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Diversity of endophytic fungi of *Myricaria laxiflora* grown under pre- and post-flooding conditions

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ABSTRACT. *Myricaria laxiflora* is distributed along the riverbanks of the Yangtze River valley. The Three Gorges Dam has dramatically changed the habitat of *M. laxiflora*, which has evolved to develop increased resistance to flooding stress. In order to elucidate the relationship between plant endophytic fungi and flooding stress, we isolated and taxonomically characterized the endophytic fungi of *M. laxiflora*. One hundred and sixty-three fungi were isolated from healthy stems, leaves and roots of *M. laxiflora* grown under pre- and post-flooding conditions. Culture and isolation were carried out under aerobic and anaerobic conditions. Based on internal transcribed spacer sequence analysis and morphological characteristics, the isolates exhibited abundant biodiversity; they were classified into 5 subphyla, 7 classes, 12 orders, 17 families, and 26 genera. Dominant endophytes varied between pre- and post-flooding plants, among different plant tissues, and between aerobic and anaerobic culture conditions. *Aspergillus* and *Alternaria* accounted for more than 55% of all isolates. Although the number of isolates from post-flooding plants

was greater, endophytes from pre-flooding plants were more diverse and abundant. Endophytes were distributed preferentially in particular tissues; this affinity was constrained by both the host habitat and the oxygen availability of the host.

Key words: *Myricaria laxiflora*; Endophytic fungi; Diversity; Dominant; Flooding

INTRODUCTION

Myricaria laxiflora is an endangered evergreen shrub that is distributed in the low-altitude region of the Yangtze River valley (Liu et al., 2006; Wang et al., 2009). Due to long-term evolutionary adaptation to the natural dynamics of seasonally fluctuating water levels, *M. laxiflora*is capable of surviving prolonged immersion in water, for 3 to 5 months in summer (Liu et al., 2009; Chen and Xie, 2009). Under flooding conditions, plants have limited access to oxygen and light, and undergo oxidative stress (Wang et al., 2009). After October, plants rapidly resume growth until the following April (Wang et al., 2009).

For nearly half a century, the reported distribution of *M. laxiflora* was restricted to the region upstream of the Three Gorges Dam (Wu et al., 1998). It was predicted that *M. laxiflora* would completely disappear following construction of the dam, as this would elevate water levels and cause year-round submergence of plants, and the entire natural habitat (Liu et al., 2006). For this reason, a number of studies were carried out to better understand morphological characteristics; natural distribution and habitat; coexisting plant community structure; ecological adaptability; and propagation methods, for the *ex situ* conservation for *M. laxiflora* (Wu et al., 1998; Wang et al., 2003).

Surprisingly, following the construction of the Three Gorges Dam, *M. laxiflora* plants have been located in a downstream area, where a summer flooding habitat still exists (Bao et al., 2010). This indicates that *M. laxiflora* has a high resistance to flooding stress, but that its growth and propagation are still dependent on summer flooding (Wu, 1998). Besides summer flooding stress, *M. laxiflora* plants must overcome other abiotic stresses in the natural habitat, such as infertility, drought, and salinity; all are adversities that strongly influence plant growth and development (Wang et al., 2009).

Recent studies have revealed that symbiotic, endophytic fungi play a critical role in host plant survival (Rodriguez et al., 2009; Aly et al., 2011; Saikkonen et al., 2013). Endophytic fungi may accelerate plant growth and improve survival of biotic or abiotic stresses, such as plant diseases; insect pests; drought; salinity; and extremes of temperature (Mei and Flinn, 2010; Radhakrishnan et al., 2013). Endophytes of plants from special or extreme environments, in particular, have multiple roles in host ecological adaptability (Gostincar et al., 2010; Khan et al., 2011; Stępniewska and Kuźniar, 2013). For example, endophytes of mangroves in marine swamps can improve host salt and alkali resistance (Yin et al., 2014; Ali et al., 2014), while those of desert plants can enhance host tolerance to high temperatures (Márquez et al., 2007; McLellan et al., 2007). This highlights the importance of co-existence between endophytes and hosts living in extreme habitats (Rodriguez and Redman, 2008; Gibert, 2012), and suggests that endophytes may be important to the conservation of endangered plants (Johnston et al., 2012).

