MYCOBIOLOGY
2018, VOL. 46, NO. 2, 85–91
https://doi.org/10.1080/12298093.2018.1454008

RESEARCH ARTICLE

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Diversity and Antifungal Activity of Endophytic Fungi Associated with *Camellia oleifera*

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ABSTRACT

Endophytic fungi strains ($n = 81$) were isolated from the leaves, barks, and fruits of *Camellia oleifera* from Hunan province (China) to delineate their species composition and potential as biological control agents of *C. oleifera* anthracnose. The fungi were identified by morphological and phylogenetic analyses. Fungal colonization rates of the leaves, barks, and fruits were 58.02, 27.16, and 14.81%, respectively. The isolates were identified as 14 genera, belonging to two subdivisions, Deuteromycotina and Ascomycotina; 87.65% of all isolates belonged to Deuteromycotina. The dominant species, occurring with a high relative frequency, were *Pestalotiopsis* sp. (14.81%), *Penicillium* sp. (14.81%), and *Fusarium* sp. (12.35%). The Simpson's and Shannon's diversity indices revealed the highest species diversity in the leaves, followed by the barks and fruits. The similarity index for the leaves versus barks comparison was the highest, indicating that the number of endophytic fungal species shared by the leaves and barks was higher than barks and fruits or leaves and fruits. Based on the results of dual culture experiments, only five strains exhibited antifungal activity against *C. oleifera* anthracnose pathogen, with isolate ty-64 (*Oidium* sp.) generating the broadest inhibition zones. Our results indicate that the endophytes associated with *C. oleifera* could be employed as natural agents controlling *C. oleifera* anthracnose.

ARTICLE HISTORY
Received 30 October 2017
Revised 26 December 2017
Accepted 2 January 2018

KEYWORDS
Antifungal activity; *Camellia oleifera*; distribution; diversity; endophytic fungi

1. Introduction

Endophytic fungi have been studied since 1898 [1], but only a limited number of endophytic fungi were reported in the years 1898–1980s [2]. Due to their antifungal and anticancer activity, endophytic fungi have attracted increasing attention, and are considered as promising biological control agents because of their intracellular colonization of healthy plants, and typically in the absence of any visible symptoms of disease [3,4]. Endophytic fungi have been identified in nearly 300,000 plant species [5], these fungi dwell in almost every organ (the root, stem, leaf, flower, fruit, and seed) of the host plant; some of them produce bioactive metabolites but only a few of these fungi have been studied.

Camellia oleifera is an endemic oil plant of China, widely distributed in southern China. The most valuable *C. oleifera*-based product is camellia seed oil, which has been shown to enhance human immunity, reduce blood pressure, and prevent cardiovascular and cerebrovascular diseases [6]. In addition to camellia seed oil, *C. oleifera* also produces a variety of secondary metabolites, such as saponins, polyphenols, and flavonoids, which are also widely used [7]. In recent years, forest pests have been severely endangering the acreage of *C. oleifera*. Although chemical pesticides would protect the plant effectively, pesticide use may lead to serious environmental pollution that might threaten human health. With the increasing awareness of environmental protection and food safety, biological control methods are attracting increasing public attention. Because of their biocontrol potential, the use of endophytic fungi to control plant diseases has become an important and promising approach of biological control. In recent decades, several endophytic fungi had been isolated from such plants as cotton, rice, potato, tomato, and pepper [8]; some endophytes were shown to be able to control plant diseases [9–13], but the biological control capability of the endophytic fungi associated with *C. oleifera* remains unclear.

To understand the ecological importance of the endophytic fungi of *C. oleifera*, we investigated the distribution and species diversity of endophytic fungi associated with this plant and assessed their biocontrol capacity against pathogenic fungi. This work is the first-ever report describing both the

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C. oleifera endophytic fungal diversity in different organs and their potential biocontrol ability.

2. Materials and methods

2.1. Sample collection

The samples were collected from the major *C. oleifera*-producing area in the Hunan province and the National Germplasm Resources Pool of *C. oleifera* from 2013 to 2015. Healthy *C. oleifera* trees of different ages were sampled; for each tree, a branch of four position with fresh leaf samples, five bark samples, and two peel samples from the same fruit were collected. Five trees were sampled at every sampling site. All samples were sealed in vacuum bags, kept at 4 °C, and used within 48 h of collection.

