

# Effects of the symbiosis between fungal endophytes and *Atractylodes lancea* on rhizosphere and phyllosphere microbial communities

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**Abstract** Tissue-cultured plantlets of *Atractylodes lancea* were inoculated with the endophytes AL4 (*Cunninghamella* sp.) and AL12 (*Gilmaniella* sp.), and subsequently transplanted into soil after hardening of the tissue-cultured plantlets. We investigated rhizospheric and phyllospheric microbial communities using culture-based and culture-independent methods. Energy spectrum analysis, high performance liquid chromatography, and other assay methods were employed to quantify the elements in the leaves, and the soluble sugars, free amino acids and organic acids in the rhizosphere. The results showed that the endophytes enhanced the diversity and size of the rhizospheric microbial populations. In the phyllosphere, AL4 (*Cunninghamella* sp.) enhanced the diversity and size of bacterial populations, while AL12 (*Gilmaniella* sp.) enhanced the diversity and size of fungal populations. The dominant bacterial genera were *Microbacterium*, *Kocuria* and *Sphingomon* in the endophytes-inoculated groups, and *Acinetobacter* and *Bacillus* in the endophytes-free group. While *Acremonium* and *Curvularia* were the dominant fungal genera in the phyllosphere of endophytes-inoculated groups, *Fusarium* and *Penicillium* were most common in the endophytes-free group.

AL4 (*Cunninghamella* sp.) enhanced the rhizospheric microbial population size and diversity by increasing rhizospheric free amino acids, while AL12 (*Gilmaniella* sp.) altered the rhizospheric microbes by changing concentration of soluble sugars in the rhizosphere. Elemental levels in the phyllosphere and the nutrients in the rhizosphere varied among the treatments and may also have influenced the microbial communities.

**Keywords** Fungal endophytes · *Atractylodes lancea* · Leaf leachates · Root exudates · Microbial communities

## Abbreviations

PCR-DGGE	Polymerase chain reaction-denaturing gradient gel electrophoresis
HPLC	High performance liquid chromatography
DAP	4',6-Diamidino-2-phenylindole
SEM	Scanning electron microscope
QS	Quorum sensing

## 1 Introduction

No plants are completely axenic under natural conditions (Partida-Martínez and Heil 2011). Plants co-exist with a variety of symbiotic microbes, among which are fungal endophytes (Rodríguez et al. 2009; Yuan et al. 2010). These non-systemic endophytes live in the phytosphere and are transmitted horizontally. Their interactions with the host plants are generally characterized as highly variable balanced antagonisms and this differentiate them from the clavicipitaceous endophytes of grass (Rodríguez et al. 2009; Porras-Alfaro and Bayman 2011). Most of fungal endophytes help plants to grow and survive (Rodríguez et al. 2009; Porras-Alfaro and Bayman 2011). They can induce multiple responses in the host plants, such as the nitric oxide burst or the accumulation of salicylic acid, jasmonic acid, hydrogen peroxide and volatile oils (Wang

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et al. 2011; Wang et al. 2012; Ren and Dai 2012). The endophyte *Phomopsis liquidambari* B3 expressed special enzymes to optimize the soil environment around the host plant in an in vitro system (Chen et al. 2011, 2013a, b). The enzymes effectively altered the balance of soil microbes in the rhizosphere and even degraded allelochemicals such as phenolic acids and N-heterocyclic indole. The endophytic fungus *P. liquidambari* also triggered the metabolism of saprophytes which promoted the cycling of organic matter in the litter after the senescence of the host tissues (Chen et al. 2012). Thus, in both in vitro and in vivo settings, fungal endophytes show specific and key ecological functions (Harman 2011). However, it is unknown whether endophytes which are important members of the phytospheric microbial communities can affect communities of other microbes in the rhizosphere and phyllosphere of the hosts in the natural environment (Yang et al. 2013). If so, what are the mechanisms underlying these effects?

To try to answer these questions, we focused on the interaction between *A. lancea* and two of its endophytes. *A. lancea* is an important traditional medicinal plant that is widely distributed in China (Hasada et al. 2010; Meng et al. 2010). It grows in association with various fungal endophytes (Chen et al. 2008), among which are AL4 *Cunninghamella* sp. and AL12 *Gilmaniella* sp. In previous studies, these endophytes were shown to promote plant growth and survival, induced plant defense responses, and modified the medicinal components of the plant (Fang et al. 2009a, b; Wang et al. 2011, 2012; Ren and Dai 2012). Most importantly, both were able to form a stable symbiosis with tissue-cultured plantlets, and altered the volatile oil components in root tissues and the distribution of metabolites in plant organs (Gao et al. 2012; Yang and Dai 2013). The above characteristics make *A. lancea* and its endophytes ideal materials for examining the ecological effects of a single factor, in this case, colonization of the host by a symbiotic fungal endophyte, on phytospheric microbial communities.

Recently, researchers used 454 pyrosequencing of the bacterial 16S ribosomal RNA gene to study the microbiota of more than 600 *Arabidopsis thaliana* plants (Lundberg et al. 2012). They found that the communities in each plant compartment were strongly influenced by soil type, and some bacteria showed quantitative differences among plants at different developmental stages and with different genotypes. We focused on the ability of fungal endophytes in vivo to affect microbial communities in the phytosphere. Extensive researches have been conducted on microbe–microbe and microbe–plant biocommunications (Oelmüller et al. 2011), but the mechanisms by which fungal endophytes may affect rhizospheric and phyllospheric microbes remained unclear.

There are many complex interphylum nutritional interactions in the phytosphere (Ghignone et al. 2012). The host plant is the only source of nutrients that support the growth of endophytes in the endosphere (Larkin et al. 2012). In contrast, rhizospheric and phyllospheric microbes are only partially

reliant on plant-derived nutrients, such as those in leachates or exudates from roots and leaves (Casas et al. 2011). Such leachates/exudates are not only nutrient sources, but may also contain substances that act as signaling molecules to regulate microbe–microbe and microbe–plant biocommunications (Oelmüller et al. 2011). In the present study, we tested the hypothesis that fungal endophytes AL4 *Cunninghamella* sp. and AL12 *Gilmaniella* sp. that form symbioses with *A. lancea* cause successional shifts of rhizospheric and phyllospheric microbial communities, and that these shifts are mediated by leachates or exudates from plant roots and leaves.

## 2 Materials and methods

We established a research system that included single endophyte inoculation, microscopic examination, hardening the plantlets and transplantation to soil (Fig. 1). This system may also be suitable for studies of endophytes in crops and other herbaceous plants.

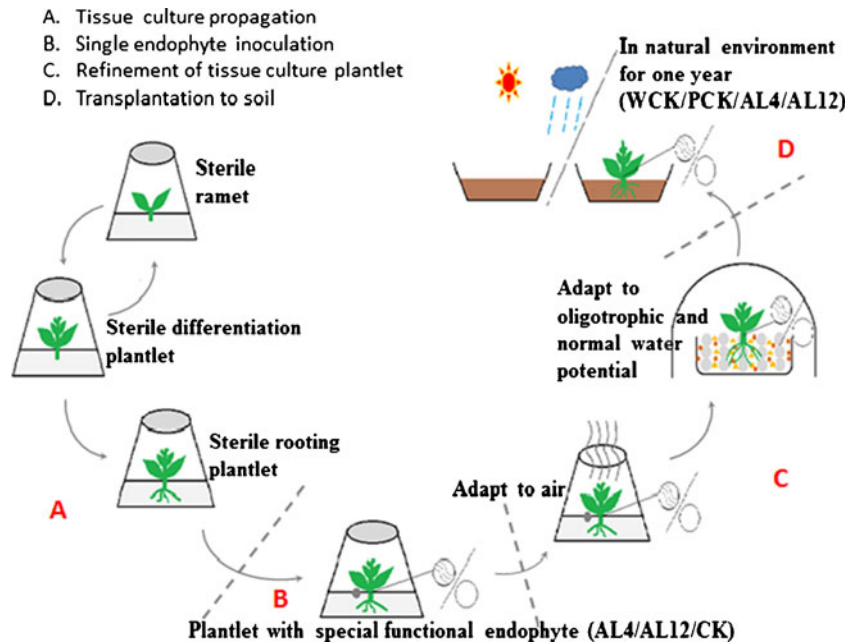
### 2.1 Plant and fungal endophyte materials

*A. lancea* was collected from the Maoshan, Jiangsu Province, China in May, 2004. The plants were identified at the Department of Botany, Nanjing Normal University, and then transplanted and grown in the university's botanic garden (Zhang et al. 2009). Endophytes of *A. lancea* including AL4 *Cunninghamella* sp. and AL12 *Gilmaniella* sp. were isolated in our laboratory and maintained on potato dextrose agar (PDA) slants at 4 °C (Chen et al. 2008).

