



Diversity and antifungal activity of fungal endophytes isolated from *Cinnomomum verum* J. Presl

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Abstract

Cinnomomum verum J. Presl, also referred to as the “true cinnamon,” is a small evergreen tropical tree belonging to the Lauraceae. It is a common spice used by different cultures around the world for several centuries. It is obtained from the inner bark of trees from the genus *Cinnomomum*. This study assesses the diversity and antifungal activity of the associated endophytic fungi resides in *C. verum*. A total 11 morphologically different fungal species have been isolated from root, leaf, and stem of *C. verum*. Identification of the isolates has been carried out by combining the morphological and molecular methods. The young leaf exhibits the highest colonization rate (54.67%) followed by bark (43.26%). Endophytic fungi isolate CZGB02 (*Lasidiplodia theobromae*) showed maximum dominant fungi percent (30.18) followed by CZYL 03 (*Aureobasidium melanogenum*). Among the dominant isolates *Lasidiplodia theobromae* exhibited more antifungal activity against most of the phytopathogens tested in dual culture study. *Lasidiplodia theobromae* exhibited maximum inhibition rate for *Aspergillus Niger* (63.64%) and *Aspergillus flavus* (68.42%), which are common storage fungi.

Keywords: *Cinnomomum Verum*, fungal endophytes, lasidiplodia theobromae, aureobasidium melanogenum

1. Introduction

Plants are considered as one of the most important sources of medicines and drugs. All plants in natural ecosystems appear to be symbiotic with fungal endophytes. This diverse group of fungi can have profound impacts on plant communities through increasing fitness by conferring both biotic and abiotic stress tolerance, increasing the biomass, disease protection, and decreasing water consumption and in the production of secondary metabolites [25]. Fungal endophytes are highly diverse and their presence in plants depends on the host, the availability of nutrients, the community composition of other microorganisms and the environment [24]. Apart from producing bioactive novel secondary metabolites, different works carried out so far regarding the role of endophytes in host plants indicate that they can stimulate plant growth, increase disease resistance, improve plant's ability to withstand environmental stress and recycle nutrients [30]. Endophytes also interact with pathogens directly in ways that can alter the manifestation of host plant disease. They are well known to contribute plant fitness benefits, facilitating adaptation of the host plant to both biotic and abiotic stress [5]. These endophytes have diverse secondary metabolites of which some are bioactive compounds [11]. Fungal endophytes have been a topic of great interest largely because of their chemical diversity rather than their biological diversity. They produce toxins either independently or in association with their host plant [23]. Endophytes including those that are derived from marine algae continue to be a rich source of structurally unique and biologically active natural products. Also, endophytes that produce host plant secondary metabolites with therapeutic value or utility have also been discovered, some examples being Camptothecin and structural analogs, Podophyllotoxin, Azadirachtin, Deoxydopodophyllotoxin, Hypericin and Emodin, Taxol [2, 9, 18, 19, 27]. Endophytes also interact with pathogens directly in ways that can alter the manifestation of host plant disease. Unlike mycorrhizal

fungi that colonize plant roots and grow into the rhizosphere, endophytes inhabit the inner plant tissues and usually grow within root, stem or leaves [28]. Currently, a lot of attention is being paid to study the bioactivity of secondary metabolites for their antibacterial, antiviral, anticancer, antioxidant, antidiabetic activities of endophytic secondary metabolites. Several studies have reported the potentials of endophytes as biocontrol agents against pathogens and insects for plant protection. *C. verum*, an eternal tree of tropical medicine, belongs to the Lauraceae family. Cinnamon is one of the most important spices used daily by people all over the world. Cinnamon primarily contains vital oils and other derivatives, such as cinnamaldehyde, cinnamic acid, and cinnamate. In addition to being used as a spice and flavoring agent, cinnamon can also improve the health of the colon, thereby reducing the risk of colon cancer [36]. Cinnamon is a coagulant and prevents bleeding [12]. Cinnamon also increases the blood circulation in the uterus and advances tissue regeneration. This plant plays a vital role as a spice, but its essential oils and other constituents also have important activities, including antimicrobial, antifungal, antioxidant, and antidiabetic, anti-inflammatory, nematicidal, mosquito larvicidal, insecticidal and anticancer agent [6, 7, 10, 17, 20, 21, 32, 33]. *Cinnomomum* species has been studied for their associated endophytes because of their promising biological activities. Moreover, active compounds have been isolated from endophytic fungi associated with this genus. *C. zeylanicum*, collected from Honduras, was the host of a novel endophyte *Muscador albus* which was reported to contain volatile organic compounds with antimicrobial activities [39]. The work by [22] reported inhibitory activity of Indian spice plant *C. zeylanicum* extracts against *Alternaria solani* and *Curvularia lunata*, the pathogenic dematiaceous moulds. Almost every part of the cinnamon tree including the bark, leaves, flowers, fruits and roots, has some medicinal or culinary use. The present study was focused on

