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Endophytic fungi from medicinal herb *Salvia miltiorrhiza* Bunge and their antimicrobial activity

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A total of 57 endophytic fungal isolates were obtained from the roots of *Salvia miltiorrhiza* Bunge (Lamiaceae). Fourteen (14) distinct isolates were selected for further taxonomical identification by morphological traits and internal transcribed spacer (ITS) rRNA gene sequence analysis. Twelve (12) genera were identified among which *Alternaria* and *Fusarium* were dominants. Eight endophytic fungi (that is, *Pleosporales* sp. Samif02, *Leptosphaeria* sp. Samif03, *Peyronellaea glomerata* Samif04, *Xylomelasma* sp. Samif07, *Bionectria ochroleuca* Samif08, *Sarocladium kiliense* Samif11, *Petriella setifera* Samif13 and *Cadophora* sp. Samif14) were separated as the endophytic fungi from *S. miltiorrhiza* for the first time. Most of the fungal isolates were observed to have antibacterial activity that suggests antibacterial compounds mainly exist in mycelia. The ethyl acetate extracts of *Alternaria* sp. Samif01, *Xylomelasma* sp. Samif07, *Fusarium redolens* Samif09, *Sarocladium kiliense* Samif11 and *Petriella setifera* Samif13 were also observed to have antifungal activity. Among the isolates, *Alternaria* sp. Samif01 and *Sarocladium kiliense* Samif11 were found to have strong antibacterial and antifungal activities. The results indicate that there is a diversity of the endophytic fungi from *S. miltiorrhiza*, and these endophytic fungi could be an excellent resource for searching natural antimicrobial compounds.

Key words: *Salvia miltiorrhiza* Bunge, endophytic fungi, ethyl acetate extracts, antimicrobial activity, antimicrobial compounds.

INTRODUCTION

Endophytic fungi, colonizing inside the normal plant tissues, are rich and potential resources for producing bioactive metabolites such as antimicrobial, insecticidal, anti-viral, anti-tumor and antioxidant compounds (Strobel, 2003; Kharwar et al., 2011; Chowdhary et al., 2012). Some endophytic fungi have the ability to produce the same or similar bioactive compounds as those that originated from their host plants (Zhao et al., 2011). Isolation of the endophytic fungi which produce certain bioactive substances has also become an efficient method to screen broad-spectrum, stable and low phytotoxic biocontrol agents (Gimenez et al., 2007).

Salvia miltiorrhiza Bunge (Lamiaceae) an important and well-known medicinal herb in Asian countries, commonly known as "Danshen" or "Tanshen" in Chinese, has been widely used as a traditional Chinese medicine (TCM) for treatment of coronary artery diseases, angina pectoris, myocardial infarction, cerebrovascular diseases, various types of hepatitis, chronic renal failure, and menstrual disorders (Wang, 2010; Wu et al., 2012).

Some endophytic fungi have been isolated from species of the genus *Salvia*. Two cytotoxic alkaloids cochliodinol and isocochliodinol were isolated from endophytic fungus *Chaetomium* sp. derived from *S. officinalis* growing in Morocco (Debbab et al., 2009).

Some endophytic fungi from *S. miltiorrhiza* were

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examined to have tanshinone IIA with TLC and HPLC (Wei et al., 2010). The endophytic fungus *Trichoderma atroviride* D16 from the roots of *S. miltiorrhiza* was screened with High-performance liquid chromatography (HPLC) and LC-HRMS/MS to contain tanshinones I and IIA though these tanshinone-producing endophytic fungi should be further verified (Ming et al., 2012).

To the best of our knowledge, there were no reports about the screening of antimicrobial activity on the endophytic fungi from *S. miltiorrhiza*. The aim of this study was to further isolate and identify the endophytic fungi from the roots of *S. miltiorrhiza* as well as to examine the antimicrobial activity of the ethyl acetate extracts on pathogenic bacteria and fungi in order to provide additional data for utilization of the antimicrobial metabolites and these fungi as biocontrol agents.

MATERIALS AND METHODS

Plant materials and isolation of endophytic fungi

The three-year old healthy roots of *S. miltiorrhiza* Bunge were collected from the Institute of Medicinal Plant Development (116°16'27" E, 40°1'59" N), Chinese Academy of Medical Sciences, Beijing, China, in July 2011. The plant was identified according to the morphological features by Prof. Yuhai Guo, a botanist from the College of Agronomy and Biotechnology, China Agricultural University. The voucher specimen (BSMPMI-201107001) of this plant 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 for processing within 24 h of collection. The isolation of endophytic fungi was performed according to the previous reports with some modifications (Li et al., 2008; Xu et al., 2008).