### 2.2 Double-culture system of *A. lancea* and a single endophyte

Sterile plantlets of *A. lancea* derived from meristem cultures (Wang et al. 2012). Plantlets were grown on Murashige and Skoog medium (Murashige and Skoog 1962) containing 6-benzylaminopurine (2 mg/L), naphthalene acetic acid (NAA) (0.3 mg/L), sucrose (30 g/L), and agar (10 g/L). The plantlets were subcultured on this medium every 45 days. To induce rooting, differentiated plantlets of a suitable size were transferred to rooting medium (half-strength MS containing NAA (0.3 mg/L)+sucrose (30 g/L)+agar (10 g/L)). After 48 days on rooting medium, well-rooted plantlets of a similar size were selected for inoculation with fungi. The two fungal endophytes were activated on PDA medium for 8 days. Then, a hole punch (9-mm diameter) was used to cut a disc of the fungus from the edge of the fresh colony. The fungal discs were placed approximately 1 cm away from the rooted plantlets. Three treatments were applied: I, the control (CK) group, with no fungal inoculations; II, plantlets inoculated with AL4; and III, plantlets inoculated with AL12. All in vitro cultures were maintained

**Fig. 1** Research system. Investigation of microbial communities in the rhizosphere and phyllosphere of *A. lancea* and measurement of basic elements in leaves were conducted at point D. Analyses of root exudates were conducted at point B



in a growth chamber under the following condition: 25/18 °C day/night, light intensity of 1500 lux, 12-h light/12-h dark photoperiod. By 28 days after inoculation, both AL4 and AL12 had established symbioses with *A. lancea* plantlets (Wang et al. 2012; Yang and Dai 2013).

### 2.3 Hardening of tissue-cultured plantlets

We used 30 tissue-cultured *A. lancea* plantlets in each treatment, and the plantlets were hardened off in four steps, as follows. First, the plastic wraps on the bottles were opened and the humidity was maintained at 90 % for 2 days. Then, the plantlets were transferred to hardening nutrient medium (perlite:vermiculite:plant-ash = 1:1:1), and the humidity was maintained at 85 % for 5 days. The humidity was decreased from 80 % to 70 % at 5 % intervals every 2 days. The plantlets were watered regularly during this process. After approximately 2 weeks, the *A. lancea* plantlets were hardened off.

### 2.4 Transplantation into soil and daily management

The hardened plantlets were transplanted into soil (yellow-brown loam soil), which was obtained from the botanic garden of Nanjing Normal University. The soil had been sterilized by a high-pressure steam treatment. The basic soil properties are shown in Table 1. The soil was mixed and added to pots (13-cm radius, 18-cm depth). Each pot contained 5 kg soil and 0.3 kg organic fertilizer (Gurun, 1.06 % N, 1.08 % P<sub>2</sub>O<sub>5</sub>, 3.42 % K<sub>2</sub>O, 49.1 % organic matter, 19 % water content) as base fertilizer. There were four pots for each treatment. Every pot contained three representative plantlets except the WCK group. The control (WCK) group had no *A. lancea* and no

fungal endophytes. The PCK treatment group consisted of *A. lancea* plantlets without endophytes. AL4 and AL12 treatment groups consisted of plantlets inoculated with each respective endophytes. The experiment was conducted in the greenhouse at Nanjing Normal University. The experiment started in May 2011 and ended in June 2012. Watering and weeding were conducted regularly during the experiment.

### 2.5 Soil and leaf samples

**Soils:** In June 2012, we sampled the rhizosphere soil of the PCK (plantlets with no endophytes), AL4 (*Cunninghamella* sp.) and AL12 (*Gilmaniella* sp.) treatment groups, and soil of WCK (control pots with no plantlets). Rhizospheric soil was collected by the method of shake-roots (Li et al. 2013). We collected *A. lancea* plantlets from soil, shaking the roots to remove loosely attached soil. Then we used sterile shear-head forceps to remove the rhizospheric soil that was tightly attached to roots. The rhizospheric soil in every pot was collected from three plantlets' roots and was mixed as one independent repetition. The soil of WCK group was collected from three different sites in one pot. We removed the soil at surface and collected the soil at a depth of 1–7 cm (this depth is closed to the length of root of *A. lancea*). Each pot was considered one replicate. We used a dilution plate coating method to analyze soil from every treatment (sieved by 2 mm), and every treatment included three replicates (pots). Every replicate included four plates. Only when four plates all contained the same microbe genus, did we regard the microbe as a representative genus and assign it a 1 in the parentheses (Table 4). The remaining soil was freeze-dried for 12 h, sieved (<2 mm) and then stored at –20 °C for DNA extraction and 4 °C for other analyses.

**Table 1** Properties of soil (yellow-brown soil) used in the experiment

Index	pH	Total N (g/kg)	Total P (g/kg)	Total K (g/kg)	Fe (g/kg)	Mg (g/kg)	Cu (g/kg)	Mn (g/kg)
Soil	6.79	0.29	0.4	15.5	26.9	3.8	1	0.02

<sup>a</sup> Soil was obtained from the botanic garden at Nanjing Normal University

**Leaves:** We sampled leaves from plantlets in the PCK, AL4 and AL12 treatment groups. Leaves were collected between 8:00 and 10:00 AM. All leaves from plants in the same pot were mixed and placed in sterile paper envelopes. The samples were returned to the laboratory within 1 h. All leaf samples were stored at 4 °C. We used a dilution plate coating method to analyze the leaf extract. Similarly, every treatment included three replicates and every replicate included four plates. Only when the same microbe genus was identified in four plates, did we regard it as a phyllospheric representative genus and assign it a 1 in the parentheses (Table 4).

## 2.6 Isolation of endophytes

Fungal endophytes were isolated from segments of leaves and stems to confirm their presences and were identified by the method of Chen et al. (2008).

## 2.7 Isolation, counting, and identification of culturable microorganisms

To culture bacteria and fungi from soil samples, 10 g fresh soil was homogenized in 90 mL sterilized water and serially diluted. Aliquots (0.1 mL) of the diluted suspension were spread on beef extract peptone medium to culture bacteria and rose Bengal medium to culture fungi (Xu and Zheng 1986). Three soil samples from different pots were analyzed in each treatment.

For leaves, 0.25 g leaf tissue was homogenized in 25 mL sterilized phosphate-buffered saline medium (pH=7.4), shaken at 200 rpm for 30 min, sonicated (Transistor/ultrasonic T7; L&R Manufacturing Co., Kearny, NJ, USA) for 5 min at medium intensity, and vortexed three times for 60 s each time at 2-min intervals. Then, aliquots (0.1 mL) of the serially diluted solutions were spread on media for isolation, counting, and identification of the culturable microorganisms as described above for soil samples.

The colony forming units (CFUs) on each medium were counted after incubation at 25 °C. Plates with 50 to 200 colonies were counted on day 3 and day 5 post inoculation. Some plates were used for microbial identification (He et al. 2008). The representative bacteria and fungi were identified according to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994) and the Manual of Determinative Mycology (Wei 1979).

