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A cultured endophyte community is associated with the plant *Clerodendrum inerme* and antifungal activity

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ABSTRACT. Fungal endophytes live in the inner tissues of *Clerodendrum inerme* and may be significant resources for new chemicals in drug discovery. A total of 242 endophytic fungi were recovered from 602 sample segments of *C. inerme*; 66 were purified. The 66 fungi belonging to 16 taxa and 11 genera (*Alternaria*, *Nigrospora*, *Bartalinia*, *Pestalotiopsis*, *Fusarium*, *Mycoleptodiscus*, *Trichoderma*, *Phomopsis*, *Diaporthe*, *Lasiodiplodia*, and *Curvularia*) were identified by morphological characteristics and fungal internal transcribed spacer sequences. The most abundant genera were *Alternaria* and *Lasiodiplodia*. Some of the endophytes exhibited tissue specificity. The colonization frequencies of endophytes in the stems were evidently higher than those in the roots and leaves. The crude ethyl acetate extracts were tested against 6 endophytes isolated from *C. inerme*. Three of 10 (33.3%) endophytes, which were identified as *Phomopsis* sp, *Curvularia* sp, and *Mycoleptodiscus* sp, displayed distinct antifungal activity against ≥3 tested fungi. To our knowledge, this is the first report of an endophytic community associated with *C. inerme* in China and its antifungal activity *in vitro*.

Key words: Endophyte; *Clerodendrum inerme*; Antifungal activity; Latent pathogen

INTRODUCTION

Endophytic fungi (i.e., endophytes) live all, or at least a significant part, of their life cycle internally and asymptomatically in plant tissues (Saikkonen et al., 1998). They comprise a diverse group and can be rich sources of biologically active secondary metabolites, which protect plants from invasion (Tanaka et al., 2005; Vega et al., 2008); others, however, are latent plant pathogens (Photita et al., 2004).

Clerodendrum inerme, a member of the genus *Clerodendrum* L. (Family: Lamiaceae), grows widely in tropical and subtropical regions. Many structurally novel metabolites have been isolated from *C. inerme* (Achari et al., 1990, 1992; Pandey et al., 2003). Studies have shown that *C. inerme* possesses potent anti-inflammatory, antidiabetic, antimalarial, antiviral, antihypertensive, hypolipidemic, and antioxidant activities that can be developed as potent remedial agents (Kalyanasundaram and Das, 1985; Somasundaram and Sadique, 1986; Mehdi et al., 1997; Guessan et al., 2010). Although interest has increased in *C. inerme*, the endophyte communities in *C. inerme* and their antifungal activities remain largely unclear.

In our present study, endophytes from the stems, roots, and leaves of *C. inerme* were isolated and identified. These endophytes were identified using a combination of morphological and internal transcribed spacer (ITS) sequence-based molecular methods. Diversity and antifungal activity of the endophyte assemblages were also evaluated. This study will provide data on the biodiversity and antifungal activity of endophytic fungi associated with *C. inerme* in China.

MATERIAL AND METHODS

Plant materials

The leaves, stems, and roots were randomly collected from 5 different *C. inerme* specimens located at different sites in Qinzhou, Beibu Gulf, Guangxi Province, China, from May to June 2012. The mean annual temperature in Qinzhou is 21.4°-22°C, and the mean annual precipitation is ~1649.1-2055.7 mm. Immediately after collection, the plant parts were washed with tap water and processed for isolation of the endophytes.

Isolation and culture of the endophytic fungi

Endophytes were isolated from healthy *C. inerme* using the method described by Wang et al. (2006), with minor modifications. For endophyte isolation, 10 different healthy segments of the leaves, stems, and roots from *C. inerme* were selected at random and washed in running tap water. The plant parts were surface sterilized successively with 70% ethanol for 3 min and then rinsed with sterile water. Then, 1% sodium hypochlorite was used to sterilize the tissues for 3 min and they were again rinsed with sterile water 5 times. Surface sterilized plant parts were dried, cut into pieces (i.e., 5 mm long and 5 mm wide), and transferred to Petri dishes (9 cm in diameter) containing potato dextrose agar (PDA) medium (amended with 50 μg/mL streptomycin and 100 μg/mL ampicillin) after taking an imprint of the dried sterile plant part. These plates were incubated at 26°C in darkness for 10 days. Hyphal tips of the developing fungal colonies were immediately transferred to fresh PDA agar plates to get a pure culture. The strains were preserved on PDA slants and stored at 4°C.

