size, shape and appearances, including the colony diameter, texture, and elevations were recorded during their growth in the nutrient media using microscopic tools, techniques and standard taxonomic monographs (Watanabe 1993, Sarmah et al. 2006, Dhayanithy et al. 2019, Jayatilake et al. 2020).

Determination of plant growth-promoting traits

Isolation of phosphate solubilizers

To isolate the phosphate solubilizing endophytic fungal isolates, the individual fungal colonies grown on PDA media were picked up and subsequently inoculated onto Pikovskaya's agar medium (Pikovskaya 1948, Bhattacharyya 2012, Doilom et al. 2020, Goenadi et al. 2020) containing insoluble tricalcium phosphate and incubated at 30 ±1°C for 5–7 days. A positive test for phosphate solubilization was indicated by the formation of transparent halos surrounding the microbial colonies on Pikovskaya agar medium after 4–5 days of incubation. All of the possible phosphate solubilizers had their phosphate solubilization index (SI) determined, and the pure cultures were examined for growth patterns and colony morphology in accordance with Lelapalli et al. (2021).

Phosphate solubilization index (SI) = $\frac{\text{Total diameter of the halo zone + microbe colony diameter}}{\text{Total microbial colony diameter}}$

Starch hydrolysis

For the starch hydrolysis test, the fungi were first inoculated on PDA media supplemented with starch and then incubated at 37 ±1°C for 4–6 days. The development of a clear zone around the microbial colonies indicated the hydrolysis of starch (Bhattacharyya 2012).

Determination of Zn solubilization efficiency *in vitro*

Actively growing cultures of each isolated strain were spot-inoculated (approx. 3 μL) onto the PDA plates and incubated at 30 ±1°C for 72–96 h. The halo zone around the microbial colony was determined. Repeated streaking on culture media was done for confirmation of the halo zone and to determine the zinc solubilizing potential. The diameter of the microbial colony (B) and the clear zone around the colony (A) were measured to calculate the solubilization efficiency as a percentage and area in mm^2. Zn solubilization efficiency was calculated using the following formula (Bhattacharyya et al. 2022):

$$\text{Solubilization efficiency (SI)} = \frac{\text{Solubilization diameter (A)}}{\text{Diameter of the colony growth (B)}} \text{ X } 100$$

Quantitative Analysis

Quantitative analysis of the parameters like density, frequency and abundance of the endophytic fungal populations were determined in accordance with Curtis & McIntosh (1950), Verma et al. (2019) and Harrison & Griffin (2020).

(a) Density

Endophytic population density is calculated using the following equation

$$\text{Density} = \frac{Total\ number\ of\ individuals\ of a\ species\ in\ all\ compartments}{Total\ number\ of\ compertments\ studied}$$

(b) Frequency (%)

The following formula was used to determine the colonization frequency.

I. Percentage of frequency (%) Compartment-wise:

$$\text{Frequency (\%)} = \frac{Total\ number\ of\ compartments\ in\ which\ the\ species\ occured}{Total\ number\ of\ compertments\ studied} \times 100$$

II. Percentage of frequency (%) Sample-wise:

$$\text{Frequency (\%)} = \frac{Number\ of samples\ in\ which\ a\ particular\ species\ was\ recorded}{Total\ number\ of\ samples} \times 100$$

(c) Abundance

Endophytic fungal abundance is represented by the following equation:

$$\text{Abundance} = \frac{Total\ number\ of\ individuals\ of\ a\ species\ in\ all\ compertments}{Number\ of\ compertments\ in\ which\ the\ species\ occured}$$

(d) Percentage of occurrence (%)

The density and distribution of arbuscular mycorrhizal (AM) fungi in rhizosphere soil were

expressed in terms of percentage of occurrence in accordance with Hu et al. (2020).

$$\text{Percentage of occurrence}(\%) = \frac{Total\ number\ of spores\ of\ individual\ species}{Total\ number\ of\ fungal\ spores} \times 100$$

Determination of colonizing frequency (CF %)

The colonizing frequency (CF %) of endophytic fungal species was calculated using Hata and Futai (1955) and Debbarma et al. (2024)

$$\text{colonizing frequency (CF \%)} = \frac{Number\ of\ segments\ colonized\ by\ an\ endophytic\ fungal\ species}{Total\ number\ of\ segments} X\ 100$$

Species richness and diversity indices

The total number of species recovered and endophytic fungal species richness were determined after identifying the fungal spores using the following formula:

$$S = n + \left(\frac{n-1}{n}\right) k$$

Where, S = Species richness
n = Total number of species present in the sample population
k = Number of "unique" species

Diversity index

Diversity index was computed using the following formula:

$$D = \frac{\sum n(n-1)}{N(N-1)}$$

Where, D = Diversity Index
N = Total number of organisms of all species found
n = Number of individuals of a particular species

Simpson's index of diversity = 1–D

Where, D = € $(n / N)^2$
n = Total number of organisms of a particular species.
N = Total number of organisms of all the species.

Shannon-Wienner diversity index, H_s = – € (Pi) (ln Pi)

i = 1

Where, H_s = Symbol for the diversity in a sample of S species or kind.
S = The number of species in the sample.
Pi = Measures the relative abundance of i^{th} species or kinds = ni/N
N = The total number of individuals of all kinds.
ni = The number of individuals of i^{th} species.
ln = log to base 2.

