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Evaluation of anti-microbial activities of extracts of endophytic fungi from *Artemisia annua*

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Abstract

The endophytic extracts of 11 fungi associated with asympomatic *Artemisia annua* Linn., were evaluated for antimicrobial activity against three human pathogenic microbes, *Escherichia coli*, *Staphylococcus aureus* and *Trichophyton rubrum*, and two plant pathogens, *Rhizoctonia cerealis* and *Magnaporthe grisea*. The results showed that these endophytic extracts had different inhibitory effects on microbial pathogens at 100 mg/mL. Among these fungal endophytes, three strains *Aspergillus* spp. SPS-02, SPS-04 and SPS-01 respectively showed the strongest antimicrobial activities against *E. coli*, *S. aureu*, *T. rubrum*. An endophytic *Mucor* sp. SPS-11 had the most pronounced effect on *R. cerealis*. Two strains *Aspergillus* sp. SPS-02 and *Cephalosporium* sp. SPS-08 exhibited the strongest antimicrobial activities against *M. grisea*. These anti-pathogenic endophytes could be applied as new sources of antibiotics in agriculture and pharmaceutical industry.

Introduction

Infectious diseases are the world's leading cause of premature deaths, killing almost 50 thousand people every day. Moreover, the increasing emergence of resistant pathogenic strains to the existing drugs and new infectious diseases has necessitated the need for searching novel molecules with better anti-microbial properties than the existing ones (Bhagat et al., 2012). So, substantial resources have been invested in the research of new antimicrobials. Natural products, mainly those from microbial origins, have provided the pharmaceutical industry with some of its most important sources of lead compounds in the search for new anti-microbials (Simoes et al., 2009).

Endophyte, a group of microorganism colonized in inter- and intra-cellular host plants without causing any symptomless disease, plays important physiological and ecological roles, such as growth promotion and adaptability improvement (Tan and Zou, 2001). Many evidences have shown that endophytic microbe has abundant biological diversity and become a rich source of natural products with a broad spectrum of bioactivities (Zhang et al., 2006). Till now, more than 10 thousand endophytic strains had been isolated and characterized, including bacterium, fungus and actinomycete. In our continuous investigation of biodiversity and chemodiversity of endophyte from medicinal plants (Zhang et al., 2005; 2008; 2010), 11 endophytic fungi were isolated from *Artemisia annua* Linn., a traditional Chinese medicine used to treat malaria. The present study focused on evaluating antimicrobial activity of extracts of these endophytic fungi in order to screen and discover functional endophytes from *A. annua* Linn.

Materials and Methods

Plant material

The whole living plant materials of *A. annua* Linn. were collected from Tianmu Mountain, East China, and

cultivated at campus of Zhejiang University of Technology, China. Within 24 hours after harvest, some plant materials of *A. annua* Linn. were subjected to endophyte isolation.

Isolation of endophytic strains

Endophytic fungus was separated from the healthy stems of *A. annua* Linn. according to the following procedure. Firstly, the stems were washed with running tap water, sterilized with 75% ethanol for 1 min and 2.5% sodium hypochlorite for 15 min, then rinsed in sterile water for three times and cut into 1 cm. Both borders of sterilized segments were cut off, and the rest was incubated at 28°C on potato dextrose agar (PDA, Sigma-Aldrich) medium in Petri plates supplemented with 200 µg/mL ampicillin and 200 µg/mL streptomycin to inhibit bacterial growth until the mycelium or colony originating from the newly formed surface of the segments appeared. The mycelium was further purified at the same condition. Another segment of the same origin without surface sterilization was cultured as a negative control to check the presence of contaminated microbes on the segment surface. 11 purified endophytic fungi were respectively numbered as SPS-A –K and transferred to PDA slants separately and were kept at 4°C after being cultured at 28°C for 7 days.

Identification of endophytes

The isolated endophytic strains were preliminary identified according to their morphological characters (Wei, 1979). The morphological examination was performed by scrutinizing the fungal culture, the mechanism of spore production and the characteristics of the spores. For inducing sporulation, each fungal isolate was separately inoculated on five culture media, including PDA medium, corn meal agar (CMA), carrot agar (CA), Winogradsky's salt-solution agar (WSA) and plate count agar (PCA). All experiments and observations were repeated at least twice.

Preparation of endophytic extracts

Each strain was cultured on PDA for 7 days at 28°C, respectively, and then to provide the culture broth in 1,000 mL Erlenmeyer flasks each containing sterilized 400 mL of potato dextrose broth (PDB). The flasks were incubated at 28°C on a rotary shaker at 150 rpm for 20 days. Furthermore, the mycelium and the culture broth of all endophytes were separated by centrifugation at 10,000 rpm. The culture broth was partitioned with ethyl acetate (2 × 400 mL, Merck) and the upper solvent was removed under reduced pressure to yield extract. Each afforded extract was dissolved in dimethyl sulfoxide (DMSO, Merck) and its final concentration reached 100 mg/mL followed by preserving at 4°C.

Test microorganisms

Three human pathogens, *E. coli*, *S. aureus* and *T. rubrum*, and two plant pathogens, *R. cerealis* and *M. grisea* were obtained from China Center for Type Culture Collection. *E. coli* and *S. aureus* were maintained on nutrient agar (NA) slants at 4°C. And three fungal pathogens were kept on PDA slants at 4°C. NA and PDA were used for testing the antibacterial and antifungal activity.

