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Full Length Research Paper

Endophytic fungi from *Dracaena cambodiana* and *Aquilaria sinensis* and their antimicrobial activity

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Single mycelium method was used to isolate endophytic fungi from surface-sterilized tissues of two medicinal plants. Of the 300 isolates recovered, 172 were from *Dracaena cambodiana* and 128 from *Aquilaria sinensis*. According to morphological characteristics, 174 (58%) isolates were identified and belonged to 41 taxa in 30 genera. The remaining 126 (42%) isolates did not sporulate and were segregated into 8 morphological groups. There were colonization rates (34 - 80%) and isolation rates (0.62 - 2.06) in two plants. And some endophytes showed certain level of host specificity or organs specificity. 21 (8.3%) isolates showed antimicrobial activity, moreover, some of them exhibited broad-spectrum antimicrobial activity and the inhibition zones ranged from 7 to 27 mm. The active isolates were identified to 17 taxa. *Fusarium* spp. were the most dominant genera in two plants and showed the most potent antimicrobial activity.

Key words: Antimicrobial activity, *Aquilaria sinensis*, Chinese medicinal plants, *Dracaena cambodiana*, endophytic fungi.

INTRODUCTION

Dragon's blood is a deep red resin, which has been used as a famous traditional medicine since ancient times in many countries. In China, the red resin of *Dracaena cambodiana* Pierre ex Gagnep. (Agavaceae) as one main source of Chinese dragon's blood is used to promote blood circulation and the treatment of traumatic, inflammation, diarrhea and diabetes (Zhang et al., 2005). *Aquilaria sinensis* (Lour.) Gilg. (Thymelaeceae) is the main plant species for the production of agarwood (also called Chen Xiang in Chinese) used as traditional Chinese drugs since 16th century (Liu, 1999). Chinese dragon's blood and agarwood cannot generate in the normal wood tissues but may form in the wounded tissues. It was suggested that Chinese dragon's blood and agarwood are pathological products formed as the results against fungal infection (Jiang et al., 1995; Qi et al., 1995). In such a special living environment, there may exist special endophytic fungi. Therefore, it is important to study the endophytic fungi isolated from *D. cambodiana* and *A. sinensis*.

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Increasing consumption of Chinese dragon's blood and agarwood in recent years, over-exploitation and over-cutting of original plants in China has caused depletion of the natural resources. *D. cambodiana* and *A. sinensis* are listed as protected plants in the "List of Wild Plants under State Protection". Co-evolving with medicinal plants, some endophytic fungi have developed the abilities to produce same or similar bioactive substances as or to what their host plants produce (Stierle et al., 1993; Strobel et al., 1996; Li et al., 1998). Thus endophytic fungi of *D. cambodiana* and *A. sinensis* will be selected as a source for the production of bioactive compunds of host plants.

It is known that endophytic fungi existing in plant are the important potential sources of antimicrobial substances (Strobel, 2003; Vicente et al., 2003; Kim et al., 2004). Some endophytic fungi possess antimicrobial activity that may be involved in a symbiotic association with a host plant (Yang et al., 1994). Studies indicated that Chinese dragon's blood had wide-spectrum antimicrobial activities. Therefore, the endophytic fungi will have a huge potential to exploit novel and highly active antimicrobial agents.

Therefore, it is necessary to carry out the systematic investigation of endophytic fungi. It not only can provide

useful ecological information to us, but also is a way of finding new bioactive strains. However, few studies of endophytic fungi of these plants have been reported. The objectives of this study were to isolate, identify and screen for antimicrobial activity of endophytic fungi from *D. cambodiana* and *A. sinensis* collected from Yunnan province, southwest China.

MATERIALS AND METHODS

Collection of plant materials

Plant materials of *D. cambodiana* and *A. sinensis* were collected randomly in April 2006 from Jinghong city, Xishuangbanna prefecture, Yunnan province, China. Samples were taken from three parts of tree, including leaf, root and stem. Samples were transported in paper bags in a cooler, stored overnight at 4℃ and processed on the following day.