***In vitro* antimicrobial bioassay**

Preliminary screening of antimicrobial agent-producing endophytic microorganisms (AAPEMs) was made using a modified cross-streak method, as mentioned in Jemimah et al. (2012). For this, a loopful of microbial inoculums (endophytic fungi) was streaked in the centre of the petri dish containing PDA medium and incubated at 28 ±1ºC for 4–5 days. The 48–72h-old target pathogen (here, *P. theae*, responsible for grey blight disease in tea) was cross-inoculated into the growth line of the fungi in the same petri dish at 28 ºC for 48–72 h. The inhibitory zones produced by the endophytic fungus against the target pathogen were measured in millimeters (mm). The pathogen isolated by a team of tea microbiology experts and well maintained at Microbial Culture Collection Laboratory (MCL), Tocklai Tea Research Institute, TRA, Jorhat, Assam, India was used in the current investigation to perform an antagonism study. Light to dark brown disease patches in tea leaves with a greyish centre on the upper surface marked with concentric zonation almost from the centre to the very edge in field survey were selected for pathogen isolation (Fig. 3), characterization and further antimicrobial bioassay. On old leaves, the pathogen usually enters through damage incidences like cut, any injury or break or a bruise on the leaf blade, while on young leaves, the symptomatic patch is usually dark brown to almost black rather irregular in shape and not marked with concentric rings.

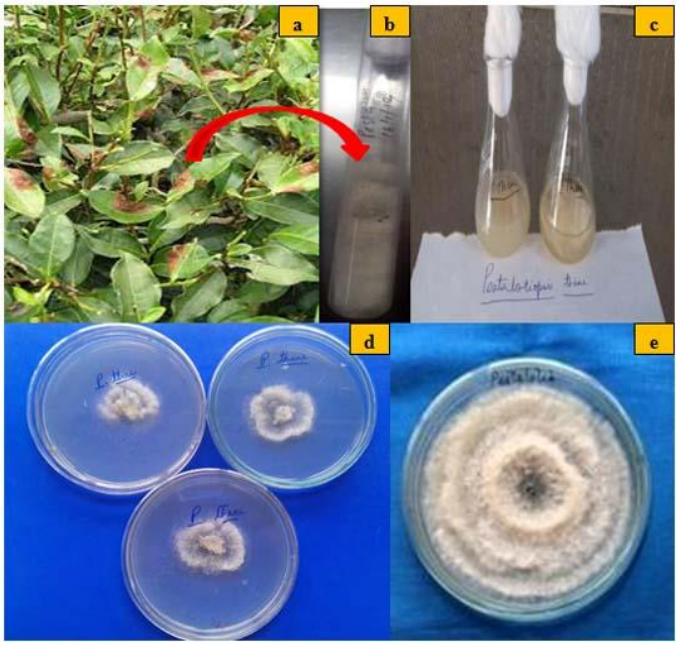


Fig. 3 – Field characterization of *P. theae* infected tea leaves and growth pattern of the phytopathogen on PDA plates *in vitro*. a Infected tea leaves with *P. theae*. b Growth behaviour in laboratory conical flasks. c–d Pure culture of the phytopathogen ready for *in vitro* antimicrobial bioassay. e A full-grown culture of the target pathogen *P. theae.*

The poisoned Food technique as described by Balouiri et al. (2016) and Dutta et al. (2018) was followed to assess the antimicrobial activity of the potent endophytic fungal strains against *P. theae*. For this, the endophytic microbial cultures showing antagonistic properties on agar plates were grown on 250 ml PDB medium and incubated in a BOD shaking incubator at 28±1 °C up to 15–21 days with periodic shaking at 250 rpm, after which the fungal cultures were macerated using waring blender for 10 min. The macerate was then filtered through a double-layered muslin cloth and centrifuged properly. Whatman No. 1 filter paper (110 mm) was used to filter the supernatant. The obtained culture filtrate served as the crude extract with 100% spore concentration. An inoculum potential of 5% densities for each microbial culture was prepared using 10^6 spore/ml of the sample and plated accordingly to measure the fungal growth. The final concentration of the desired density (at 5% spore concentration) was incorporated into the molten agar and mixed well.

The medium was then poured into petri dishes followed by overnight incubation. Pathogen mycelia disc (5 mm) was then inoculated into the centre of the plate. Dimethyl sulfoxide (DMSO) served as negative control. The plates were incubated at 28±1°C for five days. A plate devoid of antifungal agents served as control. Three replicates were maintained for each of the treatments.

The antifungal efficacy of the biocontrol agent was estimated using the formula as mentioned in Dutta et al. (2018).

$$\text{Antifungal activity (\%)} = \frac{(Dc - Ds)/Dc)}{Dc} X\ 100$$

Here, Dc is the diameter of fungal growth in the control plate, and Ds is the diameter of fungal growth in the plate containing the tested antifungal agent.

Results

During the present investigation, a total of 17 endophytic fungal isolates belonging to 14 families and three phyla were recovered from various parts of the *E. latifolia* plant, as depicted in Table 1. All the isolates were microscopically observed while following standard taxonomy and monographs, as mentioned earlier. The fungal species were classified in their respective family and phylum. The colony morphology, agar slant culture characteristics, sporulation types, and microscopic examination reports, along with the taxonomy of the isolated fungal strains, have been represented in Table 2. Figure 4 (A–L) depicts a systematic examination for exploring the endophytic microflora of different parts of the plant *E. latifolia* using a culture-based approach. The growth habits of endophytic microbes on culture plates are shown in Fig 5 (a–i).

