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BRIEF COMMUNICATION

Endophytic fungi from a pharmaceutical plant, *Camptotheca acuminata*: isolation, identification and bioactivity

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Abstract About 174 endophytic fungi were isolated from the pharmaceutical plant, *Camptotheca acuminata*. Of the 18 taxa obtained, non-sporulating fungi (48.9%), *Alternaria* (12.6%), *Phomopsis* (6.9%), *Sporidesmium* (6.3%), *Paecilomyces* (4.6%) and *Fusarium* (4.6%) were dominant. ITS rDNA assay indicated that most of the non-sporulating fungi belonged to the Pyrenomycetes and Loculoascomycetes ascomycetes or their anamorph Coelomycetes.The results of the bioactivity test showed that 27.6% of the endophytic fungi displayed inhibition against more than one indicator microorganism. 4.0% and 2.3% of the endophytic fungi showed cytotoxicity and protease inhibition, respectively. The endophytic fungi with bioactivities were distributed in more than 12 taxa including non-sporulating fungi, which are reliable sources for bioactive agents.

Keywords Endophytic fungi · Identification · Bioactivity · *Camptotheca acuminata*

Introduction

Recently, endophytic fungi from plants have been widely accepted as an important source of drugs. A large number of compounds with new structures and various bioactivities are continuously isolated (Strobel and Daisy 2003). It is known that many bioactive agents produced by plants, such as taxol, could be also produced by endophytic fungi (Stierle et al. 1993; Strobel et al. 1996). Thus, when producing certain phytochemical drugs, the application of endophytic fungi could solve existed problems such as the slow growth of plants and environmental damage.

Camptotheca acuminata, under second-level protection by national law in China, is a kind of pharmaceutical plant. It is rich in the anticancer material camptothecin, with the highest distribution in the new leaf of up to 0.4%. In such a special living environment, there may present special endophytic fungi. Meanwhile, endophytic fungi in *C. acuminata* may take part in the synthesis or transformation of camptothecin. Therefore, it is important to study bioactive materials of the endophytic fungi isolated from *C. acuminata*. In this paper, the isolation and identification of endophytic fungi in *C. acuminata* as well as the screening of strains with antimicroorganism, antitumor and protease inhibition activities are reported.

Materials and methods

Source of endophytic fungi

Twig, bark and root of *C. acuminata* were gathered at Shaowu Jiangshi nature conservation area (The plant ca.10 years old) and Mingxi Jinzi Mountain nature conservation area (The plant ca.8 years old), Fujian Province, southeast China, at an altitude of about 200~400 m. The current-year twig (8~12 × 1~2 cm, length × diameter), root (4~6 cm × 2~3 cm,

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length × diameter) and bark (8~12 × 3~5 × 0.3~1.5 cm, length × width × thickness) were sealed with Parafilm to prevent drying out during transportation.

Isolation and identification of endophytic fungi

The isolation of endophytic fungi was performed by the method described by Huang et al. (2001). Fungal identification was made by traditional and molecular methods. The former includes culture characteristics and the morphology of fruiting bodies and spores (Ainsworth et al.1973) The latter uses ITS sequence of rDNA to characterize some non-sporulating group. ITS1 primer (sequence: 5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 primer (sequence: 5′-TCCTCCGCTTATTGATATGC-3′) were used and the ITS1-5.8S-ITS2 segment amplified by PCR and subsequently sequenced, was compared with the sequence reported in GeneBank.

Fermentation and preparation of crude extract

The fermentation and treatment of the fermentation broth were performed by the methods described in our previous work (Huang et al. 2001). The strains showing cytotoxic activity in a preliminary screening were selected and fermented in potato dextrose medium for 7 days at 25°C. The cultures were extracted with mixtures of 80% ethyl acetate, 15% methanol and 5% formic acid. The organic compound extracted was evaporated under reduced pressure yielding a crude solid extract. The crude extract was applied to determine IC_{50} cytotoxicity after diluted with DMS.