Isolation, culture and identification of endophytic fungi

Each plant material was washed under running water and cut into 5 × 5 mm size pieces. A total of 300 pieces were cut from six materials from the two plants. Plant materials were surface-sterilized according to method described by Guo et al. (2000). Samples were incubated on potato dextrose agar (PDA, 2%). Petri dishes were incubated in the dark at 25℃ for 2 months and observed daily for hyphal development. Pure cultures were obtained by transferring hyphal tips to new Petri dishes with PDA and also with corn meal agar (CMA, 2%). All cultures were incubated at 25℃, with a light regime of 12 h: 12 h light: dark. Isolates, which did not sporulate under these incubation conditions, were incubated at 4℃ for 2 months to induce sporulation.

Isolates on different media were examined periodically and identified according to the morphology and the mechanism of spore production (Barnett and Hunter, 1987). The cultures which failed to sporulate after the treatments were named mycelia sterilia and divided into different "Morphospecies" according to cultural characteristics (i.e. colony colour, texture, habit and growth rate) on PDA (Shan et al., 2002). Living cultures are deposited in The Institute of Medicinal Plant Development (IMPLAD), the Chinese Academy of Medical Sciences (CAMS) and Peking Union Medical College (PUMC).

Data analysis

Data were processed using Microsoft Access v. 2003 and statistical analysis was carried out with Microsoft Excel v. 2003. Colonization rate (CR), isolation rate (IR) and relative frequency (RF) were calculated using the formula given by Taylor et al. (1999) and Li et al. (2007).

$$CR = \frac{\text{the total number of samples yielding } \geq 1 \text{ isolate}}{\text{the total number of samples in that trial}}$$

$$IR = \frac{\text{the total number of isolates yielded in a given trial}}{\text{the total number of samples in that trial}}$$

$$RF = \frac{\text{the total number of samples yielding } \geq 1 \text{ isolate}}{\text{the total number of samples in that trial}}$$

Colonization rate was calculated and used to demonstrate the infection degree of different plant or different organ of same plant by endophytic fungi and was expressed as percentage. Isolation rate was calculated and used to demonstrate the richness of endophytic fungi of plant and the degree of multiple colonization of plant organ but was not expressed as percentage. Relative frequency was expressed as percentage of a given endophytic fungi.

Screening of antimicrobial activities

Antagonists for a preliminary investigation of the antimicrobial spectrum here were 6 clinical microbial pathogens including 3 bacteria, 2 yeasts and one filamentous fungus *Aspergillus fumigatus* (Af) As 3.2910. The 3 bacteria strains were *Bacillus subtilis* (Bs) As 1.308, *Escherichia coli* (Ec) As 1.355 and *Staphylococcus aureus* (Sa) As 1.72. The 2 yeasts strains were *Candida albicans* (Ca) As 2.538 and *Cryptococcus neoformans* (Cn) As 2.1490.

The inoculum and assay plates for bacteria and yeast strains were prepared as described by Peláez et al. (1998). The conidial suspension and assay plate of *A. fumigatus* were prepared according to method described by Vicente et al. (2001). Endophytic fungi grown on PDA were used to screen the antimicrobial bioactivities according to agar block method (Zhou, 2006). Each combination of endophytic fungi/pathogen was repeated 10 times. All Petri dishes were incubated in the dark and randomly distributed. After incubation at 37℃ for 24 h for bacteria, and 25℃ for 24 h for yeasts and 48 h for *A. fumigatus,* the diameter (mm) inhibition zones were observed, measured and recorded. The experiment was replicated 3 times.

RESULTS

Endophytic fungi

A total of 300 fungal isolates were recovered from 300 pieces. 172 and 128 isolates came from *D. cambodiana* and *A. sinensis*, respectively. The colonization rates (%) and isolation rates for endophytic fungi recovered in each organ are given in Table 1. There were high infection degree (34 - 80%) and multiple colonization (0.62 - 2.06) of plant organ in this experiment.

