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Original Article

Analysis of microbial diversity in the root of *Astragalus mongholicus*

Análise da diversidade microbiana em raízes de astrágalo

Q. Zhao[a], H. Li[a]*, Z. Guo[a] and M. Gao[a]

[a]Xianyang Normal University, School of Chemistry and Chemical Engineering, Xianyang, Shaanxi, China

Abstract

The dry root of *Astragalus mongholicus* has therapeutic effects such as tonifing the middle - jiao, replenishing qi, solidifing the surface, promoting diuresis, dispelling sepsis outwards and nourishing muscle. There are some slices having black spots after slicing the root of astragalus. The diversity of endophytic fungi between slices with black spots and normal slices was analysed in this paper. The endophytic fungal sequences obtained by high-throughput sequencing were 298,044 and 297,396, and the 116 OTU subsets obtained after clustering belonged to 3 phyla, 9 classes, 22 orders, 38 families and 46 genera. The dominant classes were Eurotiomycetes and Leotiomycetes. The dominant order is Eurotiales and Helotiales. The dominant families are Helotiales_fam_Incertae_sedis and Aspergillaceae. The dominant genera are Cadophora and Aspergillus. There are some peculiar fungal flora in both normal slices and spotted slices. The study on endophytic fungi diversity of astragalus slices will provide some help for drug development of this plant.

Keywords: *Astragalus mongholicus*, slices, endophytic fungi, microbial diversity.

Resumo

A raiz seca de astrágalo tem a função de tonificar o zhongjiao, qi benéfico, superfície sólida, diurético, remoção externa de veneno e fortalecimento muscular. Depois de cortar o astrágalo em fatias finas, algumas fatias têm pontos pretos. Neste trabalho foi analisada a diversidade de fungos endofíticos em fatias de ponto negro versus fatias normais. O sequenciamento de alto rendimento resultou em 314.998 e 315.051 sequências de fungos endofíticos, respectivamente, que após agrupamento resultaram em 116 subgrupos de OTU, pertencentes a três filos, nove classes, 22 ordens, 38 famílias e 46 gêneros. As classes dominantes são Eurotiomycetes e Leotiomycetes. A ordem principal é euclides e hilllows. As famílias predominantes foram aspergilaceae e aspergilaceae. Os gêneros predominantes foram leucostilla e aspergillus. Tanto as seções normais como as secções manchadas apresentam uma flora fúngica distinta. O estudo da diversidade de fungos endofíticos em fatias de astrágalo pode contribuir para o desenvolvimento de fármacos para esta planta.

Palavras-chave: astrágalo, fatias, fungos endofíticos, diversidade microbiana.

1. Introduction

Astragalus is the dry root of *A. membranaceus* (Fisch.) Bge. or *A. mongholicus* Bge. It tastes sweet and has a mild nature. It enters the Spleen and Lung meridians. It has therapeutic effects such as tonifing the middle - jiao, replenishing qi, solidifing the surface, promoting diuresis, dispelling sepsis outwards and nourishing muscle (Hu and Zhang, 2021). Astragalus has cylindrical root with some branches. The upper of the root is thicker and slightly twisted. The length of astragalus root is 30 ~ 90 cm. The diameter of astragalus root is 0.7 ~ 3.5 cm. The surface of astragalus root is alabaster or light brown, has irregular longitudinal furrow and horizontal long skin hole. The cork is easy to peel off and the yellowish white cortex was exposed. The astragalus root has tough quality with strong fiber section. It tastes light sweet and has beany flavor. It is usually to dig astragalus root in spring or autumn. After cleaning and removing fibrous root, we drying the root to sixty percent dry and arranging straightly. Then the root was bundling and dried completely. The processing method of astragalus was mainly sliced since the Song Dynasty. The normal astragalus slices had uniform yellow color, and were smelled faintly medicine fragrance, tasted sweet. While there are some slices having black spots. The black spots are uniform distributed in the cortex. Except the black spots, these slices have no differences with normal slices in color and smell. We analyzed the endophytic fungi diversity between the normal astragalus

*e-mail: lihuaizhu121@126.com
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slices and slices with black spots to investigate the reason of these dark spots formation.