Determination of antimicrobial activity and cytotoxicity

Antimicrobial activity was tested with the double layer technique (Gauthier 1975) with indicator organisms including *Bacillus subtilis* ATCC 9372 (BS), *Escherichia coli* ATCC 25922 (EC), *Staphylococcus aureus* ATCC 25923 (SA), *Candida albicans* As 2.538 (CA) and *Aspergillus niger* ACCC 30005 (AN). Cytotoxic activity was tested by MTT method (Mosmann 1983; Huang et al. 2001). Human tumor cell lines KB, Raji and HepG2 were obtained from the Cell Line Bank of the Chinese Academy of Science. Cisplatin (Jinzhou Pharmaceutical Factory, China) was used as positive control.

Determination of protease inhibitory activity

Benzoylarginine-*p*-nitroanilide (BRpNA) (Sigma Co.) was used as a specific synthetic substrate for the determination. The reaction mixtures contained 40μl Tris–HCl buffer (pH 8.5), 150 μl of 0.8 mg ml^{-1} BRpNA (dissolved in 10% dimethylformamide), 20 μl 0.2 mg ml^{-1} trypsin (250N.F. U mg^{-1}; Shengong, Co., China) and 20μl tested ferment broth. The mixture was incubated at 37°C for 30 min, then 30 μl acetic acid was added to stop the reaction. The reaction system without enzyme and tested fermentation broth was regarded as blank control, while the one without tested fermentation broth was regarded as negative control. Trypsin inhibitor from soybean (7000 BAEE U mg^{-1}, Amresco, USA) was regarded as positive control. The optical density of the well was measured with a microplate reader (M + 3550, Bio-Rad) at 405 nm (Liang et al. 2003). Protease inhibitory rate was calculated by the following equation and ID_{50} was defined as the dilution of fermentation broths that resulted in (at least) a 50% inhibition of protease activities.

$$\text{Inhibition} = \left(1 - \frac{\text{OD treated} - \text{OD blank control}}{\text{OD negative control} - \text{OD blank control}}\right) \times 100\%$$

Results and discussion

About 174 endophytic fungi were isolated from 18 trees, 486 tissue samples (Table 1). The result of identification by traditional morphological methods showed that endophytic fungi were abundant in *C. acuminata*, and non-sporulating fungi were the largest group (48.9%) among all isolates. *Alternaria* (12.6%), *Phomopsis* (6.9%), *Sporidesmium* (6.3%), *Paecilomyces* (4.6%) and *Fusarium* (4.6%) are the dominant genera.

The result of bioactivity tests showed that 48 strains (27.6%) displayed antimicroorganism activity against at least one indicator organism. Among them, 54.2%, 20.8% and 25.0% were isolated from current-year twig, root and bark, respectively. 7 fungi (4.0%) showed cytotoxic activity with IC_{50} from 2 mg ml^{-1} to 393 mg ml^{-1} against tested tumor cell lines (IC_{50} of cisplatin are 1.5,1.5 and1.0 μg ml^{-1} against KB, HepG2 and Raji cell lines, respectively). 4 fungi (2.3%) show protease inhibition with ID_{50} 11.5–45 (inhibitory rate

Table 1 Number (*N*) and percentage (%) of endophytic fungi isolated from *C. acuminata* twigs, barks and roots. Distributed by taxon and localities[a]

Taxon	Samples			
	Twig *N*(%)	Bark *N*(%)	Root *N*(%)	Total *N*(%)
Non-sporulating fungi	48(56.5)	25(29.4)	12(14.1)	85(48.9)
Alternaria	10(45.5)	3(13.6)	9(40.9)	22(12.6)
Phomopsis	6(50.0)	1(8.3)	5(41.7)	12(6.9)
Sporidesmium	0(0)	11(100)	0(0)	11(6.3)
Paecilomyces	8(100)	0(0)	0(0)	8(4.6)
Sphaeropsidaceae	3(60)	0(0)	2(40)	5(2.9)
Fusarium	8(100)	0(0)	0(0)	8(4.6)
Helicoon	0(0)	0(0)	2(100)	2(1.1)
Rhizoctonia	2(100)	0(0)	0(0)	2(1.1)
Blastomyces	1(100)	0(0)	0(0)	1(1.1)
Cunninghamella	0(0)	1(100)	0(0)	1(1.1)
Dictyosporium	0(0)	0(0)	1(100)	1(1.1)
Heteropatella	1(100)	0(0)	0(0)	1(1.1)
Monochaetia	1(100)	0(0)	0(0)	1(1.1)
Pestalotia	1(100)	0(0)	0(0)	1(1.1)
Pleurophonella	1(100)	0(0)	0(0)	1(1.1)
Stilbaceae	1(100)	0(0)	0(0)	1(1.1)
Trichoderma	1(100)	0(0)	0(0)	1(1.1)
No identification	7(70.0)	1(10.0)	2(20.0)	10(11.2)