A total of 174 isolates yielded 41 taxa in 30 genera. The remaining 126 isolates did not sporulate and were segregated into 8 morphological groups (MG) on the basis of their cultural and morphological characteristics. The composition of taxa occurring at relative frequencies of >1% in each organ was given in Table 2. The distribution of endophytic fungi in various plants in this study was not uniform and there was a large degree of difference in the quantity of endophytic fungi. *Mycelia sterilia* sp. 6 (14.3%), *Fusarium* sp. 4 (7.1%), *Cladosporium edgeworthrae* (5.7%), *Fusarium* sp. 1 (5.7%) and *Glomerularia* sp. (5.7%) were most commonly isolated from *A. sinensis*. Meanwhile, *Fusarium* sp. 1 (7.5%), *Mycelia sterilia* sp. 4 (6.0%) and *Mycelia sterilia* sp. 6 (5.2%) were the most dominant endophytes in *D. cambodiana*.

Antimicrobial assay

Out of 300 endophytes isolated, 21 strains (12.2%) from *D. cambodiana* and 4 strains (3.1%) from *A. sinensis* could inhibit some of tested human pathogenic bacteria

Table 1. Colonization, isolation and multiple infection rates in each organ.

Parameter	*Dracaena cambodiana*				*Aquilaria sinensis*			
	Leaves	Stems	Roots	Total	Leaves	Stems	Roots	Total
Amount of samples	50	50	50	150	50	50	50	150
Amount of samples with isolates	25	35	40	100	17	22	20	59
Amount of isolates recovered	31	103	38	172	33	63	32	128
Colonization rate (%)	50	70	80	66.67	34	44	40	39.33
Isolation rate	0.62	2.06	0.76	1.15	0.66	1.26	0.64	0.85

Table 2. Relative frequencies of endophytic fungi species (RF ≥1%)[a].

Taxa	*Dracaena cambodiana*				*Aquilaria sinensis*			
	Leaves	Stems	Roots	Total	Leaves	Stems	Roots	Total
Botryosphaeria rhodina							2.9	2.9
Calcarisporium sp.		2.2		2.2				
Cephalosporium sp. 1		2.2	1.5	3.7		1.4		1.4
Cephalosporium sp. 2						4.3		4.3
Cephalosporium sp. 3						4.3		4.3
Cladophialophora sp.							1.4	1.4
Cladosporium edgeworthrae							5.7	5.7
Colletotrichum sp.					1.4			1.4
Epicoccum sp.			3.0	3.0			1.4	1.4
Fusarium oxysporum							2.9	2.9
Fusarium sp. 1		6.0	1.5	7.5			5.7	5.7
Fusarium sp. 2		2.2		2.2	2.9			2.9
Fusarium sp. 3		1.5		2.2			2.9	2.9
Fusarium sp. 4						1.4	5.7	7.1
Geotrichum sp.						4.3		4.3
Glomerularia sp.		1.5	1.5	3.0		1.4	4.3	5.7
Gonytrichum sp.		1.5		1.5				
Guignardia manqiferae					1.4			1.4
Monilia sp.							1.4	1.4
Mortierella sp.					1.4			1.4
Ovulariopsis sp. 1					1.4			1.4
Ovulariopsis sp. 2		2.2		3.0	2.9			2.9
Ovulariopsis sp. 4		2.2		2.2				
Penicillium sp.						2.9		2.9
Pleospora sp.					1.4			1.4
Rhinocladiella sp.						1.4		1.4
Mycelia sterilia sp. 1	1.5	1.5	2.2	5.2			2.9	2.9
Mycelia sterilia sp. 2		1.5	2.2	4.5		1.4		1.4
Mycelia sterilia sp. 3	1.5	3.0		5.2				
Mycelia sterilia sp. 4		6.0		6.0				
Mycelia sterilia sp. 5	3.0	1.5		5.2	2.9			2.9
Mycelia sterilia sp. 6	3.0	5.2		9.0	14.3		2.9	17.1
Mycelia sterilia sp. 7	3.0	4.5	4.5	10.4	1.4	4.3		5.7
Mycelia sterilia sp. 8		3.7		3.7				
Rare isolates	2.9	5.7	8.8	14.5				
Total	14.9	60.4	24.6	100	31.4	27.1	41.4	100

