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Full Length Research Paper

Isolating fungal endophyte from *Paris polyphylla* Smith var. *yunnanensis* and identifying their antibacterial ability

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Eighteen fungal endophytes strains parasitizing the famous Chinese medicinal plant *Paris polyphylla* Smith var*. yunnanensis (Franch.)* Hand*.* Mazz were isolated from surface-sterilized plant tissues, such as rhizome, root, stem, leaf and flower. The effect of endophytes on the growth of human pathogenic microbes was evaluated *in vitro,* using disc diffusion assay. According to the characteristics of cultures and DNA sequences, the WRF7 and WRF7' belonged to the species *Penicillium chrysogenum*. The extract of WRF7' inhibited a broad range of human -pathogenic bacteria, but that of WRF7 almost had no similar effect. And the metabolites of WRF7' could not stop the growth of test fungus *Candida albicans*. The study indicates that *P. chrysogenum* becomes fungal endophyte of *Paris polyphylla* Smith var. *yunnanensis* and its isolate WRF7' makes metabolites that are inhibitory to all test bacteria *in vitro*. Therefore, the host plant suppressing pathogens owing to its endophytes and inhibitory mechanisms of endophytes to test bacteria and fungi are different.

Key words: Fungal endophyte, *Paris polyphylla* Smith var. *yunnanensis* (*Franch*.) Hand*.* Mazz, isolating, identifying, antibacterial ability.

INTRODUCTION

Some few decades ago, antimicrobial drug in human bacterial pathogens is a continuing worldwide issue and as a consequence, effective treatment and control of such organisms remains an important challenge. Bacterial resistance has appeared for every major class of antibiotic (Mccarrell et al., 2008). Antimicrobial substances have been found in many endophytes parasitizing Chinese traditional medicines, for example, five Chinese herb medicines were tested and demonstrated that their endophytes could restrict the growth of bacteria such as *Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas aerugimosa* (Zhou et al., 2007).

Paris polyphylla Smith var. *yunnanensis*, a famous Chinese traditional medicine, is widely used to treat traumatic injuries, snake bite, abscess, parotitis and mastitis (Huang et al., 2005). In recent years, it has been proved to restrain the growth of some gram positive and negative bacteria (Wu et al., 2004). However, the plant cannot be cultivable so far, and its overexploitation has led it to be one of the endangered species, which prevents it from widely being used (Meng et al., 2005). So it is important to find alternative sources.

The main aim of this study was to investigate fungal endophytes producing broad-spectrum anti-microbe substances. The ability of ethyl acetate (EtOAc) extracts of fungi for suppressing human pathogenic microbes was tested. Fungal endophytes which had certain broad-spectrum antibacterial abilities were identified. Among them, an isolate of *Penicillium chrysogenum* clearly exhibited antibacterial ability. The consequence is that *P. chrysogenum* is an endophyte of the Chinese herb and antimicrobial activities of the plant are related to its endo-

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phytes.

MATERIALS AND METHODS

Sampling

Samples were collected in March and July of 2007 from Wu-ding Yunnan, China (102° 25'E and 25° 32' N). Annual average temperature of the area is 15.1°C and annual rainfalls is about 1001 mm. Disregarding age or size, the plants were chosen randomly, taken to the laboratory and planted in garden pots. Fungal endophytes were isolated within 24 h after sampling.

Preparation of media

Solid medium: Potato dextrose agar (PDA) and Czapek medium were used to isolate the colony and identify fungal endophytes. Nutrient agar was used to incubate test bacteria. Mueller-Hinton agar (MH agar) was used to examine the antibacterial ability of fungal endophytes. Sabouraud's medium was used to incubate test fungus and examine antifungal ability. Liquid medium: potato dextrose liquid medium (PD) with natural pH value was used for fermentation.

Isolation of fungal endophytes

Briefly, plant samples were rinsed in H_2O, wiped by sterilized napkins and sequentially surface sterilized in 75% ethanol for 3 - 5 min, and then in a 0.1 % solution of $HgCl_2$ for 0.5 - 1 min. After immersed twice in sterile distilled water, plant tissues were cut edge and placed on PDA supplemented with 4 ml/L streptomycin sulfate (North China Pharmaceutical Group Co.) in Petri dishes in the dark at 27°C. Fungal endophytes growing from the plant tissues, usually after 3 - 7 days, were picked with hyphal tips and transferred to PDA plates to determine culture purity. Pure fungi were sub-cultured on PDA slopes after incubation for up to 7 days. Individual fungi were grouped according to the gross morphology of colonies. Appropriate controls were also set up in which no cutting sterilized plant tissues were inoculated for 7 days and no microbe appeared. Isolated fungi were placed in 20% (vol/vol) glycerol and stored at -70°C.

Extracts preparation

Spores or mycelia of every strain were inoculated in 250 ml potato dextrose liquid medium flasks on a rotary shaker. After 7 days at 120 r/min and 27°C, the cultures were filtrated by filter paper (Hangzhou Paper Industry Co., Ltd.). The mycelia were frozen 30 min, ground and mixed with the filtrate. The mixture was exhaustively extracted with an equal volume of EtOAc for 40 h (Liu et al., 2008). The organic phase was concentrated by an evaporator and the final extract was stored in a little bottle.