## 2.8 Microbial DNA extraction, PCR, and DGGE analysis

Total DNA was extracted from 0.5 g soil samples (from each of WCK, PCK, AL4, and AL12) using an UltraClean Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. Microbial DNA was also extracted from the leaf samples of three plantlets (from PCK, AL4, and AL12) as follows: 2.5 g fresh leaf tissue (1.5×1.5 cm) was added to a 50-mL falcon tube containing 20 mL sterilized phosphate-buffered saline medium (pH=7.4). The tubes were shaken at 200 rpm for 30 min, then sonicated for 5 min at medium intensity and vortexed three times (60 s each time) at 2-min intervals. The leaves were removed with forceps and the liquid centrifuged at 8,000g for 30 min. DNA was extracted from the precipitate using the UltraClean Soil DNA isolation kit (MoBio Laboratories). DNA extracted from soil and leaf extracts was separated by electrophoresis on 1.5 % agarose gels and was stained by Ethidium Bromide.

The PCR reaction (50 µL total volume) consisted of 5 µL 10×PCR buffer (Mg<sup>2+</sup>-free), 4 µL 2.5 mM dNTPs, 0.5 µL Taq polymerase (5 U µL<sup>-1</sup>, Takara Biotechnology, Shiga, Japan), 1 µL each primer at 50 mM, and 2 µL DNA template (containing 10–15 ng DNA). Bacterial and fungal PCR-DGGE conditions are shown in Table 2. The same PCR-DGGE conditions were used for the rhizosphere and phyllosphere samples. The bacterial target bands were 250 bp and the fungal target bands were 350 bp.

To compare bacterial and fungal communities in the phytosphere, we combined the four rhizospheric soil/soil groups and three phyllosphere groups. Due to the quantitative limitation of gel-sheet sample-holes, we chose two replicates for every treatment randomly. Similarly every replicate represented all mixed genetic information from one pot. Then we used the CBS-DGGE system (CBS Scientific Corporation) with a voltage of 80 V. The electrophoresis times, polyacrylamide gel concentrations, and denaturing gradients are shown in Table 2. Gels were stained with 1:10,000 SYBR Green I (Invitrogen Molecular Probes, Eugene, OR, USA) for 30 min and scanned and analyzed using the GelDOC-ItTS imaging system (Ultra Violet Products, Upland, CA, USA).

## 2.9 Microscopic observation and spectrometry analysis

For soil microbes, 2 µL of a 0.01 g soil/mL solution was spotted onto a sterile glass slide, and then oven-dried at 37 °C

for 30 min. The dried samples were stained with 4',6-diamidino-2-phenylindole (DAPI) (1 µg/mL) (Sigma) for 5 min and then washed with sterile double-distilled water. The samples were observed under a fluorescence microscope (Nikon TI-FL542942) and analyzed using NIS-Elements D 3.0 imaging software.

Phyllosphere microbes were observed under a scanning electron microscope (SEM) (JSM-5610LV, JOEL, Japan). To prepare samples, leaves were fixed in 2.5 % glutaraldehyde (0.1 M phosphate-buffered saline, pH 7.0) at 4 °C for 24 h, and then dehydrated with an ethanol gradient (50 %, 70 %, 80 %, 90 %, 95 %, 100 % ethanol) before fixing to the objective table with double-sized tape. The samples were platinumized as described elsewhere (Baruah et al. 2012) before observations. At the same time, we used energy dispersive X-ray spectrometry (NORAN-VANTAGE) to analyze the basic elements in each leaf surface. The accelerating voltage was 30 KeV, the take-off angle was 25.6934°, the live time was 100 s, and the dead time was 9.366 s.

#### 2.10 Measurement of total soluble saccharides, free amino acids and organic acids concentration in root exudates

After establishing the double culture system between *A. lancea* and each single endophyte, we selected plantlets, washed them in sterile double distilled water to remove the MS medium, and added each plantlet to 15 mL sterile double distilled water (Wang and Weathers 2007). There are another forty-five plantlets which included three treatments (the plantlets inoculated by AL4; the plantlets inoculated by AL12 and the sterile plantlets without any endophytes). These plantlets were separated from plantlets described immediately prior to this, but they were clones of the tissue culture plantlets and were genetically identical to the other tissue culture plantlets. They were used for measuring total soluble saccharides, free amino acids and organic acids concentration in root exudates (Fig. 1). The plantlets were kept in a growth shaker for 14 days (25 °C, 90 rpm, 12-h light/12-h dark photoperiod). Then, the culture solution was collected to measure the concentration of free amino acids, soluble saccharides, and organic acids. Soluble saccharides were detected by the anthrone colorimetry method (Zhou 2001). Free amino acids were detected by the ninhydrin method (Zhou 2001). Organic acids were quantified using a colorimetric method as described by Montgomery et al. (1962) and Liu and Mo (1985).

Each 15-mL root exudate sample was concentrated to 1 mL under reduced pressure. The amino acids were analyzed by autosampler-assisted precolumn derivatization by o-phthalaldehyde, separation by a high performance liquid chromatography (Agilent 1100 Series, USA), and detection by ultraviolet spectrophotometry at 338 nm and 390 nm (Di-Martino et al. 2003). A standard mix of seventeen amino acids was obtained from the waters company of America. Ortho-

**Table 2** PCR-DGGE conditions

Target	Primer sequences	PCR conditions	DGGE conditions		
			Electrophoresis time	Concentration of polyacrylamide in gel	Denaturing gradient
Bacteria	357F-GC: CGCCCGCCGGCGGGCGGGGGGGCGG GGGGACGGGGGGCCCTACGGGAGGCAGCAG 517r-ATTACCGCGGCTGCTGG (Muyzer et al. 1993)	94 °C 4 min; 94 °C 1 min, 65 °C (decrease 0.5 °C every cycle) 1 min, 72 °C 1 min for 20 cycles; 94 °C 1 min, 55 °C 1 min, 72 °C 1 min for 10 cycles; 72 °C 10 min	12 h	8 %	40 %~70 %
Fungi	NSI:GTAGTCATATGCTTGTCTC GCFung: CGCCCGCCGGCCCGCCCGGGCC CGCCCGCCCGCCCAATCCCGGTTACCCGTTG (May et al. 2001)	94 °C 4 min; 94 °C, 30 s, 55 °C 30 s, 72 °C 75 s for 35 cycles; 94 °C 10 min	16 h	7 %	20 %~50 %

phthalaldehyde (OPA) and mercaptopropionic acid were obtained from the sigma company of America. The preparation process of mobile phase A was as follows. We weighed 4.80 g sodium acetate and added 1000 mL pure water and 220  $\mu$ L triethylamine. We mixed them and adjusted pH to  $7.20 \pm 0.02$  by 5 % glacial acetic acid. The solution was filtered using a 0.22  $\mu$ m filter membrane. Finally we took out 5 mL solution and added 5 mL tetrahydrofuran. The preparation process of mobile phase B was as follows. We weighed 4.40 g sodium acetate and added 200 mL pure water. We mixed the sodium acetate solution and adjusted pH to  $7.20 \pm 0.02$  by 1–2 % glacial acetic acid. The solution was filtered using a 0.22  $\mu$ m filter membrane. Finally we added 400 mL chromatographically pure methanol and acetonitrile respectively. The initial ratio of the mobile phase was A: B=94 %: 6 %. Then mobile phase B ranged from 10 % to 100 %. Stop time was 35 min. The chromatography column was Hypersil ODS C<sub>18</sub> 4.6  $\times$  250 mm. Column temperature was 40  $^{\circ}$ C and column pressure was 87–150 bar. The flow velocity was 1–1.2 mL/min.