The isolated fungal endophytes are identified as *Absidia* sp., *Arthrobotrys* sp., *Aspergillus candidus*, *Aspergillus* sp., *Basidiobolus* sp., *Chrysosporium* sp., *Cladosporium* sp., *Cunninghamella* sp., *Fusarium* sp., *Mortierella* sp., *Nigrospora* sp., *Paecilomyces* sp., *Penicillium capsulatum*, *Penicillium chrysogenum*, *Pythium* sp., *Rhizopus* sp., and *Scopulariopsis* sp. There are also sterile forms recorded based on the microbial culture characteristics in the growth medium. Table 3 represents the diversity analysis of the fungal endophytes isolated from different parts of the target plant, *E. latifolia* along with the colonization frequency, fungal population abundance and percentage of occurrence. Out of all the endophytic microbial isolates, *Fusarium* sp., *Nigrospora* sp., *Penicillium chrysogenum* and *Rhizopus* sp. showed the maximum isolation frequency (up to 67%), followed by the rest of the species with 33% each. The investigation showed the highest endophytic fungal abundance (4.0) for *Basidiobolus* sp., followed by *Aspergillus candidus* and *Cunninghamella* sp. (3.0 for each) and the lowest (0.5) in *Nigrospora* sp. While the percent natural occurrence was measured as highest (maximum up to 12%) in cases of *Fusarium* sp., *Nigrospora* sp., *Penicillium chrysogenum* and *Rhizopus* sp., respectively (Table 3). Likewise, the highest species density (0.5) was observed in the case of *Nigrospora* sp. and the lowest (0.13 for each) in *Arthrobotrys* sp., *Aspergillus* sp., *Chrysosporium* sp., *Cladosporium* sp., *Cunninghamella* sp., *Paecilomyces* sp., *Penicillium capsulatum* and *Pythium* sp., respectively (Fig. 6). Regarding the occurrence of fungal endophytes in different parts of the target plant, it was evident that more fungal isolates were obtained from the root portion (47% colonization frequency), followed by stem (41%) and leaves (29%), respectively as depicted in Fig. 7. Isolation frequency of endophytic microbes using different media is represented in Table 4. The table indicates the highest isolation frequency of endophytic microbes in SNA media (12.01 cfu/ml) in the root portion and the lowest (6.97 cfu/ml) in the leaves. Species richness and diversity index showed maximum values in the stem (15.0); whereas minimum was recorded in the leaves (9.0) (Table 5). The highest Shannon and Simpson diversity index was observed in stems (2.02 and 0.860, respectively); followed by roots (1.979 and 0.847, respectively) and least in the leaves (1.494 and 0.75, respectively). During the present investigation, isolate EF09 showed positive results for different PGP parameters like starch hydrolysis, phosphate solubilisation, zinc solubilisation and IAA production, while the rest

of the strains are either able to hydrolysis starch, IAA production or solubilise phosphorous or zinc separately (Table 6). The isolates when examined for preliminary antagonistic screening by cross streak method using *P. theae* as the target pathogen, four isolates, EF03, EF04, EF09 and EF14 showed positive responses in growth and pathogen inhibition. Therefore, these microbes were, further, selected for antimicrobial evaluation against *P. theae* using the poisoned food technique as mentioned above. The growth habit and morphology of the endophytic fungal isolates selected for antagonistic screening are represented in Fig. 8 (A–D). Amongst the screened endophytic isolates, maximum antifungal potential against *P. theae* was exhibited by EF09 (87.1%) (Fig. 9), followed by EF03 (*Aspergillus candidus*) (43.4%), EF04 (*Aspergillus* sp.) (39.34%) and EF14 (*Penicillium chrysogenum*) (30.3%). Microbial and culture characterization of the potent endophyte is confirmed as *Fusarium* sp.

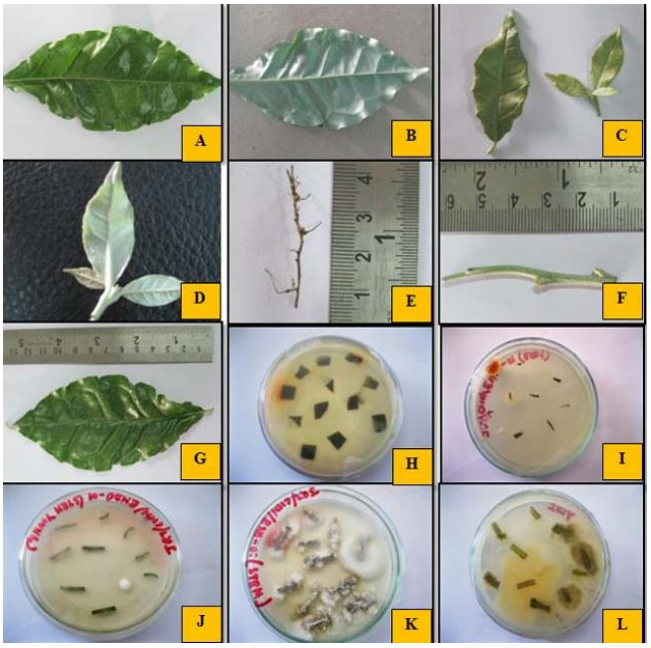


Fig. 4 – Different stages for preparing the plant parts for isolation of endophytic microflora using a culture-based approach

Table 1 Natural occurrence and diversity of endophytic fungi recorded in different parts of *E. latifolia*.

Isolate No.	Taxon	Phylum	Family	Stem	Leaves	Roots
EF01	*Absidia* sp.	Zygomycota	Cunninghamellaceae	+	-	-
EF02	*Arthrobotrys* sp.	Ascomycota	Orbiliaceae	-	-	+
EF03	*Aspergillus candidus*	Ascomycota	Trichocomaceae	-	+	-
EF04	*Aspergillus* sp.	Ascomycota	Trichocomaceae	-	-	+
EF05	*Basidiobolus* sp.	Zygomycota	Basidiobolaceae	-	-	+
EF06	*Chrysosporium* sp.	Ascomycota	Onygenaceae	-	+	-
EF07	*Cladosporium* sp.	Ascomycota	Cladosporiaceae	-	+	-
EF08	*Cunninghamella* sp.	Zygomycota	Cunninghamellaceae	+	-	-
EF09	*Fusarium* sp.	Ascomycota	Nectriaceae	+	-	+
EF10	*Mortierella* sp.	Zygomycota	Mortierellaceae	+	-	-
EF11	*Nigrospora* sp.	Ascomycota	Trichosphaeriaceae	+	+	-
EF12	*Paecilomyces* sp.	Ascomycota	Thermoascaceae	+	-	-
EF13	*Penicillium capsulatum*	Ascomycota	Aspergillaceae	-	-	+
EF14	*Penicillium chrysogenum*	Ascomycota	Aspergillaceae	-	+	+
EF15	*Pythium* sp.	Oomycota	Pythiaceae	-	-	+
EF16	*Rhizopus* sp.	Zygomycota	Mucoraceae	+	-	+
EF17	*Scopulariopsis* sp.	Ascomycota	Microascaceae	+	-	-

"+"= Present, "-" = Absent

Table 2 Morphological characteristics and microscopic examination of the isolated fungal strains.

