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Endophytic Fungi Diversity of Aquatic/Riparian Plants and Their Antifungal Activity *In Vitro*

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Two hundred and fourteen endophytic fungi were isolated from 500 segments of aquatic/riparian plants *Ottelia acuminata*, *Myriophyllum verticillatum*, *Equisetum arvense*, *Cardamine multijuga*, and *Impatiens chinensis*. They were identified to 31 taxa in which *Cladosporium*, *Fusarium*, and *Geotrichum* were the dominant genera. Among all isolates, 169 (79%) were anamorphic fungi, 1 (0.5%) was an teleomorphic ascomycete and 44 (21%) were sterile mycelia. There were significant differences in the colonization frequency of endophytes between the five plant species (X~2=51.128, *P*<0.001, Chi-square test). The riparian plants harboured more endophytes than the submerged plants. The antifungal activity of these isolates against *Fusarium solani* and *Phytophthora nicotianae in vitro* were tested and 28 (13.1%) isolates showed antifungal activities with more than 30% growth inhibition rate against the two pathogens.

***Keywords*:** aquatic plants, riparian plants, fungal endophytes, diversity, antifungal activity

Fungi causing asymptomatic infections in living plant tissues have been called fungal endophytes (Hyde and Soytong, 2008). They comprise a diverse group of fungi and may protect the host plant against the insect pests and phytopathogens (Arnold *et al*., 2003; Arnold and Lewis, 2005; Herre *et al*., 2007), increasing host fitness in extreme environments (Redman *et al*., 2002). In addition, fungal endophytes have been recognized as a repository of novel secondary metabolites, some of which have beneficial biological activities (Strobel and Daisy, 2003; Huang *et al*., 2008; Raghukumar, 2008).

There have been numerous studies on endophyte communities of temperate, tropical and subtropical plants (Kumar *et al.*, 2005; Arnold and Lutzoni, 2007; Oses *et al*., 2008; Rungjindamai *et al*., 2008; Sánchez Márquez *et al*., 2008; Tao *et al*., 2008) and there have been several studies in China (Hu *et al*., 2007; Li *et al*., 2007; Wei *et al*., 2007a). Recently, a few studies on endophyte communities of marine plants and halophytes have been carried out (Cornick *et al*., 2005; Lin *et al*., 2008; Maciá-Vicente *et al*., 2008), however, there has been less research on endophyte communities of aquatic plants (Sridhar and Bärlocher, 1992; Sati and Belwal, 2005).

The Yunnan-Guizhou Plateau is the middle tier on the eastern slope of the Himalayas. There are many plateau lakes, rivers, and streams located in this area. In order to understand the endophytic fungi diversity of aquatic and riparian plants in this environment, we examined the endophyte communities of five aquatic and riparian plants growing in a stream of Kunming City, Yunnan Province, Southwest China.

Materials and Methods

Sampling site and plants

Sampling site: Songming County, 25°25′N and 103°10′E, which is located in Northeast of Kunming City, Yunnan Province, Southwest China. The altitude is 1978 m. The stream which plants collected is clean and transparent.

Plants: *Ottelia acuminata*, *Myriophyllum verticillatum*, *Cardamine multijuga*, *Equisetum arvense*, and *Impatiens chinensis*. The first three are submerged plants and the last two are not aquatic plants in general meanings, but in the sampling sites, they are most common and occur next to the stream and part of their stem was in water (riparian plants). Ten healthy plants of each plant species were collected at randomly at October 2007.

Fungal endophytes isolation

For endophytic fungi isolation, 20 healthy leaves and 20 healthy stem segments were selected from each plant species at randomly, and washed in running tap water and processed as follows: samples were cut into segments (about 5×5 mm) and were surface sterilized by sequentially dipping into 0.5% sodium hypochlorite (2 min) and 70% ethanol (2 min), and rinsed with sterile water, then allowed to surface-dry under sterile conditions (Arnold *et al*., 2000). Finally, 100 leaf segments and 100 stem segments of each plant species were deposited on a Petri dish containing potato dextrose agar (PDA) medium amended with 0.5 g/L streptomycin sulfate, incubated at 25°C and checked every other day for 21 days, fungi growing out from the plant tissues were transferred to other plates with PDA. All strains are stored in Faculty of Life Sciences and Technology, Kunming University of Science and Technology.

Fungal endophytes identification

The sporulating isolates were identified to genus level based on their morphology and the mechanism of spore production (Barnett and Hunter, 1987; Ellis, 1988). Non-sporulating isolates were placed onto autoclaved carnation leaves for sporulation and the plates were continuously monitored for spore formation by stereo and light microscopy. After two months, sterile isolates were identified as sterile mycelia and sorted into different groups on the basis of colony

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surface texture, hyphal pigmentation and growth rates.