[a]Taxa occurring at <1% RF in the each organ:
Leaves of *Dracaena cambodiana*: *Cephalosporium* sp. 3, *Ovulariopsis* sp. 2, *Rhizatonia* sp., *Mycelia sterilia* sp. 2.
Stems of *Dracaena cambodiana*: *Acrostalagmus* sp., *Alternaria alternata*, *Botryosphaeria rhodina*, *Cephalosporium* sp. 2, *Fusarium oxysporum*, *Fusarium proliferatum*, *Fusarium* sp. 5, *Geotrichum* sp., *Lunulospora* sp., *Ovulariopsis* sp. 1, *Ovulariopsis* sp. 3, *Paecilomyces* sp., *Penicillium* sp., *Phialophora* sp., *Rhizatonia* sp., *Scytalidium lignicola*.
Roots of *Dracaena cambodiana*: *Aspergillus* sp., *Fusarium proliferatum*, *Fusarium* sp. 3, *Fusarium* sp. 4, *Humicola* sp., *Paecilomyces* sp., *Septonema* sp., *Schizophyllum commune*, *Mycelia sterilia* sp 3, *Mycelia sterilia* sp. 6.

Table 3. Antmicrobial spectra of selected endophytic fungi[a].

Strain No.	Taxa	Diameter of inhibition zone on assay plate (mm)				
		B. subtilis	***S. aureus***	***A. fumigatus***	***Cryp. neoformans***	***C. albicans***
DC[c]-1-1	*Mycelia sterilia* sp. 4	_[b]	_	8.83 ± 0.98	_	_
DC-1-5	*Mycelia sterilia* sp. 6	_	_	17.00 ± 1.79	_	_
DC-1-6	*Calcarisporium* sp.	_	_	_	8.08 ± 0.20	_
DC-1-9	*Scytalidium lignicola*	_	_	20.00 ± 2.53	_	_
DC-1-12	*Mycelia sterilia* sp. 4	_	_	9.25 ± 0.75	_	7.00 ± 0.00
DC-1-13	*Mycelia sterilia* sp. 4	_	_	12.50 ± 1.38	_	_
DC-1-21	*Fusarium* sp. 2	_	_	_	7.58 ± 0.38	_
DC-1-22	*Cephalosporium* sp. 1	_	_	14.08 ± 1.02	_	_
DC-1-23	*Fusarium* sp. 2	16.25 ± 0.52	_	15.42 ± 1.02	_	_
DC-1-24	*Ovulariopsis* sp. 1	20.08 ± 1.02	_	21.33 ± 1.03	_	13.33 ± 0.52
DC-1-27	*Fusarium* sp. 2	18.83 ± 1.04	11.83 ± 0.29	19.00 ± 0.00	18.33 ± 1.15	_
DC-1-42	*Fusarium proliferatum*	13.00 ± 1.00	_	_	16.67 ± 0.58	8.33 ± 0.58
DC-1-59	*Cephalosporium* sp. 1	16.33 ± 0.58	10.00 ± 1.00	14.33 ± 1.53	13.00 ± 0.00	_
DC-1-68	*Ovulariopsis* sp. 4	10.33 ± 0.58	_	_	_	_
DC-1-78	*Mycelia sterilia* sp. 4	_	_	27.00 ± 1.00	_	_
DC-2-9	*Mycelia sterilia* sp. 3	9.00 ± 0.00	_	_	_	_
DC-2-12	*Epicoccum sp.*	_	_	14.33 ± 1.15	_	_
DC-2-27	*Fusarium* sp. 2	19.00 ± 0.00	13.5 ± 0.50	_	_	_
DC-2-29	*Humicola* sp.	18.50 ± 0.50	_	18.00 ± 1.73	_	_
DC-2-30	*Fusarium proliferatum*	19.83 ± 0.29	11.00 ± 1.73	21.67 ± 2.52	11.00 ± 0.00	17.33 ± 0.58
DC-2-32	*Fusarium* sp. 1	22.00 ± 0.00	15.33 ± 0.58	_	8.00 ± 0.00	9.00 ± 0.50
AS[d]-2-12	*Fusarium oxysporum*	12.17 ± 0.50	_	_	_	_
AS-2-13	*Fusarium oxysporum*	15.17 ± 0.29	_	_	_	_
AS-2-16	*Fusarium* sp. 4	11.67 ± 0.58	_	_	_	_
AS-3-8	*Fusarium* sp. 1	10.33 ± 0.29	_	_	_	_

[a]All isolates failed to show antimicobial activity to *E. coli*.
[b]No antimicrobial activity.
[c]Endophytic fungi isolate from *Dracaena cambodiana*.
[d]Endophytic fungi isolate from *Aquilaria sinensis*.