Morphological identification of the endophytic fungi

The endophytic fungal strains were cultured on PDA plates at 26°C for 7 days to observe colonial morphology. Macroscopic characteristics such as size, shape, surface texture, odor, and color were described according to the method established by Wipornpan et al. (2005). Many similar colonies were observed for microscopic features with light microscopes, allowing them to be segregated into distinct isolates. Microscopic features such as size and shape of the hyphae, conidia, and conidiophores were measured by the Olympus CX31 microscope, which was equipped with a Sony digital camera A77 for capturing images. The isolates sporulated on PDA media were segregated into distinct isolates, and the remaining sterile fungal isolates were subjected to identification via molecular methodology.

DNA extraction, PCR amplification, and DNA sequencing of the fungal ITS1-5.8S-ITS2 region

The endophytes were grown in potato dextrose broth (PDB), and the mycelia were harvested by filtration and grinded with liquid nitrogen thoroughly. Total genomic DNA of the endophytes was extracted using the DNeasy Plant Mini Kit (Qiagen, Germany), according to the manufacturer protocol. The ITS region of the rDNA was amplified with universal primers [ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC -3')] (White et al., 1990). The PCR mixture (50 μL) consisted of 100 ng genomic DNA, 5 μL 10X PCR buffer, 1.5 μM $MgCl_2$, 0.5 μM of each primer, 200 μM deoxyribonucleotide triphosphate, and 1 U Taq polymerase and autoclaved with double-distilled water.

PCR was performed by pre-heating at 95°C for 2 min; followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min, and 72°C for 10 min. After the reaction, 5 μL PCR mixture was separated on 1.5% (w/v) agarose gel containing GoldView I (5 μL/100 mL) and photographed with a Tocan 360 digital camera. The PCR products were sequenced by the Beijing Sanboyuanzhi Company Ltd. (Beijing, China). The ITS sequences of representative isolates from this study were submitted to the NCBI GenBank database with the following accession Nos.: KC623554, KC623555, KC623556, KC623557, KC623558, KC623559, KC623560, KC623561, KC623562, KC623563, KC623564, KC623565, KC623566, KC623567, KC623568, and KC623569.

Phylogenetic analysis

The ITS sequences of the endophytic fungi were compared to the data available in NCBI using BLAST (www.ncbi.nlm.nih.Gov/BLAST) to estimate the phylogenetic relationships of the endophytes (Altschul et al., 1997). Isolates were considered most closely to a certain genus when its identity was found to have a ≥95% match to that genus in the database. Moreover, when the similarity was <95%, the strains were considered unidentified (Sánchez et al., 2007). These sequences were aligned using the CLUSTALX program (Thompson et al., 1997). To construct the relevant phylogenetic tree, the MEGA 5.0 software was employed (Tamura et al., 2011). The alignment data were subsequently analyzed by the neighbor-joining (NJ) method (Kimura two-parameter distance calculation). The bootstrap value was calculated using 1000 replications to assess the reliability to the nodes of the tree.

Fermentation and preparation of endophytic fungus extracts

The isolates were inoculated into 500-mL Erlenmeyer flasks containing 250 mL PDB and cultured at 150 rpm and 26°C for10 days in a rotary shaker. The contents were mixed thoroughly with a glass rod and then filtered. The filtrate was extracted thrice with ethyl acetate at 60°C and filtered. The combined filtrates were evaporated until dry under reduced pressure on a rotary evaporator. Next, the dry ethyl acetate extract was dissolved in methanol.