the isolation of endophytic fungus associated with *C. verum* and to evaluate their antifungal activity.

2. Materials and methods

Collection of samples

The samples were collected from *C. verum* plants (Figure 1 - A) growing in different locations of Palakkad districts, Kerala, India. Leaves at different stages of maturity viz; senescent, mature and young and also root and bark were taken as samples for isolating endophytes. The samples were brought to the laboratory, washed thoroughly in running water and whipped with sterile cotton.

Isolation of endophytes

Freshly collected, washed and airdried samples were surface sterilized before inoculation into the media. Sterilization was done by washing the samples in 70% ethanol for 2 minutes and sodium hypochlorite solution (2% available chlorine) for 5 minutes followed by 70% ethanol for 30 seconds and was rinsed two times in sterile distilled water. Surface sterilized samples were cut into 1cm² pieces and placed on sterilized petri-plates (90 mm diam.) containing Potato Dextrose Agar Medium. Medium was amended with Streptomycin (250 mg/L) to slow down the bacterial growth. Petriplates were incubated in a light chamber at 28±1° C for 1-12 days. Fungi growing out from the plant tissues were screened with the help of Stereo binocular microscope and were transferred on to fresh PDA medium. Each culture was purified by repeated sub-culturing and hyphal tip culture. Final pure cultures were transferred on to PDA slants in test tubes and stored in refrigerator. Endophytic populations were identified on the basis of cultural characteristics and morphology of spores using standard fungal identification manuals^[31]. Colonization rate and dominant fungi percentage was determined through regular observation of the cultures.

$$\text{Colonization rate (CR)} = \frac{\text{Number of segments yielding isolates}}{\text{Total number of segments incubated}} \times 100$$

Dominant fungi percentage of an endophytic fungus is defined as the percentage of number of tissues segments colonized by a fungus divided by total number of segments plated

$$\text{Dominant fungi \%} = \frac{\text{Number of tissue segments colonized by a fungus}}{\text{Total number of segments plated}} \times 100$$

Identification of endophytic fungus

Endophytic fungi were preliminarily grouped into genus or species level based on their microscopic characteristics. These endophytic fungal strains were subjected to molecular identification by amplification of ITS-rDNA sequence, ITS1 and ITS4. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. ITS sequences of the isolates were subjected to sequence similarity comparison of the sequences from the NCBI GenBank database (www.ncbi.nlm.nih.gov) using BLASTn search.

Determination of antifungal activity by Dual culture assay

Endophytic fungi were studied for their antifungal activity against pathogenic fungi isolated from various diseased samples. Direct opposition method (Dual culture) using simultaneous inoculation of the pathogen and the endophytic fungi was adopted to study the interaction. 5mm discs, each of the endophytic fungi and the opposing fungus, cut from 2-day old cultures were placed in Potato Dextrose Agar medium in 90 mm petri dishes in such a manner that they lay opposite to each other at 50 mm distance. Three replicas were maintained in each case and the paired cultures were incubated under 24±1°C. Plates inoculated with pathogen alone served as control. Radial growth of pathogen was measured towards test endophytic fungi after 3 days and 5 days of growth. Growth inhibition was calculated based on the growth of the pathogen in the control plate.