Antagonistic study

The antifungal activity of endophytes on 2 phytopathogenic fungi *Fusarium solani* and *Phytophthora nicotianae* was determined by Contact assay (Alvarez *et al*., 2001). Fungal endophytes were inoculated in the centre of the PDA plates. Two 5 mm diameter discs of the test phytopathogens were cut from the periphery of less than 1 week old cultures on PDA plates and placed mycelia surface down on opposite edges of the test plates against the sides of the dishes. The plates were incubated in the dark at 25°C for 3 to 6 days, extension of pathogenic hyphae towards the central endophyte was measured from the inner edge of the inoculum discs to the leading edges of colonies at a point nearest the central endophyte. Mean growth measurements were calculated from 4 replicates of each of the tested phytopathogenic fungi.

Growth inhibition (GI%) of the treatment against the control was measured by percentage, using the formular (C-T/C)×100 where C is hyphal extension (mm) of control and T is hyphal extension (mm) of endophytic treated plates (Alvarez *et al*., 2001).

Data analyses

The colonization frequency (CF), expressed as percentage, was calculated according to Petrini *et al*. (1982) as follows: %CF=(no. of tissue segments colonized by a fungus / total no. of tissue segments plated)×100. Frequency of colonization of endophytes was calculated as total number of segments yielding given fungus divided by total number of segments incubated.

SPSS 13.0 was used for statistical analysis. The chi-square test was used to compare the difference in colonization frequency of endophytes between five plant species.

Results and Discussion

Isolates (214) were recovered from 500 plant segments. The overall colonization frequencies (CF%) varied with the host species from 18% to 63% (Table 1), and showed significant difference between five plant species ($X^2=51.128$, $P<0.001$, Chi-square test) (Fig. 1). In addition, it was found that the CF% of riparian plants was higher than that of submerged plants (Table 1). Similarly, some researchers reported that the arbuscular mycorrhizal fungi colonization in emerged aquatic plants was generally higher than that in submerged ones (Miller, 2000; Šraj *et al*., 2006). This may due to the metabolic requirements of fungal endophytes and mycorrhizal fungi for oxygen, their activity decreases as environmental moisture increases (Turner and Friese, 1998).

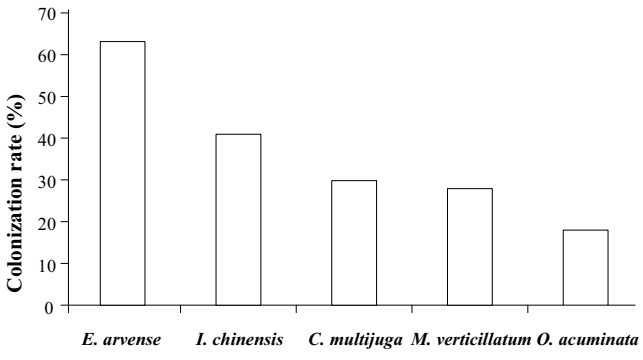

Fig. 1. The overall colonization rates of endophytic fungi of five plant species.

Colonization frequencies of terrestrial plant endophytes are known to vary with altitude, humidity, density of canopy, precipitation, and host (innate host susceptibility) (Wang *et al*., 2007; Hoffman and Arnold, 2008; Naik *et al*., 2009; Rudgers and Swafford, 2009). In the present study, the variation of CF% could be due to the differences in oxygen supply and host preference.

The CF% is an indication of the number of endophytic fungi. The reported colonization frequencies of terrestrial plants had a great variation, e.g., *Licuala ramsayi*, *Euterpe oleraceae* Mart., *Azadirachta indica*, *Trachycarpus fortunei*, *Musa acuminate*, *Amomum siamense*, and *Centella asiatica* with 12.5%, 21%-30%, 31.5%-45.5%, 23%-57%, 41.7%-56.5%, 70%-83.3% and 78% colonization frequencies, respectively (Rodrigues and Samuels, 1990; Rodrigues, 1994; Taylor *et al*., 1999; Bussaban *et al*., 2001; Photita *et al*., 2001; Verma *et al*., 2007; Rakotoniriana *et al*., 2008). The CF% of aquatic/riparian plants in this study within the range of terrestrial plants CF%, the results suggested that fungal endophytes colonization may also be abundant in plants of aquatic environments.