The current study addressed the question: do endophytic fungi of the riparian shrub, *M. laxiflora*, have a beneficial effect on plant resistance to the oxidative stress pathway? Many studies have demonstrated that endophytes improve host plant survival through the flooding stress period (Khan and Lee, 2013; Khan et al., 2013; Oberhofer et al., 2014). However, there are few reports on the association between endophyte diversity and host resistance to flooding stress (Sandberg et al., 2014). In order to address this, we isolated and identified fungi from *M. laxiflora* grown under pre- and post-flooding conditions, and described fungal distribution, frequency and composition, under aerobic and anaerobic culture conditions. Based on internal transcribed spacer (ITS) sequence and phylogenetic analysis, the dominant fungi populations were determined for *M. laxiflora* grown in non-flooding or flooding conditions. Findings from the study will contribute to a better understanding of the symbiotic relationship between endophytic fungi and the host under flooding stress, and the ecological adaptation of *M. laxiflora*. The current study provides a foundation for further studies on plant conservation and flooding tolerance.

MATERIAL AND METHODS

Plant sampling

M. laxiflora plants were collected from a small island, Yanzhiba, located 43 kilometers downstream of the Three Gorges Dam. The island is located in a water level fluctuation zone 109°32'E to 110°52'E and 30° 53'N to 31 °3'N. In July 2011, just prior to the flooding period, 20 samples of stems, leaves, and roots were collected from 10 healthy *M. laxiflora* plants older than 2 years. Sampled plants were spaced at least 50 meters apart. Roots were sampled at a depth of 10-20 cm below ground. Samples were either transported immediately to the laboratory or transiently stored at 4°C for no longer than 24 h. Sampling was repeated in October 2011, in the period immediately following recession of flood-waters. At the second sampling time point some *M. laxiflora* plants were still partly immersed, while others had emerged from the water (Figure 1).

Figure 1. *Myricaria laxiflora* plants of pre- and post-flooding. **A.** Pre-flooding in May. **B.** Post-flooding in October. **C.** A month post-flooding in November.

Isolation of endophytic fungi

Plant samples were washed thoroughly in running tap water for 10 min, and then cut into 0.3 x 0.3 cm pieces using a sterile scalpel. Surface sterilization was carried out with 75% alcohol for 1 min, followed by rinsing 4-5 times. All tissues were sterilized a second time using 3% (v/v) NaClO immersion for 1 min (stems and leaves) or 40 s (roots), as previously described (Pu et al., 2013). Samples were rinsed 4-5 times with sterile water, then sub-cut and plated on Petri dishes containing potato dextrose agar (PDA) medium with 25 μg/mL ampicillin to suppress bacterial growth. According to the method described by Pu et al. (2013), water from the final rinse step was also plated on culture medium to test for bacterial growth, and to confirm that the endophytes isolated were from *M. laxiflora* tissues and were not surface contaminants.

The PDA dishes were inverted and incubated at 28°C for 10 to 20 days, under aerobic and approximately anaerobic conditions. In the latter, dishes were sealed in a bag containing nitrogen. Microorganisms cultured on dishes were separated on the basis of morphological characteristics. To obtain pure cultures, isolates were sub-cloned in PDA and incubated at 28°C for approximately 1 week. All isolates were preserved in liquid paraffin at 4°C.

DNA extraction, PCR, and sequencing

Following morphologic observation and microscopic examination, ITS sequence analysis was carried out on each isolate. Total genomic DNA was extracted from cultures at 7-14 days, using a microbe genomic DNA isolation kit (Karroten Life Scientific, China). ITS ribosomal DNA, containing the ITS1, ITS2, and 5.8S regions, was amplified using the primers ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC GCT TAT TGA TAT GC 3') (Wäli et al., 2007). PCR was performed in 20 μL volume reactions, containing 2 μL DNA template; 1 μL of each primer; 2 μL PCR buffer; 2 μL dNTPs; 0.2 μL Ex-Taq Polymerase (Takara, Japan) and water. Reaction conditions were: 94°C for 5 min; 33 cycles at 94°C for 1 min, 55°C for 30 s, and 72°C for 1.5 min; and a final extension at 72°C for 10 min. Products were analyzed on 1% agarose (w/v) gel stained with ethidium bromide, then purified using an Agarose Gel DNA purification kit following the manufacturer protocol (TaKaRa). Each target fragment was sequenced twice at Sangon (Wuhan, China), using forward and reverse primers respectively. Sequences were reassembled using the DNASTAR software (DNASTAR Inc., WI, USA), and then used for blast analyses in GenBank.

Diversity analysis

On the basis of genus identification, five biodiversity indices were calculated. Isolation frequency (IF) was used to determine the dominant population, designated as a ratio of the specific endophyte number to the total strains (Wäli et al., 2007). Fungal dominance was determined by Camargo's index (1/S), where S represents species richness according to Rivera-Orduña et al. (2011). If IF>1/S, that species was defined as dominant (Camargo, 1992).

The Shannon-Wiener diversity index (*H'*) (Shannon and Weaver, 1949) was calculated as:

$$H' = -\sum_{i=1}^{S} Pi \ln Pi \qquad \text{(Equation 1)}$$

In the above formula, Pi = Ni/N, where Ni is the number of genera, and N is the total number of isolates obtained from *M. laxiflora* plants.