The growth inhibition was determined by applying the formula^[26].

$$\frac{C - C_1}{C} \times 100$$

Where 'C' is the growth of the pathogen in the control plate and 'C₁' is the growth of the pathogen in dual culture plate towards the endophytic fungi. Triplicates of plates were maintained. Clear inhibition zone (if any) was recorded. The hyphal interactions between endophytes and phytopathogens were assessed using a classification system ranged from type A to F: (1) Type A: Mutual intermingling growth, where both fungi grew into each other without any macroscopic signs of interaction (2) Type B: Mutual inhibition on contact or space between colonies small (2mm) (5) Type E: Inhibition of one species on contact, the inhibitor species continuing to grow at a reduced rate through the inhibited colony (6) Type F: Inhibition of one species on contact or at a distance, the inhibitor species then continuing to grow at an unchanged rate through or over the inhibited colony^[34]

3. Results and Discussion

Isolation and colonization rate of endophytes

A total of eleven morphologically distinct endophytic isolates were obtained from 150 plant samples of bark, root, senescent, mature and young leaves of *C. verum*. The isolates were classified into sporulating and non-sporulating ones. Majority of them were non-sporulating. Isolation rate and colonization frequency of endophytes were evaluated. Colonization rate was higher for young leaf tissue. Colonization rate of endophytes of young leaf was 54.67% (Table: 1) and that of in root and bark were 20.80 and 43.26% respectively. Colonization rates of endophytes in senescent leaves and mature leaves were 10.88 and 15.78 respectively.

Table 1: Isolation and colonization rates of endophytic fungi from *C. verum*

Tissue	Colonization rate (%)
Root	20.80
Bark	43.26
Senescent leaf	10.88
Mature leaf	15.78
Young leaf	54.67

Colony Characteristics of endophytes from *C. verum*

The isolated endophytic fungi were primarily characterized on the basis of colony characters such as colour, growth rate, elevation, opacity and pigmentation. Some common

characters exhibited by the colonies include rough texture, creamish white or grey pigmentation etc. The colony characters of endophytic fungi from *C. verum* are given in Table 2.

Table 2: Colony characteristics of endophytic fungi from *C. verum*

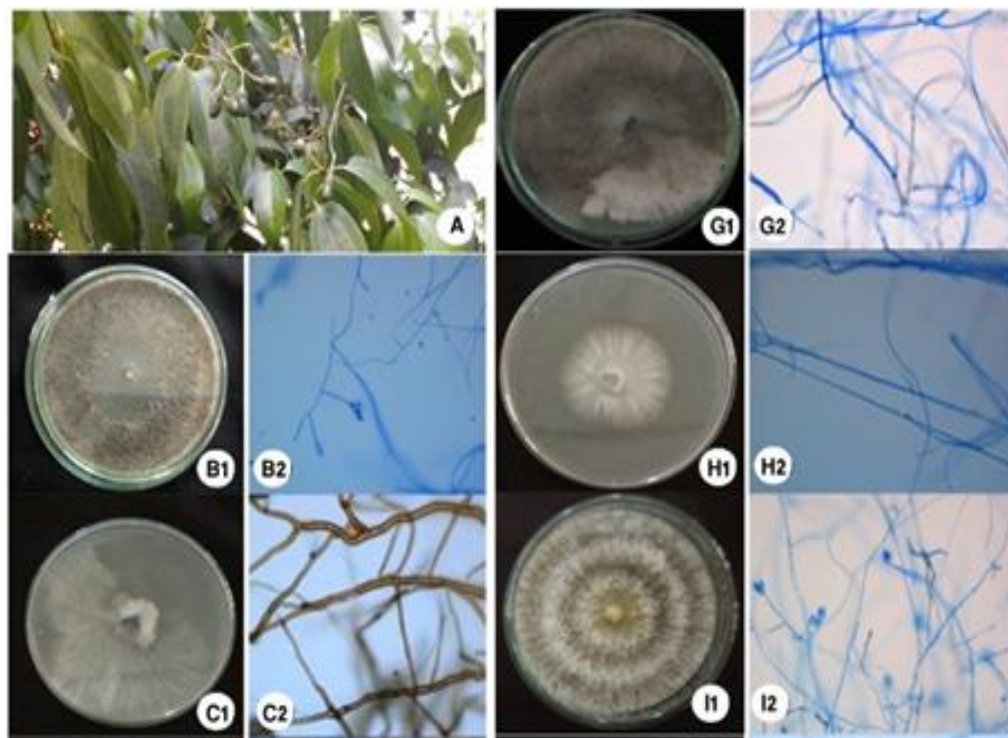
Isolates	Growth rate per day (mm)	Upper surface	Margin	Elevation	Opacity	Pigmentation
CZR01	3	Rough	Entire	Raised	Translucent	Greyish white
CZR(WA)05	2	Rough	Wavy	Flat	Translucent	Grey
CZR06	1	Rough	Entire	Flat	Translucent	Creamish white
CZGB02	2	Glistening	Filiform	Raised	Translucent	White
CZGB04	1	Rough	Filiform	Flat	Translucent	Greyish white
CZSL01	2	Powdery	Curled	Raised	Translucent	Creamish white
CZSL04	3	Filamentous	Irregular	Umbonate	Translucent	White
CZML01	1.5	Rough	Entire	Raised	Translucent	White
CZML03	2	Floccose	Entire	Umbonate	Translucent	White
CZYL(WA)01	2	Powdery	Entire	Crateriform	Translucent	Brownish
CZYL03	0.5	Smooth	Curled	Flat	Translucent	Blackish