### 2.11 Statistical analysis

The DGGE profile data were analyzed using Gelcompar II software (Applied Maths, Austin, TX, USA). Based on relative band intensity data from the Gelcompar II results, we used Primer 5 for windows (version 5.2.4) to calculate for the number of bands and Shannon's index and SPSS 17.0 to do principal component analysis (PCA) for microbial communities. When doing PCA, we used KMO and Bartlett spherical test to do related matrix analysis. The significance of differences among mean values was tested by one-way ANOVA and Duncan's tests using SPSS 17.0. The results were considered as significant at  $P < 0.05$ .

## 3 Results

### 3.1 Quantity of culturable bacteria/fungi and dominant representative genera

As shown in Table 3, the phyllospheric bacterial populations were ranked, from largest to smallest, as follows: AL4 (*Cunninghamella* sp.) treatment > AL12 (*Gilmaniella* sp.) treatment/PCK (plantlets with no endophytes), while the phyllospheric fungal populations were: AL12 > PCK > AL4. In phyllosphere, AL4 treatment had the largest bacterial population, while AL12 treatment had the largest fungal population. On the other hand, the rhizospheric bacterial/fungal populations were ranked, from largest to smallest, as follows: AL4 > AL12 > PCK. In the rhizosphere, the endophytes-inoculated groups had larger microbial populations than PCK group. In particular, the AL4 treatment contained the largest population of microbes in rhizosphere, but had the

smallest fungal population in the phyllosphere. In addition, there were significant differences in the fungal population sizes between WCK (pots with no plantlets) group and PCK (plantlets with no endophytes) group.

The representative genera of culturable fungi and bacteria in every treatment were identified by molecular and morphological methods (Table 4). Inoculated treatments showed distinctive representative genera that differed from those of the PCK group.

**Bacterial Communities:** Specifically, for the rhizospheric bacteria, the dominant genera in the PCK group were *Bacillus* and *Acinetobacter*. Soil from the WCK treatment (no plantlets, no endophytes) contained abundant bacterial genera, the dominant ones being *Advenella* and *Stenotrophomonas*. The dominant bacterial genus in the rhizosphere of AL4 (*Cunninghamella* sp.) and AL12 (*Gilmaniella* sp.) groups was *Microbacterium*. For the phyllospheric bacterial communities, AL4 and AL12 treatment groups contained the same dominant genera: *Kocuria* and *Sphingomonas*. The dominant genus in the PCK group was *Acinetobacter*. The bacterial genus *Sphingomonas* was ubiquitous in PCK, AL4, and AL12 treatment groups. *Kocuria* was present only in the endophytes-inoculated groups. *Bacillus* and *Acinetobacter* were also ubiquitous in all groups.

**Fungal Communities:** For the rhizospheric fungi, the dominant genera in the PCK group were *Penicillium*, *Fusarium* and *Acremonium*. The dominant genera in WCK group were *Penicillium*, *Fusarium* and *Rhizopus*. *Penicillium* was the common dominant fungal genus in AL4 (*Cunninghamella* sp.) group and AL12 (*Gilmaniella* sp.) group. For the phyllospheric fungi, *Penicillium* and *Fusarium* were dominant in PCK, and *Acremonium* and *Curvularia* were dominant in AL4 and AL12, respectively. *Penicillium* and *Fusarium* were present in all treatment groups, while *Acremonium* was present in rhizosphere and phyllosphere of all treatment groups. *Penicillium* and *Fusarium* were the dominant genera in all samples except for the phyllosphere of the endophytes-inoculated groups.

### 3.2 DGGE profiles of bacterial and fungal communities in rhizosphere and phyllosphere

The DGGE profiles and PCA analysis of the bacterial communities are shown in Fig. 2a and c. The phyllosphere and rhizosphere were easily distinguishable. For the phyllospheric bacteria, the endophytes-inoculated treatments AL4 and AL12 grouped together, and then grouped with the PCK group. For the rhizospheric bacteria, although cluster analysis showed the endophyte-inoculated treatments AL4 and AL12 grouped together, and then grouped with the PCK group. Based on PCA analysis, the rhizospheric bacteria in the endophytes-inoculated treatment and endophytes-free treatment were not easily distinguishable. The rhizosphere soil

**Table 3** Culturable bacterial and fungal colony forming units (CFUs) in host plant rhizosphere and phyllosphere

Treatment group	Rhizospheric or soil bacteria( $10^7$ CFUs $g^{-1}$ dw)	Rhizospheric or soil fungi( $10^5$ CFUs $g^{-1}$ dw)	Phyllospheric bacteria( $10^5$ CFUs $g^{-1}$ dw)	Phyllospheric fungi( $10^4$ CFUs $g^{-1}$ dw)
WCK	4.10±0.50 <sup>a</sup>	1.80±0.26 <sup>a</sup>	-	-
PCK	4.53±0.57 <sup>a</sup>	4.67±0.57 <sup>b</sup>	2.40±0.66 <sup>a</sup>	3.37±0.25 <sup>ab</sup>
AL4	12.40±0.83 <sup>c</sup>	8.90±0.60 <sup>d</sup>	12.3±3.21 <sup>b</sup>	2.43±0.87 <sup>a</sup>
AL12	7.10±0.20 <sup>b</sup>	6.90±0.40 <sup>c</sup>	3.77±0.67 <sup>a</sup>	3.97±0.31 <sup>b</sup>

WCK: soil only (no plantlets, no endophytes); PCK: rhizosphere and phyllosphere with sterile *A. lancea* plantlets; AL4: rhizosphere and phyllosphere with *A. lancea* plantlets inoculated with AL4 (*Cunninghamella* sp.); AL12: rhizosphere and phyllosphere with *A. lancea* plantlets inoculated with AL12 (*Gilmaniella* sp.). Values are means ± SD,  $n=3$ . Values followed by different letters differed significantly at  $P \leq 0.05$  (Duncan's test)

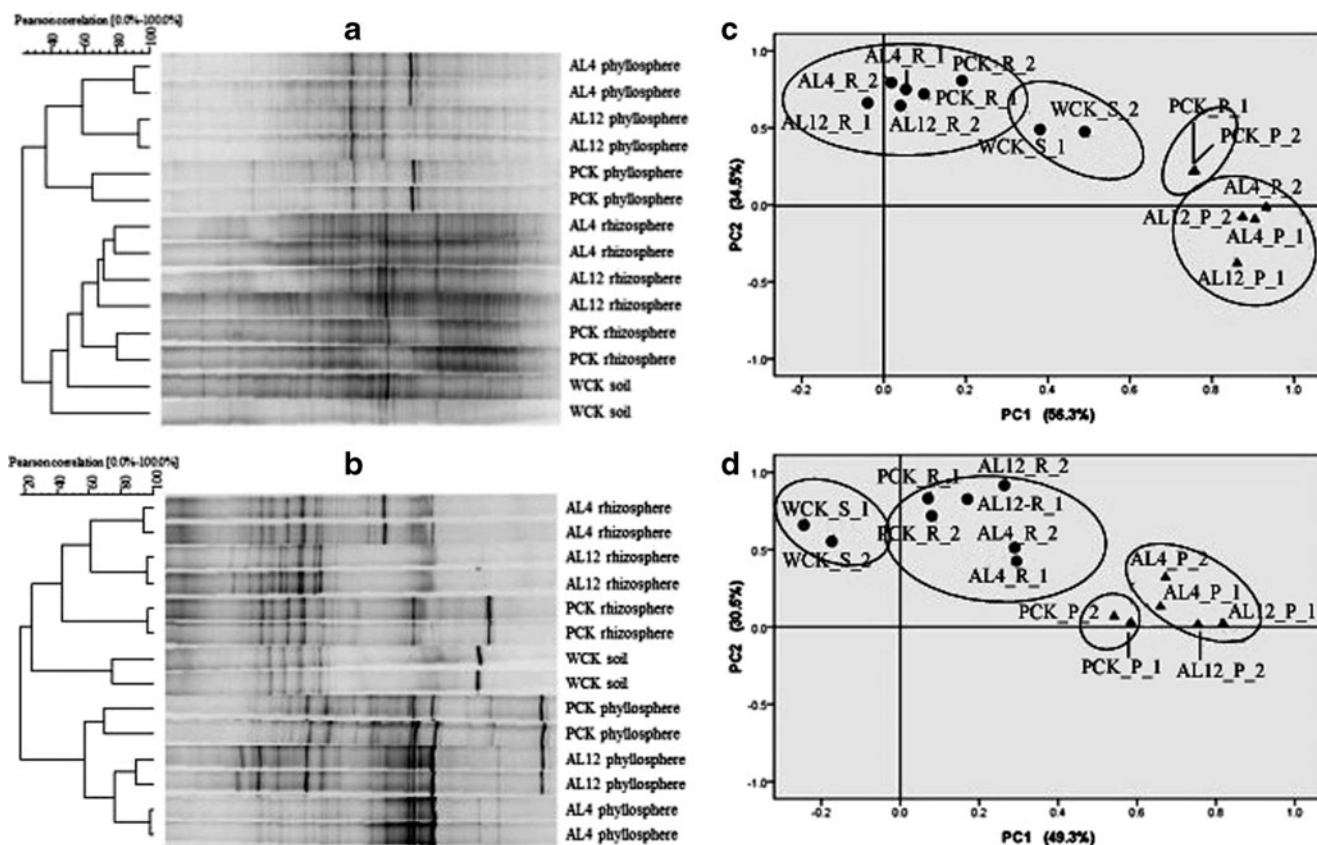
and soil were easily distinguishable. The DGGE profiles and PCA analysis of the fungal communities are shown in Fig. 2b and d. Similarly, the phyllosphere and rhizosphere were easily distinguishable. Compared with the cluster analysis of bacterial communities, there are smaller differences in fungal communities among rhizospheric replicates, implying that for a given treatment, the fungal communities among different pots were more stable than bacterial communities in the rhizosphere. Specifically, for phyllospheric and rhizospheric fungi, the endophytes-inoculated treatments AL4 and AL12 grouped together, and then grouped with the PCK group. The PCA analysis also showed that WCK and PCK groups were easily distinguishable from one another.