2.2. Isolation and purification of the endophytic fungi

The samples were washed under running water to remove the dust attached to the surface and rinsed three times with distilled water. To eliminate the epiphytic microorganisms, the samples were surface-sterilized by 75% ethanol for 30–60 s, followed by a 5% sodium hypochlorite bath for 10–12 min, and finally rinsed five times with distilled water and dried on a sterile tissue paper. The dried samples were cut with a sterile blade into 1 × 10 mm segments; each segment was placed onto a potato dextrose agar (PDA) plate. To prevent potential contamination, the final-rinse distilled water was plated on a fresh PDA plate as a negative control. All plates were incubated in the dark at 25 °C and examined daily for 7 d. When a mycelium appeared round the edge of the sample on the PDA plate, the hyphal tips were transferred onto a fresh PDA plate, and the procedure was repeated several times until a pure culture was obtained.

2.3. Morphological and phylogenetic analyses of the endophytic fungi

The endophytic fungi were identified using a traditional morphological identification method and by molecular methods. The hyphae and conidia were picked from the purified colonies, placed onto a slide, and observed under an optical microscope. The morphological characteristics of the hyphae and conidia were recorded daily, and the taxonomic status was determined using a fungus identification handbook [14].

For strains that did not produce spores, pure mycelium was inoculated into a liquid PDA medium and cultured with shaking (120 rpm) for 2 d at 25 °C. After freeze-drying, genomic DNA (gDNA) was extracted using a fungal genomic DNA extraction kit (Sangon, Shanghai, China). Next, for obtained the ITS region, gDNA was amplified using primers ITS1 (5′-TCCGTAGGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [15]. The polymerase chain reaction (PCR) reactions were performed in a final volume of 50 µL; the reaction mixtures contained 5 µL of 10× PCR buffer (10 mM), 1 µL dNTP mixture (10 mM), 0.5 µL Taq polymerase (5 U/µL), 2 µL of each primer (10 mM), 5 ng gDNA, and double-distilled water. The following PCR cycling protocol was used: 94 °C for 5 min; followed by 35 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 30 s; and finally, 72 °C for 5 min. In the negative control reactions, gDNA was replaced by double-distilled water. The PCR-amplified products were analyzed by gel electrophoresis on 1% agarose in 1× TAE (40 mM Tris base, 40 mM acetic acid, and 2 mM ethylenediaminetetraacetic acid). DNA sequencing was performed at Sangon Biotech Co., Ltd. (Shanghai, China). The obtained sequences were used as a query to search for similar sequences in GenBank using the BLAST program (Basic Local Alignment Search Tool, https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the classification of unidentified species.

2.4. Evaluation of endophytic fungi diversity

The diversity of endophytic fungi was analyzed based on the total number (n) of isolates. The isolation rate (IR, %) was calculated by dividing the total number of isolates from specific tissues by the overall number of endophytic isolates; the relative frequency (RF, %) was calculated by dividing the total number of isolates representing a single taxon by the total number of taxa obtained from all tissues. The Camargo's index (1/S) was used as a fungal dominance determination index, and a species was defined as dominant if $P_i > 1/S$, where P_i defined as the number of competing species present in the community, and S represents species richness [16]. The Simpson (D) and Shannon (H) diversity indices were calculated for each organ [17,18]. The species evenness (E) was calculated by the following formula: $E = H/Ln(S)$ [19]. The Jaccard similarity coefficient (CJ) was calculated by the formula: $CJ = C/(A + B–C)$ [20]; where, A and B are the total number of species isolated from any two organs, and C is the number of species shared by these two organs.

2.5. Screening for the antifungal activity of the endophytic fungi

To determine the endophytic fungi with antifungal activity, isolate cultures were screened for their ability to against the *C. oleifera* anthracnose pathogenic

fungi. The anthracnose phytopathogenic fungi were isolated in Changsha (China). The antifungal activity of the endophytic fungi was tested using a dual culture method. The phytopathogenic fungi were transferred to the center of a fresh PDA plate; then, two different endophytic fungal isolates were placed 2.5 cm away from the phytopathogenic fungi. All plates were incubated in the dark at 25 °C for 7 d, and each experiment was repeated three times. The width of the inhibition zones between the pathogen and the endophytic fungi was measured in millimeters and interpreted as the antifungal activity. The width of the inhibition zone (T) was classified as follows: (−), T = 0 mm; (+), 0 < T ≤ 2 mm; (++), 2 < T ≤ 5 mm; and (+++), T > 5 mm.