Screening assay

Overnight cultures of the gram positive strains *S. aureus* and the gram negative strains *Salmonella typhi, Proteus vulgaris, Shigella dysenteriae* and *E. coli* were prepared on nutrient agar plates and *Candida albicans* on Sabouraud's Medium. All test bacterial isolates were suspended in sterilized normal saline and were spread onto MH agar plates and test fungus onto Sabouraud's Medium. The extract (5 L) was then spotted onto sterile filter paper discs (5 mm diameter) placed on the plates which were incubated at 37°C for 16.5 h prior to recording zones of inhibition. Penicillin G (1 unit, Beijing Tiantan Biological Products Co., Ltd.) was used as a positive control of inhibiting *S. aureus*; Gentamycin (1 unit, Beijing Tiantan Biological Products Co., Ltd.) as a positive control of inhibiting *S. typhi, P. vulgaris, S. dysenteriae* and *E. coli*; and EtOAc as a negative control of all inhibiting tests.

Identification of fungal endophytes possessing antimicrobial activities

Isolates were identified based on their macroscopic characteristics including their morphology and characteristics grown on Czapek's medium and on their molecular identifications.

Macroscopic characteristics

Isolates were incubated for 7 day at 27°C. The experimental design was completely randomized with 2 replicates. Colonies were analyzed with respect to their average diameter (cm), the aspect of their borders, the coloration of the mycelium, the coloration of the reverse of the Petri dish and the coloration of the medium.

Genomic DNA extraction

Mycelia of WRF7 and WRF7 inoculated potato dextrose liquid media were prepared and DNAs of WRF7 and WRF7' were extracted using Biospin Fungus Genomic DNA Extraction Kit (Bioer Technology Co., Ltd., Hangzhou, P.R. China) . The DNAs were transferred to the new tubes and stored at -20°C respectively.

Agarose gel electrophoresis

DNA detection concentration was performed by electrophoresis on a 2% (wt/vol) agarose gel stained with ethidium bromide. A volume of 10 µl of DNA and 2 µl of Ficoll dye was loaded in each lane. Electrophoresis condition was 110 V for 50 min in 1× TE buffer.

PCR and DNA sequencing

TaKaRa Fungi Identification PCR Kit (Code No. D317) was used in amplifying internal transcribed spacer regions (ITS) of fungal DNA with primers ITS4 (5'-TCCTCCGCTTATTGATATGS-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3'). An initial denaturating step (94°C for 5 min) was followed by 30 cycles (with each cycle consisting of DNA denaturating at 94°C for 30 s, primer annealing at 55°C for 30 s and elongation at 72°C for 1 min) and a final extension step at 72°C for 5 min. A no-template negative control was included in each PCR run. The PCR products were purified with a TaKaRa Agarose Gel Purification Kit Ver.2.0 (Code No. DV805A, TaKaRa Biotechnology (Dalian) Co., Ltd.). Sequence analysis was performed with an automated sequence analyzer (ABIPRISM^{TM} 3730XL DNA Sequencer; Applied Biosystem). Sequence similarities were assessed by a search with GenBank using the BLAST search program.

All human-pathogenic microbes used in the bioassay test system were obtained from Medical Microbiology and Immunology Department of Kunming Medical University. All test bacteria were grown on nutrient agar, at 36°C for 24 h and test fungus were grown on Sabouraud's Medium.

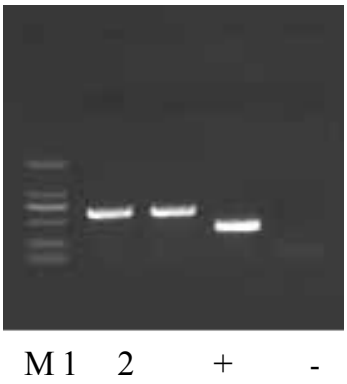

Figure 1. Agarose gel electrophoresis of PCR-amplified ITS regions of 26s rDNA. All products were electrophoresed in 3% agarose gels. M, DL2,000 DNA Marker; 1, WRF7-PCR PRODUCT; 2, WRF7'-PCR product; +, Positive control; -, Negative control.

RESULTS

Eighteen morphological types were isolated from rhizome, root, stem, leaf and flower of *P. polyphylla Smith var. yunnanensis.* The disc diffusion assay was applied to compare the antimicrobial activity of the extracts following 16.5 h incubation against a panel of microbes. The gram positive bacterium, *S. aureus*, demonstrated the largest zone of inhibition against 1 unit of penicillin G, but the gram negative bacteria, *S. typhi* and *S. dysenteriae*, shared larger zone of inhibition against 1 unit of gentamycin (Figure 1).