Table 5 showed Shannon diversity indices and the number of bands in rhizosphere and phyllosphere. The number of bands and Shannon index in rhizosphere were higher than those in phyllosphere. For phyllospheric bacteria, the Shannon index and the number of bands could be ranked from highest to lowest as follows: AL4 > AL12 > PCK. For phyllospheric fungi, they could be ranked from highest to lowest as follows: AL12 > PCK > AL4. The results were similar to that from the colony forming units. For rhizospheric fungi and bacteria, Shannon index and the number of bands could be ranked from highest to lowest as follows: AL4 > AL12 > PCK > WCK. There were small differences in the number of bands among every treatment, but the number of

**Table 4** Representative bacterial and fungal genera identified by conventional culturing methods

Treatment group		Bacteria	Fungi
Rhizosphere or soil	WCK	<i>Advenella</i> (3), <i>Stenotrophomonas</i> (3), <i>Delftia</i> , <i>Alcaligenes</i> , <i>Tetrathiobacter</i> , <b><i>Acinetobacter</i></b> , <b><i>Bacillus</i></b>	<b><i>Penicillium</i></b> (2), <b><i>Fusarium</i></b> (3), <i>Rhizopus</i> (2), <i>Aspergillus</i> , <i>Mucor</i>
	PCK	<b><i>Bacillus</i></b> (3), <b><i>Acinetobacter</i></b> (3), <i>Paenibacillus</i> , <i>Delftia</i> , <i>Alcaligenes</i>	<b><i>Penicillium</i></b> (2), <b><i>Fusarium</i></b> (2), <i>Cladosporium</i> (2), <b><i>Acremonium</i></b> , <i>Aspergillus</i> , <i>Mucor</i>
	AL4	<b><i>Microbacterium</i></b> (3), <i>Klebsiella</i> , <i>Delftia</i> , <i>Alcaligenes</i> , <b><i>Bacillus</i></b> , <i>Tetrathiobacter</i> , <b><i>Acinetobacter</i></b>	<b><i>Penicillium</i></b> (3), <b><i>Fusarium</i></b> , <b><i>Acremonium</i></b> , <i>Mucor</i> , <i>Microsphaera</i> , <i>Physoderma</i> , <i>Coremium</i>
	AL12	<b><i>Microbacterium</i></b> (3), <i>Alcaligenes</i> , <b><i>Bacillus</i></b> , <i>Tetrathiobacter</i> , <b><i>Acinetobacter</i></b> , <i>Advenella</i>	<b><i>Penicillium</i></b> (2), <b><i>Fusarium</i></b> , <b><i>Acremonium</i></b> , <i>Rhizopus</i> , <i>Mucor</i> , <i>Microsphaera</i> , <i>Physoderma</i>
Phyllosphere	PCK	<b><i>Acinetobacter</i></b> (3), <b><i>Sphingomonas</i></b> , <i>Enterobacter</i> , <i>Arthrobacter</i> , <i>Alcaligenes</i> , <b><i>Bacillus</i></b>	<b><i>Penicillium</i></b> (3), <b><i>Fusarium</i></b> (2), <b><i>Acremonium</i></b> , <i>Alternaria</i> , <i>Aspergillus</i> , <i>Verticillium</i> , <i>Cephalosporium</i> , <i>Gliocladium</i>
	AL4	<b><i>Kocuria</i></b> (3), <b><i>Sphingomonas</i></b> (3), <i>Enterobacter</i> , <i>Kineococcus</i> , <i>Arthrobacter</i> , <b><i>Bacillus</i></b> , <b><i>Acinetobacter</i></b>	<b><i>Acremonium</i></b> (3), <i>Curvularia</i> , <b><i>Fusarium</i></b> , <b><i>Penicillium</i></b> , <i>Mucor</i> , <i>Absidia</i> , <i>Aspergillus</i>
	AL12	<b><i>Kocuria</i></b> (3), <b><i>Sphingomonas</i></b> (3), <i>Enterobacter</i> , <i>Alcaligenes</i> , <b><i>Bacillus</i></b> , <b><i>Acinetobacter</i></b>	<i>Curvularia</i> (3), <b><i>Fusarium</i></b> , <b><i>Penicillium</i></b> , <b><i>Acremonium</i></b> , <i>Mucor</i> , <i>Botrytis</i> , <i>Aspergillus</i> , <i>Gilmaniella</i>

All the listed microbes were the representative bacteria or fungi for every treatment group. Every repetition (flowerpot) contained four plates. Only when there were the same microbe genus on four plates, we recorded it as 1 in parenthesis. But we usually omit 1 in parenthesis. Numbers in parentheses indicated number of repetitions detected. The biggest was 3 repetitions, which meant there were the same microbes on twelve plates for every treatment group. The microbes which were font-bold were abundant in every plate. WCK: (control) soil without plantlets or endophytes; PCK: rhizosphere and phyllosphere with sterile *A. lancea* plantlets, AL4: rhizosphere and phyllosphere with *A. lancea* plantlets inoculated with AL4 (*Cunninghamella* sp.); AL12: rhizosphere and phyllosphere with *A. lancea* plantlets inoculated with AL12 (*Gilmaniella* sp.)