The root samples were rinsed thoroughly with tap water to remove soil residues and dust, sterilized successively with 75% ethanol for 2 min and immersed in 0.2% mercuric chloride for 20 min, then rinsed in sterile distilled water for four times. After surface sterilization, both root epidermis and remnant tissues were cut into small pieces of 0.5 cm × 0.5 cm respectively, placed on potato dextrose agar (PDA) plates containing 500 µg/mL of streptomycin sulfate and incubated at 25°C until mycelia were apparent on PDA plates. Pure cultures were finally isolated by hyphal tip isolation on PDA plates without antibiotics and stored at 4°C.

Morphological characterization

The isolated fungi were observed and described according to the methods of Ainsworth et al. (1973), Photita et al. (2005), and Li et al. (2008), including colony morphology and microscopic observation of mycelia and asexual/sexual spores.

Colonization frequency of fungal endophytes

The colonization frequency (CF, %) of endophytes was calculated according to the method of Hata and Futai (1995):

$$CF\ (\%) = (N_{COL}/N_t) \times 100$$

Where, N_{COL} is the number of segments colonized by each fungus and N_t is the total number of segments.

DNA extraction, ITS-rDNA amplification and sequence analysis

Endophytic fungi were also identified based on the analysis of the ITS sequences of rDNA regions. Total genomic DNA of the fungal isolates was extracted according to the protocols described by Wang et al. (1993) and Jasalavich et al. (2000). The ITS regions were amplified by the polymerase chain reaction (PCR) with the primer pair ITS1 (5'- TCCGTAGGTGAACCTGCGG -3') and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Li et al., 2008; Xu et al., 2008; Zhong et al., 2011). For identification, the PCR products were purified using the QIA quick gel purification kit (Qiagen, Hilden, Germany) as described by the manufacturer's protocol and sequenced using the primer pair ITS1 and ITS4 on the ABI PRISM 3730 sequencer (Applied Biosystem, USA).

The sequences of the endophytic fungal strains were run by BLASTN program against the database (National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov), and then they were submitted to GenBank database where the accession numbers were obtained.

The sequences were aligned using the CLUSTAL×2.0 program and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0. The Kimura two-parameter model was used to estimate evolutionary distance. The phylogenetic reconstruction was done by using the neighbor-joining (NJ) algorithm (Naruya and Masatoshi, 1987), with bootstrap values calculated from 1,000 replicate runs, using the software routines included in the MEGA software.

Mycelia suspension culture and ethyl acetate extract preparation

Three mycelia plugs from the edge of the actively growing colony were inoculated into 500 mL Erlenmeyer flasks containing 200 mL potato dextrose broth (PDB). The cultures were incubated at 150 rpm on a rotary shaker at 25°C for 20 days. After suspension culture, the fermented broth was filtrated under vacuum to afford the filtrate and mycelia. The filtrates were extracted thrice with an equal volume of ethyl acetate (1:1, v/v). The mycelia were dried and powdered, followed by extraction with ultrasound in ethyl acetate for three times. The ethyl acetate extracts from the mycelia and filtrate were obtained by evaporation under vacuum, respectively.

Detection of antimicrobial activity of the ethyl acetate extracts

The antimicrobial activities of the ethyl acetate extracts were detected by TLC-bioautography assay (Zhao et al., 2008). Four bacterial strains including two Gram-positive (*Bacillus subtilis* ATCC 11562 and *Clavibacter michiganensis* LP-0301) and two Gram-negative (*Agrobacterium tumefaciens* ATCC11158 and *Pseudomonas lachrymans* ATCC11921) bacteria were selected for antibacterial assay. The TLC plate covered with the test bacterium was incubated at 28°C for 12 h, then 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 successively for another 10 min. The antibacterial activity of the ethyl acetate extracts 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 microorganism (Bernas and Dobrucki, 2000). Antibacterial activity was detected as the white inhibition zones against a purple background, and the length of each antibacterial area was also measured in order to calculate its R_f value:

$$R_f = D_1/D_2,$$

Where, D_1 is the distance (mm) between the antimicrobial area and

Table 1. Colonization frequency (CF) of the endophytic fungi, 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
Samif01	14.52	KC878695	*Alternaria* sp. KC139492	100	*Alternaria* sp.
Samif02	4.84	KC878696	*Pleosporales* sp. JN859326	99	*Pleosporales* sp.
Samif03	4.84	KC878697	*Leptosphaeria* sp. GU934537	99	*Leptosphaeria* sp.
Samif04	3.22	KC878698	*Peyronellaea glomerata* KC33977	99	*Peyronellaea* sp.
Samif05	3.22	KC878699	*Phoma pedeiae* GU237770	99	*Phoma* sp.
Samif06	4.84	KC878700	*Phoma eupyrena* HQ115670	100	*Phoma* sp.
Samif07	8.06	KC878701	*Xylomelasma* sp. FR837913	99	*Xylomelasma* sp.
Samif08	6.45	KC878702	*Bionectria ochroleuca* JQ794833	100	*Bionectria* sp.
Samif09	6.45	KC878703	*Fusarium redolens* HQ443207	100	*Fusarium* sp.
Samif10	9.68	KC878704	*Fusarium* sp. JX243851	100	*Fusarium* sp.
Samif11	4.84	KC878705	*Sarocladium kiliense* JX499275	100	*Sarocladium* sp.
Samif12	6.45	KC878706	*Aspergillus* sp. JX029073	100	*Aspergillus* sp.
Samif13	8.06	KC878707	*Petriella setifera* JX501314	100	*Petriella* sp.
Samif14	6.45	KC878708	*Cadophora* sp. JN859262	96	*Cadophora* sp.

initial sample point, and D_2 is the distance (mm) between the developing solvent front and initial sample point on a TLC plate (Zhong et al., 2011).

Two phytopathogenic fungi *Fusarium oxysporum* f.sp. *niveum* and *Magnaporthe oryzae* were also selected for antifungal assay. The TLC plate was spread with the test fungal conidia. Then, it was incubated at 25°C for 4 to 7 days; the inhibition zone of mycelia growth was visible, and the R_f value of the antifungal area was determined without MTT treatment. All the test bacteria and fungi were provided by the Department of Plant Pathology, China Agricultural University, and TLC-bioautography assay was performed in three times.

RESULTS AND DISCUSSION

Identification of the endophytic fungi

A total of 57 endophytic fungal isolates were separated from the root epidermis and remnant tissues of *S. miltiorrhiza*. According to their morphological characters (the shape of conidia, type of conidiophores, growth rate, colony color and texture), 14 representative fungal isolates were selected for further macro and microscopic identification. They were identified as 12 genera including *Alternaria* (Samif01), *Pleosporales* (Samif02), *Leptosphaeria* (Samif03), *Peyronellaea* (Samif04), *Phoma* (Samif05 and Samif06), *Xylomelasma* (Samif07), *Bionectria* (Samif08), *Fusarium* (Samif09 and Samif10), *Sarocladium* (Samif11), *Aspergillus* (Samif12), *Petriella* (Samif13) and *Cadophora* (Samif14) (Table 1). Among them, *Alternaria* (Samif01) and *Fusarium* (Samif09 and Samif10) were two dominant genera with their colonization frequency (CF) as 14.52% and 16.13%, respectively.

The ITS1-5.8S-ITS4 partial sequences of 14 distinct isolates were submitted to the GenBank to obtain their accession numbers (i.e. KC878695 - KC878708), and the closest related species were got by BLAST analysis (Table 1). Except for Samif14, other isolated endophytic fungi had homology greater than or equal to 99% to their closest related species. Fourteen (14) isolates were identified on the basis of morphological traits and ITS rRNA gene sequence analysis. The molecular characters of the endophytic fungi were basically coincident with their morphology. For example, isolate Samif03 had a dense colony with white to grey mycelia, and spherical shaped conidia. It was tentatively identified as *Leptosphaeria* sp. (Camara et al., 2002). The closest sequence similarity of isolate Samif03 was 99% to the fungus *Leptosphaeria* sp. (GU934537) in GenBank. In agreement with the morphology-based diagnosis, isolate Samif03 was clustered in a clade containing *Leptosphaeria* sp. (GU934537) with 100% NJ bootstrap support (Figure 1). On the basis of the ITS sequence and morphological traits, isolate Samif03 was considered as the member of the genus *Leptosphaeria*, and identified as *Leptosphaeria* sp. (Ainsworth et al., 1973; Camara et al., 2002).