Among 214 endophytic isolates, 44 (21%) were sterile mycelia and the other 170 were identified to the genus level, in which 169 (79%) were anamorphic fungi and 1 was an ascomycete (Table 1). In total, 31 taxa were recovered. However, the number of taxa recovered from each plant species was different. The maximum of 16 taxa was isolated from *Equisetum arvense* and the minimum of 9 from *Myriophyllum verticillatum*. Some taxa were common to all 5 plant species such as *Cladosporium*, *Geotrichum*, and Sterile mycelia 2, whereas, some only recovered from 1 plant species such as *Ascochyta*, *Pestalotia*, and *Trichoderma* (Table 1). Although each aquatic plant host harboured many endophytes, only one to three endophyte species were dominant in each host (Table 1). The same results had been found in mangrove hosts and terrestrial hosts (Kumaresan and Suryanarayanan, 2001).

Cladosporium had been reported as the dominant genus of *Azadirachta indica* (Verma *et al*., 2007). In present study, It was the most frequently isolated endophyte (26.6%) and it was also the dominant genus for 5 plant species examined (Table 1). The result differed from the earlier reports on terrestrial plants that the dominant endophyte of different hosts was usually different (Petrini *et al*., 1982; Kumaresan and Suryanarayanan, 2001). Our result suggested that some fungal endophyte such as *Cladosporium* and *Geotrichum* may mainly transmit horizontally through its large amounts of asexual spore in aquatic environments. Moreover, *Cladosporium* may contribute to plants stress resistance to aquatic environments. It is interesting the *Pestalotiopsis* species were not isolated as these have previously been shown to be common terrestrial endophytes in various parts of China (Hu *et al*., 2007; Tejesvi *et al*., 2007; Wei *et al*., 2007b). This may be due to the fact that the endophytes were isolated from aquatic, as compared to terrestrial plants.

All 214 isolates were tested antifungal activities against pathogenic fungi *Fusarium solani* and *Phytophthora nicotianae*. The isolates whose growth inhibition rate against pathogens

Table 1. No. of isolates, taxa, and colonization frequency of endophytic fungi from aquatic/riparian plants

Taxa	No. and CF (%) of endophytic fungi					Total
	Impatiens chinensis	*Ottelia acuminata*	*Myriophyllum verticillatum*	*Equisetum arvense*	*Cardamine multijuga*	
Ascomycete		1 (1.0)				1 (1.0)
Mitosporic fungi						
Alternaria sp.		1 (1.0)	1 (1.0)			2 (2.0)
Aposphaeria sp.		2 (2.0)		1 (1.0)		3 (3.0)
Ascochyta sp.	1 (1.0)					1 (1.0)
Aspergillus sp.	1 (1.0)	1 (1.0)				2 (2.0)
Catinula sp.	1 (1.0)			2 (2.0)		3 (3.0)
Cephalosporium sp.				5 (5.0)		5 (5.0)
Chaetomella sp.		1 (1.0)				1 (1.0)
Chaetophoma sp.				6 (6.0)		6 (6.0)
Cladosporium cladosporioides	18 (18.0)	5 (5.0)	11 (11.0)	8 (8.0)	1 (1.0)	43 (43.0)
Cladosporium sp. 1	5 (5.0)		1 (1.0)	2 (2.0)	6 (6.0)	14 (14.0)
Fusarium sp. 1	1 (1.0)		3 (3.0)	14 (14.0)	4 (4.0)	22 (22.0)
Fusarium sp. 2				7 (7.0)		7 (7.0)
Fusicoccum sp.					1 (1.0)	1 (1.0)
Geotrichum sp.	8 (8.0)	1 (1.0)	7 (7.0)	6 (6.0)	5 (5.0)	27 (27.0)
Gloeosporium sp.				2 (2.0)		2 (2.0)
Hainesia sp.			1 (1.0)			1 (1.0)
Melasmaia sp.					1 (1.0)	1 (1.0)
Mucor sp.		4 (4.0)	2 (2.0)		1 (1.0)	7 (7.0)
Oedocephalum sp.	1 (1.0)				1 (1.0)	2 (2.0)
Penicillium sp.	1 (1.0)	2 (2.0)				3 (3.0)
Pestalotia sp.				2 (2.0)		2 (2.0)
Phyllosticta sp.		1 (1.0)		1 (1.0)		2 (2.0)
Sirodesmium sp.	1 (1.0)			1 (1.0)	1 (1.0)	3
Sphaceloma sp.					1 (1.0)	1 (1.0)
Torula sp.				2 (2.0)		2 (2.0)
Trichoderma sp.					4 (4.0)	4 (4.0)
Umbelopsis sp.					1 (1.0)	1 (1.0)
Sterile mycelia 1	4 (4.0)		5 (5.0)	6 (6.0)	5 (5.0)	20 (20.0)
Sterile mycelia 2	4 (4.0)	1 (1.0)	7 (7.0)	7 (7.0)	4 (4.0)	23 (23.0)
Sterile mycelia 3	2 (2.0)					2 (2.0)
Total taxa recovered	13	11	9	16	14	31
No. of isolates recovered	48	20	38	72	36	214
No. of segments yielding more than one fungus	41	18	28	63	30	180
Total fragments plated	100	100	100	100	100	500
Total CF (%)	41	18	28	63	30	36

