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ANTIMICROBIAL ACTIVITY OF ENDOPHYTIC FUNGUS *FUSARIUM* SP. ISOLATED FROM MEDICINAL HONEYSUCKLE PLANT

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Abstract: Endophytes of plants have a wide range of strains comprising important sources of various bioactive metabolites. An endophytic fungus was isolated from honeysuckle, an important Chinese medicinal plant. The phylogenetic and physiological characterization indicated that the isolated strain JY2 corresponded to *Fusarium* sp. The culture filtrate of JY2 displayed antagonism activity against some pathogenic bacteria owing to the existence of antimicrobial compounds. The filtrate revealed the strongest *in vitro* antimicrobial activity on *Pseudomonas aeruginosa* by increasing the permeability of cell membranes. The antibacterial extract was fractionated and purified using silica gel chromatography. Five different bioactive compounds were isolated by bioactivity-guided fractionation from the culture extracts of JY2, and preliminarily identified by HPLC-MS spectral data. These results suggest that *Fusarium* sp. features a potentially remarkable antimicrobial activity and could be valuable to discover the new drugs or agents for antimicrobial purposes.

Key words: Endophyte; antimicrobial activity; *Fusarium* sp.; culture broth

INTRODUCTION

Endophytes are endosymbiotic microorganisms (e.g. bacteria or fungi) that live within plants for at least part of their life without causing apparent disease. Endophytes ubiquitously existing in plants have a wide range of antimicrobial strains comprising important sources of various bioactive metabolites, including antimicrobial, antiviral, anticancer and antidiabetic compounds (Strobel and Daisy, 2003). Bioactive natural compounds produced by endophytes have been showing some promising potential as remedies for human health concerns. The antimicrobial compounds are involved in defense against pathogens (Aly et al., 2011). In addition, antimicrobial compounds may also reduce cell toxicity towards higher organisms because the plant itself serves as a natural selection system (Yang et al., 1977). Plants are generally hosts to one or more endophytes (Schulz et al., 1995). Hence, endophytes have a remarkable potential as a source of novel, highly active antimicrobial compounds with low toxicity.

The dried flower buds of honeysuckle (*Lonicera japonica* Thunb.) have been utilized in traditional Chinese medicine for over 1000 years (Chen et al., 2005; Li et al., 2003). This plant is commonly cultivated as a highly valued medicinal and garden plant in eastern Asia, particularly China (Chai et al., 2005). Interestingly, the available literature provides no information on the isolation of any endophytes from honeysuckle or identification/proof of bioactive substances with any biological activities.

In the light of this, the purpose of our study was to isolate, select and identify the endophytic fungus *Fusarium sp*. JY2 from honeysuckle. Gram-negative *Pseudomonas aeruginosa* was selected as a test bacterium to investigate the bacterial lytic activity on the culture filtrate of strain JY2.

MATERIALS AND METHODS

Isolation of endophytes

Honeysuckle leaves were obtained from the campus of Henan University of Technology. Samples were transported in paper bags, stored overnight at 4°C and processed the following day. Plant materials were surface-sterilized according to the method described by Schulz et al. (1995) with some modifications. Briefly, honeysuckle leaves were washed in running water, and then successively sterilized with 75% ethanol for 1 min and 5% sodium hypochlorite for 5 min. After being rinsed in sterile water three times, the outer tissues of samples were removed with a sterile knife blade and the remainder was cut into 1-cm-long bars. The bars were vertically halved and incubated on PDA medium at 28±1°C until mycelia or colonies appeared around the segments. The mycelia or colonies were purified and cultured under the same condition. A similar procedure without surface sterilization was conducted as a negative control to check the surface-contaminating microbes.

Identification and characterization of the JY2 strain

Fungal identification was carried out on the basis of microscopic morphology and molecular taxonomy. Total DNA of the endophytic fungal isolate was extracted from fungal mycelia grown in PDA using a DNeasy Plant Mini Kit (QIAGEN GmbH; Hilden, Germany). Primers ITS5 (GGAAGTAAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) were used to amplify the ITS1-5.8S-ITS2 ribosomal RNA gene region from total cellular DNA (White et al., 1990). The thermal cycle program was as follows: 3 min at 95°C followed by 30 cycles of 50 s at 95°C, 40 s at 45°C and 40 s at 72°C, with a final extension period of 10 min at 72°C. The amplified DNA was purified and directly subjected to sequencing reactions using the ITS5 and ITS4 primers. The ITS sequences were compared to rDNA-ITS gene sequences in the database?? using the BLASTN program.