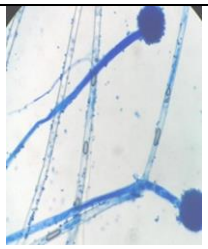
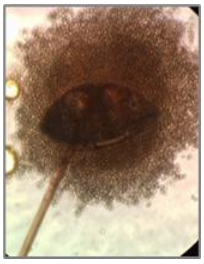
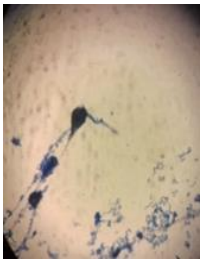
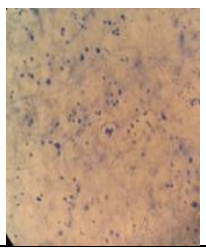
Sl. No.	Colony morphology, microscopic features and sporulation types	Microscopic observation	Identification	Taxonomy and systematic position
1.	Colonies exhibit distinct morphological features. Their growth behaviour is found to be rapid and fast-growing. Colonies are initially white, while at maturity, they become pale grey. Mature colonies are elevated and have a cottony texture. Sporangiophores are hyaline (translucent) to faintly pigmented, simple in nature. Sporangia are deliquescent-walled and have a stalk-like structure (apophysate)		Absidia sp.	Domain: Eukayota Kingdom: Fungi Division: Mucoromycota Class: Mucoromycetes Order: Mucorales Family: Cunnighamellaceae Genus Absidia sp.
2.	Colony appearance on agar slant is typically white to off-white in colour. Colonies have cottony growth. Rapidly growing fungi on media at optimum growth conditions like 25 ± 1°C. Sporangiophores are hyaline and can be simple or branched. Sporangia have a stalk-like appearance.		*Arthrobotrys* sp.	Domain: Eukaryota Kingdom: Fungi Division: Ascomycota Class: Orbiliomycetes Order: Orbiliales Family: Orbiliaceae Genus: *Arthrobotrys* sp.
3.	The colonies of this fungus show white velvety growth that becomes powdery over time. Fast-growing aerobic fungus, forming colonies within 24–48 hours on PDA media under optimal temperature, pH and suitable growth conditions. Conidia are round, rough and white in texture.		*Aspergillus candidus*	Domain: Eukaryota Kingdom: Fungi Division: Ascomycota Class: Eurotiomycetes Order: Eurotiales Family: Aspergillaceae Genus: *Aspergillus* Species: *A. candidus*
4.	Colonies are typically black or light bluish on the upper surface and reddish-gold on the lower surface. The spore surface is powdery and velvety. It is a fast-growing fungus, forming colonies within 7 days at optimum temperatures of 25 °C. The conidia are characteristically green. Spores are globose to sub-globose and yellow-green in colour.		*A. flavus*	Domain: Eukaryota Kingdom: Fungi Division: Ascomycota Class: Eurotiomycetes Order: Eurotiales Family: Aspergillaceae Genus: *Aspergillus* sp.
5.	Colony appears as flat, thin, and waxy. During maturity, the colonies turn greyish-brown. The fungal colonies are covered by fine, white, powdery mycelium. The reverse side of the colony appears as white or pale. Fast-growing fungi, forming colonies within a few days to week.		*Basidiobolus* sp.	Domain: Eukaryota Kingdom: Fungi Division: Entomophthoromycota Class: Entomophthoromycetes Order: Entomophthorales Family: Basidiobolaceae Genus: *Basidiobolus* sp.
6.	Fast growing species. Colonies are typically white in colour. Spores have a powdery texture. The reverse side of the colony is white to pale-yellow in appearance. Spores are powdery and have cottony growth characteristics.		*Chrysosporium* sp.	Domain: Eukaryota Kingdom: Fungi Division: Ascomycota Class: Eurotiomycetes Order: Onygenales Family: Onygenaceae Genus: *Chrysosporum* sp.

Table 2 Continued.