Morphological identification and colonization frequency of endophytes

Among 11 isolates obtained three (3) were from root samples. Of these two were non-sporulating and remaining one was morphologically identified as *Daldinia* sp. Two isolates were obtained from bark and were non-sporulating in PDA. These were named as NSF3 and NSF4. Two isolates were obtained from senescent leaf and one was morphologically identified as *Colletotrichum* sp. The second isolate was non-sporulating. The mature leaf yielded two isolates; of which one was morphologically identified to be *Geotrichum* sp. and the other was non sporulating in PDA (Plate:1,B-L). Similarly, young leaf also yielded two isolates, one showing yeast like cells and the other was morphologically identified to be *Aspergillus* sp. (Table 3).

Table 3: Morphological identification and dominant fungi percentage of endophytes from *C. verum*

Tissue	Code of isolate	Morphological Identification	Dominant fungi %
Root	CZR01	<i>Daldinia</i> sp.	10.25
	CZR(WA)05	NSF1	2.35
	CZR06	NSF2	4.68
Bark	CZGB02	NSF3	30.18
	CZGB04	NSF4	3.56
Senescent leaf	CZSL01	<i>Colletotrichum</i> sp.	2.39
	CZSLO4	NSF5	2.14
Mature leaf	CZML01	<i>Geotrichum</i>	2.45
	CZML03	NSF6	4.6
Young leaf	CZYL03	Yeast like cells	22.34
	CZYL(WA)01	<i>Aspergillus</i> sp.	7.33



