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Antioxidant Activity *In vitro* of Endophytic Fungi from *Myricaria laxiflora*, A Riparian Plant with Strong Tolerance Ability of Flooding

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Endophytic fungi are considered as an important source of antioxidants. In order to evaluate the antioxidant capacity of 163 endophytic fungi from *Myricaria laxiflora,* a plant grows under flooding and other oxidative stress, we analyzed the total antioxidant capacity *in vitro*. The screening results showed that the antioxidant activity of endophytes from different tissues differ according to before- or after- flooding stress of the host, under aerobic or anaerobic isolating condition. Although the endophytes from stems were more than other tissues, antioxidant activity was always the lowest. In contrast, endophytic fungi from leaves were fewer, whereas relatively high and stable antioxidant activity was presented. After flooding, the antioxidant activity of endophytic fungi from stems was significantly greater than before, whereas the leaves were just the reverse. In addition, oxygen content when strains isolated had also a significant impact in antioxidant activity, while the effects could be zero, positive, or negative as for different types of tissue. Overall, *Aspergillus*, *Alternaria*, *Penicillium*, *Trametes* and *Rhizopus* have a relatively higher antioxidant activity than other genus in *Myricaria laxiflora*. Particularly, *Aspergillus* was not only the most abundant but also the most active antioxidant genus. Among the endophytes, phenolics could be an important factor resulted in high antioxidant activity.

Key words *Myricaria laxiflora*, endophytic fungi, antioxidant capacity, flooding, *Aspergillus.*

All kinds of human diseases, 90% were directly or indirectly resulted from reactive oxygen and oxidative stress (Zhi et al. 2010). Hence, exploring more and more available, security preparation with specific antioxidation has become a hotspot in the past years (Su et al. 2010). Screening natural products with antioxidant effect from animals and plants, is an effective approach in medicine and food (Finkel et al. 2000; Judith et al. 2013; Hosseini et al. 2013).

Endophytic fungi may be a potential material, in which substances with the same antioxidant activity as the host plants are generated (Sterlea 1993; Abdelqader et al. 2012). Owing to the extraordinary diversity in species and richness in natural products, endophytic fungi have been a great interest producer of novel antioxidant (Tomita 2003; Strobel 2003).

Myricaria laxiflora is an endangering evergreen shrub distributed entirely in the Yangtze River valley (Liu et al. 2006). These plants are traditionally used to treat the burn and scald (Wu et al. 1998). In summer of each year, *M. laxiflora* has to confront over 4 months flooding stress, under which the plants are supplied limited oxygen and light, thus constrained into oxidative stress

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pathway (Wang et al. 2006). In addition, *M. laxiflora* plants must survive infertility, drought and salinity adversities, which could be looked on as a partly oxidative stress (Chen et al. 2009). Thus, the specific riparian habitat endows *M. laxiflora* high ability of anti abiotic stress, and this ability would be negatively related with the concentration of reactive oxygen in plants (Liu et al. 2010), indicating that *M. laxiflora* would be rich in antioxidant, through which the plants pull through oxidative stress period.

Recent studies revealed that endophytic fungi play a role to the host plants, and help them survive biotic or abiotic stresses (Mei et al. 2010; Radhakrishnan et al. 2013). Especially, the endophytes from extreme habitat have multiple functions in the ecological adaptability of the host plants (Gostincar et al. 2010, Stepniewsk et al. 2013). Are the endophytic fungi from *M. laxiflora* helpful to the host in oxidative stress pathway? In the previous study, we isolated and characterized 163 endophytic fungi from heathy roots, stems and leaves of *M. laxiflora*. Under aerobic or anaerobic conditions before and after flooding, 26 genera of fungi acquired (another paper in press).

As reported, many endophytes could be developed as important sources of antioxidant, which maybe caused by peptides, polysaccharide and most probably, phenolics (Qin et al. 2010; Jin et al. 2014; Yoon et al. 2014). *Aspergillus niger* fermented by soy germ had a high-efficiency bio-transformation of phenolics and flavonoids and resulted in high antioxidant activity (Sheih et al. 2014).

In order to further clarify the relationship between the endophytes from *M. laxiflora* plants and the resistance capacity to oxidative stress of the host, here we evaluated the antioxidant activity of the fungi in vitro by total antioxidant capacity (T-AOC) analysis, and found *Aspergillus*, *Alternaria*, *Penicillium*, *Trametes* and *Rhizopus* had antioxidant activity. All the studies showed that the endophytes from *M. laxiflora* plants have high antioxidant activity, and could be a promising source of antioxidant in the future. Moreover, these findings can also help to understand the symbiotic relationship between endophytic fungi and the host under oxidative and flooding stress.