The Simpson diversity index (D) was calculated as (Simpson, 1949):

$$D = 1 - \sum_{i=1}^{S} Pi^2 \qquad \text{(Equation 2)}$$

Two diversity indices, where Pi has the same meaning, were estimated for each tissue and the total population, according to the method of Rivera-Orduña et al. (2011).

The Margalef richness index (R) was calculated as: $R = (S-1)/Log_2(N)$ (Zmudzki and Laskowski, 2012), where S is the number of genera and N is the total number of the isolates. The evenness index (E) was calculated as: $E = H'/In(S)$, where H' is the Shannon-Wiener index, as defined above (Zmudzki and Laskowski, 2012).

RESULTS

Isolation and identification of endophytic fungi

In total, 163 strains were isolated from root, stem, and leaf samples from plants grown under pre- and post-flooding conditions. On the basis of BLAST results from NCBI, all strains grouped into 5 subphyla, 7 classes, 12 orders, 17 families, and 26 genera (Tables 1 and 2; Figure 2). Over 90% of the isolates were Deuteromycotina or Basidiomycotina. Seven, 3 and 2 isolates belonged to Ascomycotina, Zygomycotina, and Mastigomycotina, respectively (Table 2). The genera were *Aspergillus*, *Penicillium*, *Trichoderma*, *Cladosporium*, *Aureobasidium*, *Alternaria*, *Fusarium*, *Colletotrichum*, *Pestalotiopsis*, *Epicoccum*, *Phomopsis*, *Candida*, *Trametes*, *Ceriporia*, *Bjerkandera*, *Irpex*, *Polyporus*, *Fomitopsis*, *Coprinellus*, *Schizophyllum*, *Botryosphaeria*, *Neurospora*, *Chaetomium*, *Rhizopus*, *Lichtheimia*, and *Pythium*. Based on morphological characteristics and ITS sequences analysis, endophytes were classified as 6 *Aspergillus*, 3 *Penicillium*, 2 *Alternaria*, 3 *Fusarium* species and other genera species. Altogether, at least 36 species of endophytes were detected in *M. laxiflora* plants (Table 1), indicating an abundant biodiversity of endophytic fungi in this plant species.

Endophyte genera from *M. laxiflora* were detected at different frequencies (Figure 2). *Aspergillus* occupied more than 40% of the total isolates (Table 1 and Figure 2), and *Alternaria* accounted for 15.34%. *Penicillium* and *Phomopsis* each accounted for 4.91%, while *Fusarium* and *Trametes* each accounted for 6.14% (Table 1 and Figure 2). However, according to Camargo's index (0.038; Table 3), we were unable to conclude that these 6 genera would be the dominant endophytes of *M. laxiflora*.

Endophyte diversity from different tissues

The number of endophytes varied between root, stem, and leaf samples (Table 3). The greatest fungal diversity, with 63 isolates, was detected from stems. Fifty-seven strains were obtained from the roots, and 49 from the leaves (Table 3). The species richness (S) for all tissues was 26: with 17 in stems; 14 in roots; and 12 in leaves (Table 3 and Figure 3A). According to Camargo's index, *Aspergillus* and *Alternaria* were the dominant genera

in all tissues (Table 3 and Figure 3A). *Penicillium* and *Fusarium* also had a relatively high abundance and a wide distribution in all tissues (Table 3). These findings indicate that these 4 genera may be systemic endophytes of *M. laxiflora*.

Table 1. ITS sequences of the endophytes in GenBank.

