Endophytic Fungi from Tea Cultivars: Identification and In Vitro Antagonism Against *Fusarium ambrosium*, a Symbiont of Shot Hole Borer (*Euwallacea fornicatus*)

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Abstract. Tea (*Camellia sinensis*) is one of the most widely consumed beverages globally and plays a vital role in Sri Lanka's economy. Shot Hole Borer (SHB), *Euwallacea fornicatus*, is a key pest that cause substantial economic damage in Sri Lankan tea. Successful colonization and brood development of SHB depend on its obligate fungal symbiont, *Fusarium ambrosium* (*Neocosmospora ambrosia*), Conventional management of SHB predominantly depends on chemical insecticides; however, their effectiveness is limited by the beetle's cryptic behavior and emergence of insecticide resistance. Given the limitations of chemical control, alternative management strategies are urgently needed. Targeting the symbiotic fungus *F. ambrosium* offers a promising approach to disrupt the SHB life cycle. Due to their intimate association with host plants, fungal endophytes represent a valuable source for biological control approaches. This study aimed to isolate and identify endophytic fungi from selected tea cultivars and evaluate their antagonistic potential against *F. ambrosium* through dual culture assays. Among 30 isolates screened, six exhibited significant antagonistic activity, reducing fungal growth by 74.69% to 50.60%. Molecular characterization based on ITS rDNA sequences identified four isolates as *Diaporthe* spp. and two as *Coprinellus disseminatus*. These findings demonstrate that tea-associated endophytic fungi confer resistance to SHB by suppressing its fungal symbiont, offering a promising and sustainable biocontrol strategy.

Keywords: *Coprinellus disseminates*, *Diaporthe* spp., *Fusarium ambrosium*

1 Introduction

Tea is one of the most consumed beverages in the world and economically important for Sri Lanka, which is the third-largest tea exporter globally [1]. Among the major threats to tea cultivation in Sri Lanka, the Shot Hole Borer (SHB), *Euwallacea fornicatus* Eichhoff (formerly *Xyleborus fornicatus* Eichhoff), is an insect pest prevalent at elevations of 150–1400 meters above mean sea level (amsl), with peak infestations occurring between 600 and 900 meters amsl [2,3]. The SHB causes extensive damage to tea plants by boring galleries into the stems, leading to branch breakage and considerable yield reduction [4]. These galleries also serve as entry points for wood-decaying fungi and termites, which exacerbate bush deterioration and can ultimately result in plant death [5,6]. A key feature of SHB's biology is its obligate symbiotic relationship with the fungus *Fusarium ambrosium* (current name *Neocosmospora ambrosia,* Biosynonym: *Monacrosporium ambrosium*), which functions as the primary nutritional source for developing beetle larvae [4].

Conventional SHB management relies heavily on chemical insecticides, including Fipronil and lime-sulfur treatments applied at susceptible plant stages. However, these methods have shown limited efficacy due to the SHB's cryptic habits and resistance development [7,8]. Although over 15 chemical and biological agents including *Beauveria bassiana* have been evaluated, none have demonstrated significant control [9]. Moreover, chemical control raises environmental concerns, health risks, and issues related to pesticide residues, particularly Maximum Residue Limits (MRLs), which threaten the competitiveness of Sri Lankan tea in global markets [10]. Due to the limited availability of SHB-tolerant cultivars and the shortcomings of chemical control, alternative strategies such as biological control have gained attention. Targeting the fungal symbiont *F. ambrosium* could be a promising approach to disrupt the SHB life cycle [11]. Studies indicate that certain microbial biocontrol agents can effectively suppress fungal pathogens, offering a potential pathway for managing SHB through the inhibition of its symbiotic fungus.

Plant-associated microorganisms, including epiphytes and endophytes, are gaining attention for their role in sustainable crop protection. Endophytes microorganisms that colonize internal plant tissues

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without causing harm have been shown to produce diverse and bioactive secondary metabolites and these compounds exhibit antifungal, antibacterial, and plant growth-promoting properties [12,13]. Tea plants host a diverse array of endophytic fungi across different tissues, including roots, stems, leaves, shoots, and flowers [14]. Studies have shown that roots and stems share similar fungal community structures, while older leaves harbor a more diverse fungal population [15,16]. Moreover, each tissue type tends to support a distinct microbial community [17]. Disrupting the establishment of the fungal symbiont is a critical strategy that can directly affect the life cycle of the SHB. Despite growing interest, the endophytic fungi of Sri Lankan tea cultivars remain largely unexplored. Given the ability of these fungi to enhance host resistance against abiotic and biotic stresses, including fungal pathogens, they present a promising avenue for biocontrol applications in tea. Given the challenges posed by current management approaches for SHB and the promising role of endophytic fungi as biological control agents, this study aimed to isolate and identify endophytic fungi from selected Sri Lankan tea cultivars. In addition, the antagonistic activity of these fungal isolates against SHB associated *F. ambrosium* fungi was evaluated under *in vitro* conditions. The results are intended to support the development of an integrated pest management strategy that fosters sustainable tea production.