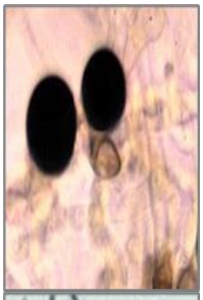
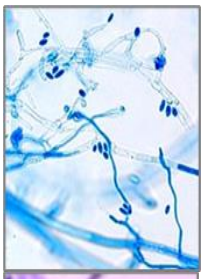
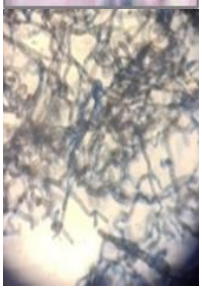
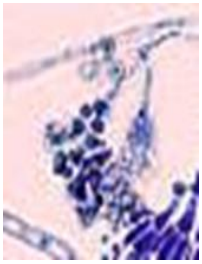
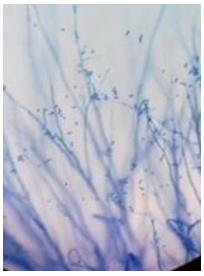
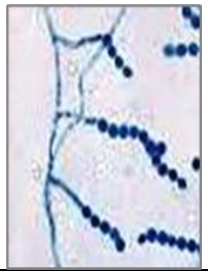
Sl. No.	Colony morphology, microscopic features and sporulation types	Microscopic observation	Identification	Taxonomy and systematic position
7.	Colonies are initially white while becomes grey and powdery in maturity. The reverse side of the colony is usually white to pale brownish-yellow with age. The fungus produces erect, straight, branched conidiophores.		*Cunninghamella sp.*	Domain: Eukaryota Kingdom: Fungi Division: Mucoromycota Class: Mucoromycetes Order: Mucorales Family: Cunninghamellaceae Genus: Cunninghamella sp.
8.	White to off-white zonation on culture media indicates its growth behaviour. The colonies are powdery or granular in nature. The reverse side of the colony is typically white or pale in appearance. Moderately fast-growing fungi. Aerial conidiophores develop.		*Mortierella* sp.	Domain: Eukaryota Kingdom: Fungi Division: Mucoromycota Class: Mortierellomycetes Order: Mortierellales Family: Mortierellaceae Genus: *Mortierella* sp.
9.	Colonies are white in appearance. Rapid growth of the fungus takes place after culturing for 3-5 days. White to grey colonies develops that possess woollen or hairy in texture. Conidiophores are translucent and produce conidia.		*Nigrospora* sp.	Domain: Eukaryota Kingdom: Fungi Division: Ascomycota Class: Sordariomycetes Order: Trichosphaeriales Genus: *Nigrospora* sp.
10.	Colony appears as white masses that becomes grey at maturity. Moderate growth rate at PDA, Colony surface is velvety. The reverse side of the colony is black. According to microscopic and micro morphometric observation, PDA media could provide abundant nutrient resources for vigorous growth and sporulation.		*Cladosporium* sp.	Domain: Eukaryota Kingdom: Fungi Division: Ascomycota Class: Dothideomycetes Order: Capnodiales Family: Davidiellaceae Genus: *Cladospora* sp.
11.	Colonies are white to pink or red both in front and reverse order. Exhibits woolly or cottony texture. Rapidly growing on PDA media. Conidiophores are specialized structures producing microconidia and macroconidia.		*Fusarium* sp.	Domain: Eukaryota Kingdom: Fungi Division: Ascomycota Class: Sordariomycetes Order: Hypocreales Family: Nectriaceae Genus: *Fusarium* sp.
12.	*Paecilomyces* sp. is a filamentous fungus. Known for its rapid growth and thermophilic nature. Colonies grow rapidly and gets mature within 3 days of incubation. *Paecilomyces* are initially white that turn into yellow or brown as they mature. The surface of the growth type is powdery or velvety in nature.		*Paecilomyces* sp.	Domain: Eukaryota Kingdom: Fungi Division: Ascomycota Class: Eurotiomycetes Order: Eurotiales Family: Thermoascaceae Genus: *Paecilomyces* sp.

Table 2 Continued.

Sl. No.	Colony morphology, microscopic features and sporulation types	Microscopic observation	Identification	Taxonomy and systematic position
	While the reverse order of the colony is yellow to brown in coloration. Abundant sporulation is obtained when grown on PDA media.			
13.	Colonies are green in colour. Due to the presence of large numbers of conidiophores, the microscopic fruiting bodies appears as dense. Rapid growth has been observed after inoculation on agar slants followed by clear conidiophore development. Reverse coloration appears as white or pale in appearance.		*Penicillium capsulatum*	Domain: Eukaryota Kingdom: Fungi Division: Ascomycota Class: Eurotiomycetes Order: Eurotiales Family: Aspergillaceae Genus: *Penicillium* Species: *P. capsulatum*
14.	Colonies are typically white or cream-colored. The surface is slimy or gelatinous due to the production of zoospores. Rapid growth appears on suitable media. The reverse side of the colony is usually white or pale in appearance. Non-septate hyphae are other characteristics of the fungus.		*Pythium* sp.	Domain: Eukaryota Kingdom: Fungi Division: Ascomycota Clade: Diaphoretickes Clade: SAR Clade: Stramenopiles Phylum: Oomycota Order: Peronosporales Family: Pythiaceae Genus: *Pythium* sp.
15.	Colonies are white in appearance, although at maturity, it becomes blue-green in colour. The growth surface is dense, velvety and often described as fluffy mass. The reverse side of the colony is yellow in appearance. It's a fast-growing mold and forms colonies within a few days. PDA media supports maximum growth and sporulation of the fungus.		*Penicillium chrysogenum*	Domain: Eukaryota Kingdom: Fungi Division: Ascomycota Class: Eurotiomycetes Order: Eurotiales Family: Aspergillaceae Genus: *Penicillium* Species: *P. chrysogenum*
16.	*Rhizopus* sp. are known for its rapid growth and distinctive colony morphological properties. The colonies on agar plate appears as white or grey while becomes greyish brown at maturity. The surface of the fungi shows cottony appearances. The reverse side of the colony is white or pale in observation.		*Rhizopus* sp.	Domain: Eukaryota Kingdom: Fungi Division: Mucoromycota Class: Mucoromycetes Order: Mucorales Family: Mucoraceae Genus: *Rhizopus* sp.
17.	Colonies appears as whitish in coloration. However, at maturity, the colonies become brown. The surface of the colony exhibits cottony or velvety growth behaviour. This is a fast-growing fungus. The reverse side of the colony is white or pale in appearance.		*Scopulariopsis* sp.	Kingdom: Fungi Division: Ascomycota Class: Sordariomycetes Order: Microascales Family: Microascaceae Genus: *Scopulariopsis* sp.