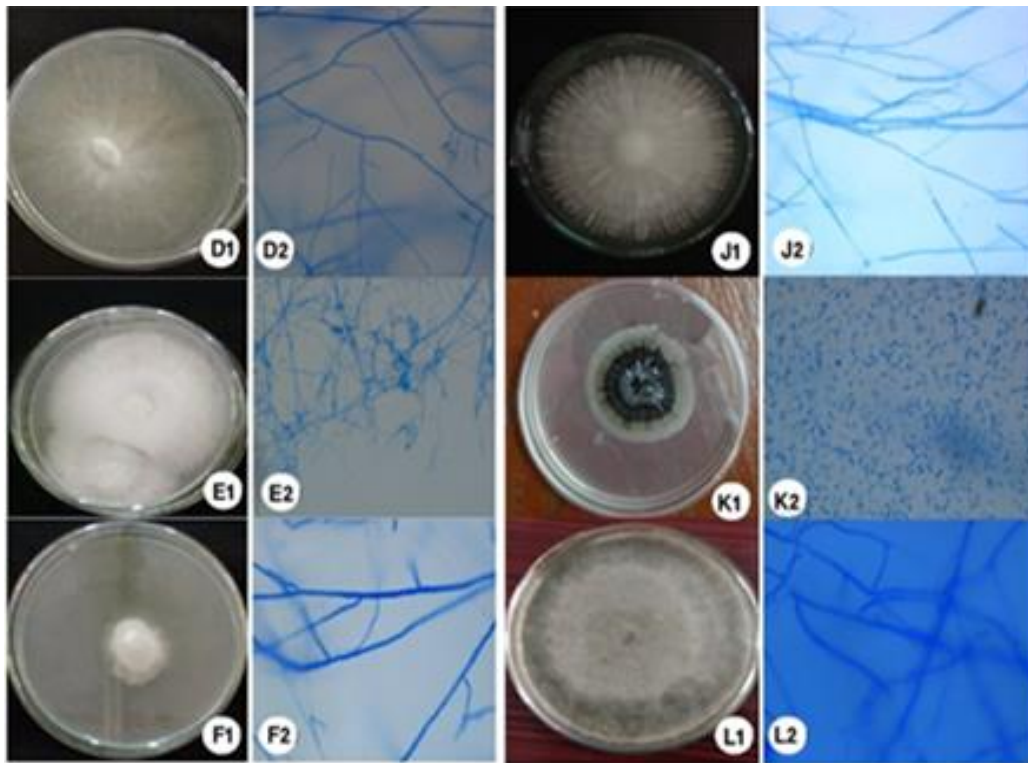


Plate 1: A. Habit of *Cinnamomum verum* J. Presl. B - K: Endophytic fungi isolated from different stages of different vegetative parts of *Cinnamomum verum* J. Presl. B-D: Root endophytes Bi & B2. CZR01; Cl & C2. CZR (WA) 05; D1 & D2. CZR06; E-F: Mature leaf endophytes; E1 & E2. CZML01; F.1 & F2. CZML03; G-H: Bark endophytes; G1 & G2. CZGB02; H1 & H2. CZGB04; I-J: Senescent leaf endophytes; I1 & I2. CZSL01; .11 & J2. CZSL04; K - L: Young leaf endophytes: K1 & K2. CZYL03; Li & L2. CZYL (WA) 01

Molecular characterization of selected isolates

A total of 4 endophytic isolates showing high dominant percentage were selected for molecular characterization. This includes CZGB02, CZR01, CZYL03, and CZYL (WA) 01 with colonization frequency of 9.37%, 6.25%, 12.5% and 6.25% respectively. The PCR amplification of ITS (ITS1 & ITS4) region of rDNA sequences resulted in around 500-600 amplicons in all samples. Sequencing of ITS region for sample CZR01 resulted in 552b.p long sequence and when this sequence was subjected to BLASTn, it showed 99% similarity towards *Daldinia eschscholtzi*. Sequencing of ITS

region for sample CZGB02 resulted in 531b.p long sequence and when this sequence was subjected to BLASTn, it exhibited 97% similarity towards *Lasiodiplodia theobromae*. Sequencing of ITS region for sample CZYL (WA) 01 resulted in 595 b.p long sequence and when this sequence was subjected to BLASTn, it exhibited 97% similarity towards *Aspergillus flavus*. Sequencing of ITS region for sample CZYL03 resulted in 564b.p long sequence and when this sequence was subjected to BLASTn, it exhibited 97% similarity towards *Aureobasidium melanogenum* (Table: 4)

Table 4: Molecular characterization of fungal endophytes

Tissue	Code of isolate	Accession number	Identification
Root	CZR01	MW03166	<i>Daldinia escholtzi</i>
Bark	CZGB02	MW03117	<i>Lasiodiplodia theobromae</i>
Young leaf	CZYL03	MW031167	<i>Aureobasidium melanogenum</i>
	CZYL(WA)01	MW024065	<i>Aspergillus flavus</i>

Dual culture test

The endophytic fungi showing good dominant fungi percent, viz., *L. theobromae* and *A. melanogenum* were selected for testing antifungal activity against phytopathogens. The inhibition percentage shown by *L. theobromae*, against all the pathogens was higher than that of *A. melanogenum* (Plate: 2).