**Fig. 2** DGGE profile and analysis of rhizospheric and phyllospheric microbial communities **a**: Cluster analysis of bacterial communities; **b**: Cluster analysis of fungal communities; **c**: Principal component analysis of bacterial communities; **d**: Principal component analysis of fungal communities. WCK: (control) soil without plantlets or endophytes; PCK: rhizosphere and phyllosphere with sterile *A. lancea* plantlets,

AL4: rhizosphere and phyllosphere with *A. lancea* plantlets inoculated with AL4; AL12: rhizosphere and phyllosphere with *A. lancea* plantlets inoculated with AL12. In **c** and **d**, “filled triangle” indicates the microbial communities in phyllosphere, while “filled circle” indicates the microbial communities in rhizosphere or soil

**Table 5** Genotypic diversity indices of bacteria and fungi in rhizosphere and phyllosphere

	Treatment group	Bacteria		Fungi	
		S	H'(log e)	S	H'(log e)
Phyllosphere	AL4	29	2.869	18	2.257
	AL12	28	2.759	21	2.646
	PCK	26	2.654	19	2.517
Rhizosphere or soil	AL4	50	3.614	26	2.92
	AL12	48	3.556	23	2.75
	PCK	47	3.469	22	2.684
	WCK	41	3.372	20	2.54

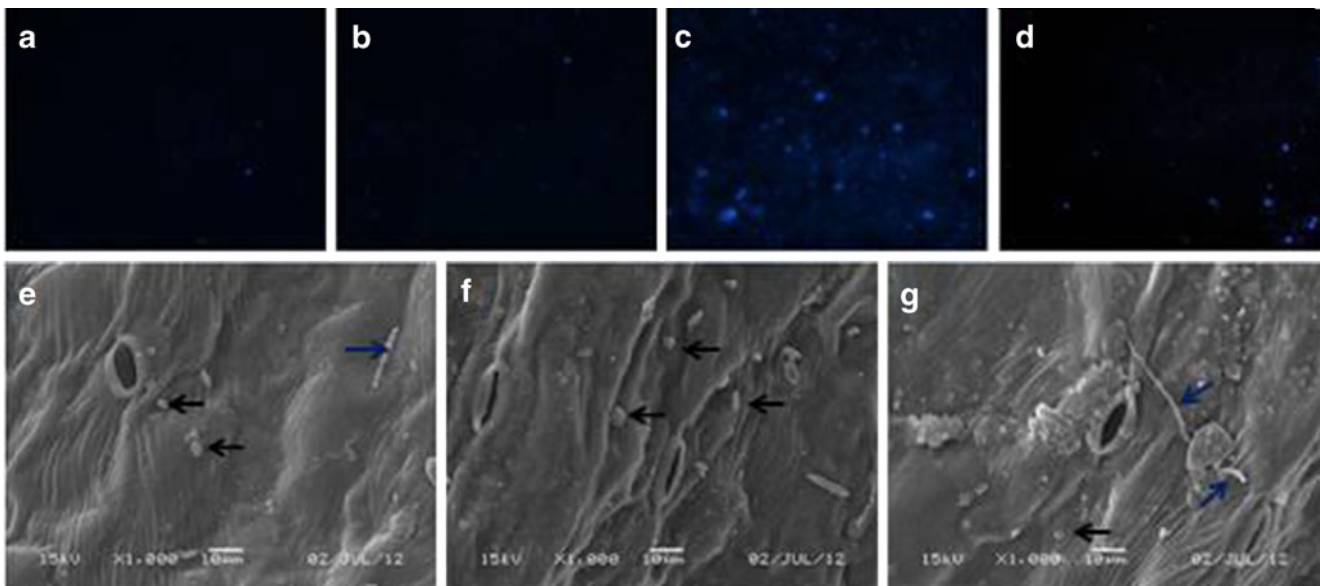
Shannon’s index:  $H' = -\sum (P_i \cdot \log_e P_i)$ , S: the number of bands in every lane. All the values were from the Gelcompar II results. The repetition number is two, but every repetition contained three individual samples’ mixture. WCK: (control) soil without plantlets or endophytes; PCK: rhizosphere and phyllosphere with sterile *A. lancea* plantlets, AL4: rhizosphere and phyllosphere with *A. lancea* plantlets inoculated with AL4 (*Cunninghamella* sp.); AL12: rhizosphere and phyllosphere with *A. lancea* plantlets inoculated with AL12 (*Gilmaniella* sp.)

bacterial bands in the WCK group was significantly smaller than the PCK group.

### 3.3 Microscopic observation of microbial communities in rhizosphere and phyllosphere

We used fluorescence microscopy and scanning electron microscopy to analyze the microbial communities in the different treatment groups. As shown in Fig. 3, in rhizospheric soil the largest quantity of total microbes was in the AL4 (*Cunninghamella* sp.) treatment, while the smallest was in the WCK group. The intense blue staining indicated the large amount of microbial DNA in the rhizosphere. This result was similar to those obtained from the spread-plate method and DGGE profile analyses. Although the dehydration steps during SEM analyses caused some deformation of bacterial and fungal morphologies, it was clear that there were more bacteria in the phyllosphere of the AL4 group than in that of the PCK group, and more fungi in the phyllosphere of the AL12 group than in those of the other treatment groups, similar to the results shown in Table 3.





**Fig. 3** Microbes in the rhizosphere and phyllosphere of *A. lancea* **a**: WCK soil; **b**: rhizospheric soil of PCK; **c**: rhizospheric soil of AL4; **d**: rhizospheric soil of AL12; **e**: phyllosphere of PCK; **f**: phyllosphere of AL4; **g**: phyllosphere of AL12. Soil samples were stained by DAPI and

observed under a fluorescence microscope. Phyllosphere samples were observed by SEM. Blue arrows mark representative fungi, black arrows mark representative bacteria

### 3.4 Elemental content of leaves

We analyzed elemental levels in the phyllosphere by energy dispersive X-ray spectrometry. As shown in Table 6, for endophytes-inoculated treatments (AL4 *Cunninghamella* sp. and AL12 *Gilmaniella* sp.), they had lower levels of nitrogen, calcium and aluminum and higher levels of copper. In addition, different endophyte-inoculated treatments had different effects on elemental content of leaves. The AL4 phyllosphere had higher levels of carbon, oxygen, sodium, silicon and iron compared with those in other treatments, while the AL12 phyllosphere had lower levels of carbon, oxygen, sodium, silicon and potassium. It was notable that nitrogen was detected only in the PCK phyllosphere. These findings indicated that the endophyte symbiosis with the host plant resulted in changing the elemental levels in the phyllosphere.

### 3.5 Soluble saccharides, free amino acids and organic acids in root exudates

The results obtained from the spread-plate method and DGGE profile analyses showed the rhizospheric microbial communities were affected by endophytes. Rhizospheric exudates are a rich source of nutrition for microbes. Soluble saccharides, free amino acids, and organic acids are the three main kinds of carbon and nitrogen sources in plant rhizospheric exudates. The effects of endophytes on the concentration of these substances in each treatment are shown in Table 7. Inoculation with AL4 (*Cunninghamella* sp.) significantly decreased the concentration of soluble saccharides and increased that of free

amino acids in the root exudates. Inoculation with AL12 (*Gilmaniella* sp.) significantly increased the concentration of soluble saccharides. Both AL4 and AL12 groups showed significantly lower organic acids concentration in root exudates compared with the PCK group.

In endophytes-inoculated groups, the effect of AL4 on rhizospheric microbes was most significant, and the concentration of free amino acids in AL4 group was larger than the sum of those in AL12 group and PCK group. One question was whether high levels of free amino acids were related to rhizospheric microbial communities' changes? To answer this the concentration of 16 free amino acids was determined by HPLC analysis (Table 8). It was found that there were differences between endophytes-inoculated treatments and endophytes-free treatment. Seven kinds of free amino acids which were detected in all treatments and histidine was not detected in any groups. Endophytes strongly affected free amino acids. The AL4 group lacked glycine and cysteine. AL12 and PCK groups lacked methionine. The AL12 group also lacked alanine, tyrosine, valine, leucine and lysine. The kind of free amino acids in AL12 group was significantly smallest. We conducted a significant difference analysis. The concentrations of two kinds of acidic amino acids (glutamic acid and aspartic acid) were larger in the endophytes-inoculated groups than the PCK group. For the concentration of other amino acids, the three treatment groups could be ranked, from highest to lowest, as follows: AL4 > PCK > AL12. In the AL12 group, serine was the only amino acid which was significantly at higher levels than that in other groups.