MATERIALS AND METHODS

Endophytic fungi strains, media and growth conditions

A total of 163 endophytic fungi were isolated from the healthy roots, stems and leaves of *M. laxiflora* plants. 74 strains were obtained before *M. laxiflora* suffered flooding in May 2012, and 89 after flooding in September. Among the 163 isolates, 55 were from roots, 63 from stems and 45 from leaves. Under aerobic condition of isolation and culture, we acquired 138 endophytes, whereas only 25 fungi were found in anaerobic condition. All the endophytic fungi used in this study listed in table 1. According to ITS sequence analysis and morphological characterization (Pimenta et al. 2012), the total strains were molecularly classified into 26 genera (Table 1).

All the strains were cultured in PDA solid medium (potato, 200 g; dextrose, 20 g; agar, 20 g; H_2O, 1,000 ml) with 25µg/ml-50µg/ml of ampicillin or 10µg/ml-50µg/ml of tetracyyline. Endophytes grew under 28±1°C for 3-7 d until single colony appeared according to Xiong et al. (2012). Hyphae or spores were picked into PDA liquid medium at (28±1) °C and 150 r/min for about 5 d. In anaerobic conditions, the dishes were put into a sealed bag full of nitrogen.

Main reagents, method for determination of T-AOC

Fungi fermentation suspension was filtrated by gauze to remove the thalli, then centrifuged at 10000 rpm for 15 min. The supernatant was used to measure total AOC by T-AOC assay kit (Jiancheng BioEngineering Institute (Nanjing, China) according to the method (Niu et al. 2013). In the supernatant, antioxidants can turn Fe^{3+} into Fe^{2+}, which was formed a stable clathrate with phenanthroline and then was measured by colorimetry at 520 nm. Each sample was measured 3 times. A T-AOC unit is defined as the absorbance (OD) value increase 0.01 per minute per milliliter at 37 °C, and calculated by the equation:

$$Total\_AOC = \frac{ODu - ODc}{0.01} \times N \times n/30$$

Here, OD_U is the absorbance value of the sample, OD_C is the absorbance value of the control, N is dilution of the reaction system, and n is the dilution of the sample.

Determination the content of total phenols

To evaluate the relationship between the antioxidant activity and the phenol materials, we selected 5 endophytic fungi with higher activity to determine the total phenol contents (TPC) through Folin"Ciocalteu Reagent method described by He et al (2013) with some revision. Fermentation liquid was disposed with absolute ethyl alcohol to precipitate protein and eliminate sugar, ethyl acetate to get crude extracts. After the prepartion, 1 ml extract or a blank was added to a 25 ml volumetric flask containing 6-7 ml water, then added Folin-Ciocalteu reagent (750 µL) and after 8 min, 4.5 ml of 10% Na_2CO_3 (w/v) was added. Added water to 25 ml and shaked the flask to mix evenly, then kept the flask at 30°C for 2h. Absorbance was read at 765 nm. Results were expressed as milligrams of gallic acid equivalent per gram crude extract (mg GAE/g).

RESULTS

Antioxidant activity of endophytic fungi from different *M. laxiflora* tissues

55 endophytic isolates from roots, 63 from stems and 45 from leaves were used in this study (Table 1 and Fig 1). Using the same method, Xie et al reported that the AOC maximum of endophytic fungi obtained from *Eucommia ulmoides Oliv* was 20.70 U/ml and sequenced to be *Chaetomium globosum* Kunze eventually (Xie et al. 2009). Shi et al found that the endophytic fungi isolated from *Sophora japonica* L showed 12.54 U/ml as maximum (Shi et al. 2007). Here, from leaf we got a strain identified as *Aspergillus niger*, showed the highest AOC as 31.48 U/ml, indicating that the endophytes from *M. laxiflora* plants could be as a potent antioxidant.