Early understanding of microbial diversity was mainly based on pure strain separation and pure culture technology, mainly using plate separation method, fluorescence staining method, phospholipid fatty acid map analysis method, etc (Gao et al., 2016). However, most microorganisms are active but not culturable. The traditional methods have missed a large number of microorganisms that cannot be culturable, which vastly underestimated the number and diversity of microbes and limited our comprehensive understanding of microorganisms. Recently, the development of high-throughput sequencing technology enables us to have a more comprehensive understanding of microbial community composition and ecological functions (Delmotte et al., 2009; Redford et al., 2010; Cordier et al., 2012; Ritpitakphong et al., 2016). ITS1 was located between 18S and 5.8s of the eukaryotic ribosomal rDNA sequence, while ITS2 was between 5.8s and 28S of the eukaryotic ribosomal rDNA sequence. As the ITS region is cut off during ribosomal RNA processing and does not play a functional role, it has less selection pressure in the process of evolution, and ITS evolution rate is about 10 times that of 18S rDNA, which is a moderately conserved region. It can be used to study species and sub-species taxonomic orders. In this study, the diversity of endophytic fungi was analyzed based on ITS1 sequence between the normal astragalus slices and slices with black spots.

2. Material and Methods

2.1. Materials

Normal astragalus slices (referred to as "NS") and slices with black spots (referred to as "SS") of *A. mongholicus*are were all got from Zizhou County, Yulin City, Shaanxi Province. Both samples of *A. mongholicus*are were of the same variety and grew in the same environment. The photograph of two materials were showed in Figure 1.

2.2. Total DNA extraction and library construction

250-500mg NS and SS samples were collected, and DNA was extracted using MN NucleoSpin 96 Soil kit (Macherey-Nagel Inc, MN, Germany). The two-step library building method is adopted. In the first step, DNA is used as the template. Primers with connectors are designed for PCR. The purpose of primer connectors is to add barcode/index conveniently during the library building in the second step. The amplification primer is Fungi ITS1 F: CTTGGTCATTTAGAGGAAGTAA; Fungi ITS1 R: GCTGCGTTCTTCATCGATGC. Amplification system (10 μL): 5×GC Buffer 5 μL, dNTP (2 mmol /L) 2 μL, Forward Primer (10 μmol/L) 0.3 μL, Reverse Primer (10 μmol/L) 0.3 μL, DNA Template 50 ng, ddH2O 8.75 μL, KOD FX Neo DNA Polymerase (TOYOBO) 0.2 μL, KOD FX Neo Buffer 5 μL. Amplification parameters: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 40 s, 25 cycles, and the final elongation was 7 min at 72°C. In the second step, PCR products of the first step were used as templates. Amplification system (20 μL): Target region PCR purified product 5 μL, MPI-A (2 μmol/L) 2.5 μL, MPI-B (2 μmol/L) 2.5 μL, 2×Q5 HF MM 10 μL (New England Biolabs). Amplification parameters: pre-denaturation at 98°C for 30 s, denaturation at 98°C for 10 s, annealing at 65°C for 30 s, extension at 72°C for 30 s, 10 cycles, and the final elongation was 5 min at 72°C.

PCR products were mixed according to the results of electrophoresis quantification (ImageJ software) and the mass ratio was 1:1. After mixing, OMEGA DNA purification column was used for column purification. Electrophoresis was performed in 1.8% agarose gel at 120V for 40min. Then the target fragment was cutted and recovered.