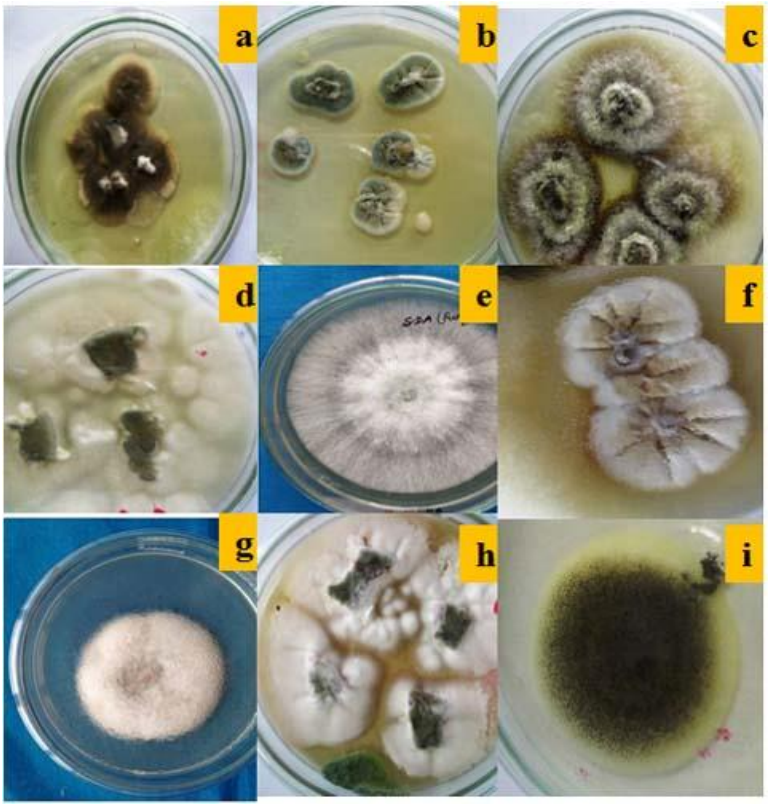


Fig. 5 – Culture characteristics of certain isolated endophytic strains.

Table 3 Diversity analysis of fungal endophytes in different plant parts of *E. latifolia*.

Taxon	Phylum	Frequency (%)	Abundance	Percentage of occurrence (%)
Absidia sp.	Zygomycota	33	1.0	6.0
Arthrobotrys sp.	Ascomycota	33	1.0	6.0
Aspergillus candidus	Ascomycota	33	3.0	6.0
Aspergillus sp.	Ascomycota	33	1.0	6.0
Basidiobolus sp.	Zygomycota	33	4.0	6.0
Chrysosporium sp.	Ascomycota	33	2.0	6.0
Cladosporium sp.	Ascomycota	33	1.0	6.0
Cunninghamella sp.	Zygomycota	33	3.0	6.0
Fusarium sp.	Ascomycota	67	1.0	12
Mortierella sp.	Zygomycota	33	2.0	6.0
Nigrospora sp.	Ascomycota	67	0.5	12
Paecilomyces sp.	Ascomycota	33	1.0	6.0
Penicillium capsulatum	Ascomycota	33	2.0	6.0
Penicillium chrysogenum	Ascomycota	67	1.5	12
Pythium sp.	Oomycota	33	1.0	6.0
Rhizopus sp.	Zygomycota	67	2.0	12
Scopulariopsis sp.	Ascomycota	33	1.0	6.0

Table 4 Isolation frequency of endophytes based on sources of isolation using different media.

Media types	Sources of isolation	Mean (cfu/ml)	Standard Deviation (±)
synthetic low-nutrient agar (SNA)	Root	12.01	1.9
	Stem	9.97	0.9
	Leaves	7.12	1.1
Carnation leaf agar (CLA)	Root	10.45	1.4
	Stem	8.89	1.1
	Leaves	6.97	1.7

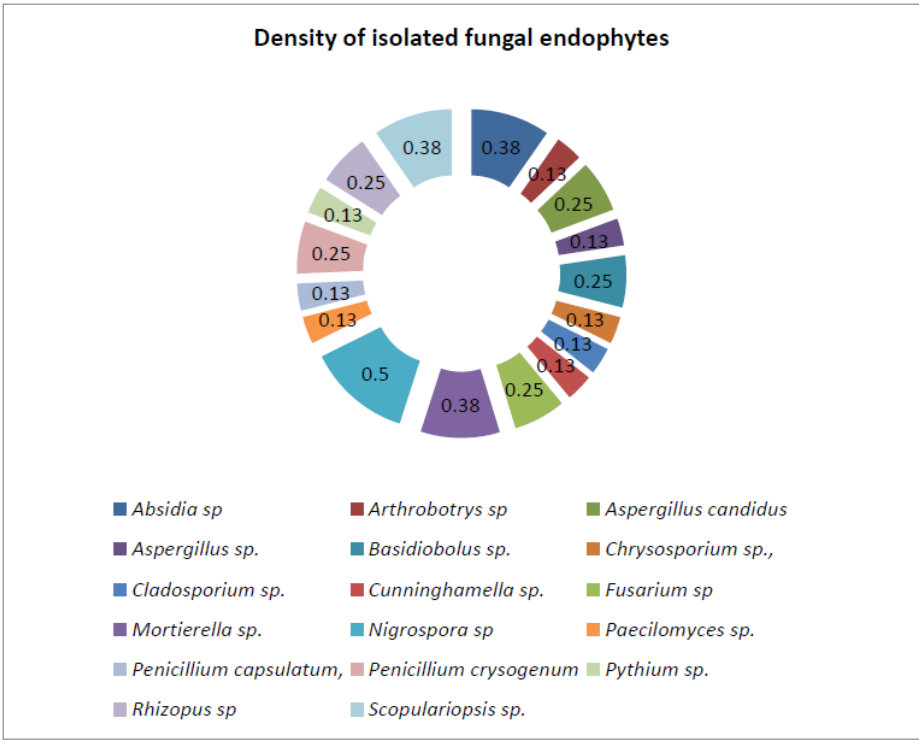


Fig. 6 – Density of fungal endophytes isolated from different plant parts of *E. latifolia*.