Table 1. Endophytic fungi used in this study

Habitat	Before flooding						After flooding						
Tissue	Roots		Stems		leaves		Roots		Stems		leaves		
Condition	Aero	Anaero	Aero	Anaero	Aero	Anaero	Aero	Anaero	Aero	Anaero	Aero	Anaero	Total
Aspergillus	11	0	8	1	5	1	10	0	15	2	14	1	68
Penicillium	2	0	2	0	0	0	2	0	0	0	2	0	8
Trichoderma	0	0	0	0	0	0	0	0	0	0	1	0	1
Cladosporium	0	0	2	0	0	0	0	0	0	0	0	0	2
Aureobasidium	0	0	0	0	0	0	0	0	0	1	0	0	1
Alternaria	1	2	2	2	2	0	8	1	2	0	5	0	25
Fusarium	1	0	0	0	4	0	0	2	2	1	0	0	10
Colletotrichum	0	0	0	1	0	0	0	0	0	1	0	0	2
Pestalotiopsis	1	0	0	0	0	0	0	0	0	0	0	0	1
Epicoccum	0	0	0	0	0	0	0	1	1	0	0	0	2
Phomopsis	0	3	0	0	0	0	0	0	4	1	0	0	8
Candida	0	0	0	0	0	0	0	0	0	1	0	0	1
Trametes	2	0	5	0	3	0	0	0	0	0	0	0	10
Ceriporia	1	0	1	0	0	0	1	0	0	0	0	0	3
Bjerkandera	0	0	0	0	0	0	0	0	2	0	1	0	3
Irpex	1	0	0	0	0	0	0	0	1	0	0	0	2
Polyporus	0	0	1	0	0	0	0	0	0	0	0	0	1
Fomitopsis	1	0	0	0	0	0	0	0	0	0	0	0	1
Coprinellus	0	0	0	0	1	0	0	0	0	0	0	0	1
Schizophyllum	0	0	0	0	1	0	0	0	0	0	0	0	1
Botryosphaeria	0	0	1	0	0	0	1	0	1	1	0	0	4
Neurospor	0	0	0	0	2	0	0	0	0	0	0	0	2
Chaetomium	0	0	0	0	0	1	0	0	0	0	0	0	1
Rhizopus	0	0	1	0	0	0	0	1	0	0	0	0	2
Lichtheimia	0	0	0	0	1	0	0	0	0	0	0	0	1
Pythium	0	0	0	0	0	0	1	1	0	0	0	0	2
Total	21	5	23	4	19	2	23	6	28	8	23	1	163

The T-AOC analysis showed that the antioxidant activity of endophytes from different tissues varied according to the environment (Fig 2). Before flooding, the endophytes from leaves showed the most active antioxidant as for the T-AOC average values, the next were roots, and the fungi from stems were the lowest (Fig 2A). Whereas after flooding, the ability order of antioxidant was: roots > leaves > stems (Fig 2B). The results indicated that in different tissue sources, various genera of endophytic fungi were isolated, and the antioxidant activity would be affected by the flooding stress of the host due to the species difference.

When enough oxygen supplied, the ability order of antioxidant was: roots > leaves > stems (Fig 2C). However, the endophytes isolated under anaerobic condition showed: leaves > roots > stems (Figure 2D). Although the endophytes from stems were more than other tissues, the average T-AOC value was always the lowest, weheras endophytic fungi from leaves small in number, showed relatively high and stable antioxidant activity (Fig 2), particarlally in anaerobic condition (Fig 2D).

Antioxidant activity of endophytic fungi from the host before- and after- flooding stress

Respectively, we isolated 74 endophytes before *M. laxiflora* suffered flooding stress and 89 after flooding (Table 1 and Fig 2A). Whether before flooding or after flooding, the average T-AOC of endophytes in roots have no significant difference (Fig 3A and 3B). However, the antioxidant activity of endophytic fungi from stems after flooding stress was significantly greater than before (Fig 3A and 3B). In leaves, we got an opposite result (Fig 3A and 3B). Since each tissue type had many genera of endophytes with different