No. of isolates	Closest species in GenBank	Accession No.	Genus	Similarity (%)	Score
11	*Aspergillus flavus*	JX157882	*Aspergillus*	99	981
13	*Aspergillus awamori*	EU846237	*Aspergillus*	99	1433
16	*Aspergillus niger*	HQ850370	*Aspergillus*	95	472
13	*Aspergillus fumigatus*	KC430930	*Aspergillus*	99	1007
11	*Aspergillus tubingensis*	KF435033	*Aspergillus*	99	1029
4	*Aspergillus oryzae*	JQ724414.1	*Aspergillus*	96	845
5	*Penicillium oxalicum*	KC344971	*Penicillium*	98	996
1	*Penicillium pinophilum*	HQ671180	*Penicillium*	100	968
2	*Penicillium verruculosum*	JN676121	*Penicillium*	99	946
1	*Trichoderma longibrachiatum*	JN039083	*Trichoderma*	99	1077
2	*Cladosporium* sp	KC110616	*Cladosporium*	97	869
1	*Aureobasidium pullulans*	KF986552	*Aureobasidium*	99	974
8	*Alternaria pharbitidis*	JX418344	*Alternaria*	99	959
17	*Alternaria alternate*	AY751456	*Alternaria*	98	915
5	*Fusarium culmorum*	KC311482	*Fusarium*	98	808
2	*Fusarium equiseti*	N038467	*Fusarium*	99	961
3	*Fusarium concentricum*	HQ379635	*Fusarium*	99	994
2	*Colletotrichum gloeosporioides*	KC010544	*Colletotrichum*	98	848
1	*Pestalotiopsis microspora*	KF941280	*Pestalotiopsis*	98	909
2	*Epicoccum* sp	J176473	*Epicoccum*	98	898
8	*Phomopsis* sp	JQ809669	*Phomopsis*	99	963
1	*Candida carpophila*	KC119205	*Candida*	99	1004
10	*Trametes hirsute*	JX867226	*Trametes*	98	977
3	*Ceriporia lacerate*	KC414240	*Ceriporia*	99	1096
3	*Bjerkandera adusta*	AB592333	*Bjerkander*	99	1020
2	*Irpex* sp	JX290578	*Irpex*	100	1149
1	*Polyporus* sp	AY322496	*Polyporus*	98	1019
1	*Fomitopsis palustris*	AB733120	*Fomitopsis*	96	1022
1	*Coprinellus radians*	FJ462761	*Coprinellus*	98	1035
1	*Schizophyllum commune*	KC505580	*Schizophyllum*	99	1020
4	*Botryosphaeria dothidea*	FJ790846	*Botryosphaeria*	98	966
2	*Neurospora tetrasperma*	JX136749	*Neurospora*	97	809
1	*Chaetomium globosum*	HQ529775	*Chaetomium*	97	900
2	*Rhizopus oryzae*	AY211273	*Rhizopus*	99	1038
1	*Lichtheimia ramosa*	HQ285653	*Lichtheimia*	99	1487
2	*Pythium* sp	KF889741	*Pythium*	98	828

Penicillium was also the dominant endophyte detected in roots, with an IF value of 0.073 (1/S value 0.071). *Phomopsis* was the third most dominant genera in stems, with an IF value of 0.079 (1/S value 0.059). *Fusarium* was another dominant fungus detected in leaves (Table 3).

The Simpson diversity and Shannon diversity indexes were much higher in stems than in roots and leaves, as were the Margalef richness and evenness indexes (Table 3). This indicates that the stems of *M. laxiflora* have more abundant endophytic diversity than other tissues. Roots ranked second with slightly higher endophytic diversity than leaves.

Many endophytes were found in only one tissue (Table 3). *Pestalotiopsis*, *Fomitopsis*, and *Pythium* were isolated only from roots. *Cladosporium*, *Aureobasidium*, *Colletotrichum*, *Candida*, and *Polyporus* were isolated only from stems. *Trichoderma*, *Coprinellus*, *Neurospora*, *Chaetomium*, and *Lichtheimia* were isolated specifically from leaves. Thus, endophytic taxa in *M. laxiflora* have obvious preferences for specific tissues.

Table 2. Taxonomy of endophytic fungi from *Myricaria laxiflora* plants.

Subphylum	Class	Order	Family	Genus	Total
Deuteromycotina	Hyphomycetes	Moniliales	Moniliaceae	*Aspergillus*	68
				Penicillium	8
				Trichoderma	1
			Dematiaceae	*Cladosporium*	2
				Aureobasidium	1
				Alternaria	25
			Tuberculariaceae	*Fusarium*	10
		Melanconiales	Melanconiaceae	*Colletotrichum*	2
				Pestalotiopsis	1
	Deuteromycetes	Sphaeropsidales	Discellaceae	*Epicoccum*	2
		Sphaeropsidales	Sphaerioidaceae	*Phomopsis*	8
	Blastomycetes	Crytococcales	Cryptococcaceae	*Candida*	1
Basidiomycotina	Basidiomycetes	Polyporales	Polyporaceae	*Trametes*	10
				Ceriporia	3
				Bjerkandera	3
				Irpex	2
				Polyporus	1
			Fomitopsis	*Fomitopsis*	1
		Agaricales	Psathyrellaceae	*Coprinellus*	1
			Schizophyllaceae	*Schizophyllum*	1
Ascomycotina	Ascomycetes	Pleosporales	Botryosphaericeae	*Botryosphaeria*	4
		Sordariales	Sordariaceae	*Neurospora*	2
		Chaetomiales	Chaetomiaceae	*Chaetomium*	1
Zygomycotina	Zygomycetes	Mucorales	Mucoraceae	*Rhizopus*	2
			Radiomycetaceae	*Lichtheimia*	1
Mastigomycotina	Oomycetes	Peronosporales	Pythiaceae	*Pythium*	2