3. Results

3.1. Identification of the endophytic fungi

In total, 183 endophytic fungi isolates were obtained from 360 segments of the leaves, barks, and fruits. The endophytic fungi were identified on the basis of morphology and by phylogenetic analysis. The isolates were divided into 81 phenotype groups based on their morphological characteristics and classified into 29 fungal taxa according to phylogenetic analyses based on the ITS region sequencing. From the 29 fungal taxa, 23 taxa containing 61 phenotype groups were identified to species level; five taxa containing 19 phenotype groups were identified to genus level; and one taxon was identified to family level. The isolates represented two subdivisions: Deuteromycotina and Ascomycotina; 87.65% of the isolates belonged to the former subdivision (Table 1). The Deuteromycotina strains were divided into two classes: Coelomycetes and Hyphomycetes; the Coelomycetes endophytic fungi (29 strains) represented five genera: *Pestalotia*, *Pestalotiaopsis*, *Colletotrichum*, *Phoma*, and *Macrophoma*; Hyphomycetes isolates (42 strains) represented seven genera: *Penicillium*, *Penicilliopsis*, *Paecilomyces*, *Trichothecium*, *Oidium*, *Alternaria*, and *Fusarium*. Ten strains of endophytic fungi classified into Deuteromycotina represented three families: Sacoharomycetaceae, Endomycetaceae, and Xylariaceae.

3.2. Distribution and diversity of the endophytic fungi

Differences in the fungal isolation frequencies and relative frequencies in various plant organs were calculated; 58.02% of the isolates were obtained from the leaf tissues, 27.16% from the barks, and 14.81% from fruits. The dominant species occurring with a high relative frequency were *Pestalotiopsis* sp. (14.81%), *Penicillium* sp. (14.81%), and *Fusarium* sp. (12.35%); *Pestalotiopsis* sp. and *Penicillium* sp. were isolated from all three organs, and *Fusarium* sp. was isolated from the leaves and barks. Differences in the species of endophytic fungi isolated from various plant organs were also observed. *Xylaria* sp. were isolated exclusively from the barks, and *Pichia* sp. were isolated exclusively from the fruits. The detail results of these endophytic fungi were summarized in Table 2.

To characterize the diversity of the isolated endophytic fungi, we calculated the Camargo's index, Simpson's diversity index, and Shannon diversity index. The overall Camargo's index was 0.034, since the value of species richness for the three organs was 29. The Camargo's index revealed the highest species diversity in the leaves (1/S = 0.053), which was higher than in the barks (1/S = 0.100) and fruits (1/S = 0.200). The overall Camargo's index (0.034) reflected high species diversity. The Simpson's and Shannon's diversity indices showed the same tendency, i.e., the values were highest in the leaves (1–D = 0.930, H = 2.786), followed by the barks (1–D = 0.888, H = 2.240) and fruits (1–D = 0.764,

Table 1. Distribution of endophytic fungi in *Camellia oleifera*.

			No. of isolates			
Order	Family	Genus	Total	Leaves	Barks	Fruits
Melanconiales	Melanconidaceae	*Pestalotia*	2	1	1	
		Pestalotiaopsis	12	5	3	4
		Colletotrichum	5	3	2	
Sphaeropsidales	Sphaeropsidaceae	*Phoma*	3	3		
		Macrophoma	7	5		2
Moniliales	Moniliaceae	*Penicillium*	12	6	4	2
		Penicilliopsis	5	5		
		Paecilomyces	4	4		
		Trichothecium	3	3		
		Oidium	3			3
	Dematiaceae	*Alternaria*	5	5		
	Tuberculariaceae	*Fusarium*	10	6	4	
Endomycetales	Sacoharomycetaceae	*Pichia*	1			1
	Endomycetaceae	–[a]	1	1		
Sphaeriales	Xylariaceae	*Xylaria*	8		8	
Total			81	47	22	12

[a]"–" represent the unidentified genera.

Table 2. Diversity of endophytic fungi in *Camellia oleifera*.

Genera	Species	Leaves	Barks	Fruits	Total
Pestalotia	*P. pezizoides*	1	1		2
Pestalotiaopsis	*P. clavispora*	5			5
	P. heterocornis		3		3
	P. versicolor			4	4
Colletotrichum	*C. boninense*	3			3
	C. gloeosporioides		2		2
Phoma	*P. destructiva*	2			2
	P. cucurbitacearum	1			1
Macrophoma	*M. kawatsukai*	4			4
	M. musae			2	2
	M. abensis	1			1
Penicillium	*P. digitatum*	3			3
	P. expansum	2	1	2	5
	P. italicum	1	3		4
Penicilliopsis	*P. lilacinus*	2			2
	P. sinensis	1			1
	P. varioti	2			2
Paecilomyces	–[a]	4			4
Trichothecium	–	3			3
Oidium	–			3	3
Alternaria	*A. mzcrospora*	2			2
	A. longipes	3			3
Fusarium	*F. moniliforme*		2		2
	–	6	2		8
Pichia	–			1	1
Xylaria	*X. carpophila*		3		3
	X. juruensis		2		2
	X. cubensis		3		3
Unidentified species	–	1			1
Number of total fungal isolates (*n*)		47	22	12	81
Species richness (S)		19	10	5	29
Camargo's index (1/S)		0.053	0.100	0.200	0.034
Simpson's index of diversity (1 − D)		0.930	0.888	0.764	
Shannon index of diversity (H)		2.786	2.240	1.517	
Species evenness (E)		0.724	0.725	0.611	

[a]"–" represent the unidentified genera or species.

