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Full Length Research Paper

Antibacterial activity of the endophytic fungi from medicinal herb, *Macleaya cordata*

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A total of 48 endophytic fungal isolates were separated from the healthy roots of *Macleaya cordata* R. Br. (Papaveraceae), a traditional medicinal herb mainly distributed in China. Nine distinct isolates (Macof01 to Macof09) were selected for further taxonomical identification by morphological traits and internal transcribed spacer (ITS) rRNA gene sequence analysis. Seven genera namely *Acremonium*, *Alternaria*, *Aspergillus*, *Bionectria*, *Cladosporium*, *Neosartorya* and *Penicillium* were identified on the basis of their morphological characterizations. Fungal isolates Macof02 (*Bionectria ochroleuca*), Macof03 (*Neosartorya fischeri*) and Macof05 (unidentified) have not been previously reported as the endophytic fungi according to their ITS-rDNA sequences compared with those available in the GenBank database. Most of the fungal isolates displayed antibacterial activity. The extracts obtained from Macof02 (*B. ochroleuca*), Macof06 (*Aspergillus* sp.) and Macof08 (*Acremonium* sp.) exhibited strong inhibition on test bacteria. The endophytic fungi from *M. macleaya* could be an alternative source for producing antimicrobial agents.

Key words: *Macleaya cordata*, endophytic fungi, *n*-butanol extract, TLC-bioautography assay, antibacterial activity.

INTRODUCTION

Plant endophytic fungi are microorganisms that reside in internal tissues of living plants without causing any immediate overt negative effects or external symptoms, but may turn pathogenic during host senescence (Rodriguez et al., 2009). They have been regarded as an important and novel resource of natural bioactive compounds with great potential applications in agriculture, medicine and food industry (Verma et al., 2009; Zhao et al., 2011). In the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities have been successfully obtained from the endophytic fungi. These bioactive compounds could be mainly classified as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols and lactones (Aly et al., 2010; Zhou et al., 2010). Many pathogenic microorganisms have developed resistance due to the misusage or long-term usage of the same class of antibiotics. An intensive search for newer and more effective antibiotics to deal with these problems is now underway. Endophytes, which occupy a unique biotope with global estimation up to one million species, are a great choice to avoid replication in the study of natural products to assist in solving not only plant diseases, but also human and animal health problems (Strobel and Daisy, 2003; Strobel et al., 2004; Gimenez et al., 2007).

Since the endophytes can be found in nearly all living plant species, a scientific basis in plant selection is necessary for the study of endophytes in order to isolate microorganisms with pharmaceutical potential (Tong et al., 2011; Zhao et al., 2011). *Macleaya cordata* R. Br. (Papaveraceae) also named as plume poppy or *Bocconia cordata* is a perennial herb mainly distributed in the southeast area of China (Wu, 1999). It has been used as an important and traditional Chinese medicine (TCM) for its analgesic and anti-inflammatory properties in humans (Xinrong, 2003). The phytochemical and pharmacological studies conducted on this medicinal herb have successfully isolated several alkaloids with a wide range

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of biological activities including antimicrobial, cytotoxic and molluscicidal activity (Ulrichova et al., 1996; Sedo et al., 2002; Liu et al., 2009; Kosina et al., 2010; Ming et al., 2011).

To the best of our knowledge, there is no reported study on the endophytic fungi associated with *M. cordata*. The purpose of this study was to isolate and identify the endophytic fungi from the roots of *M. cordata* as well as to examine their antibacterial activity in order to provide additional data for the utilization of the antimicrobial metabolites from the endophytic fungi.

MATERIALS AND METHODS

The healthy roots of *M. cordata* R. Br. were collected from Changsha situated in Hunan Province of China in June 2008, and were authenticated by Prof. Fanglu Du from Hunan University of Chinese Medicine of China. A voucher specimen was deposited in the Herbarium of the Institute of Chinese Medicinal Materials, China Agricultural University. The plant samples were stored in the sealed plastic bags at 4°C until required.

Isolation and culture of the endophytic fungi

The isolation of fungi was performed following the process described by Xu et al. (2008) with modifications. Briefly, the healthy roots of *M. cordata* were washed thoroughly under running tap water first, then surface sterilized by dipping them in 75% ethanol for 30 s, followed by immersing in 0.2% mercuric chloride for 20 min, then rinsed in sterile distilled water thrice (5 min for each time), and finally dried on the sterile tissue paper. The epidermis of each root explant was removed with a sterile scalpel. The sterilization process was confirmed by placing the sterile epidermal tissues on potato dextrose agar (PDA) plate. After sterilization, each root (without epidermis) was cut approximately into 8 × 8 × 8 mm cubes which were individually placed on PDA plates supplemented with streptomycin sulfate (500 mg/L) to suppress bacteria growth. After the plates were incubated in the dark at 25°C for 7 to 14 days, the number of fungi was counted, and each fungal colony was isolated and subcultured to get a pure culture at last. The colonization frequency (CF) of each endophyte was calculated according to the method of Hata and Futai (1995): CF (%) = (N_{COL}/N_t) × 100, where N_{COL} is the number of cubes colonized by each fungus and N_t is the total number of cubes. All the isolated fungi were deposited at the Department of Plant Pathology, China Agricultural University.