When *L. theobromae* and *A. melanogenum* were tested against *A.niger*, mean inhibition percent of the *A. Niger* by *L. theobromae* (63.54) was high compared to *A. melanogenum* (19.89) and when tested against *A. flavus* mean inhibition percent of the *A. flavus* by *L.*

theobromae (68.42) was high compared to *A. melanogenum* (17.04). When *L. theobromae* and *A. melanogenum* were tested against *Pestalotiopsis palmarum* mean inhibition percent of the *Pestalotiopsis palmarum* by *L. theobromae* (62.60) was high, while *A. melanogenum* did not show any inhibition and when *L. theobromae* and *A. melanogenum* were tested against *Alternaria alternata* and *Fusarium oxysporum* mean inhibition percent of 54.61 % and 52.20 % were recorded.

Mean inhibition percent of the *Penicillium digitatum* by *L. theobromae* (48.59) was high, while *A. melanogenum* did not showed any inhibition (Table: 5).

Table 5: Antifungal activity of selected endophytes against phytopathogenic fungi

Phytopathogenic Fungi	Percentage of Inhibition of phytopathogens by					
	<i>L. theobromae</i>			<i>A. melanogenum</i>		
	After 3 Days	After 5 Days	Mean inhibition %	After 3 Days	After 5 Days	Mean inhibition %
<i>Alternaria alternata</i>	51.48* (61.22)	57.87 (71.73)	54.61 (66.47)	14.32 (6.12)	13.92 (5.79)	14.11 (5.95)
<i>Fusarium oxysporum</i>	51.94 (62.83)	58.08 (72.05)	52.20 (67.44)	24.20 (16.81)	9.87 (2.94)	18.31 (9.87)
<i>Aspergillus niger</i>	60.98 (76.47)	66.51 (84.12)	63.64 (80.29)	26.56 (20)	10.25 (3.17)	19.89 (11.58)
<i>Aspergillus flavus</i>	66.62 (84.26)	70.35 (88.7)	68.42 (86.48)	20.57 (12.35)	12.69 (4.83)	17.04 (8.59)
<i>Pestalotiopsis palmarum</i>	62.68 (78.72)	62.02 (78.94)	62.60 (78.83)	0 (0)	0 (0)	0 (0)
<i>Penicillium digitatum</i>	47.81 (54.9)	49.38 (57.62)	48.59 (56.26)	0 (0)	0 (0)	0 (0)

C.V% = 1.93 CD (P= 0.05) for fungal isolates = 1.28 CD (P= 0.05) for time intervals = 0.44 Transformation used: $\sin^{-1} \sqrt{p}$ where 'p' is the inhibition per cent*figure in Parentheses are actual %