2 Materials and methods

2.1 Isolation of endophytic fungi

Endophytic fungi were isolated from the following tea cultivars: 15, 16, 22, 19, 397, 23, R80, CY-9, DT-1, 1530, 1082, 1520, 20, R95, TRI 2025, TRI 2026, TRI 2142, TRI 2151, 34, GR, 1005, TRI 2043, TRI 3069, 108, as well as from SHB-resistant cultivars TRI 2023 and TRI 4042. Isolations were performed in triplicate for each cultivar. Immature, healthy tea leaves were collected from the selected tea cultivars maintained in the germplasm collection of the Tea Research Institute of Sri Lanka and the St. Coombs Estate, Talawakelle (6.9382° N, 80.6615° E; altitude ≥1200 m). These leaves were washed off under running water. Then, leaf surface was sterilized by immersing them in 75% ethanol solution for 2 min followed by dipping them in 5% NaOCl for 1 min. Subsequently, the leaves were again submerged into 75% ethanol solution for 30 sec. Finally, they were washed thoroughly with sterile distilled water. After drying the surface sterilized leaves under sterile conditions, small pieces were cut off and plated on potato dextrose agar (PDA) supplemented with chloramphenicol (100 mg/L) to inhibit bacterial contamination. The petri dishes were incubated at room temperature (22±2 °C) for 2-3 weeks. Emerging fungal colonies were sub-cultured to obtain a pure culture of endophytic fungi [12].

2.2 Isolation of *F. ambrosium*

Pencil-thick (≈7 mm) tea branches exhibiting symptoms of SHB infestation were collected from the highly susceptible cultivar TRI 2025 at Ettampitiya (6.9365° N, 80.9878° E, altitude:1,141 m), Sri Lanka. The branches were carefully split open to extract adult female beetles. Six beetles were surface sterilized using 75% ethanol, rinsed with sterile distilled water, and dried on sterile filter paper under aseptic conditions. The sterilized beetles were then placed on PDA plates and incubated at 25°C for 3 to 4 days. Fungal growth was monitored daily, and fungus emerging specifically from the head region of the beetles were isolated. Theisolate was subsequently sub-cultured to obtain a pure culture of *F. ambrosium*. Morphological identification of *F. ambrosium* was performed using standard taxonomic keys [18,19].

2.3 Molecular characterization of endophytic fungi

The endophytic fungi were grouped based on their morphological similarity and 6 representative isolates (fungal isolates obtained from tea cultivars 1530, GR, 34, 1005, TRI and TRI 3069) were selected for further studies. Single spore isolates of selected endophytic fungi were grown on PDA plates. The mycelium free of culture medium of each isolate was separately scraped into Eppendorf tubes. DNA from the fungal mycelium was extracted using the Qiagen DNA extraction kit as per the manufacturer's protocol. DNA was amplified using fungal-specific internal transcribed spacer (ITS) regions of the fungal rDNA gene primers, ITS1 and ITS4 ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3) and ITS-4 (5' TCC TCC GCT TA T TGA TAT GC-3') or ITS1 and ITS2 (ITS2-5'- GCT GCG TTC TTC ATC GAT GC) [20]. Amplification of rDNA was performed in a total volume of 25 µL containing of 1 µl DNA (20 ng), 8.75 µL deionized water, 0.16 mM $MgCl_2$ (Promega, USA), 5 µL PCR buffer, 0.6 U of Taq polymerase enzyme, 0.04 mM of dNTPs, 0.2 mM of forward and reverse primers. The PCR conditions were an initial denaturing at 94°C for 5 min., followed

by 30 cycles of denaturation at 94 °C for 30 sec., annealing at 55 °C (ITS1 & ITS 4 pairs) or 58 oC (ITS1 &ITS 2) for 30 sec and extension at 70°C for 2 min and the final extension at 72 °C for 7 min in a thermal cycler (VeritiTM, Thermofisher, USA). The amplicons of size about 500 bp and 230 bp were obtained from ITS1 and ITS 4 and ITS 1 and ITS 2 primer pairs, respectively were Sanger sequenced.