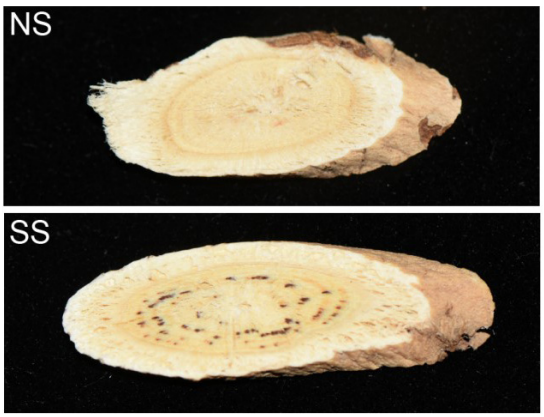

Figure 1. Photos of the NS and SS samples.

2.3. High throughput sequencing and quality assessment of sequencing data

Illumina HiSeq 2500 sequencing platform was used for double-terminal sequencing at Biomarker Technologies Corporation. Splicing the original data (FLASH, Version 1.2.11) (Magoc and Salzberg, 2011), quality filtering was performed on the spliced sequences (Trimmomatic, Version 0.33) (Bolger et al., 2014). Chimeras were removed (UCHIME, Version 8.1) to get high-quality Tags sequence (Edgar et al., 2011). Files containing reads sequence were deposited in the BIG Submission of China National Center for Bioinformation with accession number CRA006101.

2.4. Species annotation and taxonomic analysis

Sequence clustering and operational taxonomic unit (OTU) division were performed at the 97% similarity level (USEARCH, Version 10.0) (Edgar, 2013), and 0.005% of all sequenced sequences were used as the threshold to filter OTU (Bokulich et al., 2013). The sequence with the highest abundance in each OTU was selected as the representative sequence of this OTU.

The fungal species annotation of ITS sequence was performed based on UNITE database (Koljalg et al., 2013). The software Mothur (version V.1.30) was used for Alpha diversity analysis of samples to analysis the difference and correlation at the species level (Schloss et al., 2009). Alpha pleomorphism refers to the pleomorphism within a specific domain or phylogeny. The indexes of abundance included Chao 1 richness estimator and Ace richness

estimator which indicate the biomass of species. The larger the index, the higher the community richness. The indexes of diversity included Shannon-wiener diversity index and Simpson diversity index. The larger Shannon index is, the smaller Simpson index is, indicating the higher community diversity.

2.5. Analysis of ecological function group

OTU sequences with a abundance greater than 10 were selected to classify the trophic modes and guilds of endophytes based on the FUNGuild online database platform (Nguyen et al., 2016).

3. Results

3.1. Sequencing data quality analysis

After sequencing, 298,044 valid sequences were obtained for NS, with an average length of 289bp, while 297,396 valid sequences were obtained for SS, with an average length of 304bp. The length distribution of clean sequences of each sample is shown in Table 1. The number of valid sequences annotated to species for each taxonomic level in the two samples were also calculated (Table 2).

The OTU (Operational Taxonomic Unit) was obtained by clustering the sequences at 97% similarity level, and the OTUs were annotated Taxonomic. As shown in OTU Venn diagram (Figure 2), a total of 116 OTUs were obtained from all samples. There were 93 OTUs obtained by both NS and SS. 21 OTUs were unique to SS and 2 OTUs were unique to NS. The rarefaction curve (Figure 3) showed that the number of OTU tended to be stable with the increase of the number of sequence in both samples, indicating that the sequencing depth met the requirements of microbial diversity analysis.

Table 1. The length distribution of valid sequences.