Indicator microorganisms and culture conditions

Ten types of bacteria were utilized to test the antimicrobial activities of the isolate (Table 1). These microorganisms were purchased from the China Committee of Culture Collection for Microorganisms (CCCCM). Bacteria were inoculated in the nutrient agar (NA) at 37°C for 24 h. Indicator strain cultures were always adjusted to OD_{600} = 0.5 before inoculation on the media for stainless cylinder diffusion assays.

Assay of antimicrobial spectrum of strain JY2

The strain JY2 was inoculated into PD broth (50 mL) in a 250-mL Erlenmeyer flask and incubated in a rotary shaker (180 rev/min) at 28°C for 7 d. Cell-free supernatant was obtained by centrifugation (6000*g*, 10min) and filtered on a 0.45-μm Millipore filter. The antimicrobial activity of the culture filtrate was evaluated using the method of stainless steel cylinders (Li et al., 2008). The suspension of each indicator strain (0.5 mL) was mixed with 10 mL of suitable agar media at 55°C, and the mixture was immediately poured into

Table 1. Antimicrobial activity of the culture filtrate of endophytic Fusarium sp. JY2.

	Inhibition zone in diameter on petri plates (mm)		
	Culture filtrate	Honeysuckle extract	1% streptomycin sulfate
Gram-positive bacteria			
Staphylococcus aureus	26.4±0.5	14.4±1.0	19.5±0.3
Bacillus anthracis	13.5±0.4	8.8±0.6	18.3±0.6
α-hemolytic streptococcus	22.1±0.4	10.8±0.6	11.2±0.8
Gram-negative bacteria			
Escherichia coli	31.6±0.3	12.5±0.3	20.5±0.3
Pseudomonas aeruginosa	35.2±0.2	18.2±0.3	24.1±0.3
Bacillus proteus	15.1±0.3	8.2±0.3	7.2±0.6
Salmonellae enteritis	28.9±0.4	15.7±0.5	16.7±0.7
Eberthella typhi	25.6±0.3	14.8±0.7	12.6±0.4
Shigella dysenteriae	31.9±0.2	16.6±0.5	12.7±0.6
Helicobacter pylori	26.5±0.7	16.3±0.4	10.9±0.5

Values are means±standard deviation of four disks.

a 9-cm-diameter Petri dish. Upon solidification, four sterilized stainless cylinders (6 mm internal diameter and 10 mm high) were equidistantly placed open end up on each plate. Filter-sterilized culture filtrate (200 μL) of JY2 was added to the cylinders. After incubation, the diameter of a round inhibition zone against the indicator strains was measured with calipers.

Determination of minimum inhibitory concentration (MIC)

A broth microdilution method was used to determine the MIC (Wiegand et al., 2008). Serial doubling dilutions of the culture filtrate were prepared in a 96-well plate, ranging from 0.0328 to 12.4 mg/mL. The final concentration of test strain was adjusted to 1×10^6 CFU/ml. Plates were incubated at 37°C for 24 h for bacteria and 30°C for 24 h for fungus. The MIC was defined as the lowest concentration of essential oil at which the microorganism does not demonstrate visible growth. The microorganism growth was indicated by the turbidity.

Determination of minimum bactericidal concentration (MBC)

To determine MBC, broth was taken from each well and incubated in PDA at 37°C for 24 h for bacteria. The MBC were determined by serial subcultivations of 20 μL into microtiter plates containing100 μL of broth per well and further incubation for 24 h at 37°C. The MBC was defined as the lowest concentration of essential oil at which the incubated microorganism was killed. Each test was carried out in triplicate and repeated three times (Wiegand et al., 2008).

Alkaline phosphatase and β-galactosidase assays

To test for bacterial lytic activity on the culture filtrate by the strain JY2, the culture filtrate at MIC was added in PD medium with the test bacterium (adjusted to 1×10^6 CFU/mL). After incubation for different time points at 37°C, the broth was centrifuged at 5000×*g* for 5 min and 50 μl of the supernatant were transferred into one plate in which alkaline phosphatase and β-galactosidase assays were performed by alkaline phosphatase activity colorimetric assay kit (Nanjing Jiancheng Technology Co. Ltd, Nanjing, China) and β-galactosidase staining kit (Genmed Scientifics, Inc, USA), respectively (Seeber, 2000) according to Malamy and Horecker (1964). The lytic activity was defined as the OD value at 520 nm and the relative fluorescence intensity (detected by a Bio-Rad Model 680 microplate reader (USA)) using the alkaline phosphatase and β-galactosidase assay kits, respectively.