2.4 Dual culture assay

A 5 mm diameter disc was excised from the actively growing margin of *F. ambrosium* from 7-day-old culture and placed on one side of PDA plate. A 5 mm diameter agar disc of 7-day-old endophytic fungal isolate was positioned on the opposite side of the PDA plate to facilitate dual culturing of the fungus and the antagonist. Control plates were inoculated solely with *F. ambrosium*. All plates were incubated at 25°C for 14 days. Three replicate plates were maintained for each endophytic fungal isolate and the experiment was repeated thrice. After incubation, the diameter of *F. ambrosium* colony was measured and the antagonistic activity was represented as Percentage Inhibition of Radial Growth (PIRG) in comparison to the control [21].

2.5 Crude extract of fungal secondary metabolites

A conical flask (1 L) containing 250 mL of potato dextrose broth (PDB) was inoculated with the selected isolates of endophytic fungus separately and incubated at 25 °C for 28 days. The culture broth was filtered, and the cell free culture filtrate and mycelial biomass were separately extracted, each three times with ethyl acetate. Ethyl acetate was evaporated using the rotary evaporator (BUCHI rotavapor R-20, Switzerland) until the crude extract was obtained [13].

2.6 Agar well diffusion assay

Mycelial suspension of endophytic fungi was prepared by scraping mycelia of 7-day-old fungal culture into 5 ml of sterile distilled water separately. PDA plates were inoculated by evenly spreading 1 ml mycelial suspension of an endophytic fungi over the entire agar plate and let it air dry. Wells measuring 5 mm in diameter were cut in each plate using a sterile cork borer. An ethyl acetate extract from an endophytic fungal isolate was dissolved in sterile distilled water to prepare a 1000 ppm solution, and 20 µl of this solution was added to the wells. The plates were left at room temperature for 1 hour to allow pre-incubation. Subsequently, they were incubated at 25°C and the diameter of the inhibition zones along the two axes at right angles to each other were measured and recorded [10,22].

2.7 Disk diffusion assay

Sterile disk of filter papers (diameter 7 mm) was soaked in fungal extracts dissolved in a solvent (MeOH/EtOAc) in order to get 200 µg of the sample per disk. Air dried disks (05) were placed on spread plates of *F. ambrosium* prepared as described above. The plates were transferred into an incubator and incubated at 25°C. The diameter of the inhibition zones was measured along the two axes at right angles to each other [23].

2.8 Statistical Analysis

The descriptive statistics and one-way ANOVA of the antagonistic data was performed in SAS software (version 9.1). The Tukey test was employed for the calculation of the mean separation.

3 Results and Discussion

In this study, a total of 76 endophytic fungi were isolated from healthy young leaves of tea plants from 26 tea cultivars including SHB-resistant cultivars, and SHB-susceptible Based on morphological similarity, only 30 representative isolates were selected for dual culture analysis. Among them only 6 isolates showed antagonistic activity against *F. ambrosium*. Table 1 and Fig. 1 show the antagonistic activity of the 6 endophytic fungi against *F. ambrosium.*

Table 1. Percentage growth inhibition (%) of *F. ambrosium* by fungal endophytes in dual culture on PDA after 14 days. The data represent mean ± standard error of the mean.

Tea cultivar	Endophytic fungal isolate code	Growth inhibition (%)
1005	1005	74.69±2.36[a]
TRI 3069	3069-1	61.44±3.27[b]

TRI 3069	3069-2	63.85±1.98[b]
GR	GR	59.03±2.91[c]
34	34	50.69±1.26[d]
1530	1530	50.60±2.03[d]

The means, that do not share the same subscription letter (within a column), are significantly different at 95% confidence (α=0.05).