Region	NS	SS
0-100bp	0	0
100-130bp	0	0
130-160bp	14	13
160-190bp	221	190
190-220bp	54093	2305
220-250bp	1777	4783
250-280bp	1930	3264
280-310bp	239718	276068
310-340bp	262	10625
340-370bp	16	114
370-400bp	10	33
400-430bp	3	1
430-460bp	0	0
460-490bp	0	0

3.2. Alpha diversity index of sample fungal communities

Alpha diversity can be used to analyze microbial richness and diversity in environmental samples. In order to compare the richness and diversity of fungal communities in NS and SS, Chao1, Ace, Simpson and Shannon indices were selected for analysis. Chao1 and Ace index indicate the biomass of species. The larger the index, the higher the community richness. Shannon and Simpson indexes are used to measure species diversity. The larger Shannon index is, the smaller Simpson index is, indicating the higher community diversity. In addition, OTU Coverage was also

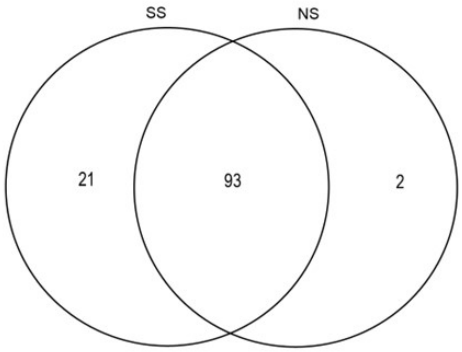

Figure 2. OTU Venn diagram of the NS and SS samples.

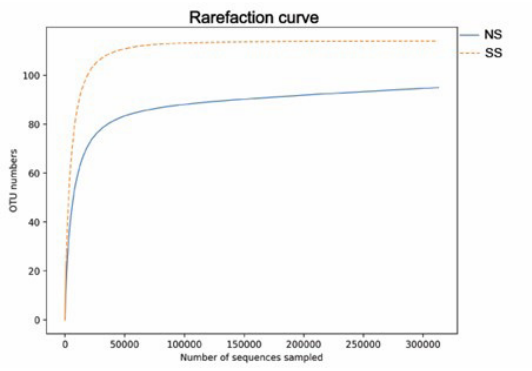

Figure 3. Rarefaction curve of the NS and SS samples.

Table 2. Statistic table of per taxonomic level valid sequences.

Sample	Kindom	Phylum	Class	Order	Family	Genus	Species
SS	293,393	17,849	17,342	17,251	6,928	15,938	13,929
NS	296,277	56,472	55,745	55,602	55,621	55,614	53,820

counted. The higher the value, the higher the probability of a species being detected in the sample.

Differences in Alpha diversity index of fungal communities between NS and SS were shown in Table 3. The Chao1 and Ace indices of the SS were higher than those of the NS, indicating that the fungal richness of SS was higher than NS. Shannon and Simpson index showed that the diversity of fungal community in SS was lower than that in NS.

3.3. Fungal community composition of samples

In our results, the species annotated of the OTU with the largest number of matching valid sequences (OTU1) was the Fungi kingdom, and the other taxa were all Unclassified. The valid sequences of this OTU accounted for 80.73% in the NS and 93.45% in the SS, respectively. It is speculated that the number of microbial species recognized by us now accounts for less than 10% of the number of microorganisms in the natural environment (Jones et al., 2011). While the existing of OTU1 indicates that there are unknown species in the endophytic fungi of astragalus. Considering the large proportion of OTU1 in the fungi sequences of astragalus, the fungi species OTU1 annotated may have some critical impact to astragalus. The study of these unclassified fungi species will help us to have a deeper understanding of the physiological and pharmacological mechanism of astragalus.

For the convenience of analysis, the OTU1 with the highest number of sequences was removed and then the abundance of the remaining sequences was calculated. The known taxa annotated by the two samples included 3 phyla, 9 classes, 22 orders, 38 families and 46 genera. At phylum level, astragalus endophytic fungi belong to *Ascomycota*, *Basidiomycota*, and *Mortierellomycota*. The relative abundance of sequences annotated to *Ascomycota* in NS (96.45%) was 9.59% higher than that in SS (86.86%), while the relative abundance of sequences annotated to *Basidiomycota* (0.61%) was 1.61% lower than that in SS (2.22%). The relative abundance of *Mortierellomycota* in SS (0.66%) was higher than that in NS (0.26%).