Antifungal activity of extracts from endophytic fungi

The fungal extracts were tested for bioactivity against 6 different endophytic fungi by the agar well diffusion method, with slight modifications (Perez et al., 1990). Cultures that were 48 h old and grown on PDA were used for inoculation of the fungal strains onto PDA plates. After solidification of the molten PDA, the appropriate wells were made on agar plates using a cork borer (size 6.0 mm). A total of 0.05 mL methanolic extract was introduced into the agar wells. An incubation period of 24-48 h at 28°C was maintained for observation of antifungal activity of the fungal extracts. The antifungal activity was evaluated by measuring the inhibition zones surrounding the extracts. The complete antifungal analysis was carried out under strict aseptic conditions. The zones of inhibition were measured with an antibiotic zone scale (mm), and the experiment was carried out in triplicate. Natamycin and 0.05 mL methanol were added to the wells of each Petri dish as the positive and negative controls, respectively.

RESULTS

Isolation of endophytic fungi

A total of 602 sample segments from the leaves, roots, and stems of *C. inerme* were collected from the Beibu Gulf, Guangxi, China. Sixty-six isolates from 242 recovered strains were randomly selected and purified. The number of endophytes isolated from the different tissues is listed in Figure 1 and Table 1. Fifty of total 66 endophytes were recovered from the stems (75.7% occurrence). On the contrary, it was found that many of the leaf samples presented minimal fungal growth (only 3 isolates, 4.5% occurrence); endophytes from the root samples were identified in 19.6% of the total purified fungi. Among the 66 endophytic fungi, 16 morphotypes were recognizable based on colony or hyphal morphological characteristics, characteristics of the spores, and conidiophores (Figure 2). Among them, 28 strains produced spores in the culture medium and were identified as *Alternaria* sp, while the others needed further identification based on morphological and molecular methods.

To confirm the reliability of morphological identification, all 16 morphotypes were subjected to molecular identification via ITS sequence analysis (Table 2). Filamentous *Ascomycota* dominated all of the fungal endophytic communities in *C. inerme*, representing 100% of the isolates. Within the *Ascomycota*, 2 genera were particularly common, including *Alternaria* (42.4% of all isolates) and *Lasiodiplodia* (15.1% of all isolates). The genera *Nigrospora* and *Diaporthe* presented fewer fungi, 9.0 and 7.6%, respectively. Other identified genera, including *Bartalinia*, *Pestalotiopsis*, *Fusarium*, *Mycoleptodiscus*, *Trichoderma*, *Phomopsis*, and *Curvularia* were rarely isolated, exhibiting frequencies ranging from 3.0 to 4.5% (Table 1).

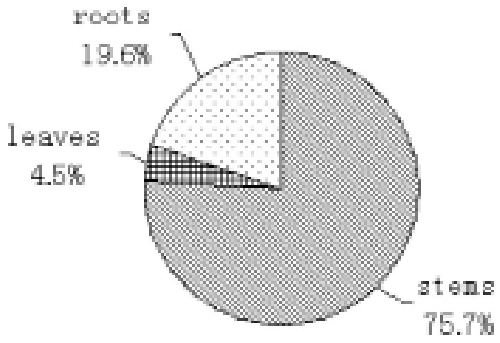

Figure 1. Endophytic fungi isolated from different tissues and their percentage.

Table 1. Endophytic fungi isolated from leaves, stems, and roots of *Clerodendrum inerme*.

Endophytic fungi	Leaf		Stem		Root		Total
	I	CF%	I	CF%	I	CF%	CF%
Alternaria sp	-	-	28	42.4	-	-	42.4
Nigrospora sp	-	-	3	4.5	3	4.5	9.0
Bartalinia sp	-	-	2	3	-	-	3
Pestalotiopsis sp	-	-	3	4.5	-	-	4.5
Fusarium sp	-	-	-	-	3	4.5	4.5
Trichoderma sp	-	-	-	-	2	3	3
Mycoleptodiscus sp	-	-	2	3	-	-	3
Phomopsis sp	-	-	2	3	-	-	3
Diaporthe sp	-	-	5	7.6	-	-	7.6
Lasiodiplodia sp	3	4.5	2	3	5	7.6	15.1
Curvularia sp	-	-	3	4.5	-	-	4.5

I = number of isolated strains; CF% = colonization frequency.

