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Endophytic Bacteria from *Myricaria laxiflora* Before- and After- Flooding Stress and the Antioxidant Activity

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Abstract

Myricaria laxiflora is restricted to the riverbanks of the Yangtze River valley and will be deprived of the original habitat due to the construction of the Three Gorges Dam. These plants grow under flooding and other oxidative stress. Recently, Endophytic bacteria are considered as an important source of antioxidants. In order to dissect the relationship between the endophytic bacteria diversity and the oxidative stress, we isolated totally 84 bacteria from healthy stems, leaves and roots of *M. laxiflora* before- and after- flooding stress. The antioxidant capacity in vitro analysis showed that 10 endophytic bacteria have strong activity. Most highly active strains were from the host after flooding. Whether before- or after- flooding, the ability order of antioxidant from different tissues was: roots > stems > leaves. All the endeavors are helpful to understand the symbiotic relationship between endophytic bacteria and the host under oxidative stress.

1. Introduction

Myricaria laxiflora is an endangering evergreen shrub distributed entirely in the Yangtze River valley, traditionally used to treat the burn and scald (Liu et al., 2006). *M. laxiflora* is capable of surviving water immersion as long as 3 to 5 months in summer, due to long evolutionary adaptation to the natural dynamics of seasonal fluctuations in water level (Liu et al. 2009, Chen and Xie, 2009). Under flooding stress, the plants are supplied limited oxygen and light, and thus are constrained into oxidative stress pathway (Wang et al. 2009). After October, the plants resume growth rapidly until the next April (Wang et al. 2009). For nearly half a century scientists only found these plants in the upstream of the Three Gorges Dam (Wu et al. 1998). It is reported years ago that *M. laxiflora* would completely disappear due to the construction of the dam, which lifts the water level and submerges the plants and the whole natural habitat all the year round (Liu et al. 2006). Hence, it has been the hotspot in understanding the morphological characters of *M. laxiflora*, natural distribution, habitat, and coexisting plant community structure, ecological adaptability, and propagation method for *ex situ* conservation (Wu et al. 1998; Wang et al. 2003).

However, after the construction of the dam, *M. laxiflora* plants were surprisingly found in the downstream, where a summer flooding habitat still exists (Bao et al. 2010). It indicates on one hand that *M. laxiflora* plants have a strong capacity to resist waterlogging stress, on the other, the growth and the propagation would be dependent on summer flooding (Wu 2012). Furthermore, besides the summer flooding stress, *M. laxiflora* plants must confront other abiotic stresses in the natural habitat. Infertility, drought and salinity

are also adversities that strongly influence on the plant growth and development (Wang *et al.* 2009).

Through long term evolution in the specific riparian habitat, *M. laxiflora* gains the capacity to resist waterlogging stress (Liu *et al.*, 2009). In addition, all over the year *M. laxiflora* plants must confront infertility, drought and salinity adversities, which could be looked on as a partly oxidative stress (Wang *et al.*, 2009). Therefore, the specific riparian habitat endows *M. laxiflora* high ability of anti abiotic stress, indicating that *M. laxiflora* would be rich in antioxidant, through which the plants pull through oxidative stress period (Chen and Xie, 2009).

Recent studies showed that endophytic bacteria may be a potential material, in which substances with the same antioxidant activity as the host plants are generated (Sterlea, 1993; Strobel, 2003; Yoon *et al.*, 2014). Owing to the extraordinary diversity in species and richness in natural products (Shi *et al.*, 2007), endophytic bacteria have been a great interest producer of novel antioxidant (Zhi and Guo, 2010). Furthermore, endophytic bacteria can play a role to the host plants (Tomita, 2003), and help them survive biotic or abiotic stresses (Finkel and Holbrook, 2000; Xiong, 2012; Stepniowski and Kuźniar, 2013).

Here, we isolated 84 endophytic bacteria from *M. laxiflora* plants and dissect the total antioxidant activity (T-AOC). Higher capacity of antioxidant strains were found than previous report, indicating a promising source of antioxidant in the future. All the studies bring a light on exploring a novel antioxidant material and help to understand the symbiotic relationship between endophytic bacteria and the host under oxidative stress.

2. Materials and Methods

2.1. Plants Sampling

M. laxiflora plants were collected at a small island called Yanzhiba, 43 kilometers away downstream the Three Gorges Dam. This island locates in the water level fluctuation zone from 109°32'E to 110°52'E, 30° 53'N to 31 °3'N. In July 2012, just before the flood water came, 20 healthy samples of stems, leaves and roots from 10 *M. laxiflora* plants older than 2 years were collected, and each plant was at least 50 meters apart. Then the samples were immediately transported to the laboratory. The roots were from 10-20 cm below ground. Some samples were transiently stored at 4°C less than 24 hrs. And these samples were taken as before flooding stress. While in Oct. 2011, just after the flood water receded, some *M. laxiflora* plants were still in partly immersion, and some plants emerged from water, the samples were collected again and taken as after flooding stress.

