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): 

\[
CF (\%) = \left( \frac{N_{cc}}{N} \right) \times 100, 
\]

where *N* is the number of cubes colonized by each fungus and *N* 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 Pholita 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'- TCCGTAAGTGAACCTGCGG -3') and ITS4 (5'- TCCTCGGTTATGTATGCTGC -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 extraction 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 and treated with streptomycin sulfate (500 mg/L) to suppress bacteria growth. After the filtration was confirmed by placing the sterile epidermal tissues on the plates, the filtrates were stored at 4°C until required.

**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 (Bennas 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.

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>CF (%)</th>
<th>GenBank accession number</th>
<th>Closest related species</th>
<th>Similarity (%)</th>
<th>Macro- and microscopic identification</th>
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<tbody>
<tr>
<td>Macof01</td>
<td>15.8</td>
<td>HQ731620</td>
<td>Penicillium janthinellum</td>
<td>100</td>
<td>Penicillium sp.</td>
</tr>
<tr>
<td>Macof02</td>
<td>22.1</td>
<td>HQ731621</td>
<td>Bionectria ochroleuca</td>
<td>99</td>
<td>Bionectria sp.</td>
</tr>
<tr>
<td>Macof03</td>
<td>19.0</td>
<td>HQ731622</td>
<td>Neosartorya fischeri</td>
<td>100</td>
<td>Neosartorya sp.</td>
</tr>
<tr>
<td>Macof04</td>
<td>12.6</td>
<td>HQ731623</td>
<td>Aspergillus ustus</td>
<td>97</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td>Macof05</td>
<td>6.3</td>
<td>HQ731624</td>
<td>Peyronellaea glomerata</td>
<td>96</td>
<td>Unidentified</td>
</tr>
<tr>
<td>Macof06</td>
<td>2.1</td>
<td>HQ731625</td>
<td>Aspergillus sp.</td>
<td>99</td>
<td>Aspergillus sp.</td>
</tr>
<tr>
<td>Macof07</td>
<td>2.1</td>
<td>HQ731626</td>
<td>Cladosporium Cladosporioides</td>
<td>100</td>
<td>Cladosporium sp.</td>
</tr>
<tr>
<td>Macof08</td>
<td>4.2</td>
<td>HQ731627</td>
<td>Acremonium sp.</td>
<td>100</td>
<td>Acremonium sp.</td>
</tr>
<tr>
<td>Macof09</td>
<td>6.3</td>
<td>HQ731628</td>
<td>Alternaria sp.</td>
<td>99</td>
<td>Alternaria sp.</td>
</tr>
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</table>

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 hylaline or melanised, but the hyphae are usually hylaline (Summerbell et al., 2011). Acremonium sp. has been isolated from some plants such as Brachiaaria brizantha (Kelemu et al., 2001), Festuca arizonic (An et al., 1992), Stipa robusta (Petroski et al., 1992) and Taxus globosa (Soca-Chafre et al., 2011). Cladosporium cladosporioidies (Macof07) has been isolated from Dendrobium thyrsiflorum (Xing et al., 2011), Hyperzia 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 azedarch (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 isouquinoline 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.

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<tr>
<td>Macof01</td>
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<td>Macof07</td>
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<td>Macof08</td>
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<td>Macof09</td>
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M, Mycelia n-butanol extract; F, filtrate n-butanol extract; A.t., Agrobacterium tumefaciens; B.s., Bacillus subtilis; E.c., Escherichia coli; P.l., 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.

**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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