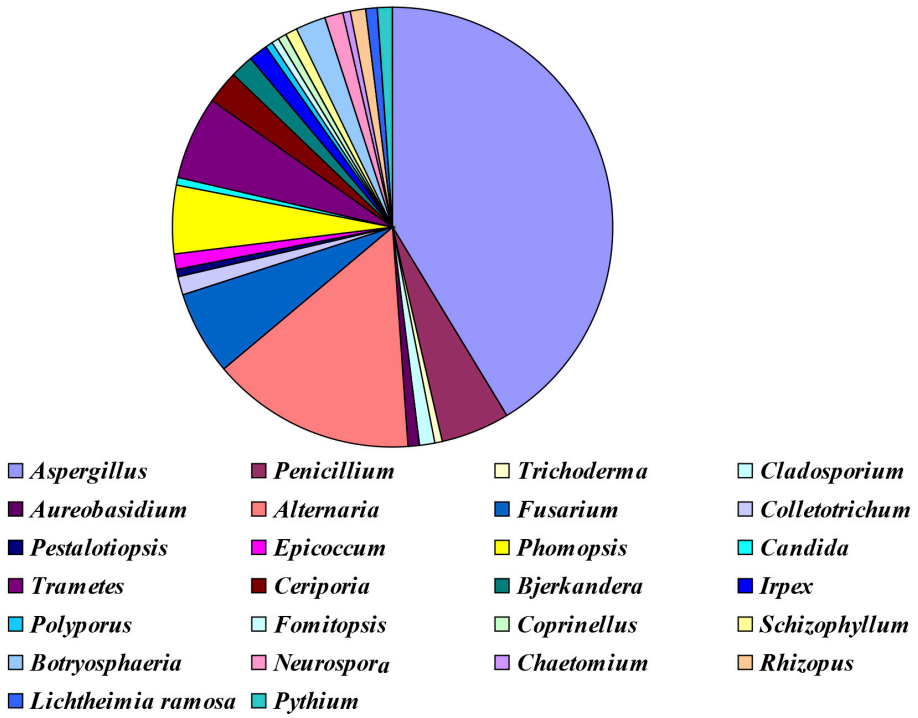

Figure 2. Composition of endophytic fungi from *Myricaria laxiflora*.

Table 3. Diversity analysis of the endophytes from different *Myricaria laxiflora* tissues.

Genus or index	Tissues of *Myricaria laxiflora*			
	Roots	Stems	Leaves	Total isolates
Aspergillus	21	26	21	68
Penicillium	4	2	2	8
Trichoderma	0	0	1	1
Cladosporium	0	2	0	2
Aureobasidium	0	1	0	1
Alternaria	12	6	7	25
Fusarium	3	3	4	10
Colletotrichum	0	2	0	2
Pestalotiopsis	1	0	0	1
Epicoccum	1	1	0	2
Phomopsis	3	5	0	8
Candida	0	1	0	1
Trametes	2	5	3	10
Ceriporia	2	1	0	3
Bjerkandera	0	2	1	3
Irpex	1	1	0	2
Polyporus	0	1	0	1
Fomitopsis	1	0	0	1
Coprinellus	0	0	1	1
Schizophyllum	0	0	1	1
Botryosphaeria	1	3	0	4
Neurospora	0	0	2	2
Chaetomium	0	0	1	1
Rhizopus	1	1	0	2
Lichtheimia	0	0	1	1
Pythium	2	0	0	2
Total fungal isolates	55	63	45	163
Species richness (S)[a]	14	17	12	26
Camargo's index (1/S)	0.071	0.059	0.083	0.038
Simpsons index (D)	0.211	0.202	0.261	0.212
Simpsons index of diversity (1-D)	0.789	0.798	0.739	0.787
Shannon index of diversity (H′)	2.006	2.180	1.825	2.220
Margalef richness index (R)	2.248	2.677	2.003	3.402
Evenness index (E)	0.760	0.769	0.734	0.681

[a]Number of S was counted by genus, not by species and the same in the following tables.