[a] 18 trees, each trees, 9 twigs, barks and roots fragments were analyzed, respectively

Table 2 The taxa with antimicroorganism, antitumor and protease inhibitory activity

Taxa	Number of tested strains	Number of strains with activity (%)	Bioactivity[a]		
			AM	AT	PI
Non-sporulating fungi	85	27(31.2)	23	5	1
Alternaria	22	3(13.6)	3	0	0
Phomopsis	12	4(33.3)	3	0	1
Sporidesmium	11	1(9.1)	0	0	1
Paecilomyces	8	6(75.0)	6	1	0
Fusarium	8	3(37.5)	3	0	0
Sphaeropsidaceae	5	2(40.0)	0	1	1
Rhizoctonia	2	2(100.0)	2	0	0
Cunninghamella	1	1(100)	1	0	0
Rhizoctonia	2	2(100.0)	2	0	0
Heteropotella	1	1(100)	1	0	0
Pleurophonella	1	1(100)	1	0	0
The others	10	3(60.0)	3	0	0
Total	168	56(33.3)	48	7	4

[a] AM antimicroorganism; AT antitumor; PI protease inhibition

of 12.2 U ml^{-1} trypsin inhibitor from soybean was found to be 56.5% against trypsin).

The bioactive strains isolated from *C. acuminata* are distributed in at least 12 taxa (Table 2). Different bioactivities were found to present in different taxa. For example, 75.0% of *Paecilomyces* strains displayed bioactivity; and in *Fusarium* and *Phomopsis*, it reached as high as 37.5% and 33.3%, respectively. Non-sporulating fungi were the largest and most widely distributed group among the endophytic fungi isolated from *C. acuminata*, and it was also the major source of bioactive strains. In the 56 strains with bioactivity, 27(48.2%) belonged to the non-sporulating group.

According to different activities and culture characteristics, the ITS rDNA of 15 non-sporulating fungi were chosen to be sequenced. The results show that all 15 strains had more than 96% similarity with some species in the GeneBank, including five genera at least. From the closest relationship, we have designated XZ-01 to *Corynespora cassiicola*, XZ-60 to *Glomerella cingulata* (both with 100% similarity) and the other strains to the corresponding genera. Our study further confirms the opinion that the classification of the non-sporulating fungi can be achieved by the method of molecular biology (Gao et al. 2001).

The phylogenetic assay of non-sporulating fungi based on ITS-rDNA (Fig. 1) showed that these 15 strains could be sorted to 4 different clades (groups). The first clade was a monophyletic group with SXZ-01 only, which has 100% similarity with *Corynespora cassiicola*. Clade 2 was composed of SXZ-08 and SXZ-16, closely related to *Batryosphaeria parva* and *B. rhodina*, respectively.

The biggest clade 3 was made up of XZ-07, SXZ-19, XZ-29, XZ-26, XZ-40, XZ-19, NXZ-05, XJ-05, XZ-05 and XZ-22, closely related to some species of *Diaporthe* and its anamorph *Phomopsis* (Mclaughlin et al. 2001). Assay of the tree showed the 10 cultures constituting a short branch and displaying close relationships among them. Clade 4 is made up of XZ-60 (100% similarity with *Glomerella cingulata*) and XJ-02 (close to a leaf litter ascomycete strain its294). According to the phylogenetic assay, clade 4 and clade 3 both belong to Pyrenomycetes and have a relatively close relationship. To sum up, the non-sporulating fungi isolated from *C. acuminata* are mostly made up of Pyrenomycetes, Loculoascomycetes ascomycetes and their anamorph. However, further tests are needed for these are only the identification of part of the group.