Preparation, determination and purification of antimicrobial substances

The filter-sterilized culture filtrate of JY2 was concentrated in a rotatory evaporator under vacuum (at 60°C) and then lyophilized. The powder was extracted three times with methanol. The residue was dissolved in pH 7.0 PBS buffer for antimicrobial assays. The combined extracts were then concentrated in a rotatory evaporator, and then dissolved in methanol. The crude extract was subjected to silica gel (200-300 mesh) column chromatography (4.2×17.8 cm) and eluted with 200 mL ethyl acetate-methanol solutions (80:20, v/v) at a rate of 5.0 mL/min to afford 90 fractions (1.0 mL/fraction). The eluent of the fractions was concentrated for antimicrobial activity assays.

LC-MS-MS analysis

LC-MS-MS analyses were performed with an Agilent 1200 series HPLC system coupled to an Agilent 6310 ion-trap mass spectrometer with an ESI source, operated under negative-ion mode. The ESI capillary voltage was set at 3500 V and the fragmentor at 170 V. The liquid nebulizer was set to 15.0 psi, and the nitrogen drying gas was set to a flow rate of 5.0 L/min. The drying gas temperature was maintained at 325°C. The acquisition rate was 1.5 spectra/s and the stored mass range was m/z 50–1,000. All data were processed using Data Analysis for 6300 series Ion Trap LC/MS Version 3.4 software.

Statistical analysis

The results were expressed as mean value±standard deviation (SD). The results were analyzed for statistical significance by one-way analysis of variance (ANOVA) using the Statistical Package of the Social Science (SPSS) version 11.0 (SPSS Inc., Chicago, IL, USA). Group means were considered to be significantly different at $P<0.05$, as determined by the technique of protective least-significant difference (LSD).

RESULTS AND DISCUSSION

Isolation and identification of the strain JY2

An endophytic fungus (strain JY2) was isolated from honeysuckle and found to produce strong antibacterial substances. The strain was able to develop perithecia and produce egg-shaped ascospores in PDA culture. Opaque and circular colonies of ca. 4-6 cm in diameter with a regular margin and cream-colored were observed to grow on nutrient agar at 37°C after 3 days (Fig. 1). Furthermore, the mycelial and conidial morphology were observed with a light microscopy. Mycelia were found to be transparent, thick and septate (Fig. 2). The rDNA-ITS gene sequences of JY2 (570 bp) were identified by polymerase chain reaction, sequencing and comparison to all sequences in GenBank. Its GenBank accession number was GU380354. The nucleotide sequence showed the closest match to that of *Fusarium* with a homology of 99%. Therefore, by combined analysis of the fungal morphological characters according to Genera of Hyphomycetes (Carmichael et al., 1980), the strain JY2 could be identified as *Fusarium* sp., the first endophyte reported to be isolated from honeysuckle. This strain was kept in the Henan Province Microbiological Culture Collection Center (HPMCC no. 252451).

Fig. 1. Colony morphology (A) and morphology of mycelial and asexual spore formation (B) of endophytic *Fusarium sp*. JY2: (A) representative images were taken after 5-day cultivation and (B) representative images were taken at 400-fold magnification.

Antimicrobial spectrum of strain JY2

There is a great deal of reports about *Fusarium* as a plant pathogenic fungus (Tian et al., 2004). Gong and Guo (2009) reported *Fusarium s*p. as one of the most dominant genera in *Dracaena cambodiana* and *Aquilaria sinensis,* illustrating also a significant antimicrobial activity of this fungal species (Gong et al., 2009). Results from Table 1 show that all bacterial indicators were found to be sensitive to antimicrobial substances

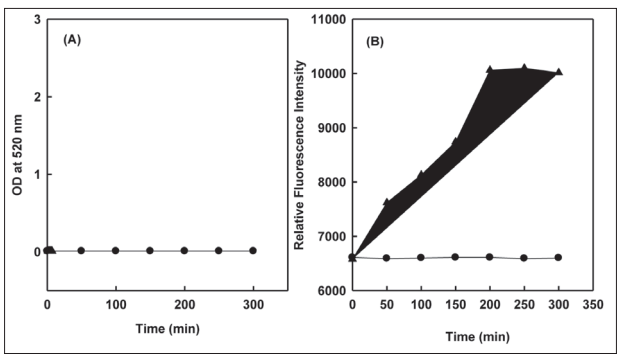

Fig. 2. Lytic activity of culture filtrate on *Pseudomonas aeruginosa* at different time points when alkaline phosphatase (A) and β-galactosidase activities (B) in the culture supernatant was measured. The enzymatic activity is described by the fluorescence intensity; (▲) – treated with the culture filtrate; (•) – control.