At the class level, the endophytic fungi of astragalus belongs to *Archaeorhizomycetes*, *Dothideomycetes*, *Eurotiomycetes*, *Leotiomycetes*, *Saccharomycetes*, *Sordariomycetes*, *Agaricomycetes*, *Tremellomycetes*, *Mortierellomycetes* (Figure 4). The sequences annotated to *Eurotiomycetes* accounted for 93.42% of the NS and 11.68% of the SS. *Eurotiomycetes* is the dominant species in NS. The dominant species in SS were *Leotiomycetes* (53.60%).

At the order level, the top ten of the 22 orders annotated by sequences of the two samples were: *Helotiales*, *Eurotiales*, *Sordariales*, *Hypocreales*, *Pleosporales*, *Capnodiales*, *Glomerellales*, *Mortierellales*, *Microascales*, and *Russulales*. The order *Eurotiales* had the highest abundance in NS, accounting for 93.40%, while the order *Helotiales* had the highest abundance in SS, accounting for 53.60%.

At the family level, the top ten families according to the abundance of sequences of NS were: *Aspergillaceae*, *Thermoascaceae*, *Trichocomaceae*, *Mortierellaceae*, *Russulaceae*, *Hypocreaceae*, *Microascaceae*, *Archaeorhizomycetaceae*, *Plectosphaerellaceae*, *Nectriaceae*, with *Aspergillaceae* beening the highest abundance in NS, accounting for 92.18%. The top ten families of SS were: *Helotiales_fam_Incertae_sedis*, *Chaetomiaceae*, *Thermoascaceae*, *Aspergillaceae*, *Trichocomaceae*, *Cordycipitaceae*, *Plectosphaerellaceae*, *Nectriaceae*, *Cladosporiaceae*, *Pleosporaceae*. The family *Helotiales_fam_Incertae_sedis* had the highest abundance in SS, accounting for 53.46%.

At the genus level, as shown in Figure 5, the dominant endophytic fungi in NS mainly belonged to *Aspergillus*, and its abundance accounted for 91.95%. The dominant fungal communities in SS mainly belong to *Cadophora*, *Myceliophthora*, *Thermoascus* and *Thermomyces*, accounting for 53.46%, 6.25%, 4.04% and 2.05%, respectively. In general, there was a large difference in the relative abundance of endophytic fungi between NS and SS.

3.4. Fungal flora unique to both samples

The NS and SS had their unique endophytic fungi at order, family, genus and species level respectively. At the order level, NS had no unique flora, while SS had two unique fungi orders: *Onygenales* and *Thelephorales*. At the family level,

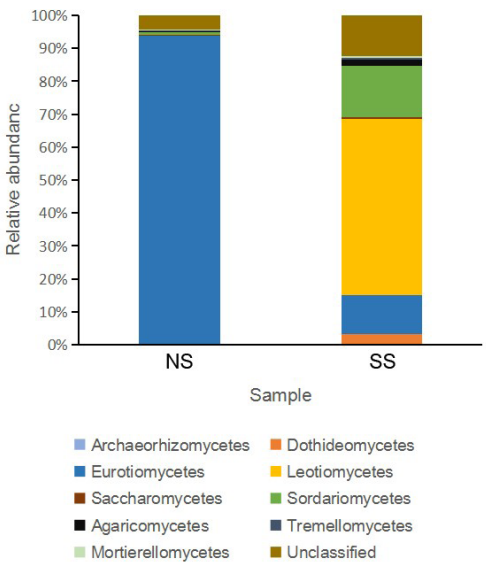

Figure 4. Species richness at the class level.

Table 3. Alpha diversity index of two samples.