and fungi. The remaining isolates appeared not to produce secondary metabolites which displayed antimicrobial activity against the test microorganisms. All active isolates failed to show antimicobial activity to *E. coli* (Table 3). Among the active isolates, 56% displayed antimicrobial activity against only one pathogen, 16% against 2 pathogens, 8% against 3 pathogens, 12% against 4 pathogens and 8% against 5 pathogens. 72% of strains showed inhibition to pathogenic fungi, while 60% displayed inhibition to test bacteria, which indicated that endophytic fungi had strong antimicrobial activity for fungi pathogen than for bacteria pathogen. The inhibition zones ranged from 7 mm to 27 mm. Three isolates exhibited strong antifungal activity with more than 20 mm inhibition zone diameter, namely DC-1-78 to Af, DC-2-30 to Af and DC-2-32 to Bs, and could be developed as biological control agents.

DISCUSSION

The isolation rates of the endophytic fungi were different in different organs. The main effect factors of the isolate rate were the plant species, the amount of sample and isolation medium. We only studied small number of samples and only one isolation media (potato dextrose agar). All these factors above limited isolation of endophytic fungi. So, more endophytic fungi will be recovered with enlargement of investigation scope and reason, increase of sample quantity and more kinds of isolation medium.

Fusarium was the most dominant genera in *D. cambodiana* and *A. sinensis*, which was also demonstrated in the research of other plants (Jiang et al., 1995; Tian et al., 2004). There is great deal of reports about *Fusarium* as plant pathogenic fungi. However, *Fusarium* could not cause apparent disease to their host. So the effect of endophytic *Fusarium* during the developmental stage of plant need to be further studied.

Nine of the genera that occurred in *D. cambodiana* were also obtained in *A. sinensis*. 19 fungal species only were observed in one organ. These data indicated that the distribution of endophytic fungi in the different organs was different and some endophytes showed certain level

of host specificity or organs specificity and supported hypothesis of Carroll et al. (1977). It is due to following reasons: there are obvious differences in the internal structure of the different organs of plant and the different tissues of the same organ, the constitute of chemistry material of plant tissue and the nutrition status to satisfy different growth, which would influence infection, growth and distribution of endophytic fungi, directly (Carroll and Petrini, 1983; Rodrigues, 1994).

Antimicrobial activities of medicinal plant endophytic fungi have been reported by a few groups (Li et al., 2005; Xu et al., 2008). Associated with previous study, there was at least 1 active isolate in each medicinal plant species. Therefore, endophytic fungi of Chinese medicinal plants should be a potential resource of bioactive antimicrobial agents.

The antimicrobial active isolates selected belonged to 17 taxa (Table 3). Most active fungi distributed in the genera of *Calcarisporium*, *Cephalosporium*, *Scytalidium*, *Fusarium*, *Epicoccum*, *Ovulariopsis* and some of *Mycelia sterilia*. Some among them such as *Fusarium* and *Ovulariopsis*, were known to have various bioactivity (Park et al., 2003; Weber et al., 2004).

In this study, the antimicrobial spectrum of endophyte exhibited certain differences between different genera and strains. Furthermore, the activity of different strains from the same genus or species also has differences, for example, *Fusarium* isolates. DC-1-42 and DC-2-30 are strains of *F. proliferatum*; DC-2-30 showed strong antimicrobial activity to 5 pathogens, while DC-1-42 was only active against Bs, Ca and Cn. Two strains of *F. oxysporum* displayed similar antibacterial activity against Bs with an inhibition zones ranging from 12 to 16 mm. At the genus level, different species of *Fusarium* showed different antimicrobial activity. This result is similar to those of Souwalak et al. (2006). Therefore, individual strains of endophytic fungi should be tested for antimicrobial activities.

In other studies (unpublished data), we observed that fungi of *Fusarium* spp., *Calcarisporium* spp., *Cephalosporium* spp. and *Ovulariopsis* spp. could improve production and accumulation of secondary metabolism products of *D. cambodiana* and *A. sinen*. These studies were, however, only preliminary, and further research is required to identify their role during the formation of Chinese dragon's blood and agarwood.

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