Comparison of the ITS-rDNA sequences obtained from

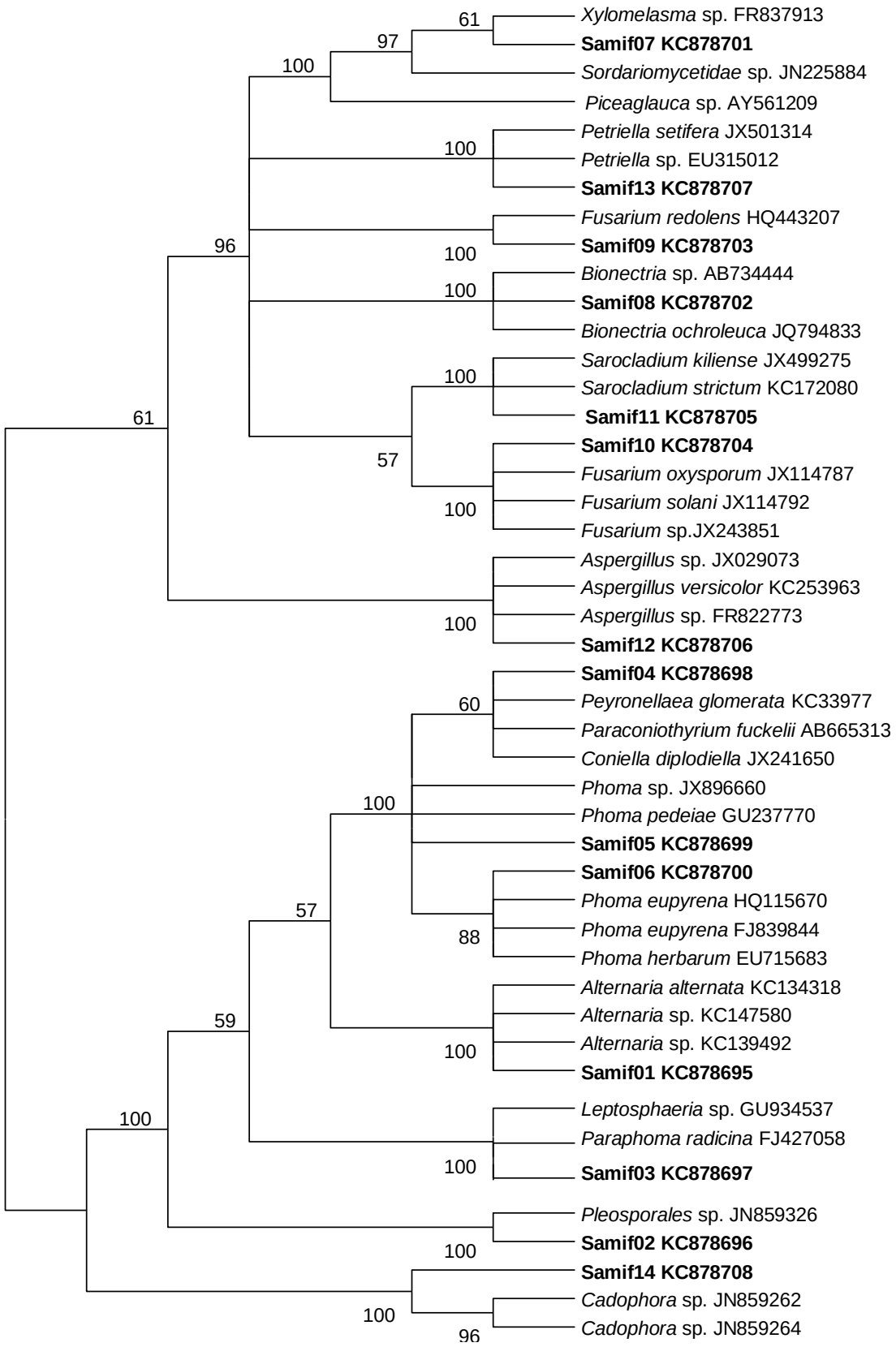

Figure 1. Phylogenetic relationship analysis of the fungal isolates Samif01 to Samif14 from *S. miltiorrhiza* Bunge to other fungi from GenBank, deduced from the ITS rDNA sequences. The numbers at the branches indicate the percentages of trees from 1000 bootstrap replication in which the branch occurs. The unrooted tree was generated using Clustal×2.0 program by Neighbor-Joining method. Phylogeny test was computed by MEGA 4.0.

Table 2. Antibacterial activity of the ethyl acetate extracts from the endophytic fungi by TLC-bioautography-MTT assay.