Morphological characterization

The morphological characters of the fungal isolates were observed and described according to the method of Photita et al. (2005). Morphological identification according to the standard taxonomic key included colony diameter, texture, color and the dimensions and morphology of hyphae and conidia (Ainsworth et al., 1973).

DNA extraction, ITS-rDNA amplification and sequence analysis

Total genomic DNA of the fungal isolates was prepared according to a modification of the rapid preparation of DNA from filamentous fungi (Raeder and Broda, 1985). Primers ITS1 (5'- TCCGTAG-GTGAACCTGCGG -3') and ITS4 (5'- TCCTCCGCTTATTGATATGC -3'), as well as ITS-rDNA amplification were referenced by previous reports (Xu et al., 2008; Li et al., 2008; Zhong et al., 2011). For identification, the PCR products were purified using the QIA quick Gel Extraction Kits (Qiagen, Hilden, Germany) and sequenced using the primer pair ITS1 and ITS4 on the ABI PRISM 3730 sequencer. Then the sequences were run by BLASTN program against the database (National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov), and they were submitted to GenBank where the accession numbers were obtained.

Mycelial suspension culture and *n*-butanol extract preparation

A 1000-ml Erlenmeyer flask containing 200 ml of potato dextrose broth (PDB) was inoculated with 2-3 agar plugs containing mycelia taken from the culture of each endophytic fungal isolate purified on PDA. All flasks were incubated at 25°C on a rotary shaker at 150 rpm for 15 days. After suspension culture, the culture broth (1 L for each fungal isolate) was filtrated in vacuum to afford the filtrate and mycelia. The filtrate was extracted with an equal volume of *n*-butanol for three times. The mycelia were lyophilized and powdered, followed by extracting with ultrasound in methanol for three times. The concentrated methanol extract was dissolved in water, and then extracted with an equal volume of *n*-butanol for three times. Finally, the above *n*-butanol solutions were concentrated in vacuum at 50°C to obtain mycelia and filtrate extracts, respectively.

Detection of antibacterial activity of the extracts

Thin layer chromatography (TLC)-bioautography assay of the samples was carried out according to the method of Zhao et al. (2008). Three Gram positive (*Bacillus subtilis* ATCC 11562, *Staphylococcus aureus* ATCC 6538 and *Staphylococcus haemolyticus* ATCC 29970) and five Gram-negative (*Agrobacterium tumefaciens* ATCC 11158, *Escherichia coli* ATCC 25922, *Pseudomonas lachrymans* ATCC 11921, *Salmonella typhimurium* ATCC14028, and *Xanthomonas vesicatoria* ATCC 11633) bacteria were selected for antibacterial assay. All these bacterial strains were provided by the Department of Plant Pathology of China Agricultural University. Twenty milligrams of each mycelia or filtrate *n*-butanol extract was dissolved in 1 ml of methanol with ultrasonic assistance. Five microliters of the sample solution was sampled on the TLC plate. Developing solvent system in TLC was chloroform-methanol (10:1, v/v). After TLC was complete, 5 µL of streptomycin sulfate (CK^+) solution (0.2 mg/ml) was sampled on the lower right of the TLC plate. The test bacterial suspension was then covered on the TLC plate and incubated at 28°C for 12 h, the color reagent was sprayed with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, purchased from Amresco, USA), and incubated for another 2 h. The presence of antibacterial activity was determined by the formation of well-defined inhibition zones made visible by spraying with MTT that was converted to a formazan dye by the living microorganisms (Bernas and Dobrucki, 2000). Antibacterial activity was detected as white inhibition zones against a purple background, and the diameter of each antibacterial area was measured (Xu et al., 2010). All tests were performed in triplicate.

RESULTS AND DISCUSSION

Identification of the endophytic fungi

A total of 48 endophytic fungal isolates were obtained from the healthy roots of *M. cordata*. According to their

Table 1. Colonization frequency (CF) of the endophytic fungi and their closest relatives based on the data from BLAST analysis and morphological identification.