The endophytic fungal isolate 1005 exhibited highest antagonistic activity against *F. ambrosium*, with a 75% growth inhibition. The fungal isolates 3069-1 and 3069-2 also showed higher growth inhibition of 61% and 64%, respectively. Meanwhile, the isolates 1530 (50.60%), 34 (50.69%), and GR (59.03%) demonstrated moderate antagonistic activity. The results show the potential of these isolates to be used in biological control approach of SHB through suppression of its fungal symbiont *F. ambrosium.*

Colonies of GR, 3069-2 and 1005 on PDA are cottony and radially spreading with abundant aerial mycelium on center and sparse in the margin. Colony of 1530 shows a tanned concentric ring of dense hyphae, white to pale yellow on reverse side. All the isolates developed stromata as dark, irregular patches scattered or aggregated on the colony surface within 14-21 days. Formed both alpha and beta conidia. Isolates 34 and 3069-2 developed white, cottony mycelial colonies. The mycelium shows creamy to yellowish pigmentation that intensifies on both the mycelium and the agar surface as the colony matures.

Based on the ITS sequences of rDNA region, the endopytic isolates 1530, GR, 3069-2 and 1005 belonged to the Genus *Diaporthe* (Ascomycota), and two isolates 34 and 3069-1 were identified as *Coprinellus disseminates* (Basidiomycota) (Table 2). The ITS region alone is not sufficient for accurate species identification. Therefore, alignment with multilocus sequencing and multiple reference species are required to achieve precise taxonomic resolution.

Diaporthe species are well documented as endophytes, pathogens or associates of tea in several Asian countries, including Sri Lanka [16,24,25]. Accordingly, the detection of *Diaporthe* as endophyte tea in the present study is consistent with previous reports. In contrast, *Coprinellus* isolates associated with tea appear to be rare and, to the best of our knowledge, have not been previously reported from this host. This observation therefore may represent a novel or uncommon record of *Coprinellus* in tea.

Table 2. Details of fungal isolates showing high sequence similarity to the endophytic fungal isolates based on BLAST analysis.

Endophyte code	Primer combination	Similarity %	NCBI accession No.	Fungal species
1530	ITS 1, ITS 4	99.5	NR_152472	*Diaporthe yunnanensis* CGMCC 3.18289
34	ITS 1, ITS 4	99.75	OR584151	*Coprinellus disseminatus* isolate DJL1-2
GR	ITS 1, ITS 4	98.56	KM979830	*Diaporthe helianthi* strain F87
1005	ITS 1, ITS 4	100	KM979830	*Diaporthe helianthi* strain F87
3069_1	ITS 1, ITS 2	96.67	OR584151	*Coprinellus disseminatus* isolate DJL1-2
3069-2	ITS 1, ITS 2	97.23	NR_147574.1	*Diaporthe tulliensis* BRIP 62248a

This is the first study to evaluate endophytic fungi against *F. ambrosium*, the symbiont of SHB in tea plants in Sri Lanka. Rabha et al. (2014) evaluated the endophytic fungus *Colletotrichum gloeosporioides*, isolated from tea leaves, for its antagonistic activity against tea pathogens *Pestalotiopsis theae* and *Colletotrichum camelliae* [26]. The study found that *C. gloeosporioides* exhibited strong inhibition against *P. theae* and moderate inhibition against *C. camelliae*. In another study, Zhu et al. (2014) assessed the biocontrol potential of two endophytic fungi, *Pseudocercospora kaki* and *Penicillium sclerotiorum*, also isolated from tea leaves in China, against the rice blast pathogen *Magnaporthe grisea* [27]. Their findings revealed that the culture broth and ethyl acetate extract from the dual culture exhibited stronger inhibitory activity against *M. grisea* than either monoculture alone.