In the general isolation of soil fungi, the ascomycete always reaches a low isolation frequency, and is considered as rare fungus. Nevertheless, the molecular evidence shows that these rare fungi are prevalent in different tissues of *C. acuminata*. Although they appear as non-sporulating fungi under common culture, we can effectively identify them through molecular biology technology. It has been reported recently that, quite a number of bioactive substances were produced by ascomycetes and indicates a potential area for drug development (Strobel and Daisy 2003). Over 400 sec-

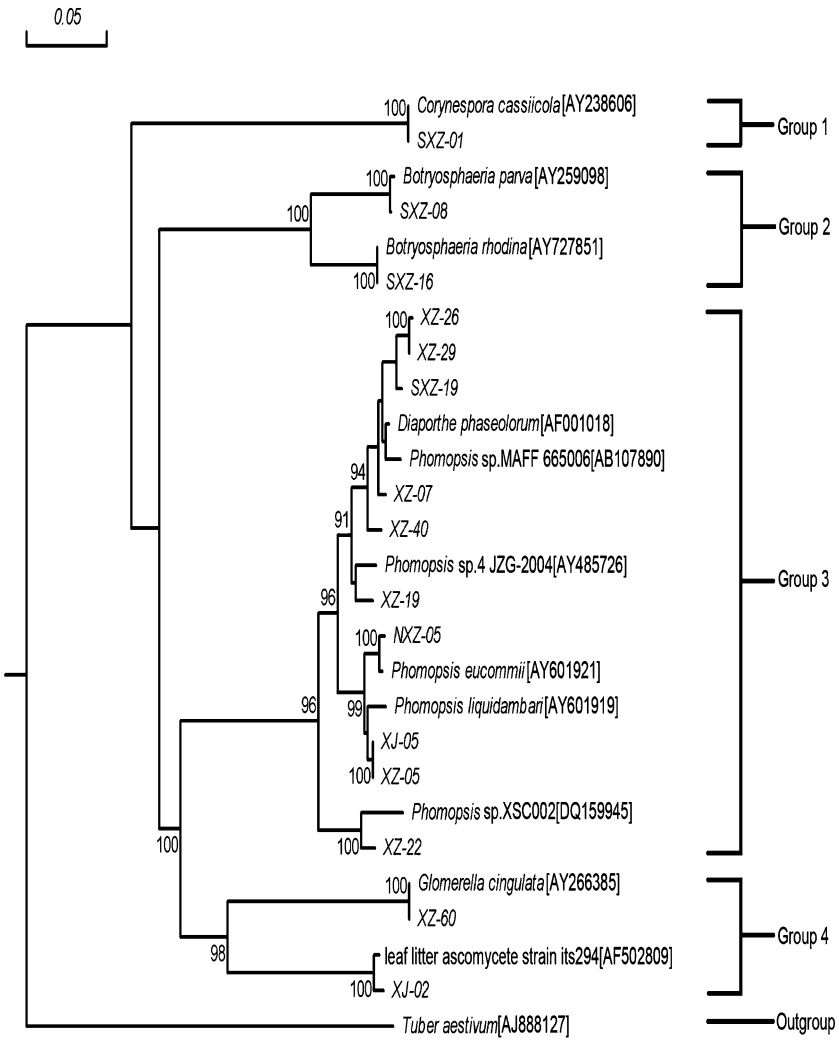

Fig. 1 Phylogentic relationship of non-sporulating fungi isolated from *C. acuminata* to other fungi from GeneBank, deduced from sequence of ITS rDNA. The numbers at branches indicate the percentages of trees from 1000 bootstrap replication in which the branch occurs

ondary metabolites were produced by Pyrenomycetes ascomycetes (Huang and Kaneka 1996). With prevalent antimicroorganism, antitumor and protease inhibitory activities, these ascomycetes isolated from *C. acuminata* are potentially reliable source of natural drugs.

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