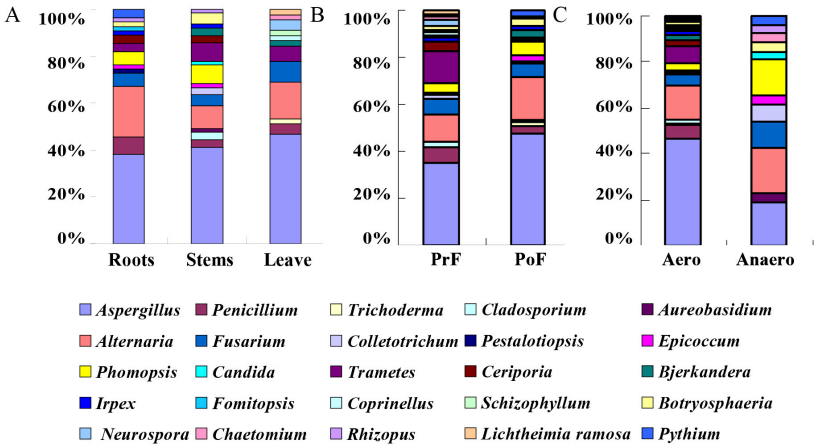

Figure 3. Relative frequencies of the endophytes. **A.** Endophytes from different tissues and their frequency. **B.** Endophytes from pre-flooding (PrF) and post-flooding (PoF). **C.** Endophytes under aerobic (Aero) and anaerobic conditions (Anaero).

Diversity of endophytes from plants grown in pre- and post-flooding conditions

A total of 74 endophytic fungi were isolated from *M. laxiflora* before flooding, whereas 89 were isolated after flooding (Table 4 and Figure 3B). However, there were 20 endophyte genera represented in plants sampled before flooding, and 16 genera in those sampled after flooding. The Simpson and Shannon diversity indexes of pre-flooding endophytes were 0.830 and 2.297, respectively (Table 4). This was higher than those for post-flooding endophytes (0.732 and 1.878 for Simpson and Shannon indexes, respectively). Moreover, the Margalef richness index and the Evenness index were both higher in endophytes of pre-flooding, compared to post-flooding plants (Table 4). Although the total number of isolates from post-flooding plants was higher than those from pre-flooding, post-flooding plants had a less diverse endophyte population. For example, 42 *Aspergillus* isolates were detected in post-flooding plants, versus 26 in pre-flooding (Table 4); this was a significant portion of the entire increment (from 74 to 89). Taken together, these results suggest that pre-flooding plants exhibited more endophyte diversity than post-flooding plants.

Table 4. Endophytes diversity of *Myricaria laxiflora* before- and after-flooding.

Genus or index	Before water-flooding stress				After water-flooding stress			
	Roots	Stems	Leaves	Total isolates	Roots	Stems	Leaves	Total isolates
Aspergillus	11	9	6	26	10	17	15	42
Penicillium	2	2	0	4	2	0	2	4
Trichoderma	0	0	0	0	0	0	1	1
Cladosporium	0	2	0	2	0	0	0	0
Aureobasidium	0	0	0	0	0	1	0	1
Alternaria	3	4	2	9	9	2	5	16
Fusarium	1	0	4	5	2	3	0	5
Colletotrichum	0	1	0	1	0	1	0	1
Pestalotiopsis	1	0	0	1	0	0	0	0
Epicoccum	0	0	0	0	1	1	0	2
Phomopsis	3	0	0	3	0	5	0	5
Candida	0	0	0	0	0	1	0	1
Trametes	2	5	3	10	0	0	0	0
Ceriporia	1	1	0	2	0	1	0	1
Bjerkandera	0	0	0	0	0	2	1	3
Irpex	1	0	0	1	0	1	0	1
Polyporus	0	1	0	1	0	0	0	0
Fomitopsis	1	0	0	1	0	0	0	0
Coprinellus	0	0	1	1	0	0	0	0
Schizophyllum	0	0	1	1	0	0	0	0
Botryosphaeria	0	1	0	1	1	2	0	3
Neurospor	0	0	2	2	0	0	0	0
Chaetomium	0	0	1	1	0	0	0	0
Rhizopus	0	1	0	1	1	0	0	1
Lichtheimia	0	0	1	1	0	0	0	0
Pythium	0	0	0	0	2	0	0	2
Total fungal isolates	26	27	21	74	28	37	24	89
Species richness(S)	10	10	9	20	8	12	5	16
Camargo's index (1/S)	0.1	0.1	0.111	0.05	0.125	0.083	0.2	0.063
Simpsons index (D)	0.225	0.185	0.166	0.170	0.250	0.249	0.444	0.268
Simpsons index of diversity (1-D)	0.775	0.815	0.834	0.830	0.750	0.751	0.556	0.732
Shannon index of diversity (H′)	1.883	1.957	1.980	2.297	1.655	1.890	1.092	1.878
Margalef richness index (R)	1.915	1.893	1.821	3.060	1.456	2.111	0.872	2.316
Evenness index (E)	0.818	0.850	0.901	0.767	0.796	0.761	0.679	0.677

According to Camargo's evaluation criterion, the IF values were greater than 1/S for 5 genera: *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Trametes* (0.05 for pre-flooding).