Table 2. The T-AOC values of intergeneric endophytic fungi

genus	amount	proportion	AVG[a]	maximum[b]
Aspergillus	68	41.72%	9.00±7.98	31.48±0.80 (HY1)
Penicillium	8	4.91%	11.18±7.58	25.99±0.47 (SY15)
Trichoderma	1	0.61%	1.79±0[c]	1.79±0.42 (MY19)
Cladosporium	2	1.23%	4.42±0.44	4.73±0.26 (SJ4)
Aureobasidium	1	0.61%	7.18±0	7.18±0.13 (HJ3)
Alternaria	25	15.34%	7.95±6.54	25.67±0.20 (MG14)
Fusarium	10	6.13%	5.84±3.26	10.18±0.33 (SY5)
Colletotrichum	2	1.23%	4.33±1.67	5.15±0.22 (HJ4)
Pestalotiopsis	1	0.61%	21.03±0	21.03±0.17 (SG6)
Epicoccum	2	1.23%	7.03±6.51	11.63±0.41 (HG4)
Phomopsis	8	4.91%	4.01±2.00	7.49±0.30 (MJ26)
Candida	1	0.61%	12.5±0	12.5±0.13 (HJ6)
Trametes	10	6.13%	7.38±5.67	17.73±1.11 (SY1)
Ceriporia	3	1.84%	5.54±1.87	8.70±0.30 (SJ12)
Bjerkandera	3	1.84%	5.24±1.63	7.83±0.16 (MY16)
Irpex	2	1.23%	4.63±3.88	7.37±0.18 (SG20)
Polyporus	1	0.61%	1.84±0	1.84±0.40 (SJ9)
Fomitopsis	1	0.61%	19.65±0	19.65±0.13 (SG16)
Coprinellus	1	0.61%	11.34±0	11.34±0.40 (SY11)
Schizophyllum	1	0.61%	5.55±0	5.55±0.48 (SY7)
Botryosphaeria	4	2.45%	5.69±2.07	7.14±0.35 (MG2)
Neurospor	2	1.23%	9.71±2.98	11.82±0.77 (SY3)
Chaetomium	1	0.61%	10.51±0	10.51±0.28 (QY2)
Rhizopus	2	1.23%	16.81±11.38	24.85±0.22 (HG2)
Lichtheimia	1	0.61%	14.02±0	14.02±0.52 (SY13)
Pythium	2	1.23%	10.28±1.94	11.65±0.34 (MG7)
Total	163	100%		

[a] means ± standard deviation; and [b] denotes the maximum of TVOA in the genus, the fungi serial number is in the bracket;[c] standard deviation is 0 since only one isolate found in the genus.

T-AOC value, the fungi with high T-AOC value were different before and after flooding stress in roots, stems and leaves (Fig 3A and 3B). In a word, the antioxidant activity of endophytic fungi from the host showed significant difference before- and after- flooding stress, but it was hard to evaluate which was better due to the specific genera isolation.

Antioxidant activity of endophytic fungi isolated under normal and low oxygen availibility

In total we got 138 endophytes under normal and 25 under low oxygen supplication (Fig 2C and 2D). Under aerobic condition, the T-AOC of endophytes isolated from roots and stems were slightly greater than that under anaerobic condition (Fig 3C and 3D), whereas isolates from leaves under low oxygen condition showed much more active in antioxidant ability, either compared with normal leaves, or with other tissues (Fig 3C and 3D). Therefore, oxygen content when strains isolated had a significant impact in T-AOC value, while the effects could be zero, positive, or negative as for different types of tissue (Fig 3C and 3D).

Antioxidant activity analysis of different genera and different species

163 endophytic fungi classified into 26 genera were used in T-AOC analysis (Table 1 and 2). Since each genus had different quantity of endophytes, we surveyed both the means and the maximum values (Table 2). Overall, *Aspergillus*, *Alternaria*, *Penicillium*, *Trametes* and *Rhizopus* have a relatively higher T-AOC value than other genus in *M. laxiflora* (Table 2). Particularly, Aspergillus was not only the most abundant but also the most active antioxidant genus. We obtained 13 *Aspergillus* of which the T-AOC value was more than 15.00 U/ml, indicating that this genus would play an important role in the host metabolic pathway.

Among the 26 genera, there were 4 genera comprising the bulk of the endophytes. Through bioinformatical analysis of ITS sequence, *Aspergilli* was consisted of 6 species: *Aspergillus flavus*, *Aspergillus awamori*, *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus tubingensis* and *Aspergillus oryzae* (Fig 4, Another paper in publishing). *Alternaria*, *Penicillium* and *Fusarium* all had 3 species (Fig 4). T-AOC analysis showed that *Aspergillus fumigatus* was significantly greater than the rest in the same genus, while one strain of *Aspergillus niger* showed the highest AOC as 31.48 U/ml (Fig 4). Similarly, *Alternaria alternaria*, *Penicillium oxalicum* and *Fusarium culmorum* have higher AOC than others in the genera (Fig 4).