2.2. Isolation of the Endophytic Bacteria

The plant samples of stems, leaves and roots were thoroughly washed in running tap water for 10 min, and then cut into 0.3×0.3 cm small pieces by a sterile scalpel.

Surface-sterilization was carried out with 75% alcohol for 1 min followed by rinsing for 4-5 times by sterile water. The stems and roots were immersed by 3% (v/v) NaClO for 1 min whereas the roots for 40 s. Rinsed for 4-5 times again with sterile water, the materials were sub-cut and placed into Petri dishes containing LB medium (Yeast extract 5g, Peptone 10g, NaCl 10g, Agar 15g, Distilled water to 1000ml, pH 7.2-7.4) culture at 37 °C for 2 d. According to the method by Pu *et al.* (2013), the last rinsed water was also poured into the medium to observe whether other microorganism grew, and make the surface sterile plant materials contact with the medium 20 min, and then remove the materials to confirm that the endophytes were isolated from the inner of *M. laxiflora*, not from the surface.

2.3. Subclone, Microexamination and Preservation of the Endophytic Bacteria

To obtain pure cultures, the isolates were sub-cloned in LB and incubated at 37°C for about 2d. All isolates were slant preservation at 4°C. The bacteria were used to direct microscopy, mainly included: the colony size, the colony color, the height of the colony and the colony edge.

2.4. Determination of T-AOC

Bacteria fermentation suspension was filtrated by gauze to remove the thalli, and 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 by Niu *et al.* (2013). Each sample was measured 3 times. The result was calculated by the equation:

$$Total_AOC = \frac{ODu - ODC}{0.01} \times N \times n/30$$

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

3. Results and Discussion

3.1. Endophytic Bacteria from Different Tissues Before- or After- Flooding of the Host

Before flooding, a total of 56 strains were isolated, 20 from roots, 24 from stems, and 12 from leaves. While after flooding, we acquired 28 strains, in which 12 from roots, 7 from stems, and 9 from leaves (Table 1).

Table 1. Endogenous bacterium from *M. laxiflora*

	Root	stem	leaf	total
Before flooding	20	24	12	56
After flooding	12	7	9	28

The endophytic bacteria were far greater before flooding than that of after flooding. This may be resulted from the external environment, and the bacteria probably also suffered the flooding or oxidative stress. The results showed that the endophytic bacteria in roots or stems were more abundant than

in leaves, and this diversity distribution was influenced by flooding stress, in accordance with the fungi (Another paper in publishing).

3.2. Morphology of the Bacteria

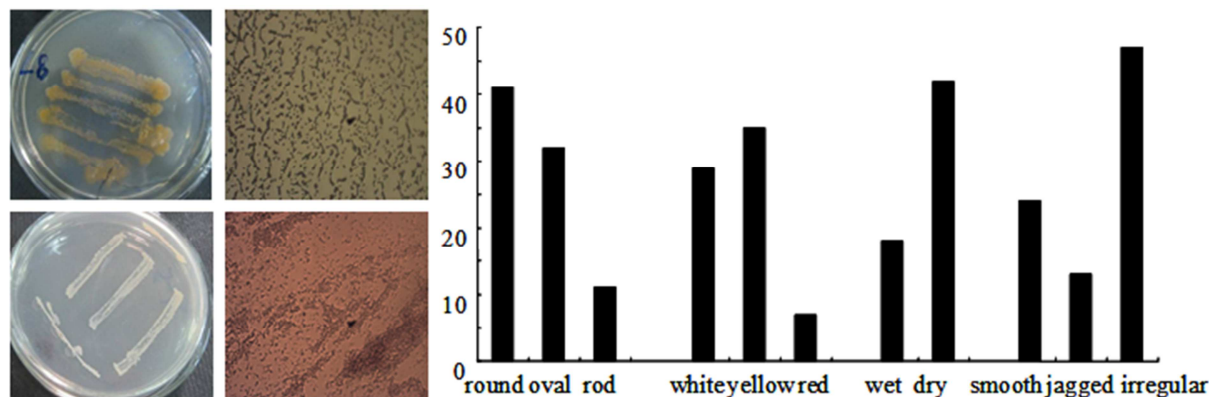


Fig 1. Morphology of the bacteria (On the Left, two representative isolates were examined by microscopy; On the right, the statistical data were shown by the morphology according to the colony size, the colony color, the height of the colony and the colony edge, and the Y-axis means the number of the clones.)

According to morphologic observation (Fig 1), such as the colony size, the colony color, the height of the colony, colony edge, most strains were classified into round, yellow, dry and irregular shape. Some typical isolates were in the left. And the statistical data was shown in Fig1 (On the right).