Sample	OTU	ACE	Chao1	Simpson	Shannon	Coverage
SS	114	114.0	114.0	0.8746	0.4122	1.0
NS	95	102.4143	109.0	0.6827	0.6127	1.0

the NS had no unique flora, while SS had six unique fungal families: *Elaphomycetaceae*, *Gymnoascaceae*, *Sordariales_fam_Incertae_sedis*, *Omphalotaceae*, *Ceratobasidiaceae*, and *Thelephoraceae*. At the genus level, the NS had unique genus *Naganishia*, while SS had seven unique genera: *Elaphomyces*, *Simplicillium*, *Microascus*, *Myceliophthora*, *Remersonia*, *Gymnopus*, and *Tomentellopsis*. At the species level, *Aspergillus penicillioides* and *Naganishia diffluens* were unique to NS, and six species of fungi were unique to SS: *Lecanicillium saksenae*, *Microascus paisii*, *Myceliophthora sepedonium*, *Remersonia thermophila*, *Gymnopus melanopus* and *Tomentellopsis bresadolana*.

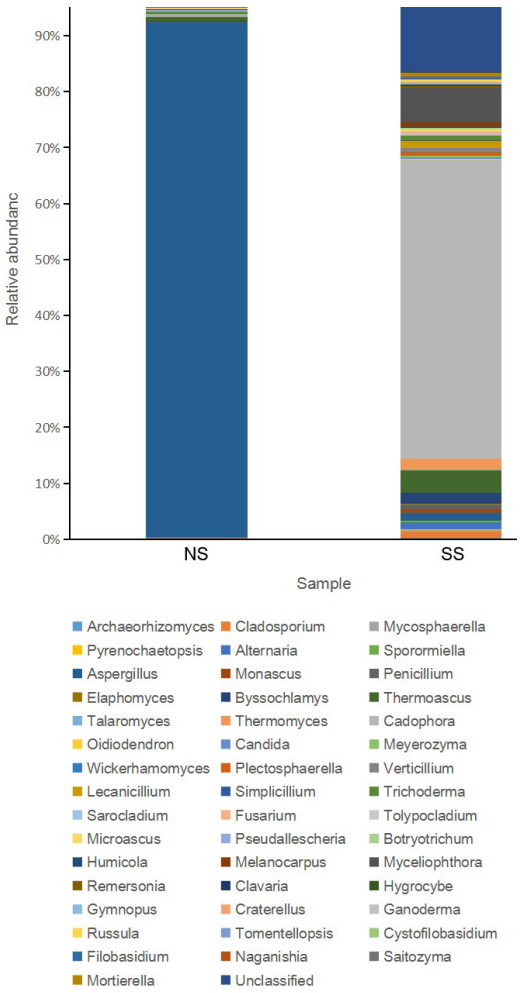

Figure 5. Species richness at the genus level.

In general, both NS and SS had their own unique endophytic fungi, among which the relative abundance of *Myceliophthora sepedonium* in SS was greater than 5%, and the relative abundance of other unique endophytic fungi was less than 1%.

3.5. Ecological function analysis of fungi

Fungi are divided into three groups according to their nutritional patterns: pathotroph, Symbiotroph and saprotroph. In our result, 7 OTUs were highly credible according to the analysis of ecological function group. The OTU45 annotated to *Filobasidium* was saprotroph, and the other 6 OTUs are symbiotroph which annotated to genera *Elaphomyces*, *Tomentellopsis*, *Cadophora*, *Russula* and *Craterellus* (Table 4). OTU3 was most abundant in SS which accounting for 53.5% in 7 OTUs, annotating to genus *Cadophora* and belonging to symbiotroph nutritional pattern. Among NS, 5 OTUs were Highly reliable, one of which annotated to genus *Filobasidium* was saprotroph, while other four OTUs annotated to genus *Cadophora*, *Russula* and *Craterellus* were symbiotroph.