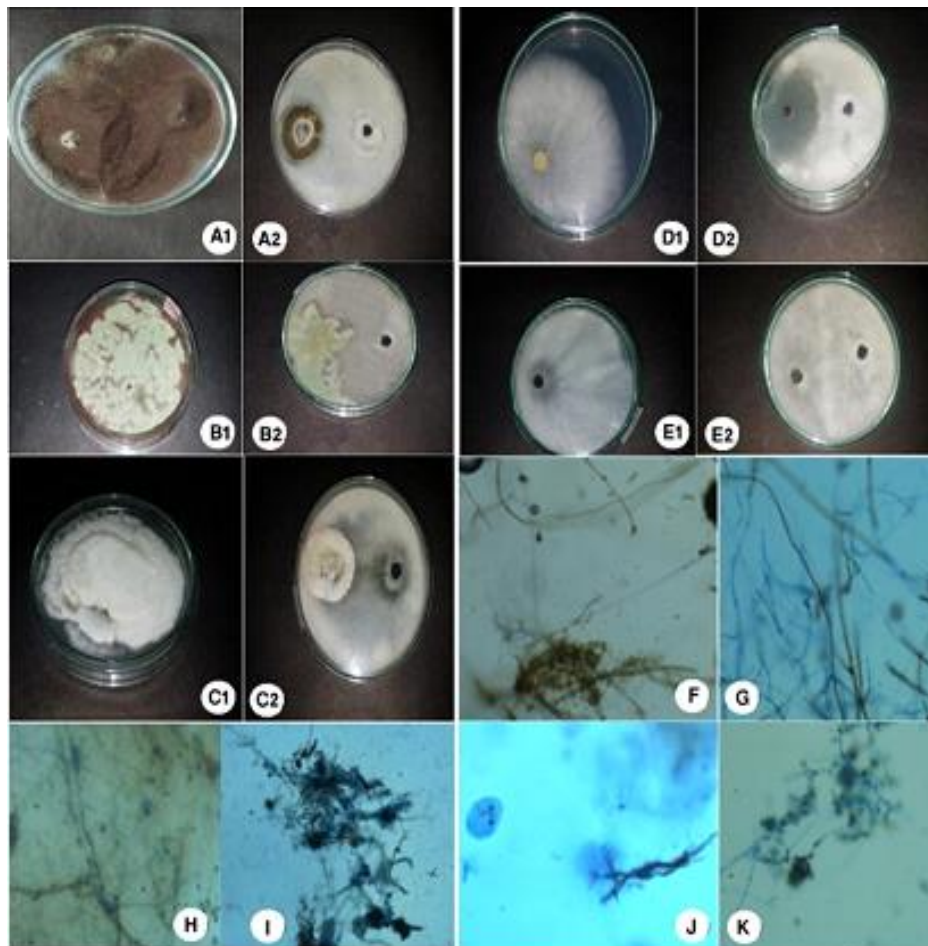


Plate 2: Antifungal activity of endophytic fungi *Lasiodiplodia theobromae* from *Cinnamomum verum* J. Presl. against phytopathogens; A1. Culture plate of *Aspergillus Niger* control; A2. *A. Niger* x *L. theobromae*; B1. Culture plate of *Aspergillus flavus* control; B2. *A. flavus* x *L. theobromae*; C1. Culture plate of *Pestalotiopsis palmarum* control; C2. *P. palmarum* x *L. theobromae*; D1. Culture plate of *Alternaria alternata* control; D2. *A. alternata* x *L. theobromae*; E1. Culture plate of *Fusarium oxysporum* control; E2. *E. oxysporum* x *L. theobromae*; F - K. Hyphal interactions between *L. theobromae* and phytopathogens: F. Hyphal coiling against *A. flavus*; G. Hyphal coiling against *A. alternata*; H. Hyphal coiling and lysis against *A. Niger*; I. Hyphal coiling against *F. oxysporum*; J. Hyphal coiling against *P. palmarum*; K. Hyphal coiling against *P. digitatum*.

Hyphal interaction studies were carried out as a part of the study for understanding the mechanism of antagonism between phytopathogens and endophyte. The morphological differences observed in the hyphae of phytopathogens were recorded for a period of 5 days in comparison with the hyphae grown separately in the control plates. Mycelial fragments of phytopathogens were observed under microscope after dual culturing with endophyte at 24 hrs intervals and observed hyphal tip lysis, hyphal swelling and lysis of protoplasm in some of the hyphae. After 96 hours of growth, the production of conidia was less in the hyphae near to the inhibition zone compared to hyphae away from inhibition zone, in the conidia producing phytopathogen.

The hyphae of *A. alternata* were denatured once coming in contact with *L. theobromae*. This may be due to the production of lytic enzymes and secondary metabolites by *L. theobromae*. By examining the contact area between *L. theobromae* and *P. palmarum*, an obvious coiling by the hyphae of *L. theobromae* around the hyphae of *P. palmarum* was noticed. *L. theobromae* also exhibited the mechanism of coiling and the disintegration of the hyphae in the case of *F. oxysporum*, *A. niger*, *A. flavus* and *P. digitatum* (Figure 2, H-M).

In the current study, a total of 11 morphologically distinct fungi have been isolated from leaf, root, and bark of *Cinnamomum verum* plant. Young leaves exhibited highest