Table 5 Species richness and diversity index of the isolated endophytes.

Sources of isolation	Total isolates	Species Richness	Diversity Index	
			Shannon	Simpson
Root	8.0	14.1	1.979	0.847
Stem	8.0	15.0	2.02	0.86
Leaves	5.0	9.0	1.494	0.75

Table 6 PGP potential of the isolated endophytic strains.

Isolates	Growth promoting potential			
	Starch hydrolysis	Phosphate solubilization	Zinc solubilization	IAA production
EF01	-	+	+	+
EF02	+	-	-	+
EF03	+	-	+	-
EF04	-	-	+	-
EF05	+	-	-	+
EF06	+	-	+	-
EF07	-	-	+	-
EF08	+	-	+	+
EF09	+	+	+	+
EF10	-	+	+	-
EF11	-	+	-	+
EF12	+	-	+	+
EF13	+	-	+	-
EF14	-	-	-	+
EF15	+	+	-	-
EF16	-	-	-	+
EF17	+	+	-	-

'+' Activity showed; '-' Not determined.

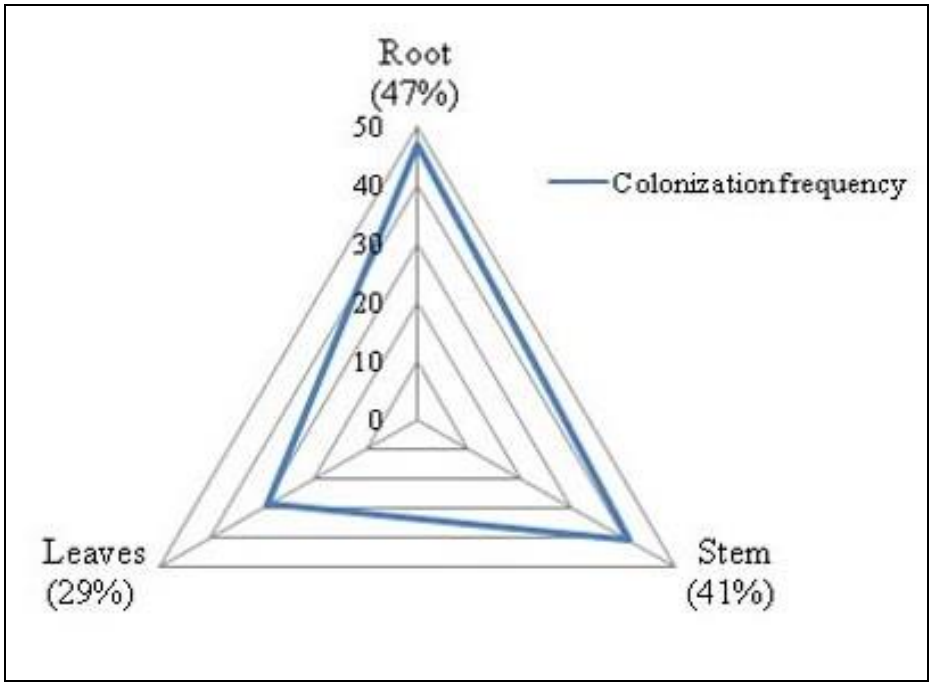


Fig. 7 – Percent occurrence of fungal endophytes in *E. latifolia*.

Fig. 8 – Morphology of the antagonistic endophytic isolates on PDA media. A endophytic microbial cultures on PDA tubes. B Broth preparation for antagonistic screening activities. C Culture characteristic of the isolate EF09. D Zinc solubilisation potential by EF09.

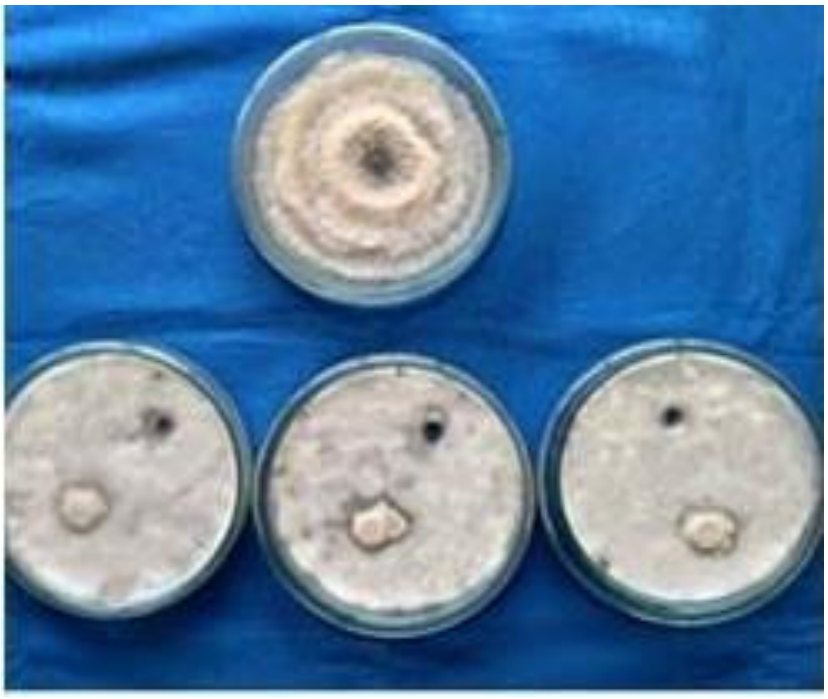


Fig. 9 – Antimicrobial evaluation of EF09 against *P. theae* using poisoned food method.