Table 2. MIC and MBC values of the culture filtrate of endophytic *Fusarium* sp. JY2.

	Culture filtrate	
	MIC (mg/mL)	**MBC** (mg/mL)
Gram-positive bacteria		
Staphylococcus aureus	0.525	1.050
Bacillus anthracis	3.100	12.400
α-hemolytic streptococcus	1.050	3.100
Gram-negative bacteria		
Escherichia coli	0.263	0.525
Pseudomonas aeruginosa	0.263	1.050
Bacillus proteus	1.050	3.100
Salmonellae enteritis	0.525	1.050
Eberthella typhi	0.525	0.525
Shigella dysenteriae	0.263	0.525
Helicobacter pylori	0.525	1.050

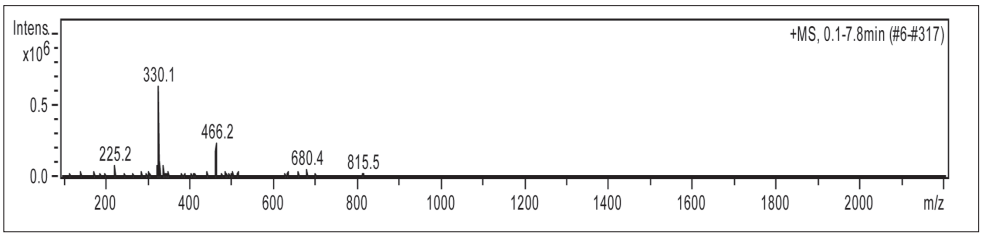

Fig. 3. HPLC-MS chromatogram of the fraction with the strongest antimicrobial activity from the broth of *Fusarium* sp. JY2.

produced by *Fusarium* sp. strain JY2, as confirmed by the inhibition zone diameter (24 h after incubation). The filtrate most strongly inhibited the growth of *P. aeruginosa* (Table 1). MIC and MBC values of the culture filtrate against *P. aeruginosa* were 0.2625 mg/mL and 1.05 mg/mL, respectively (Table 2). It was also observed that the antimicrobial substances produced by JY2 had a wide inhibition spectrum for both Gram-negative and Gram-positive bacteria.

Release of alkaline phosphatase and β-galactosidase from *P. aeruginosa* treated with culture filtrate

Alkaline phosphatase and β-galactosidase are released as a consequence of change in outer cell-wall layers and inner membrane permeability, respectively. In this study, we used *P. aeruginosa* since the bacterium was proved to be the most sensitive to culture filtrate as shown in Table 1. As shown in Fig. 2A, when cells were treated with the culture filtrate by produced by *Fusarium* sp. strain JY2, there was no release of alkaline phosphatase observed from *P. aeruginosa* cells. This result indicates that culture filtrate cannot cause damage to outer cell-wall layers. A progressive release of the cytoplasmic β-galactosidase for up to 200 min was observed (Fig. 2B). It has been assumed that the culture filtrate can increase the inner membrane permeability of Gram-negative *P. aeruginosa* (Xing et al., 2009).

Analysis of antimicrobial substances

The isolation of bioactive components from endophytes has been proved to produce various valuable substances with several biological functions in plants (Strobel and Daisy, 2003). In this study, the methanol fraction showed a distinct antimicrobial activity as compared to the residue dissolved in PBS buffer. These findings may point to the hydrophobic nature of JY2 antimicrobial substances. The antimicrobial activity experiments revealed the strongest antimicrobial activity from the 46th to the 53rd of the 90 fractions from the broth extracts. Accordingly, the combination of the fractions (46th to 53rd) was concentrated, further purified and analyzed by LC-MS/MS. LC-MS analysis showed five pseudomolecular ions [MH]+ at *m*/*z* 225.2, 330.1, 466.2, 680.4 and 815.5 (Fig. 3), which indicates the presence of five different compounds with theoretical masses of 224.2, 329.1, 465.2, 679.4, 814.5 Da, respectively.

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