**Table 6** Basic element contents in phyllosphere

Group	Net counts of basic elements									
	C	O	N*	Na	K	Ca	Fe	Cu	Al	Si
PCK	6274±106 <sup>b</sup>	2408±96 <sup>b</sup>	150±86	3696±90 <sup>b</sup>	664±109 <sup>b</sup>	1423±117 <sup>c</sup>	152±54 <sup>a</sup>	352±62 <sup>a</sup>	1018±63 <sup>c</sup>	1904±126 <sup>b</sup>
AL4	9058±129 <sup>c</sup>	3813±118 <sup>c</sup>	-	7815±120 <sup>c</sup>	602±61 <sup>b</sup>	1132±65 <sup>b</sup>	297±66 <sup>b</sup>	551±74 <sup>b</sup>	698±72 <sup>b</sup>	2858±147 <sup>c</sup>
AL12	4098±80 <sup>a</sup>	893±63 <sup>a</sup>	-	1468±56 <sup>a</sup>	427±41 <sup>a</sup>	266±43 <sup>a</sup>	61±50 <sup>a</sup>	549±63 <sup>b</sup>	154±35 <sup>a</sup>	107±42 <sup>a</sup>

“-”: Element net count lower than limit of detection. PCK: sterile *A. lancea* plantlets; AL4: *A. lancea* plantlets inoculated with AL4 (*Cunninghamella* sp.); AL12: *A. lancea* plantlets inoculated with AL12 (*Gilmaniella* sp.). The number of cation results are based upon 24 Oxygen atoms. In order to compare the differences among treatments conveniently, we used net counts instead of k-ratio. Values are means ± SD ( $n=3$ ). Values followed by different letters differed significantly at  $P \leq 0.05$  (Duncan's test)

## 4 Discussion

Previous researches on fungal endophytes focused on the interaction between plants and endophytes (Wang et al. 2011, 2012; Ren and Dai 2012) and focused on the effects of endophytes on secondary metabolites rather than primary metabolites (Tan and Zou 2001; Wang et al. 2011). Our results showed that endophytes-induced changes in primary metabolites correlate with the successional shifts of microbial communities.

### 4.1 The rhizospheric/phyllospheric microbial communities' characteristics for normal *A. lancea* plantlets

The present study is the first to investigate the rhizospheric and phyllospheric microbial communities of *A. lancea*. The phyllospheric microbial diversity was significantly smaller than that of rhizosphere. This may reflect the fact that the microbial community residing in the phyllosphere is faced with a nutrient poor and variable environment characterized by fluctuating temperature, humidity and UV radiation, while the rhizospheric microbes reside in a more stable environment and provide with nutrition from plants (Bodenhausen et al. 2013). The fungal communities in these two microhabitats were more similar than the bacterial communities. This may be because bacteria are more sensitive than fungi to environment variables. It is interesting that colonization by the fungal endophyte *Neotyphodium*

**Table 7** Concentration of total soluble saccharides, free amino acids, and organic acids in root exudates

Groups	Soluble saccharides (µg/mL)	Free amino acids (µg N content/mL)	Organic acids (µmol/15 mL)
PCK	141.65±1.41 <sup>b</sup>	3.36±0.39 <sup>a</sup>	18.4±0.97 <sup>c</sup>
AL4	80.34±4.56 <sup>a</sup>	7.06±0.4 <sup>b</sup>	13.42±0.93 <sup>b</sup>
AL12	157.37±1.68 <sup>c</sup>	3.25±0.27 <sup>a</sup>	11.59±0.69 <sup>a</sup>

Values are means ± SD ( $n=3$ ). Values followed by different letters differed significantly at  $P \leq 0.05$  (Duncan's test). PCK: sterile *A. lancea* plantlets; AL4: *A. lancea* plantlets inoculated with AL4 (*Cunninghamella* sp.); AL12: *A. lancea* plantlets inoculated with AL12 (*Gilmaniella* sp.)

in Italian rye grass caused changes in the bacterial community structure, but not in the fungal community (Casas et al. 2011).

The Gram-negative genus *Acinetobacter* and the Gram-positive genus *Bacillus* were dominant in the rhizosphere of the PCK group, while *Acinetobacter* was also dominant in the phyllosphere of the PCK group. *Acinetobacter* are important environmental microbes that are widely distributed in soil and water (Smith et al. 2007). *Bacillus* species are widely distributed in farmland ecosystems, and some are plant growth-promoting bacteria (PGPB), increasing plant health and growth

**Table 8** Concentration (µmol/15-mL root exudate) of free amino acids in root exudates of different treatment groups

Amino acid	Treatment group		
	PCK	AL4	AL12
Asp	40.94±0.57 <sup>b</sup>	39.68±0.41 <sup>b</sup>	33.09±0.85 <sup>a</sup>
Glu	75.17±0.79 <sup>c</sup>	22.15±0.23 <sup>b</sup>	14.41±0.47 <sup>a</sup>
Ser	6.50±0.22 <sup>a</sup>	16.7±0.18 <sup>b</sup>	18.32±0.36 <sup>c</sup>
Thr	4.48±0.43 <sup>b</sup>	10.56±0.35 <sup>c</sup>	2.65±0.12 <sup>a</sup>
Arg	15.79±0.19 <sup>b</sup>	31.28±0.41 <sup>c</sup>	12.63±0.22 <sup>a</sup>
Phe	4.57±0.35 <sup>b</sup>	14.66±0.37 <sup>c</sup>	3.83±0.14 <sup>a</sup>
Ile	5.27±0.17 <sup>b</sup>	13.08±0.21 <sup>c</sup>	3.12±0.11 <sup>a</sup>
His*	-	-	-
Gly*	3.87±0.25	-	3.81±0.19
Ala*	4.71±0.14	5.23±0.25	-
Tyr*	1.56±0.12	22.00±0.22	-
Cys-s*	9.19±0.21	-	7.87±0.14
Val*	1.79±0.42	2.86±0.21	-
Met*	-	3.79±0.24	-
Leu*	1.85±0.32	17.99±0.43	-
Lys*	12.80±0.21	14.51±0.43	-
∑AA	189.92	214.94	99.22

Values are means ± SD ( $n=3$ ). In every gray-deepened row, values followed by different letters differed significantly at  $P \leq 0.05$  (Duncan's test). “-”: Element net count was lower than limit of detection. PCK: sterile *A. lancea* plantlets; AL4: *A. lancea* plantlets inoculated with AL4 (*Cunninghamella* sp.); AL12: *A. lancea* plantlets inoculated with AL12 (*Gilmaniella* sp.). ∑AA, sum of concentration of the 16 amino acids listed above

(Compant et al. 2010). In both the rhizosphere and phyllosphere, *Fusarium* and *Penicillium* were the dominant fungi in the PCK group. In an investigation on the endophytic fungal community of *A. lancea*, *Fusarium* and *Penicillium* were also the dominant fungi (Chen et al. 2008), which supports our hypothesis that the endophytes of *A. lancea* come from random infection (horizontal transmission) outside.

Generally speaking, plant leachates or exudates are abundant in the rhizosphere and this accounts for the larger microbial population in the rhizosphere than that in soil. However, in the present study, the bacterial population in the WCK group (pots with soil but not plantlets) was similar to that in the PCK group (plantlets without endophytes), but the fungal population in the WCK group was smaller than that in the PCK group. It may be relevant that the rhizome of *A. lancea* rich in essential oils (Hasada et al. 2010; Meng et al. 2010). During growth lots of sesquiterpenes with anti-bacterial activity are secreted to the rhizosphere (Tholl et al. 2005; Hasada et al. 2010; Meng et al. 2010; Wawrzyn et al. 2012). They have a stronger inhibitory effects on bacteria than on fungi which weakens the bacterial “rhizosphere effect”. As mentioned above, the bacterial diversity in the PCK group (plantlets without endophytes) was significantly larger than that in the WCK group (pots with soil but not plantlets), so the presence of the plant positively affects bacterial diversity as has been reported before (Ryser et al. 2005).