Fungal isolate	M/F	R_f value of the antibacterial area (Diameter of the antibacterial area)			
		Agrobacterium tumefaciens	*Bacillus subtilis*	*Clavibacter michiganensis*	*Pseudomonas lachrymans*
Samif01	M	0-0.57 (+++)	0-0.53 (+++)	0-0.53 (+++)	0-0.45 (+++)
	F	0-0.55 (+++)	0-0.52 (+++)	0-0.52 (+++)	0-0.42 (+++)
Samif02	M	0-0.47 (++)	0-0.18(++), 0.35-0.68(+)	0.63-0.68 (+)	0-0.43 (++)
	F	0-0.13 (+)	0-0.57(++), 0.65-0.72(+)	0-0.18(++), 0.37-0.70(+)	0-0.10 (+)
Samif03	M	0-0.33(++), 0.45-0.58(+)	0-0.53 (++)	0-0.52 (++)	0-0.35 (++)
	F	0-0.20 (+)	0-0.32 (++)	0-0.30 (+)	0-0.18 (+)
Samif04	M	0-0.38 (++)	0-0.37 (++)	0-0.35 (++)	0-0.42 (++)
	F	nd	nd	nd	nd
Samif05	M	0-0.45 (++)	0-0.42 (++)	0-0.35 (++)	0-0.30 (+)
	F	0-0.03 (+)	0-0.22 (++)	0-0.20 (++)	nd
Samif06	M	0-0.35 (+)	0-0.28 (++)	0-0.27 (++)	0-0.32 (+)
	F	nd	0-0.18 (+)	0-0.18 (+)	nd
Samif07	M	0-0.15 (+)	0-0.15 (+)	0-0.15 (+)	0-0.23 (+)
	F	0-0.03 (+)	0-0.23 (+), 0.35-0.43 (+)	0-0.30 (+), 0.35-0.47(+)	0-0.17 (+)
Samif08	M	0-0.30 (++)	0-0.37 (++)	0-0.35 (++)	0-0.30 (++)
	F	0-0.45 (+)	0-0.72 (++)	0-0.70 (++)	0-0.35 (+)
Samif09	M	0-0.53 (++)	0-0.52(++), 0.57-0.65(+)	0-0.53(++), 0.57-0.67(+)	0-0.72 (++)
	F	0-0.20 (++)	0-0.27 (++)	0-0.28 (+)	0-0.38 (+)
Samif10	M	0-0.43 (++)	0-0.51 (++)	0-0.57 (++)	0-0.60 (++)
	F	0-0.18 (++)	0-0.27 (++)	0-0.28 (+)	0-0.36 (+)
Samif11	M	0-0.72 (++)	0-0.43(++),0.57-0.68(+)	0-0.68 (++)	0-0.72 (++)
	F	0-0.42 (++)	0-0.32 (++)	0-0.32 (++)	0-0.48 (++)
Samif12	M	0-0.02 (+)	0-0.05 (+)	Nd	Nd
	F	0-0.03 (+)	0-0.17 (+)	0-0.23 (+)	Nd
Samif13	M	0-0.40 (+)	0-0.42 (++)	0-0.38 (++)	0-0.42 (+)
	F	0-0.05 (+)	0-0.20 (+)	0-0.18 (+)	Nd
Samif14	M	0-0.23 (+)	0-0.63 (++)	0-0.68 (+++)	0-0.20 (+)
	F	0-0.73 (+)	0-0.18 (+)	nd	0-0.68 (+)

M, mycelia ethyl acetate extract; F, filtrate ethyl acetate extract; developing solvent system in TLC was petroleum ether-acetone (2:1, v/v); nd, antimicrobial activity was not detected; +, the diameter of the antimicrobial activity area was 0-5 mm; ++, the diameter of the antimicrobial activity area was 5-10 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.

the isolates with the sequences available in the GenBank databases allowed us to analyze the phylogenic affiliation of these fungi (Figure 1). The phylogenic relationship demonstrated that the isolates could be sorted to two groups (clades). The first group was composed of isolates Samif07, Samif08, Samif09, Samif10, Samif11, Samif12 and Samif13.

The second group was composed of isolates Samif01, Samif02, Samif03, Samif04, Samif05, Samif06 and Samif14. To the best of our knowledge, Samif02 (*Pleosporales* sp.), Samif03 (*Leptosphaeria* sp.), Samif04 (*Peyronellaea glomerata*), Samif07 (*Xylomelasma* sp.), Samif08 (*Bionectria ochroleuca*), Samif11 (*Sarocladium kiliense*), Samif13 (*Petriella setifera*) and Samif14 (*Cadophora* sp.) were isolated from *S. miltiorrhiza* Bunge for the first time.