colonization rate (54.67%) followed by bark (43.26%). The variation in the isolates diversity among plant organs may also be due to differences in the chemical composition of these organs [13]. Composition of endophytic fungi community from different part of the plant display a tissue-specific manner as most of the isolates were recovered from a specific organ. *L. theobromae* was isolated only from bark and *A. melanogenum* only from young leaf. This indicates that the isolated fungi may develop a specific relationship with their host plant organ. This finding is supported by many studies on endophytes [14]. The isolated fungi were grouped into sporulating and non sporulating based on their sporulation in PDA medium. Most of them were non sporulating. Morphological identification has been able to determine most of the isolates genera and detect the sexual phase (teleomorph) if exist. Microscopic examination, also, were able to identify the species of some genera. As many isolated fungi were reported to fail to sporulate in culture media, Nonsporulated isolates have hampered their identification. The identities of the isolated fungi have been confirmed by combining morphological with molecular identification. ITS region is one of the most frequently utilized tool to identify fungi at the species level. However, it has complications and cannot serve as the universal barcode of fungi [8]. In this study, ITS sequences of the isolates have been analyzed and subjected to sequence similarity comparison using BLASTn search. In our study, molecular identification effectively identifies fungi on the level of species with at least 99% identity and 95% query coverage. In the current study, the dominant isolate *L. theobromae* exhibited more antifungal activity against most of the phytopathogens tested in dual culture study. *L. theobromae* exhibited maximum mean inhibition percentage for *A. niger* (63.64%) and *Aspergillus flavus* (68.42%) which are common storage fungi. *L. theobromae* showed a mean inhibition percent of 62.60, 52.20 and 54.61 against *P. palmarum*, *F. oxysporum* and *A. alternata* respectively.

The Inhibition of pathogens shown by the endophytes may be due to the production of hydrolytic enzymes by the fungal isolates, or the production of antibiotics [15]. Twelve endophytic fungi were isolated from *Houttuynia cordata* and found that endophyte isolates were effective for *in vitro* antagonistic activity by inhibition of radial growth against five plant pathogenic fungi which include *F. oxysporum* and the most common mode of activity observed were mycoparasitism and competition [3]. Antagonistic activity against *Alternaria porri* by nine isolates of *Trichoderma* was reported [1]. Microorganisms have always been a source of secondary metabolites used for the benefit of human kind.

Many of the antibiotics used against pathogens originate from bacteria and fungi. Using microorganisms and their products for the control of pathogens in agriculture is not new. Some bacterial and fungal biocontrol agents (BCAs) or their secondary metabolites are registered for the control of plant disease in organic or conventional farming. Present study revealed that *L. theobromae* has ability to inhibit the growth of all fungal pathogens tested. Six sulfureous diketopiperazines designated as lasiodiplines A–F (1–6) were characterized from the culture of *L. theobromae* residing in the apparently normal flower of *Illigera rhodantha* (Hernandiaceae). Lasiodipline E (5) was demonstrated to be antibacterial against the clinical strains *Streptococcus* sp., *Bacteroides vulgates*, *Pepto*

streptococcus sp. and *Veillonella parvula*, respectively, with a minimum inhibitory concentration (MIC) range of 0.12–0.25 µg/mL [34].

Coiling and cytoplasmic degradation of pathogenic fungi by endophytic fungi (isolated from common reed (*Phragmites australis*)) has been reported [4] and found that the endophytes produced various cell wall degrading enzymes like β-1, 3-glucanases and chitinases. This forms an example of mycoparasitism by endophytes. When *F. oxysporum* was evaluated with 4 antagonistic fungi, namely: *Piriformospora indica*, *Sebacina vermifera*, *Trichoderma viride* and *Trichoderma harzianum*, hyphal coiling and hyphal lysis was observed in *F. oxysporum* [16].

4. Conclusions

Cinnamomum species have been extensively studied for their endophytic fungi with pharmacological importance. In the present study, eleven fungal endophytes of *C. verum* were isolated from the leaves, bark and roots. The isolates were classified into sporulating and non-sporulating ones. Majority of them were non-sporulating. Isolation rate was higher for young leaf tissue. The dominant fungi % of various endophytes isolated from different parts of *C. verum* showed variation. A maximum of 12.5% was recorded for *A. melanogenum* isolated from young leaf. *L. theobromae*, a non sporulating isolate obtained from bark showed dominant fungi % of 9.37%. Morphological and molecular sequencing using ITS regions confirmed the identification of four isolates as *D. escholtzi*, *L. theobromae*, *A. melanogenum* and *A. flavus*. This is the first report of these fungi in *C. verum* as endophytes. Among these fungi *L. theobromae* showed highest mean inhibition percent against the phytopathogenic fungi tested. So, this study concludes that *C. verum* harbors endophytes, particularly *L. theobromae* which possess antifungal activity and thus a potential source of antifungal compounds

5. Acknowledgments

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