Relationship between antioxidant activity and phenols content

To elucidate the relationship between the antioxidant ability and the total phenolics content, we investigated the fermentation products of 5 endophytic fungi (Figure 5). The result showed that the T-AOC value had an analogous tendency as the total phenolics content. Approximately, the

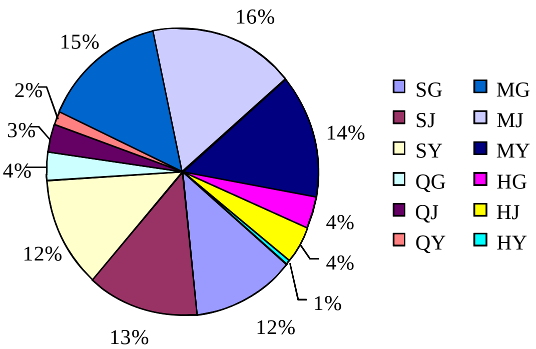

Fig. 1. Endophytic fungi have been isolated from *M. laxiflora* before and after flooding stress with disparate oxygen content in various tissues. S: before flooding with oxygen. M: after flooding with oxygen. Q: before flooding without oxygen H: after flooding without oxygen. G: root J: stem Y: leaf

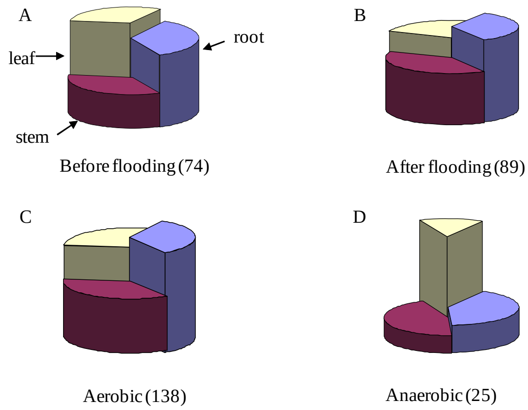

Fig. 2. Total AOC assays of endophytes isolated in different tissues and environmental conditions. The area means the number of the endophytes, and the height denotes the value of AOC. A and B showed before or after flooding, C and D showed in aerobic or anaerobic condition

endophytic fungi with high antioxidant ability possessed more phenolics, the correlation coefficient got nearly to 0.50 (Fig 5). However, if abondon the last sample (HG2), the correlation coefficient got nearly to 0.92. So in the endophytes from *M. laxiflora* plants, phenolics could be an important factor resulted in high T-AOC value.

DISCUSSION

Till now endophytes become an important resource of antioxidant. *Aspergilli. awamori* isolated from *Rauwolfia serpentine* in 2013, showed a stable antioxidant activity (Archana et al. 2013), and this species was also acquired here and showed bioactivity to a extent. From *Rhododendron tomentosum*, *Penicillium raciborskii* was selected as an antioxidant endophyte (Mysore et al. 2011; Fan 2012). Here we isolated some *Penicillium*, *Aspergillus* and *Alternaria* showed higher antioxidant activity than reported before. In addition, we found a group of fungi with high antioxidant activity never reported

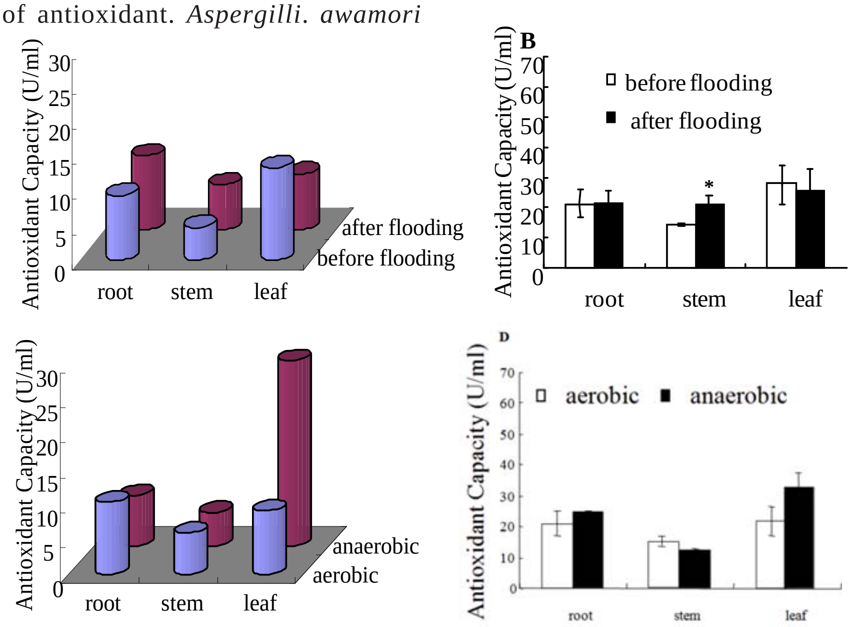

Fig. 3. The antioxidant capacity of the endophytes. Data points are the average of three separate measurements, and error bars represent standard deviation