Therefore, these endophytes were dominant for pre-flooding plants (Table 4). For post-flooding plants, only *Aspergillus* and *Alternaria* were dominant (IF greater than 1/S of 0.063). In addition, *Penicillium*, *Fusarium*, *Colletotrichum*, *Phomopsis*, *Ceriporia*, *Irpex*, *Botryosphaeria*, and *Rhizopus* were isolated from both pre- and post-flooding plants, suggesting that these 8 genera were not sensitive to flooding stress. On the other hand, *Cladosporium*, *Pestalotiopsis*, *Trametes*, *Fomitopsis*, *Coprinellus*, *Schizophyllum*, *Neurospora*, *Chaetomium*, *Lichtheimia*, and *Polyporus* were isolated only from pre-flooding plants, and *Trichoderma*, *Aureobasidium*, *Bjerkandera*, *Epicoccum*, *Candida*, and *Pythium*were found only in post-flooding plants (Table 4 and Figure 3B). Taken together, these data suggest that flooding stress could change the composition and distribution of endophytic fungi populations in *M. laxiflora* plants. In particular, *Trametes* was a dominant genus from pre-flooding plants, but was not detected at all in post-flooding plants, indicating that this fungus may have exhibit high sensitivity to flooding stress.

For all three tissue types, the diversity of the endophytes differed slightly between pre- and post-flooding plants. Twenty-six, 27 and 21 strains (from pre-flooding samples), and 28, 37, 24 strains (from post-flooding samples) were detected in roots, stems and leaves, respectively (Table 4). However, this was not the case for particular genera. For instance, for the genus *Aspergillus*, 9 isolates were found in stems from pre-flooding plants, compared to 17 for post-flooding plants (Table 4). Most *Fusarium* spp were isolated from leaves in pre-flooding samples, but from stems in post-flooding samples (Table 4). It is plausible that the biased distribution of endophytic taxa in specific tissues of *M. laxiflora* is related to flooding stress.

Endophyte diversity under conditions of normal or low oxygen supply

Under flooding stress, plants are forced to undergo the oxidative stress pathway (Chen and Xie, 2009). In line with this idea, we isolated endophytes from *M. laxiflora* under various oxygen availability conditions. Under normal oxygen supply, 138 strains were isolated; these were classified into 23 genera and 15 families. Under anaerobic conditions, only 25 strains were isolated; these were classified into 12 genera and 11 families (Table 5 and Figure 3C). The Margalef richness index under aerobic conditions was significantly larger than that under anaerobic conditions (3.095 compared to 2.368). These results suggest that endophytes are more abundant under aerobic conditions, and that most endophytes of *M. laxiflora* require oxygen.

Under both aerobic and anaerobic conditions, *Aspergillus*, *Alternaria*, and *Fusarium* were the dominant genera (Table 5). On the other hand, *Penicillium* and *Trametes* were dominant under aerobic conditions, but absent under anaerobic conditions. Meanwhile, *Trichoderma*, *Cladosporium*, *Pestalotiopsis*, *Ceriporia*, *Bjerkandera*, *Irpex*, *Fomitopsis*, *Schizophyllum*, *Coprinellus*, *Neurospora*, *Polyporus*, and *Lichtheimia* occurred only under aerobic conditions, suggesting dependence on oxygen availability of these genera. *Aureobasidium*, *Chaetomium* and *Candida* were isolated only under anaerobic conditions, indicating that these fungi might be induced by flooding stress and low oxygen conditions; these genera may play a role in the oxidative pathway of the host plants.

Oxygen availability affected the growth of endophytes in the three tissue types. Under anaerobic conditions, only 3 strains were isolated from leaves. This was in contrast to aerobic conditions, under which 42 strains were isolated, indicating that the majority of endophytes from the leaves of *M. laxiflora* plants live on oxygen.

Table 5. Endophyte diversity of *Myricaria laxiflora* under aerobic and anaerobic conditions.