Astragalus fungi were mainly divided into 27 ecological function groups which included animal pathogens, ectomycorrhizal, endophyte, ericoid mycorrhizal, leaf saprotroph, plant pathogens, soil saprotroph, undefined saprotroph, wood saprotroph, and a variety of mixed nutritional fungi, such as animal pathogen-clavicipitaceous endophyte-fungal parasite, animal pathogen-dung saprotroph-endophyte-epiphyte-plant saprotroph-wood saprotroph, endomycorrhizal-plant pathogen-undefined saprotroph, etc.

4. Discussion

The endophytic fungal sequences obtained by high-throughput sequencing were 298,044 and 297,396, and

Table 4. Nutritional pattern of highly credible OTUs.

OTU ID	Number of valid sequences		Taxon	Trophic mode
	NS	SS		
OTU119	57	0	Elaphomyces	Symbiotroph
OTU296	32	0	Tomentellopsis	Symbiotroph
OTU32	45	74	Russula	Symbiotroph
OTU92	11	51	Russula	Symbiotroph
OTU69	35	22	Craterellus	Symbiotroph
OTU45	53	11	Filobasidium	Saprotroph
OTU3	10874	2	Cadophora	Symbiotroph

the 116 OTU subsets obtained after clustering belonged to 3 phyla, 9 classes, 22 orders, 38 families and 46 genera. The dominant classes were *Eurotiomycetes* and *Leotiomycetes*. The dominant order is *Eurotiales* and *Helotiales*. The dominant families are *Helotiales_fam_Incertae_sedis* and *Aspergillaceae*. The dominant genera are *Cadophora* and *Aspergillus*. The richness of the fungal community in SS was higher than that in NS, but its diversity was lower than that in NS. The structural changes of fungal community may be closely related to the formation of black spots.

There are some peculiar fungal flora in both NS and SS, and although their relative abundance is small, in some cases, a few or rare microbial groups rather than dominant groups are the main driving factors affecting multiple physiological functions of plants (Chen et al., 2019). The relative abundance of *Myceliophthora sepedonium*, which was unique to SS, was greater than 5%, while the relative abundance of other bacteria was less than 1%. *Myceliophthora* is characterized by blastospore with narrow basal attachments (sometimes on a pothole mass). A thermophilic fungus in this genus can produce xylanase and cellulase (Badhan et al., 2007). *M. thermophthora* genome encodes a kind of carbohydrate-active enzymes (CAZymes) involved in plant biomass degradation (Kolbusz et al., 2014). The author speculated that *M. sepedonium*, which is unique to the samples of SS, could also decompose the root tissue of *A. membranaceus* to some extent, and might be one of the reasons for the appearance of black spots.

Endophytes are microorganisms (mainly bacteria and fungi) that live in the tissues or organs of healthy plants in certain or all stages of their life cycle. Some of them are attached to the surface of plants, and some live in plants. In recent years, more and more researchers in different fields have focused their attention on the microbes that live inside plants. Since Strobel first reported the isolation of a paclitaxel-producing endophytic fungus from the phloem of *Taxus brevifolia* in 1993(Stierle et al., 1993), certain progress has been made in the study of endophytic bacteria, and some strains of medical and agricultural value have been found. Such as *Lycium barbarum* is important Chinese traditional medicine, having high requirements for growing environment, generally need 3 ~ 5 years to form a gourd shape root. There were 31 species of endophytic fungi isolated from the root of *L. barbarum* in previous study, with some fungi improving the growth of host plant, some increasing the flavonoids content, and some increasing the expression of the treophyll extension protein gene (Th - exp) (Song et al., 2017). The endophytic fungi were isolated from the roots of plants growing in soil contaminated by heavy metals, which can improve the repair ability of host plants (Shi et al., 2017). The endophytic fungi isolated from *Catharanthus roseus* can synthetic vin blastine (Wen et al., 2004; Zhou, 2005; Namdeo et al., 2002). Thus the study on endophytic fungi diversity of *A. membranaceus* will provide some help for drug development of this medicinal plant.

Acknowledgements

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