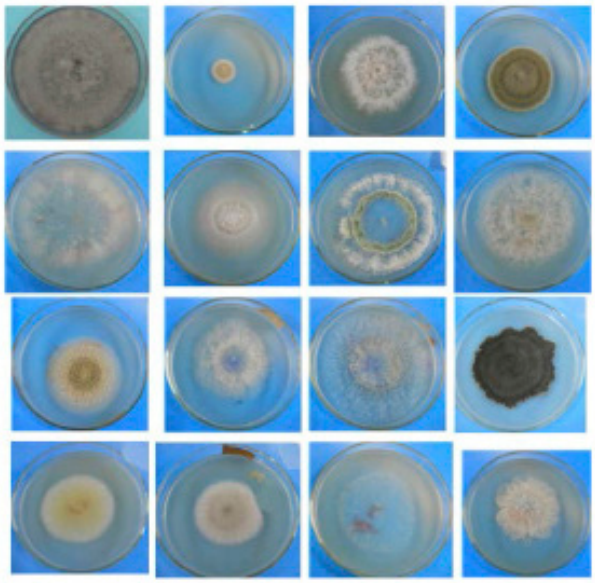

Figure 2. Colony morphology of some endophytic fungus isolates recovered from *Clerodendrum inerme* tissues.

Table 2. Culturable endophytic fungal ITS sequences closely matching to the GenBank sequences.

Isolates No.	Accession No.	Reference accession	Sequence coverage (%)	Max. identity (%)
Y1	KC623554	*Alternaria alternate* JN038452	98	99
Y2	KC623555	*Nigrospora* sp HQ832828	99	99
Y3	KC623556	*Alternaria* sp JN038452	98	99
Y4	KC623557	*Alternaria* sp KC139496	99	99
Y5	KC623558	*Bartalinia pondoensis* GU291796	99	99
Y13	KC623559	*Phomopsis phyllanthicola* FJ441623	98	99
Y15	KC623560	*Curvularia* sp HQ832834	99	99
Y16	KC623561	*Mycoleptodiscus indicus* GU980698	98	99
Y18	KC623562	*Diaporthe phaseolorum* JF441180	97	98
Y22	KC623563	*Alternaria arborescens* JQ936167	98	99
Y24	KC623564	*Lasiodiplodia pseudotheobromae* JX914479	97	99
Y25	KC623565	*Pestalotiopsis microspora* AF377296	98	99
KR1	KC623566	*Lasiodiplodia pseudotheobromae* JX914479	97	99
KR3	KC623567	*Trichoderma atroviride* JX421707	98	99
KR4	KC623568	*Fusarium oxysporum* JN232190	98	99
KR7	KC623569	*Nigrospora oryzae* HQ607943	99	99

Phylogenetic analysis

The ITS NJ tree of the endophytes is shown in Figure 3. The 16 morphospecies (i.e., Y1, Y2, Y3, Y4, Y5, Y13, Y15, Y16, Y18, Y22, Y24, Y25, KR1, KR3, KR3, and KR7) sharing a sequence max identity of ≥98% with available data in NCBI (Table 2) were grouped into 11 genera, including *Alternaria*, *Nigrospora*, *Bartalinia*, *Pestalotiopsis*, *Fusarium*, *Mycoleptodiscus*, *Trichoderma*, *Phomopsis*, *Diaporthe*, *Lasiodiplodia*, and *Curvularia*. Among these endophytes, the strains Y5, Y25, KR4, Y24, KR1, KR3, Y16, and Y4 exhibited high bootstrap support (100%) within their cluster, whereas the strains Y2, KR7, Y13, Y1, Y3, and Y22 formed their own cluster, with bootstrap values ranging from 25 to 99%. In this NJ tree, strain Y16 and reference taxa *Mycoleptodiscus indicus* UAMH 10746 (GU980698) formed a clade, with 100% bootstrap support. Strains Y1, Y3, Y14, Y22, Y15, Y18, Y13, KR3, KR1, KR4, KR7, Y2, Y25, and Y5 shared sequence max identities ranging from 98 to 100% [*Alternaria alternate* (100% bootstrap), *Curvularia* sp (100% bootstrap), *Diaporthe phaseolorum* (81% bootstrap), *Trichoderma atroviride* (100% bootstrap), *Lasiodiplodia theobromae* (71% bootstrap), *Fusarium oxysporum* (100% bootstrap), *Nigrospora oryzae* (99% bootstrap), *Nigrospora* sp (93% bootstrap), *Pestalotiopsis microspora* (100% bootstrap), and *Bartalinia robillardoides* (100% bootstrap)].