more than 30% were shown in Table 2. It was found that at least 1 active isolate was obtained from each plant species, and total 28 (13.1%) active isolates were obtained. The overall percentage of active isolates in this study was lower than that of tropical and sub-tropical plants (Huang *et al*., 2001; Li *et al*., 2005; Guimaraes *et al*., 2008). From the results, it was assumed that most of the aquatic/riparian plants endophytes may mainly play a roll in nutrient absorption and nitrogen fixation rather than protecting the host plants against pathogens as the endophytes of tropical and sub-tropical plants usually do, for nutritional components and plant species diversity in aquatic environment studied was lower when compared with terrestrial environments. However, in contrast to terrestrial plants, endophytes colonization in plants of aquatic environment and its possible role(s) is still poorly understood.

The endophytes isolated in this study were obtained by traditional methodology which incorporated taking mycelia growing out of tissue and plating them out. The mycelia sterilia obtained were not identified using molecular techniques as in other studies (Thomas *et al.*, 2008; U'Ren *et al*., 2009) and it is unlikely that we would have isolated slow

Table 2. The antifungal activities of endophytes against 2 phytopathogens

Plants	Strain no.	Taxa	Growth inhibition rate (%)[a]	
			Phytophthora nicotianae	*Fusarium solani*
I. chinensis	A8	*Cladosporium* sp.	9.25±2.62	41.91±1.87
	A9	Sterile mycelium 3	19.25±7.86	32.06±7.90
	A10	*Catinula* sp.	16.66±2.61	42.35±1.66
	A11	*Ascochyta* sp.	1.85±2.61	42.65±2.08
	A12	*Cladosporium* sp.	16.11±1.83	52.94±4.15
	A13	*Cladosporium* sp.	44±5.66	29.18±1.85
	A16	*Geotrichum* sp.	20.37±2.62	47.05±1.85
	A18	Sterile mycelium 3	32.41±6.61	32.24±5.61
	A27	*Fusarium* sp.	25±5.89	43.01±1.52
	A31	Sterile mycelium 1	31.25±4.71	29.03±3.04
	A37	*Geotrichum* sp.	27.08±2.95	24.30±1.22
	A38	*Aspergillus* sp.	32.5±5.89	39.14±5.79
O. acuminata	B10	Sterile mycelium 2	46.14±5.05	32.79±2.32
M. verticillatum	C9	*Fusarium* sp.	37.5±2.53	18.51±0.90
E. arvense	D44	*Fusarium* sp.	8.14±4.65	30.77±1.81
	D61	*Cephalosporium* sp.	64.28±6.06	59.02±2.32
	D66	Sterile mycelium 2	39.53±6.58	18.75±5.3
	D68	*Geotrichum* sp.	49.58±0.59	57.78±3.14
C. multijuga	E1	*Trichoderma* sp.	31.09±4.37	39.54±5.87
	E2	*Trichoderma* sp.	38.64±8.15	38.89±7.86
	E4	*Trichoderma* sp.	22.5±6.96	32.41±6.83
	E10	*Cladosporium* sp.	5.87±1.57	30.43±5.30
	E11	Sterile mycelium 2	14.60±5.39	34.37±4.42
	E13	*Phyllosticta* sp.	8.3±3.06	31.92±0.99
	E19	*Melasmaia* sp.	14±2.82	30.2±2.82
	E30	*Oedocephalum* sp.	44±7.35	38±2.83
	E34	*Cladosporium* sp.	21.66±7.07	26.90±0.51
	E35	*Geotrichum* sp.	30±4.71	31.22±1.98

[a] Values are Mean±SE of three replications

growing endophytes or those which cannot grow in culture (Hyde and Soytong, 2008). Future studies should incorporate direct DNA sequencing to identify mycelia sterilia and direct analysis of whole DNA (e.g. DNA cloning, DGGE or T-RLFP) (Duong *et al*., 2006; Bridge and Newsham, 2009; Fernandes *et al*., 2009; Robinson *et al*., 2009) to detect all fungi present.

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