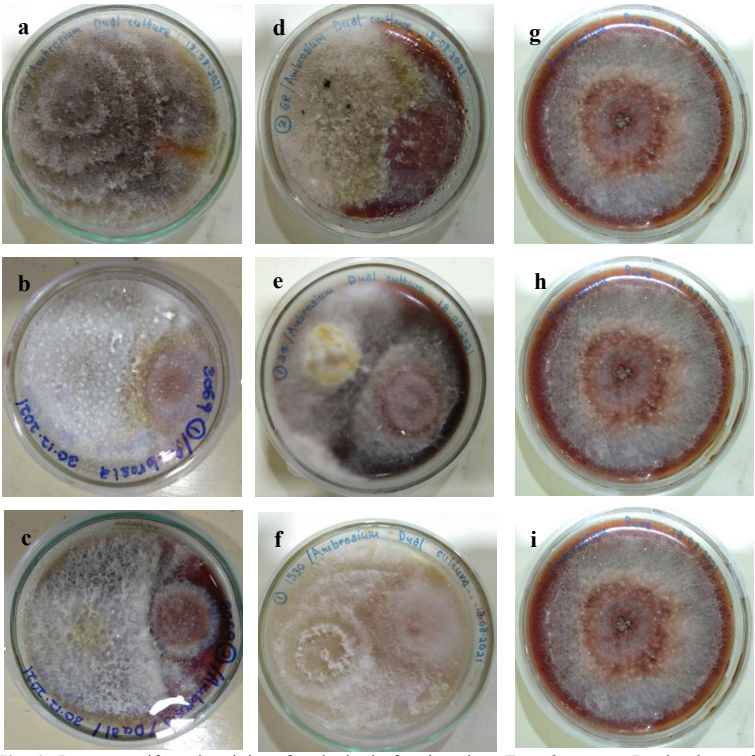

Fig. 1. *In vitro* antifungal activity of endophytic fungi against *F. ambrosium.* Dual culture of *Fusarium ambrosium* and fungal isolates a)1005, b) 3069-1, c) 3069-2, d) GR, e) 34, f) 1530, g, h ,I - pure culture of *F. ambrosium* in the absence of endophytes.

Microbial antagonists combat target fungi/organism through various mechanisms, including competition for nutrients and space, antibiosis, and direct parasitism [28]. It is reported that Ambrosia beetles *Xylosandrus crassiusculus* and *X. germanus* are among the most significant exotic pests affecting orchards and nurseries. This study assessed the potential of biological control fungi to manage these beetles by targeting both the insects and their mutualistic fungal symbionts. *In vitro* assays revealed that the mycoparasitic fungus *Trichoderma harzianum* effectively outcompeted the symbionts (*Ambrosiella roeperi* and A. *grosmanniae*) in both primary and secondary resource competition assays. In contrast, the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium brunneum* inhibited symbiont growth only during primary competition. Complementary beetle bioassays demonstrated that *T. harzianum* treatment resulted in reduced symbiont proliferation and lower brood production, with outcomes comparable to those observed in beetle-infested stems treated with high doses of entomopathogens likely due to foundress mortality before or shortly after oviposition [29]. *Xylosandrus compactus* (SHB), a major pest of robusta coffee, relying on its symbiotic *F. ambrosium* for colony establishment. *In vitro* dual culture assays showed *Trichoderma harzianum* and *Bacillus subtilis* to be the most effective antagonists against the fungus [7]. Recognized as a major pest in Turkish hazelnut orchards, the invasive ambrosia beetle *Xylosandrus germanus* Blandford was effectively suppressed by isolates of *Metarhizium anisopliae* and *Beauveria bassiana*, highlighting their potential as biological control agents [30]. These studies support the potential of

endophytic fungi to suppress *F. ambrosium*, thereby contributing to the management of SHB. However, the mechanisms by which these endophytes inhibit *F. ambrosium* require further investigation.

In the agar well diffusion assay and disc diffusion assay, the ethyl acetate extract of none of the fungal isolates demonstrated inhibition activity against *F. ambrosium*. This suggests that the observed antagonism may result from direct competition, environmental modification, or, rather than from stable, extractable, non-volatile compounds at the tested concentration [31]. It has been reported variation in biological activity among different solvent extracts of secondary metabolites and the variation is largely attributed to differences in solvent polarity, which influence the extraction efficiency of bioactive compounds. Polar solvents such as water and methanol preferentially extract hydrophilic compounds, including phenolics and flavonoids, whereas nonpolar solvents like ethyl acetate and acetone are more effective at extracting lipophilic metabolites such as terpenoids and fatty acids [32,33]. Therefore, the observed results in this study can be attributed to solvent's polarity that does not align with the target bioactive compounds; thus, those molecules may remain unextracted or under-extracted, resulting in no detectable activity. Similar observations have been reported, for example, in *Salacia chinensis*, acetone (50%) out performed water in extracting proanthocyanidins and saponins due to its intermediate polarity, yielding higher antioxidant activity [32]. Therefore, further investigation is warranted using different organic solvents, and aqueous extracts to evaluate their antifungal activity against *Fusarium ambrosium*.

4 Conclusion

In the present study 30 endophytic fungi were isolated from 26 selected tea cultivars and assessed for antagonistic activity against *F. ambrosium*. Six endophytic fungal strains exhibited higher antagonistic effects against *F. ambrosium*. Four isolates were identified as *Diaporthe*, while the remaining two were as *Coprinellus disseminates* species. These findings suggest that the potential endophytic fungi associated with tea plants may play a crucial role in enhancing host resistance to SHB infestation and hold considerable potential for further development as effective biological control agents.

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