Antifungal activity of endophytes from *C. inerme*

The crude ethyl acetate extracts of endophytes from the stems of *C. inerme* were quantitatively assessed for their inhibition against the tested fungi *Alternaria*, *Lasiodiplodia*, *Pestalotiopsis*, *Nigrospora*, *Diaporthe*, and *Phomopsis*, which were isolated in this study using the agar diffusion method. Three of 10 endophytes (i.e., *Phomopsis*, *Curvularia*, and *Mycoleptodiscus*) displayed broad antifungal activity and inhibited the growth of most of the tested fungi (Table 3). Inhibition zones were observed for *Mycoleptodiscus* sp Y16 to all 6 tested fungi. Maximal inhibition zones for the fungi sensitive to the ethyl extract of *Mycoleptodiscus* were in the range of 6-23 mm. Ethyl acetate extracts of *Phomopsis* sp Y13 inhibited the growth of *Lasiodiplodia*, *Nigrospora*, and *Alternaria*, with inhibition zones in the range of 9-15 mm. The

ethyl acetate extracts of *Curvularia* sp obviously inhibited the growth of 4 tested endophytes (i.e., *Pestalotiopsis*, *Diaporthe*, *Alternaria*, and *Phomopsis*) (Table 3).

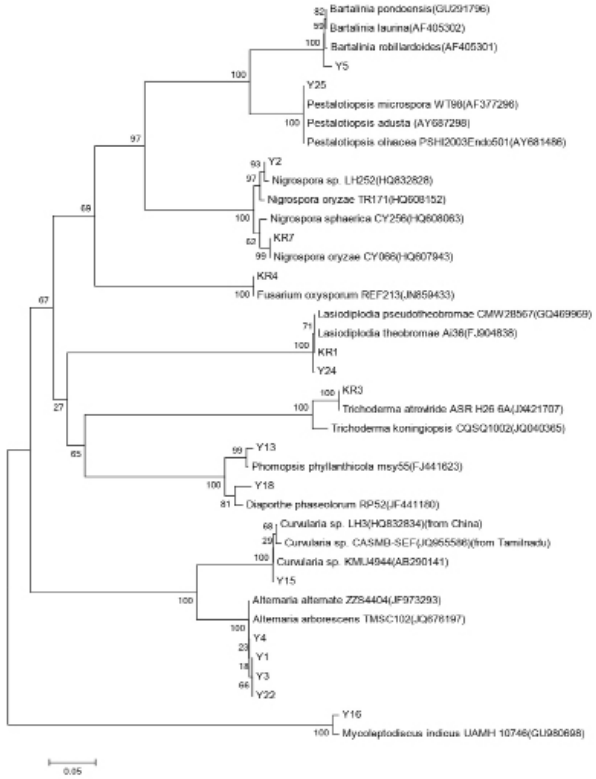

Figure 3. Neighbor-joining tree of the ITS sequences of the endophytic fungi associated with *Clerodendrum inerme inerme*. The tree was constructed based on rDNA sequence by using neighbor-joining method. The bootstrap consensus tree inferred from 1000 replicates.

Table 3. Antifungal activity of the crude ethyl extracts (50 µg/disc) of endophytes.

Culture number	Fungal isolates	Inhibition zone diameter around test disc					
		La[a]	Pe	Ni	Di	Al	Ph
Natamycin*		15	18	12	21	10	17
Negative control		-	-	-	-	-	-
Y1	*Alternaria alternate*	-	-	-	-	-	-
Y2	*Nigrospora* sp	-	8	-	-	-	-
Y5	*Bartalinia pondoensis*	-	-	-	-	5	-
Y13	*Phomopsis* sp	12	-	9	-	15	-
Y15	*Curvularia* sp	-	25	-	14	7	13
Y16	*Mycoleptodiscus* sp	8	12	14	23	17	18
Y18	*Diaporthe phaseolorum*	-	-	-	-	-	6
Y22	*Alternaria arborescens*	-	-	-	-	-	-
Y24	*Lasiodiplodia* sp	-	-	-	7	-	-
Y25	*Pestalotiopsis microspora*	-	-	6	-	-	-

(-) no activity; *10 µg/mL. Tested fungi: [a]La - *Lasiodiplodia* sp; Pe - *Pestalotiopsis* sp; Ni - *Nigrospora* sp; Di - *Diaporthe* sp; Al - *Alternaria* sp; Ph - *Phomopsis* sp.