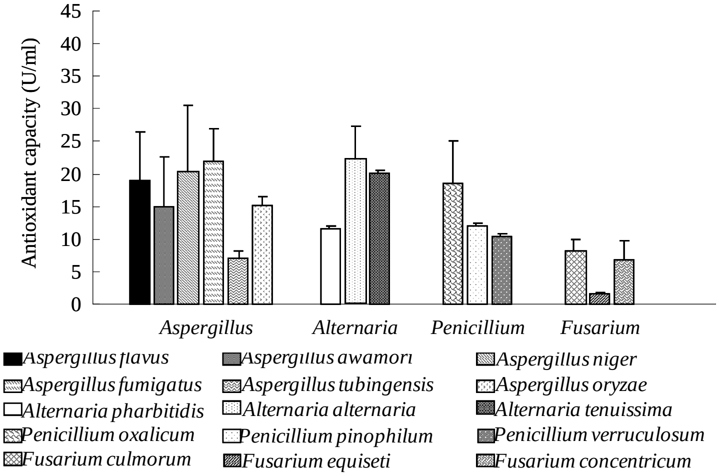

Fig. 4. Four genus, fifteen species were investigated by calculating the mean of the top three in each species. Data points are the average of three separate measurements, and error bars represent standard deviation of means

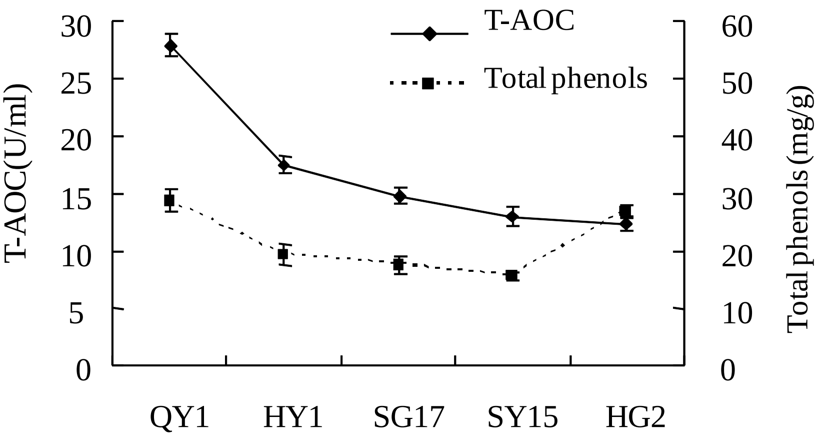

Fig. 5. The relationship between T-AOC and TPC. 5 endophytes with high antioxidant activity were named in the bottom. Data points are the average of three separate measurements, and error bars represent standard deviation

before. In this study we identified a *Phomopsis sp*., a *Rhizopus oryzae* and a *Fomitopsis palustris* showed high antioxidant activity. This may be related with the species specificity of *M. laxiflora* plants. Whether before or after the host suffered blooding, whether in aerobic or anaerobic condition, whatever tissue type used, there were many fungi isolated with high T-AOC value, suggested that the endophytes from *M. laxiflora* plants would be an effective and potential antioxidant.

The reason resulted in high antioxidant activity is very complicated. It was reported that in the fermentation products of *Alternaria alternata* and *Botrytis cinerea*, mannitol was the main antioxidant (John et al. 2013). Coincidently, the 2 endophytes were found here in the stems of *M. laxiflora*. In our study, a *Pestalotiopsis* showed a relatively higher antioxidant activity, the possible reason was the production of paclitaxel (Strobel et al. 1996; Li et al. 1996). Anyhow, phenols contents in the endophytes from *M. laxiflora* would partly account for the antioxidant activity (Fig 5), of which the substance needs further research.

Endophytes with high antioxidant activity may be related with the *complexities* ecology environment of the host. In the habitat of *M. laxiflora*, the plant must confront the oxidative stress. And this may be the reason why there were too many endophytes with high antioxidant capacity. In order to verify whether the endophytic fungi could enhance the tolerance of the host to the stress, we infected a rice species, Nipponbare, by fifteen high antioxidant ability endophytic fungi. One *Aspergillus fumigatus* was screened out that can help the rice seedlings survive drought, salt and flooding stress (another paper in publishing). To an extent, it was confirmed that *Aspergillus*, the most dominant genus, may play a positive role in the flooding stress or other oxidative stress.

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