Genus or index	Under aerobic conditions				Under anaerobic conditions			
	Roots	Stems	Leaves	Total isolates	Roots	Stems	Leaves	Total isolates
Aspergillus	21	23	19	63	0	3	2	5
Penicillium	4	2	2	8	0	0	0	0
Trichoderma	0	0	1	1	0	0	0	0
Cladosporium	0	2	0	2	0	0	0	0
Aureobasidium	0	0	0	0	0	1	0	1
Alternaria	9	4	7	20	3	2	0	5
Fusarium	1	2	4	7	2	1	0	3
Colletotrichum	0	1	0	1	0	1	0	1
Pestalotiopsis	1	0	0	1	0	0	0	0
Epicoccum	0	1	0	1	1	0	0	1
Phomopsis	0	4	0	4	3	1	0	4
Candida	0	0	0	0	0	1	0	1
Trametes	2	5	3	10	0	0	0	0
Ceriporia	2	1	0	3	0	0	0	0
Bjerkandera	0	2	1	3	0	0	0	0
Irpex	1	1	0	2	0	0	0	0
Polyporus	0	1	0	1	0	0	0	0
Fomitopsis	1	0	0	1	0	0	0	0
Coprinellus	0	0	1	1	0	0	0	0
Schizophyllum	0	0	1	1	0	0	0	0
Botryosphaeria	1	2	0	3	0	1	0	1
Neurospor	0	0	2	2	0	0	0	0
Chaetomium	0	0	0	0	0	0	1	1
Rhizopus	0	1	0	1	1	0	0	1
Lichtheimia	0	0	1	1	0	0	0	0
Pythium	1	0	0	1	1	0	0	1
Total fungal isolates	44	52	42	138	11	11	3	25
Species richness (S)	11	15	11	23	6	8	2	12
Camargo's index (1/S)	0.091	0.067	0.091	0.043	0.167	0.125	0.500	0.083
Simpsons index (D)	0.285	0.226	0.254	0.244	0.207	0.157	0.556	0.133
Simpsons index of diversity (1-D)	0.715	0.774	0.746	0.756	0.793	0.843	0.444	0.867
Shannon index of diversity (H′)	1.693	2.063	1.805	2.074	1.673	1.972	0.637	2.221
Margalef richness index (R)	1.832	2.456	1.854	3.095	1.445	2.023	0.631	2.368
Evenness index (E)	0.706	0.762	0.753	0.661	0.934	0.948	0.918	0.894

DISCUSSION

Plant colonization by endophytes may offer significant host benefits, improving ecological adaptability by enhancing tolerance to environmental stress (Khan et al., 2011; Stępniewska and Kuźniar, 2013). In the current study, the relationship between endophytic fungi diversity and flooding stress in *M. laxiflora* plants was studied. Endophytes were isolated and identified under conditions of both pre- and post-flooding stress in three plant tissues. A total of 163 isolates were detected, indicating that *M. laxiflora* harbors a wide diversity of fungal endophytes. Through phylogenetic analysis, the fungal distribution pattern of frequency and composition was established and compared between conditions of pre-/post-flooding, and aerobic/anaerobic culture conditions. Results from this study will provide a foundation for further research on symbiotic interactions between *M. laxiflora* and endophytic fungi; habitat adaptability of *M. laxiflora*; and mechanisms of flooding tolerance in plants.

On the basis of the present results, a study is currently underway to determine the relationship between endophytes and *M. laxiflora*, and whether fungal endophytes play a role in increasing the tolerance of *M. laxiflora* to water immersion. Flooding affects not only the growth of host plants, but also of endophytes. This is supported by our observation of

a decrease in the number of endophytic genera detected in plants after flooding (Table 4). The question remains: what happened to the surviving endophytes following flooding? In our study, *Aspergillus* was the most abundant genus detected, and thus, fungi in this genus may play an important role in host habitat adaptability and flood tolerance. The number of isolates from this dominant genus increased, from 26 in pre-flooding samples, to 42 in post-flooding samples. If *M. laxiflora* plants employ endophytes to respond to the flooding stress, *Aspergillus* may be a key member, which was worthy of special attention. On the basis of this possibility, we have demonstrated that an *Aspergillus* species with high antioxidant activity enhances drought resistance of rice seedlings (Zeng et al., 2015). In light of the fact that drought and flooding damage are at least partly caused by oxidative stress, *Aspergillus* with high antioxidant activity may play a role in ecological adaptability of *M. laxiflora*. Further characterization of the isolates detected in our study only in post-flooding samples is warranted.

Hormones produced by endophytes, may also benefit the host plant. In our study, we obtained an isolate that was initially identified as *Gibberella* sp, but was subsequently identified as *Neurospora* sp, in which gibberellin was found in the fermentation liquor. It is noteworthy that gibberellin has vital significance in resistance of plants to abiotic stress (Bailey-Serres and Voesenek, 2010). Furthermore, while this strain can be cultured under aerobic and anaerobic conditions, higher yields of gibberellin are obtained under anaerobic culture conditions (data not shown), perfectly matching the behavior pattern of the host. These results suggested that the endophytes help the hosts through oxygen deficiency by means of hormones.

While fungi are extraordinarily rich in species, few can be cultured in the laboratory (Hawksworth, 1991). In this study, we isolated endophytes from *M. laxiflora* using aerobic and anaerobic culture conditions, and observed that the majority of endophytes required oxygen for growth. In the presence of oxygen, most endophytes grew well even for those isolated under anaerobic conditions. This may be related with a specific chemistry or texture of different tissues, in which oxygen availability is varied. Conversely, tissue-specific endophytes may be affected by oxygen supply to tissues, and oxygen levels within the plant habitat.

Conflicts of interest

The authors declare no conflict of interest.

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