Discussion

Elaeagnus latifolia, due to its diverse applications in agriculture, forestry and medicine, needs conservation. It is established that the above and below ground parts of *E. latifolia* possess diverse structural, chemical and biotic uniqueness, facilitating a wonderful niche for effective colonization by beneficial microbiomes (Dasila & Singh 2022), including the arbuscular mycorrhizal (AM) colonization and the population dynamics of endophytic fungi. Enumeration of endophytic fungal diversity in different plant parts, including the leaves, stems, fruits and roots of four ethnomedicinal plants such as *Digitalis purpurea, D. lanata*, *Plantago ovata*, and *Dioscorea bulbifera* has been made by Ahmed (2012). Identification and characterization of antimicrobial metabolite from an endophytic fungus, *Fusarium solani*, isolated from the bark of Himalayan yew, was conducted by Tayung et al. (2011). This study indicated the potential of endophytic fungi as an alternative and sustainable antimicrobial agent in drug discovery. Unravelling the potential of endophytic fungi as popular antimicrobial agents against phytopathogens has been highlighted by Akram et al. (2023). According to the researchers, as endophytic fungi are known to produce a wide variety of antifungal secondary metabolites, including lipopeptides, antibiotics and enzymes, there appear to be chances to compete with other plant pathogenic microorganisms for available space and nutrients. During our current investigation, the endosymbionts have performed well against the grey blight pathogenesis in tea. This might be due to the development of specific genes and the production of antagonistic molecules by the antagonistic fungal strains that eventually exhibited positive antagonism and elicited defensive responses against the phytopathogen (Bhattacharyya et al. 2024c). Additionally, exhibition of antibiosis, parasitism, production of lytic enzymes and siderophores and indirect responses by induced systemic resistance (ISR) in the host plant are other mechanisms that might be mentioned here to support microbial antagonism (Bhattacharyya & Jha 2012, Segaran & Sathiavelu 2019, Agrawal & Bhatt 2023, Bhattacharyya et al. 2024d). Roy & Banerjee (2018) reported that tropical endophytes could be hyper-diverse with host preference and spatial heterogeneity. Pavithra et al. (2020) suggested that the majority of the endophytic fungi are supposed to be ascomycetes and asexual fungi. Xi et al. (2012) have made investigations on the spatial distribution and community composition of endophytic fungi in the stems of *Mussaenda pubescens* on a regional scale. Our findings are in good agreement with Xi et al. (2024), who reported the dominance of the phylum Ascomycota during their investigation. According to the

researchers, the distribution and population dynamics of endophytic fungi living inside the host plants are primarily dependent on the combination of nutrient allocation and their ability to colonize the host. Endophytes often penetrate the root region along with the lateral roots, thereby assisting in colonizing the root epidermis, root cracks and below the root hair zone (Zakria et. al. 2007). Microbial colonization of this nature can effectively establish and engineer diverse microbiomes (Xia et al. 2022) with a unique potential that, if explored properly, might be useful in industry, medicine and forestry (Bhattacharyya et al. 2023b). The PGP potential of endophytic microbial isolates to different plant growth-promoting parameters (Khan et al. 2015) might be attributing their selection for crop improvement programmes. Similar studies on determining the growth-promoting potential by endophytic fungi have been made by Nath et al. (2015) and Waqas et al. (2012). In their investigation, Souchie et al. (2006) have reported *Aspergillus* sp. and *Penicillium* sp. as efficient phosphate solubilizers. Thongsandee et al. (2012) reported the differences in endophytic population density and frequency in relation to changes in different plant parts such as young leaves, petioles and twigs of *Gingko biloba* L. According to Giri & Dudeja (2012), plants may strictly limit the growth of endophytes, and these endophytes might use several mechanisms to gradually adapt to their living environments.

Isolation, identification and conservation of endemic plant specimens and putative microbial genetic resources in threatened areas worldwide (Handique et al. 2015, Bhattacharyya et al. 2023b) using biological tools and techniques are necessary for *in situ* and *ex situ* conservation of these plant and microbial resources and their natural habitats. As one of the objectives of this study is to evaluate the antifungal activity of endophytic fungal strains isolated from *E. latifolia* against the grey blight pathogen in tea, different parts of the target plant were selected further to develop the microbe-based bioformulations in tea crop protection. The highest antifungal potential of the endophytic fungal isolate EF09 might be due to its ability to produce diverse secondary metabolites and antagonistic molecules (Mejia et al. 2008) that subsequently play significant disease reduction potential. It is, thus, imperative to explore, select, characterize and manipulate the appropriate microorganism to regulate the growth of phytopathogens.

Conclusions

Although there exist several opportunities in endophytic research in forestry, studies pertaining to endophytic fungal assemblances in economically important endemic plant species like *Elaeagnus latifolia* are still confined. It is a tragic incidence that *Elaeagnus latifolia*, due to its multiple usages and economic significance, is under threat and nearing its threshold. The current study strengthens the existing knowledge of fungal endophytes with regard to their occurrence with this actinorhizal plant species. It is apparent that endophytic fungi have profound impacts on the survival and fitness of the plants in all terrestrial ecosystems and, therefore, are likely to play a significant role in plant biogeography, evolution and community structure. The isolation of endophytic fungi from *E. latifolia* and assessing its ability to combat significant tea disease in North-East India has promising implications for the development of commercially viable biologically active agents, such as microbial biopesticides. This approach, thus, avoids the need to harvest economically important plants and disrupt environmental biodiversity, as the fungi can be easily cultured *in vitro*. Sufficient knowledge and understanding on the diversity and population dynamics of endophytic fungi along with action mechanisms of endophytes-mediated pathogen management are, thus, seems to be essential to develop integrative strategies to develop climate-smart agriculture. Further, bioprospecting of microbial metabolites and gene pool for the estimation of bioactive compounds are also found to be essential to implement sustainable strategies in plant protection in agriculture including in tea.

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