Extract of fungal endophyte WRF7' showed distinct zones of inhibition to every test bacterium, but WRF7 mostly failed to show the same activities. Meanwhile, extracts of LSF1, WSF2, WRF6, LSF9, LEF10, WRF12 and RF2 were inhibitory to some test bacteria (Table 1). The antimicrobial effect of fungal endophytes from *P. polyphylla* Smith var. *yunnanensis* on the growth of human pathogenic microbes was evaluated *in vitro,* using disc diffusion assay. The positive control of inhibiting *S. aureus* was Penicillin G (PG.). The positive control of inhibiting *S. typhi*, *P. vulgaris* , *S. dysenteriae* and *E. coli* was Gentamycin (GM) and the negative control was EtOAc. (L = the plant under forest; W = the plant from greenhouse; S = the isolate from plant's stamen; R = the isolate from plant's root; L = the isolate from plant's leaf; E = the isolate from plant's sepal; F = fungus).

The isolate WRF7' developed villiform colony with blue-greenish centre and whitish edge on PDA, but it showed pale brownish coloration on czapek's medium. The phenomenon that an isolate had different macroscopic characteristics in two kinds of media was also observed in other fungal endophytes (Figueiredo et al., 2007).

The ITS sequences obtained for the isolates were compared with the public DNA databases by using the BLAST interface at http://www.ncbi.nlm.nih.gov/BLAST/. WRF7 was proved to be 100% identical to previously reported *P. chrysogenum* sequences, and WRF7' to be 99% identical to the species.

DISCUSSION

The associations between plants and fungal endophytes are widely regarded as phases from near-pathogenic to symbiotic (Strobel et al., 2001; Li et al., 2007; Kim et al., 2007). Fungal endophytes affect the early evolution of plants, and probably also impose selective pressure on the plants. The plants have an enormous diversity of endophytes that make bioactive metabolites. Some of these compounds are inhibitory, and in many cases lethal, to various test organisms (Ezra et al., 2004). The results obtained in the present study indicate that the isolate WRF7' of *P. chrysogenum* suppressed all the test bacteria. But it could not stop the growth of test fungus *C. albicans*. The metabolites suppressing test bacteria have no effect on test fungus because the structures of bacteria vary from that of fungi (Xuan et al., 2008). It seems that antimicrobial actions of the host, *P. polyphylla* Smith var. *yunnanensis*, are related to its endophytes.

For adapting to the environment of host, fungi regulate their metabolism. Microbes can apparently even appropriate genes from "higher" organisms (Pennisi, 1999). Meanwhile, some genes of endophytes are capable of inserting into the genome of the Chinese herb. In the course of gene transfer, transposable elements existing universally, play an important part. Since *P. chrysogenum*, a basic species producing penicillin, transfers the related genes to the host, the host acquires the ability of inhibiting bacteria.

Conclusion

Overall in this study system, we found that *P. chrysogenum* becomes fungal endophyte of *P. polyphylla* Smith var. *yunnanensis* and only one of its isolate, WRF7' makes metabolites that are inhibitory to all test bacteria *in vitro*.

Though the metabolites of endophytes can inhibit human pathogenic microbes, it seems that no comparable situation involving the host inhibiting pathogens owing to its endophytes has been previously demonstrated. It is necessary to investigate the relationship between antimicrobial action and Chinese traditional drugs and their endophytes deeply. The extract of WRF7 has markedly antibacterial potential, so it is essential to further elucidate the chemical structures of the secondary metabolites.

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Table 1. Antimicrobial Activities of the EtOAc extracts of fungal endophytes from *P. polyphylla* Smith var. *yunnanensis.*

Extract of endophyte	Antimicrobial activity /mm					
	S. aureus	***S. typhi***	***P. vulgaris***	***S. dysenteriae***	***E. coli***	***C. albicans***
LSF1	0	0	14	12	0	0
WSF2	11	0	0	0	0	0
WSF3	0	0	0	0	0	0
LRF4	0	0	0	0	0	20
LLF5	0	0	0	0	0	0
WRF6	9	0	0	0	0	0
WRF7	0	0	7	0	0	0
WRF7’	18	13	19	19	12	0
LSF9	9	0	0	0	0	0
LEF10	9	0	0	0	0	0
WRF11	0	0	0	0	0	0
WRF12	11	0	0	0	9	8
RF1	0	0	0	0	0	0
RF2	11	0	0	0	0	0
RF3	0	0	0	0	0	0
RF4	0	0	0	0	0	0
PG.	36	0	0	0	0	0
GM.	0	24	20	24	21	0
EtOAc	0	0	0	0	0	0

The antimicrobial effect of fungal endophytes from *P. polyphylla* Smith var. yunnanensis on the growth of human pathogenic microbes was evaluated in vitro, using disc diffusion assay. The positive control of inhibiting *Staphylococcus aureus* was Penicillin G (PG.). The positive control of inhibiting *Salmonella typhi*, *Proteus vulgaris, Shigella dysenteriae* and *E. coli* was Gentamycin (GM) respectively. The negative control was EtOAc respectively. Symbol meaning: L, the plant under forest; W: the plant from greenhouse; S, the isolate from plant’s stamen; R, the isolate from plant’s root; L, the isolate from plant’s leaf; E, the isolate from plant’s sepal; F, fungus.

(Yunnan Baiyao Group Co., Ltd.) for collecting plant samples of *P. polyphylla* Smith var. *yunnanensis*.

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