H = 1.517). The evenness indices for the leaves (E = 0.724) and barks (E = 0.725) were close, and followed by the value for fruits (E = 0.358). The Jaccard similarity coefficient for the leaves versus barks was 0.160; for the barks versus fruits was 0.154; and for the leaves versus fruits was 0.043. These results indicated that the biodiversity of endophytic fungi in the leaves and barks was similar.

3.3. Antifungal activity of the endophytic fungi

To evaluate the potential antagonistic activity of endophytic fungi against phytopathogens, all isolates were screened using the dual culture method with the *C. oleifera* anthracnose pathogen fungi on PDA plates. Only five strains inhibited the growth of *C. oleifera* anthracnose fungi, the results are shown in Table 3. Unfortunately, most of the isolated endophytic fungi did not exhibit any antifungal activity against the tested phytopathogen. The results were recorded as no inhibition (−), and weak inhibition (+), moderate inhibition (++), or strong inhibition (+++). The strain ty-64 exhibited strong antifungal activity, the strain hj-63 showed moderate antifungal activity, and the strains lc-14, lc-20, and lc-24 exhibited weak antifungal activity.

Table 3. Identification of endophytic fungi and antifungal activity against pathogenic fungi of *Camellia oleifera* anthracnose according to the dual culture.

Strain	Identified as	Antifungal Activity
lc-20	*Aspergillus* sp.	+
lc-24	*Xylaria* sp.	+
lc-14	*Fusarium* sp.	+
hj-63	*Trichothecium* sp.	++
ty-64	*Oidium* sp.	+++

4. Discussion

Previous studies have shown a biocontrol potential of endophytic bacteria associated with *C. oleifera* [21–26], but endophytic fungi did not attract much attention until recently [27–29]. In the current study, we isolated 183 endophytic fungi in 81 phenotype groups representing 14 genera from the leaves, barks, and fruits of *C. oleifera* collected from the tree's main planting region in the Hunan province. Most endophytic fungal species obtained herein were consistent with previous studies; nine genera of endophytic fungi were found in both the current study and by Zhou et al. [27] but only 15 species from three genera identified by Zhang et al. [28] were consistent with our results. Further, this is the first report of *Pestalotia* sp. and *Macrophoma* sp. as endophytic fungi of *C. oleifera*. The numbers of endophytic fungal species are closely correlated with the sampling range of the plant age, and furthermore the probability of invasion and colonization by endophytic fungi varies depending on the surroundings; therefore, the variety of species and quantities of endophytic fungi is infinite [30]. Hence, sample collection area should be expanded, such as increasing the sampling range and collecting plant specimens of different ages, to enable the isolation of additional endophytic fungi. *C. oleifera* were widely distributed in 17 provinces in China, which 40.8% were planted in Hunan province; meanwhile, *C. oleifera* planted in Hunan province showed highest diversity. Hence, the species of endophytic fungi associated with *C. oleifera* in Hunan province may reveal higher diversity than others. In contrast with Zhou et al. and Zhang et al. [27,28], our samples were collected in the main area of *C. oleifera* origin in China and therefore the diversity of the obtained isolates was high; nevertheless, we did not collect stem or root *C. oleifera* samples, therefore some endophytic fungal species may have been underrepresented.

Surface sterilization is the first critical step of sample processing that ensures the isolation of endophytic fungi while avoiding the contaminants [31]. Based on the surface sterilization methods described elsewhere [13,32,33], we tested several sterilizing combinations to ascertain that the most suitable surface sterilization method was used in this study.