Fungal isolate	CF (%)	GenBank accession number	Closest related species	Similarity (%)	Macro- and microscopic identification
Macof01	15.8	HQ731620	*Penicillium janthinellum* AY373921	100	*Penicillium* sp.
Macof02	22.1	HQ731621	*Bionectria ochroleuca* GQ302681	99	*Bionectria* sp.
Macof03	19.0	HQ731622	*Neosartorya fischeri* AF176661	100	*Neosartorya* sp.
Macof04	12.6	HQ731623	*Aspergillus ustus* AY213637	97	*Aspergillus* sp.
Macof05	6.3	HQ731624	*Peyronellaea glomerata* AB470906	96	Unidentified
Macof06	2.1	HQ731625	*Aspergillus* sp. GQ169453	99	*Aspergillus* sp.
Macof07	2.1	HQ731626	*Cladosporium Cladosporioides* GU932679	100	*Cladosporium* sp.
Macof08	4.2	HQ731627	*Acremonium* sp. EF577238	100	*Acremonium* sp.
Macof09	6.3	HQ731628	*Alternaria* sp. GQ302684	99	*Alternaria* sp.

morphological features, nine distinct fungal isolates were selected for further taxonomical identification. Based on the results of the macro- and microscopic identification, they were identified as the members of seven genera, *Acremonium*, *Alternaria*, *Aspergillus*, *Bionectria*, *Cladosporium*, *Neosartorya* and *Penicillium* (Table 1). Their ITS1-5.8S-ITS4 partial sequences were submitted to the GenBank to obtain their accession numbers, and the closest related species were achieved by BLAST analysis. The results (Table 1) show that all the sequences had more than 96% similarity with the species in GenBank. The molecular characters of the endophytic fungi were basically coincident with their morphological ones, indicating the diversity of the fungi associated with *M. cordata*. Both isolates Macof04 and Macof05 had relatively low DNA similarities as 97% to *Aspergillus ustus* AY213637, and 96% to *Peyronellaea glomerata* AB470906, respectively which need further identification. Other endophytic fungi were in 99 or 100% of similarity to the closest related species in GenBank.

Aspergillus sp. was isolated from healthy *Cephalotaxus mannii* (Lu et al., 2008). In this study, the similarity of Macof04 was only 97% to *A. ustus* AY213637, and considerable morphological differences were found between Macof04 and Macof06, which could be in the genus *Aspergillus* but different species. The isolate Macof08 was identified as *Acremonium* sp. The characteristic morphology of the genus *Acremonium* consists of septate hyphae giving rise to thin, tapered, mostly lateral phialides produced singly or in small groups. Conidia tend to be unicellular, produced in mucoid heads or unconnected chains. They can be hyaline or melanised, but the hyphae are usually hyaline (Summerbell et al., 2011). *Acremonium* sp. has been isolated from some plants such as *Brachiaria brizantha* (Kelemu et al., 2001), *Festuca arizonica* (An et al., 1992), *Stipa robusta* (Petroski et al., 1992) and *Taxus globosa* (Soca-Chafre et al., 2011). *Cladosporium cladosporioides* (Macof07) has been isolated from *Dendrobium thyrsiflorum* (Xing et al., 2011), *Huperzia serrata* (Zhang et al., 2011), and *Taxus media* (Zhang et al., 2009). In addition, *Alternaria* sp. (Macof09) has been isolated from healthy *Lippia sidoides* (Siqueira et al., 2011). *Penicillium janthinellum* (Macof01) has been isolated from *Melia azedarach* (Marinho et al., 2005) and *Coffea* sp. (Vega et al., 2006). Other isolated fungi (Macof02, Macof03 and Macof05) have not been previously reported as the endophytic fungi.

Detection of antimicrobial activity

Antibacterial activity results of the endophytic fungal extracts against eight bacteria by using TLC-bioautography method is shown in Table 2. Most of the extracts from the fungal isolates except Macof01 and Macof07 showed strong antibacterial activity on test bacteria. For some fungal isolates (example, Macof01-Macof04, Macof06 and Macof08), the mycelia extracts showed stronger antibacterial activity than the filtrate extracts, or both mycelia and filtrate extracts showed the same antibacterial activity. Among the isolates, Macof02, Macof06 and Macof08 exhibited the strongest inhibition on test bacteria.

Interestingly, endophytic fungus *Acremonium* sp. was found to increase alkaloid production of its host *Stipa robusta* (Petroski et al., 1992). *M. cordata* is a perennial herb that produces isoquinoline alkaloids (Liu et al., 2009). Moreover, whether the endophytic fungi in this study such as *Acremonium* sp. Macof08 (Figure 1) could also enhance the alkaloid production of the host plant

Table 2. Antibacterial activity of the crude extracts from the endophytic fungi against different bacteria by TLC-bioautography-MTT test.