3.3. The Relationship Between T-AOC and the Fermentation Time

In order to determine the suitable time of measuring T-AOC value, we randomly selected 2 representative bacteria to evaluate the change of T-AOC over time (Fig 2). A bell shaped curve was observed (Fig 2), and the results showed that the highest T-AOC appeared at about 72 h, which was used in the following study to measure the T-AOC value.

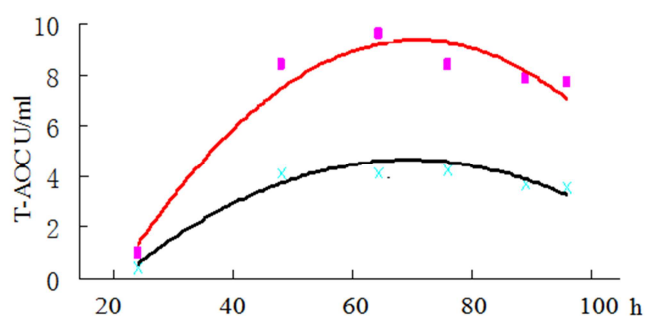


Fig 2. The changes of T-AOC over time (2 representative bacteria were used)

3.4. T-AOC Analysis

84 endophytic bacteria were used in this study for antioxidant activity. Using the same method, Xie (2009) 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, 2009) Shi et al found that the endophyte isolated from *Sophora japonica* L showed 12.54 U/ml as maximum (Shi et al., 2007).

Here, from root of *M. laxiflora* we got a endophytic bacterium strain, showed the highest AOC as 14.42 ± 1.5 U/ml, far greater than the previous report related with bacteria (Xie, 2009), indicating that the endophytes from *M. laxiflora* plants could be as a promising and potent antioxidant source.

In addition, the isolates before flooding were more active in T-AOC than that after flooding (Fig 3), although the latter was more abundant. Whether before- or after- flooding, the ability order of antioxidant from different tissues was: roots > stems > leaves (Fig 3). Hence the endophytic bacterium from roots in *M. laxiflora* plants maybe more active in antioxidant and need more attention.

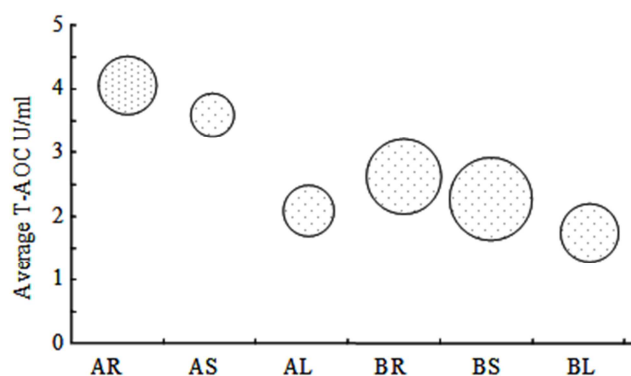


Fig 3. The average T-AOC analysis (A standed for roots after flooding and B for before flooding, R, S and L represented roots, stems and leaves accordingly. The round size means the amount of the bacteria).

There were 10 strains with T-AOC value more than 5.0 U/ml, showed strong antioxidant activity in vitro. Most of them were from after flooding of the host (Fig 4). Before flooding, there were no high antioxidant isolates in the leaves (Fig 4). And the high antioxidant isolates after flooding was far more than that of before flooding, indicating that flooding stress would promote the high antioxidant bacteria.

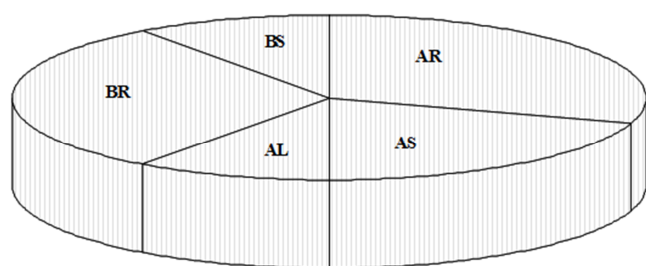


Fig 4. The distribution of higher antioxidant strains (The meaning of the abbreviation is same as the above).

4. Conclusion

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

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 (Williamson *et al.*, 2013). Here, we showed that endophytic bacterium 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 this study, we isolated totally 84 bacteria from healthy stems, leaves and roots before- and after- flooding stress. The antioxidant capacity in vitro analysis showed that 10 endophytic bacteria have strong activity. Most highly active strains were from after bleeding of the host. Whether before- or after- flooding, the ability order of antioxidant from different tissues was: roots > stems > leaves. All the endeavors are helpful to understand the symbiotic relationship between endophytic bacteria and the host under oxidative stress. This may be related with the species specificity of *M. laxiflora* plants. Whether before or after the host suffered bleeding, suggested that the endophytes from *M. laxiflora* plants would be an effective and potential antioxidant.

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