Anti-microbial activities assay

NA plates were seeded with 8 hours broth culture of different bacterial pathogens while PDA plates were seeded with 16 hours broth culture of fungal pathogens after spore suspension. The antimicrobial screening was performed by the disk diffusion method (DDM) (Zaika, 1998). 100 µL of prepared endophytic extract was carefully dropped on a standard sterilized paper disk (Φ = 6 mm) using sterilized dropping pipette and subsequently placed on NA plate or PDA plate. Then the plates of bacterial and fungal pathogens were respectively incubated at 37°C for 2 days, 28°C for 4 days. DMSO was used as the negative substance. The anti-microbial activity was evaluated by measuring the diameter of inhibition zone. All antimicrobial tests were carried out in triplicate and the mean of the diameter of the inhibition zones was calculated.

Results

Some fungal endophytes have been confirmed to be excellent producers of antimicrobial substances, such as cephalosol (Zhang et al., 2008), cytosporolides A-C (Li et al., 2010). A total of 11 endophytic strains were isolated from the stems of *A. annua* Linn. According to morphological character, these fungal endophytes were preliminary identified and shown to have four genera (Table I), which included *Aspergillus* sp., *Fusarium* sp., *Cephalosporium* sp. and *Mucor* sp. Comparatively, endophytic *Aspergillus* sp. and *Cephalosporium* sp. were the dominant groups in *A. annua*.

Anti-microbial assay showed that 11 endophytic extracts had different inhibitory effects on microbial pathogens at 100 mg/mL. As shown in Table I, four endophytic strains SPS-02, SPS-08, SPS-10 and SPS-11 had broad spectra of antimicrobial activities. Among them, three strains *Aspergillus* spp. SPS-02, SPS-04 and SPS-01 respectively exhibited the strongest activity against *E. coli*, *S. aureu*, *T. rubrum*. One endophytic strain *Mucor* sp. SPS-11 showed the most pronounced effect on *R. cerealis*. While two strains *Aspergillus* sp. SPS -02 and *Cephalosporium* sp. SPS-08 had the strongest antimicrobial activity against *M. grisea*.

Discussion

Diverse endophytic fungi exist within plant tissues, with a global estimate of up to a million undescribed species (Zhang et al., 2006). It is an urgent need to search for new antibiotics because many antibiotics

Table I

Anti-microbial effects of extracts of 11 endophytic fungi from *Artemisia annua*[a]

Strain	Genus	Antimicrobial effect				
		Escherichia coli	*Staphylococcus aureus*	*Trichophyton rubrum*	*Rhizoctonia cerealis*	*Magnaporthe grisea*
SPS-01	*Aspergillus* sp.	++	++	+++	+	+
SPS-02	*Aspergillus* sp.	+++	++	++	+++	++++
SPS-03	*Aspergillus* sp.	+	+	+	+	++
SPS-04	*Aspergillus* sp.	+	+++	+	–	+
SPS-05	*Fusarium* sp.	+	+	+	–	–
SPS-06	*Fusarium* sp.	++	++	–	+	+
SPS-07	*Cephalosporium* sp.	+	+	–	++	++
SPS-08	*Cephalosporium* sp.	++	++	+	+++	++++
SPS-09	*Cephalosporium* sp.	+	+	–	–	–
SPS-10	*Mucor* sp.	++	++	+	+++	+++
SPS-11	*Mucor* sp.	+	++	++	++++	++

[a]Expressed by the diameter of inhibition zones: –, no inhibition; +, <10 mm; ++, 10–15 mm; +++, 16–20 mm; ++++, >20 mm

have encountered drug resistance or cause severe adverse drug reactions (Rouveix, 2003). The present work showed that 11 fungal endophytes associated with *A. annua* Linn. have a wide range of antimicrobial activities against human three microbial pathogens *E.* with *A. annua* Linn. have a wide range of anti-microbial activities against human three microbial pathogens *E. coli*, *S. aureus*, *T rubrum*, and two phytopathogenic fungi *R. cerealis* and *M. grisea*.

In previous studies, endophytic *Aspergillus* spp. have been found in many medicinal plants, including *Taxus cuspidate* (Zhao et al., 2009), *Cephalotaxus mannii* (Xue et al., 2012), *Melia azedarach* (Li et al., 2012). Many anti-microbial compounds had been isolated and characterized from these endophytic fungi, such as diterpenoids (Sun et al., 2012), alkaloids (Zhan et al., 2007; Ding et al., 2012). *Cephalosporium* spp. and *Fusarium* spp. are often found fungi as endophytes from plants and known to produce bioactive metabolites, such as graphislactones (Zhang et al., 2005), cephalosol (Zhang et al., 2008). Till now, few endophytic *Mucor* sp. has been reported. However, a *Mucor* sp. strain from rapes had strong biosorption capacity of Cd and Pb from contaminated solutions (Deng et al., 2011).

This founding reinforced the assumption that endophytes could be a promising source of antimicrobial substances, which may play important roles in protecting their host plants from various microbial pathogens and pests. These 11 anti- pathogenic endophytes isolated from the stems of *A. annua* Linn. could be applied as new sources of anti-microbial agents in agriculture and pharmaceutical industry.

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