Fungal isolate	M/F	*A.t.*	*B.s.*	*E.c.*	*P.I.*	*S.a.*	*S.h.*	*S.t.*	*X.v.*
Macof01	M	+	++	+	+	++	+	+	+
	F	-	+	-	-	-	+	++	+
Macof02	M	+++	+++	+++	+++	+++	+++	+++	+++
	F	++	+++	++	+++	+++	+++	++	+++
Macof03	M	+++	+++	++	++	+++	+++	+++	+++
	F	++	++	+	+	++	++	++	++
Macof04	M	+++	+++	++	+++	+++	+++	+++	+++
	F	+	++	-	+	-	++	++	++
Macof05	M	++	++	++	+	+++	+++	+++	+++
	F	++	++	++	++	++	+	+++	++
Macof06	M	+++	+++	+++	+++	+++	+++	+++	+++
	F	++	+++	++	++	++	+++	+++	+++
Macof07	M	+	+	-	-	-	+	++	+
	F	+	+	-	-	-	-	++	+
Macof08	M	+++	+++	+++	+++	+++	+++	+++	+++
	F	++	++	++	++	++	++	++	++
Macof09	M	+++	+++	+	+	+++	+++	+++	+++
	F	+	++	+	+	+++	+	+++	++

M, Mycelia *n*-butanol extract; F, filtrate *n*-butanol extract; *A.t.*, *Agrobacterium tumefaciens*; *B.s.*, *Bacillus subtilis*; *E.c.*, *Escherichia coli*; *P.I.*, *Pseudomonas lachrymans*; *S.a.*, *Staphylococcus aureus*; *S.h.*, *Staphylococcus haemolyticus*; *S.t.*, *Salmonella typhimurium*; *X.v., Xanthomonas vesicatoria*; - sign indicate that antimicrobial activity was not observed; +, the diameter of the antimicrobial activity area was 0 to 5 mm; ++, the diameter of the antimicrobial activity area was 5 to 10 mm; +++, the diameter of the antimicrobial activity area was more than 10 mm; The positive control was streptomycin sulfate which was only sampled on the TLC plate and showed antibacterial activity.

M. cordata or produce alkaloids by themselves need further investigation. In addition, *C. cladosporioides* MD2 from *Taxus media* was screened to produce taxol (Zhang et al., 2009), and *C. cladosporioides* LF70 from *Huperzia serrata* was screened to produce huperzine A (Zhang et al., 2011). Whether the isolate Macof07 which was identified as *C. cladosporioides* also has the ability to produce the above valuable compounds needs further clarification in detail. Two *Aspergillus* species (*A. nidulans* and *A. oryzae*) isolated from the twigs of *Ginkgo biloba* L. were found to be able to produce phenolic and flavonoid compounds (Qiu et al., 2010). In addition, two *Aspergillus* species (Macof04 and Macof06) from *M. cordata* in this study should be further examined for their metabolites such as phenolics and flavonoids.

Conclusion

We first reported the endophytic fungi from medicinal plant *M. macleaya*, and detected their antibacterial activity. Some fungal isolates (example, Macof02, Macof06 and Macof08) displayed strong antibacterial activity. The endophytic fungi from *M. macleaya* could be an alternative source for producing antimicrobial agents. Studies to isolate antimicrobial compounds from these fungi as well as to taxonomically identify some fungi (example, Macof04 and Macof05) are now in progress. Furthermore, the significances of the endophytic fungi on the quality, active metabolite production, and medicinal effects of its host *M. macleaya* also need investigation.

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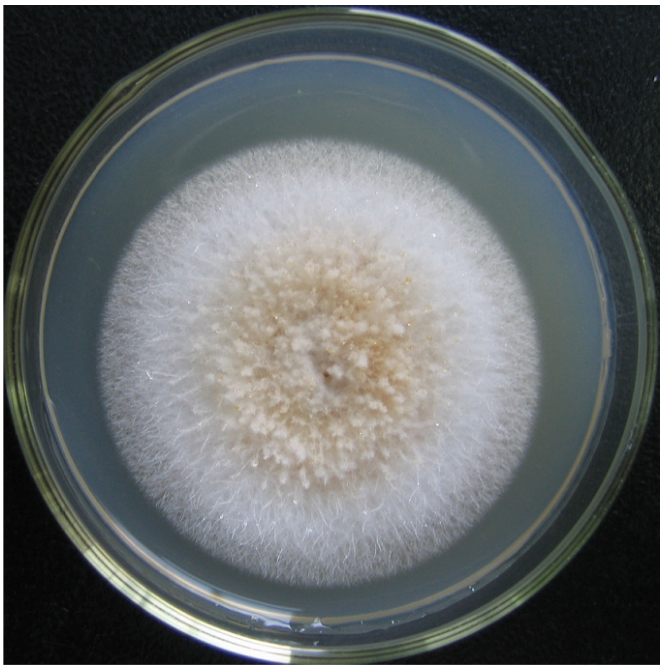

Figure 1. Colony front view of the fungal isolate Macof08 (*Acremonium* sp.). The period of culture at 25℃ on PDA was in 10 days.

(30871662 and 31071710).

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