#### 4.2 The effect of fungal endophytes on rhizospheric or phyllospheric microbes

Biotic factors such as host genus, genotype and leaf age can all affect the microbial community structure in the phytosphere (Lundberg et al. 2012), and abiotic factors such as temperature, season, water, pH and nutrition level also strongly affected them (Zimmerman and Vitousek 2012). Rhizobia, mycorrhizal fungi and *Piriformospora indica* can affect the rhizospheric or phyllospheric microbial communities of associating plants (Offre et al. 2007; Ikeda et al. 2008; Nautiyal et al. 2010; Scheublin et al. 2010; Ikeda et al. 2011). Perhaps the changes in microbial community facilitate the formation of a mutualism between the host and the plant symbiont (Frey et al. 1997; Cruz et al. 2008; Pivato et al. 2009; Rigamonte et al. 2010).

Colonization by endophytes enhanced the diversity and population size of the rhizospheric microbial communities, and endophyte AL4 (*Cunninghamella* sp.)’s effect was more pronounced. In the phyllosphere, endophytes-inoculation also changed microbial abundance and diversity differently. In plants collected from the wild, the endophytes AL4 and AL12 (*Gilmaniella* sp.) colonize the aboveground parts of *A. lancea* (Chen et al. 2008) which may be the reason why they affect the phyllosphere microbes to a greater extent. Previous research showed that when both strains (AL4 and AL12) colonize *A. lancea*, one strain antagonizes the growth

of the other (Yang and Dai 2013). More importantly, AL12 can regulate some biochemical pathways including salicylic acid, nitric oxide, hydrogen peroxide, and jasmonic acid (Ren and Dai 2012; Wang et al. 2011). Perhaps by affecting these biochemical pathways, AL12 stimulates other phyllospheric fungi to grow. Because different fungal endophytes have their own species-specific characteristics, they affected bacterial and fungal communities differently, which reveals the complexity of the effect of endophytes on other microbes.

Endophytes-inoculation changed the bacterial and fungal genera except that the dominant fungal genus in rhizospheric communities was still *Penicillium*. In the rhizosphere, the dominant bacteria of the endophytes-inoculated group was *Microbacterium* species. For the phyllosphere, the dominant bacteria of endophytes-inoculated group were *Kocuria* species and *Sphingomonas* species. Recent research showed that *Kocuria rhizophila*, *Microbacterium ginsengisoli*, and *Sphingomonas* sp. are bacteria that are closely associated with arbuscular mycorrhizal fungi (AMF) (Lecomte et al. 2011). Our research has shown that these genera are also closely associated with fungal endophytes. *Microbacterium* was the predominant bacterial genus on three endophyte-colonized prairie plants and on three endophyte-colonized agronomic crops (Zinniel et al. 2002). Some plants provide microbes with species specific nutrients (Innerebner et al. 2011), while other plants secrete special anti-microbes substance which certain microbes can resist (Rosenblueth and Martinez-Romero 2004). Why did the endophytes AL4 and AL12 allow *Microbacterium*, *Kocuria* and *Sphingomonas* to be the dominant bacteria? We will continue to research this in the future.

The dominant fungal genera in the phyllosphere were affected by the presence of the fungal endophytes. The previously dominant genera *Fusarium* and *Penicillium* were replaced by *Acremonium* and *Curvularia*, respectively. Our unpublished results indicate that *Acremonium* is the dominant endophyte genera in the Mao Shan area, but it was not isolated in the Shaanxi area of China. Our experimental tissue culture plantlets were all from Mao Shan. We hypothesize that the previous colonization of AL4 (*Cunninghamella* sp.) stimulated colonization of leaf surfaces by *Acremonium*. Similarly, the previous colonization by AL12 (*Gilmaniella* sp.) may stimulate colonization on leaf surfaces by *Curvularia*, both of which can be endophytes of other plants (Gond et al. 2007; Marquez et al. 2007).

#### 4.3 The effect of fungal endophytes on rhizospheric or phyllospheric exudates—the possible cause of change in microbial communities

Endophytes significantly affected plant epibiotic microbial communities including their abundance, community diversity and dominant genera. But the underlying mechanism was not revealed. *Neotyphodium* changed the bacterial community in

the rhizosphere, which was closely associated with changes in soil catabolic profiles (Casas et al. 2011). The endophytic fungus *Neotyphodium lolii* competed with mycorrhizal fungi *Glomus* spp. for carbohydrates and other host-derived resources (Liu et al. 2011). In these cases, symbiotic fungal infections were correlated with available nitrogen and carbon sources in their host plants. By adjusting the nutrient levels, the symbiotic microbes may form stable associations with other microbes in the phytosphere.

In the rhizosphere, both AL4 (*Cunninghamella* sp.) and AL12 (*Gilmaniella* sp.) significantly decreased organic acids concentration in root exudates. However, AL4 significantly increased the concentration of free amino acids in the root exudates, while AL12 significantly increased the concentration of soluble saccharides. In the rhizosphere, free amino acids and soluble saccharides were two main kinds of nitrogen and carbon source, respectively. These vital nutrients will stimulate the rhizospheric microbes' growth. Compared with high levels of soluble saccharides, high levels of free amino acids seem to more significantly affect the growth of rhizospheric microbes. Rhizospheric microbes preferred to utilize the soluble saccharides as a first-choice carbon source, but in the rhizosphere, many fungi and bacteria can also utilize other complex macromolecular carbon compounds (Chen et al. 2011, 2013a). In contrast, free amino acids may function as signaling molecules or food sources. Hence, the AL4 treatment contained the largest populations of microbes in the rhizosphere, and the concentration of free amino acids in AL4 group was larger than the sum of those in the AL12 group and the PCK group.

Certain kinds of amino acids are the indispensable growth factor for some heterotrophic microbes in the rhizosphere (Hartmann et al. 2009). Additionally, the rhizospheric microbes exist in a nutrient-poor environment but are able to take up amino acids rapidly (Hobbie and Hobbie 2012). AL4 (*Cunninghamella* sp.) had the most diverse and largest rhizospheric bacterial community. In addition, most bacteria can utilize quorum sensing to form tight associations with the host plants. Quorum sensing signals can be divided into two main categories: amino acids (Diggle et al. 2007) and fatty acid derivatives (Dunny and Winans 1999). Organic acids may also function as signaling molecules or as plant allelochemicals, variations in their concentrations may affect the rhizospheric microbial communities. High concentrations of allelochemicals negatively affected microbial diversity in the rhizosphere. Similarly, once quorum sensing signals reach a certain threshold concentration, the bacterial population size decreases. Hence, it is possible that the decreases in organic acids concentration resulting from colonization by AL4 or AL12 enhanced the diversity and size of the bacterial populations.

Previous research has shown that the endophyte AL12 can significantly stimulate the transfer of carbon from *A. lancea* from leaves to roots and the accumulation of carbon in roots,

including cellulose, hemicellulose, lignin, soluble sugars and volatile oils (Gao et al. 2012). This may explain why the AL12 group (*Gilmaniella* sp.) had less carbon in the phyllosphere and more soluble saccharides in rhizosphere. Interestingly, the AL12 group had the largest amount of serine, which can transform to homoserine. Homoserine lactone is a key quorum sensing signal (Yang et al. 2013). In addition, serine is the active center of many microbial metabolic enzymes. The highest level of serine may explain the effect of AL12 (*Gilmaniella* sp.) on rhizospheric microbes. The kind of detected amino acids was greatest in the PCK (plantlets with no endophytes), which implies that colonizing endophytes must use some amino acids in their nutrition from host plants. In conclusion, the endophytes AL4 (*Cunninghamella* sp.) and AL12 (*Gilmaniella* sp.) in symbiosis with *A. lancea* caused concerted and successional shifts in the rhizospheric and phyllospheric microbial communities. These shifts were probably mediated by endophytes-induced changes in leachates or exudates from plant roots and leaves.

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