DISCUSSION

This is the first report on endophyte communities from *C. inerme* in China and their antifungal activity *in vitro*. A similar study was conducted by Namasivayam et al. (2012) in which 25 endophytic fungi were recovered from *C. inerme* collected in India; however, the isolated fungi differed from those in the current study (Namasivayam et al., 2012).

Fusarium sp isolated from the roots was the most closely related to *Fusarium oxysporum*, which has a wide distribution in plants and grows in different ecosystems. Typically, *F. oxysporum* infects hosts via their roots, thus obstructing the vascular system (i.e., reducing or preventing the flow of water from the roots to the upper parts of the plant), which leads to wilting symptoms (Summere and Leslie, 2011).

The genus *Trichoderma* includes opportunistic plant symbionts that can colonize the apoplast of the plant roots (Brotman et al., 2013). The counter inhibition of *Trichoderma* against *Fusarium* has been observed *in vitro*, and *Trichoderma* spp may be used as ideal biocontrol agents (Dube et al., 2007; Poornima, 2011).

Mycoleptodiscus sp Y16 recovered from the stems of *C. inerme* displayed antifungal activity against 6 fungi. The genus *Mycoleptodiscus* comprises 15 species, including endophytes, plant pathogens, and human pathogens (Padhye et al., 1995; Watanabe et al., 1997; Rosa et al., 2012). Although the extract of *M. indicus* was found to exhibit larvicidal activity against *A. aegypti*, relatively little is known about the antifungal activity of this species. Siriwach et al. (2012) isolated a new chromone derivative, Mycoleptione, from *Mycoleptodiscus* sp MU41. Two novel reddish-orange alkaloids were also isolated from *Mycoleptodiscus* sp, which was obtained from *Desmotes incomparabilis* in Panama (Ortega et al., 2013).

Species of *Curvularia* have a wide distribution in different plants. According to Chomcheon (2010), 5 new hybrid peptide-polyketides [i.e., curvularides A-E (1-5)] were isolated from the endophytic fungus *Curvularia geniculata*, which was obtained from the limbs of *Catunaregam tomentosa*.

Phomopsis sp is often found in the asymptomatic leaves and roots of many different wild plants. Fu (2011) isolated antifungal metabolites from *Phomopsis* sp By254, an endophytic fungus in *Gossypium hirsutum*, and identified them as epoxycytochalasin H (1), cytochalasin N (2), and cytochalasin H (3). According to Wu et al (2013), 2 new steroids and 3 known steroids were isolated from the culture broth of the endophytic fungus *Phomopsis* sp, which was isolated from *Aconitum carmichaelii*. Most of these compounds showed moderate or weak antifungal activities in a broth-microdilution assay.

One attractive finding is that many endophytic fungi isolated from *C. inerme* are latent pathogens isolated in other plants. *Alternaria* spp are major plant pathogens, which cause at least 20% of agricultural spoilage throughout the world (Shipunov et al., 2008). Species of *Lasiodiplodia* are common in tropical and subtropical regions, where they cause a variety of plant diseases (Fujinawa et al., 2012). *Pestalotiopsis* are plant pathogens as well as endophytes in a wide range of hosts (Maharachchikumbura et al., 2011). Why can these latent pathogens live in plant tissues without apparent symptoms? One reason may be that endophytes produce antibiotic substances (Wang et al., 2007). Because of the increasing evidence that endophytes have apparent resistance to plant pathogens, endophytes are gaining attention as subjects for research and applications in Plant Pathology. In this study, 3 isolates showed considerable biocontrol potential against latent plant pathogens. Further studies will focus on the analysis and development of an efficient strategy for controlling plant pathogens based on these endophytic fungi.

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