Detection of antimicrobial activity

Tables 2 and 3 show the antimicrobial activity of the ethyl acetate extracts obtained from the isolated fungi by using TLC-bioautography assay. R_f values of the antimicrobial areas can indicate the relative polarity of the active compounds in the samples, and the diameters can indicate the relative antimicrobial activity of the compounds (Bernas and Dobrucki, 2000; Zhong et al., 2011). Most of the ethyl acetate extracts except the filtrate extract of isolate Samif04 showed antibacterial activity to some extent, and antibacterial compounds mainly existed in mycelia. The ethyl acetate extracts of isolates Samif01, Samif08, Samif09, Samif10 and Samif11 were found to have stronger antibacterial activity than other fungal extracts. Compared with antibacterial

Table 3. Antifungal activity of the ethyl acetate extracts from the endophytic fungi on plant fungal pathogens by TLC-bioautography assay.

Fungal isolate	M/F	R_f value of the antifungal area (Diameter of the antifungal area)	
		Fusarium oxysporum f. sp. *niveum*	*Magnaporthe oryzae*
Samif01	M	0-0.18 (++)	0-0.40 (+++)
	F	0-0.20 (++)	0-0.43 (+++)
Samif02	M	nd	0.63-0.67 (+)
	F	0.53-0.60 (+)	0.52-0.61 (+)
Samif03	M	nd	0-0.10 (+)
	F	nd	0-0.08 (+)
Samif04	M	nd	nd
	F	nd	nd
Samif05	M	nd	nd
	F	nd	nd
Samif06	M	nd	0-0.06 (+)
	F	nd	nd
Samif07	M	0-0.10 (+)	0-0.13 (+)
	F	0-0.10 (+)	0-0.13 (+)
Samif08	M	0-0.12 (+)	nd
	F	nd	nd
Samif09	M	0-0.20 (+)	0-0.03 (+)
	F	0-0.15 (+)	0-0.10 (+)
Samif10	M	nd	0-0.21 (+)
	F	nd	0-0.03 (+)
Samif11	M	0-0.10 (++)	0-0.27 (++)
	F	0-0.03 (++)	0-0.11 (++), 0.35-0.51 (+)
Samif12	M	nd	0-0.06 (+)
	F	nd	0-0.10 (+)
Samif13	M	0-0.67 (+)	0-0.52 (+)
	F	0-0.03 (+)	0-0.08 (+)
Samif14	M	0-0.05 (+)	0-0.05 (+), 0.30-0.40 (+)
	F	nd	0-0.08 (+), 0.34-0.41 (+)

The positive control was carbendazim which was only sampled on the TLC plate and showed antifungal activity. Other notes are the same as those in Table 2.

activity, only a few endophytic fungal extracts (isolates Samif01, Samif07, Samif09, Samif11 and Samif13) showed antifungal activity on two test phytopathogenic fungi (Table 3). The ethyl acetate extracts of isolates Samif01 and Samif11 were also found to have stronger antifungal activity than other extracts. The results indicate that both antifungal and antibacterial compounds mainly exist in the ethyl acetate extracts of isolates Samif01 and Samif11.

Conclusion

In this study, we reported the endophytic fungi from the roots of *S. miltiorrhiza* Bunge and the detection of the antimicrobial activities of their ethyl acetate extracts. Twelve (12) genera were identified among which both *Alternaria* and *Fusarium* were dominant endophytes. Fourteen (14) fungal isolates were identified by both morphological and molecular methods. Eight endophytic fungi such as *Pleosporales* sp. Samif02, *Leptosphaeria* sp. Samif03, *Peyronellaea glomerata* Samif04, *Xylomelasma* sp. Samif07, *Bionectria ochroleuca* Samif08, *Sarocladium kiliense* Samif11, *Petriella setifera* Samif13 and *Cadophora* sp. Samif14 were separated as the endophytic fungi from *S. mitlorrhiza* for the first time. Some fungal isolates (*Alternaria* sp. Samif01 and *Sarocladium kiliense* Samif11) displayed strong anti-bacterial and antifungal activities. The results indicate that there is a diversity of the endophytic fungi from *S. miltiorrhiza*, and these endophytic fungi will have a great potential as producers of natural antimicrobial compounds.

Further investigation will focus on the isolation of the antimicrobial compounds from these fungi as well as on their applications as biocontrol agents. Other biological activities (cytotoxic, insecticidal, and antioxidant activities) of the endophytic fungi from *S. miltiorrhiza* also should be studied in detail.

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