The preferred plant growth organ of endophytic fungi may be determined based on the appearing frequency of occurrence of endophytic fungi in different organs [34]. Our results revealed that the distribution of endophytic fungi in *C. oleifera* was organ-specific. The number of endophytic fungi isolated from the leaf tissues was the highest, accounting for 58.02% of all isolates; 22 and 12 isolates were obtained from the bark and peel tissues, respectively, accounting for 27.16% and 14.81% of all isolates, respectively. Similarly, the Simpson's and Shannon's diversity indices indicated that the highest species diversity was in the leaves, followed by the barks and fruits, accordingly. Zheng et al. [31] proposed that the colonization rate of endophytic fungi is generally significantly higher in the stems than in the leaves, but we found the opposite; nevertheless, the results of several previous studies were similar to ones obtained in the current study [11,35]. The possible reasons for these discrepancies are different organ structure, chemical composition, and even the period length of organs stay remaining in the tree, possibly leading to plant intracellular substance composition variance [27,36,37]. Although some differences in endophytic fungi isolates were observed between organs, similarities between the distribution of endophytic fungi in various organs were also noted (as per the similarity index).

In the current study, *Pestalotiopsis*, *Penicillium*, and *Fusarium* were the dominant endophytic fungal genera of *C. oleifera*, which contains the largest quantity and variety of endophytic fungi among all isolates we obtained. *Pestalotiopsis* sp. and *Penicillium* sp. were isolated from the three sampled organs, and Fusarium sp. were isolated from the leaves and barks. According to previous studies, *Pestalotiopsis* and *Fusarium* are generally commonly isolated from plants [31]. Despite the fact that *Pestalotiopsis* is considered as a pathogen that causes leaf spots, some studies revealed that some *Pestalotiopsis* isolates may act as antifungal agents against pathogens, as well as producers of bioactive substances [38–41]. *Fusarium* sp. is a confirmed biological control agent, and even the predominant genus among antifungal isolates, and it is also one of the dominant endophytic fungi genera [42,43]; previous studies also revealed its ability to promote the host plant's growth [42,44] and to induce systemic resistance in the host plant [45]. *Penicillium* is frequently isolated from such plants as *Acer ginnala* [46], *Hevea brasiliensis* [47], *Opuntia ficus-indica* [48], *Stryphnodendron adstringens* [49], *Taxus globosa* [13], and *Theobroma* spp. [50], and is often reported as a genus with antibiotic-producing [47,50] and antioxidant [51] activity. By contrast, *Xylaria* sp. representatives were only isolated from the barks, while Pichia fungi were specifically isolated from the fruits. Generally, *Xylaria* is commonly isolated from most tropical plants [31]. In the current study, even though *Xylaria* was only obtained from the barks, the isolation frequency was 9.88%, which was higher than that of other isolates, with the exception of the dominant species, consistent with earlier studies. *Xylaria* has also been reported as a promising efficient bioactive resource because of the ability to produce antimicrobial compounds [52,53] and on account of its natural antioxidant activity [54].

In the current study, 81 endophytic fungi strains were isolated from different areas of the Hunan province and identified both on the basis of morphological characteristics and by phylogenetic analyses. The data indicated that the endophytic fungal strains represented five different orders: Melanconiales, Sphaeropsidales, Moniliales, Endomycetales, and Sphaeriales. Usually, endophytic fungal colonization of the host plant was asymptomatic. In addition, many endophytic fungal species did not produce spores on PDA media; therefore, phylogenetic analysis based on rDNA sequencing was subsequently employed to identify species that could not be categorized based on their morphological characteristics. Nevertheless, in the current study, some strains could not be categorized into species or genera, e.g., fungi belonging to the Endomycetaceae family. At the same time, some fungal isolates were recalcitrant to culture on the PDA medium. Hence, cultivation on artificial media precludes the isolation of some endophytic fungi, and an accurate, rapid identification technology for these fungi is still needed.

The ability of endophytic fungi isolated from *C. oleifera* to inhibit *C. oleifera* anthracnose fungi was somewhat limited. In search of more effective biological control agents, we also investigated the endophytic bacteria of *C. oleifera*. Although previous studies have revealed the presence of several useful endophytic bacterial strains in *C. oleifera*, we identified two new strains from the genus *Bacillus*, with the initial results suggesting their high inhibitory activity toward the *C. oleifera* anthracnose fungi (data no shown).

Our study revealed the presence of a highly diverse endophytic fungal community associated with the Chinese endemic oil plant *C. oleifera*; five isolates were able to inhibit the growth of *C. oleifera* anthracnose fungi. Furthermore, we showed that the endophytic fungi of *C. oleifera* could act as biocontrol agents. The identified antifungal activities suggest the possibility of developing a novel, useful biopesticide based on the strain ty-64 or hj-63. Confirmation of the effective filed application is worthy of further investigation.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Hunan Provincial Forestry Science and Technology Research Project [XLK201606], the Youth Innovation Research Foundation of Hunan Academy of Forestry [2013LQJ03] and Hunan Provincial Science and Technology Department Key Project of Science and Technology Research [2014NK2013].

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