Community structure of arbuscular mycorrhizal fungi associated with *Robinia pseudoacacia* in uncontaminated and heavy metal contaminated soils

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Abstract: The significance of arbuscular mycorrhizal fungi (AMF) in soil remediation has been widely recognized because of their ability to promote plant growth and increase phytoremediation efficiency in heavy metal (HM) polluted soils by improving plant nutrient absorption and by influencing the fate of the metals in the plant and soil. However, the symbiotic functions of AMF in remediation of polluted soils depend on plant-fungus-soil combinations and are greatly influenced by environmental conditions. To better understand the adaptation of plants and the related mycorrhizae to extreme environmental conditions, AMF colonization, spore density and community structure were analyzed in roots or rhizosphere soils of *Robinia pseudoacacia*. Mycorrhization was compared between uncontaminated soil and heavy metal contaminated soil from a lead-zinc mining region of northwest China. Samples were analyzed by restriction fragment length polymorphism (RFLP) screening with AMF-specific primers (NS31 and AM1), and sequencing of rRNA small subunit (SSU). The phylogenetic analysis revealed 28 AMF group types, including six AMF families: *Glomeraceae*, *Claroideoglomeraceae*, *Diversisporaceae*, *Acaulosporaceae*, *Pacisporaceae*, and *Gigasporaceae*. Of all AMF group types, six (21%) were detected based on spore samples alone, four (14%) based on root samples alone, and five (18%) based on samples from root, soil and spore. Glo9 (*Rhizophagus intraradices*), Glo17 (*Funneliformis mosseae*) and Acau3 (*Acaulospora* sp.) were the three most abundant AMF group types in the current study. Soil Pb and Zn concentrations, pH, organic matter content, and phosphorus levels all showed significant correlations with the AMF species compositions in root and soil samples. Overall, the uncontaminated sites had higher species diversity than sites with heavy metal contamination. The study highlights the effects of different soil chemical parameters on AMF colonization, spore density and community structure in

contaminated and uncontaminated sites. The tolerant AMF species isolated and identified from this study have potential for application in phytoremediation of heavy metal contaminated areas.

Key words: Arbuscular mycorrhizal fungi (AMF), Environmental interactions, Heavy metal pollution, Phytoremediation, *Robinia pseudoacacia*.

1. Introduction

Soil contamination with toxic heavy metals is a serious and widespread issue resulting from both natural and anthropogenic activities. Mining, agriculture, smelting, electroplating, and other human activities are the major anthropogenic sources of heavy metals found in soils (Gómez-Sagasti et al. 2012). Heavy metals cannot be degraded easily like organic pollutants and can therefore constitute a persistent environmental hazard. Long-term impacts of heavy metal accumulation in soils include adverse effects on microbial biomass, activity, and diversity (Alguacil et al., 2011; Margesin et al., 2011). In most terrestrial ecosystems, soil microorganisms play a critical role in mineral cycling and organic material decomposition. Therefore, any adverse change in the environment impacting soil microbial communities can greatly affect ecosystem functions (Wang et al., 2010).

The arbuscular mycorrhizal fungi (AMF), belonging to the phylum Glomeromycota (Schübler et al., 2001), form mutualistic symbioses with most terrestrial plants (Smith and Read, 2008). It is well established that mycorrhizal plants take up water and mineral nutrients, especially phosphorus (P), more efficiently than non-mycorrhizal plants (Smith and Read, 2008). In addition, AMF protect host plants against diverse biotic and abiotic stresses, such as heavy metals, because of their beneficial role in enhancing metal tolerance and nutrient acquisition

(Doubková et al., 2012; Lehmann et al., 2014). However, the symbiotic functions of AMF are diverse on the level of AMF species, host plants species, soil properties and even AMF ecotypes (Hildebrandt et al., 2007; Hempel et al., 2009; Zarei et al., 2010). Distributions of AMF are also affected by many environmental parameters. Among them, mineral nutrients, especially phosphorus, seem to be more important than other environmental factors. Generally, higher content of phosphorus in soils is associated with lower mycorrhizal root colonization rates and lower AMF diversity (Cheng et al., 2013). Moebius-Clune et al. (2013) suggested that soil texture rather than phosphorus or pH may have an influence on AMF community structure. Although several studies on the effect of heavy metals on abundance and diversity of AMF have been conducted, we still have a poor understanding of the factors that determine AMF community structure, symbiotic functioning and adaptive evolution (Antunes et al., 2012). Therefore, a better understanding of AMF communities in heavy metal contaminated areas is important to fully evaluate the plant-AMF (König et al., 2010; Thoms and Gleixner, 2013), AMF-AMF (Jansa et al., 2008) and AMF-soil (Bedini et al., 2010) interactions. The effects of heavy metals on soil environments are complex because of the complex array of parameters (Wang et al., 2010), and therefore it is crucial to identify specific phylotypes or ecotypes of AMF and their relationship with specific soil environments (Zarei et al., 2010). Consequently, a comparative analysis of AMF community structure in polluted soils with different heavy metal concentrations is essential for identification of metal-tolerant AMF isolates and ecotypes to develop efficient phytoremediation techniques (Zarei et al. 2010; Hassan et al., 2011).

A variety of methods have been designed to analyze the diversity of AMF in field soils and plant roots (Sanders, 2004). Most current studies are based on spore counts and on morphological identification of AMF spores extracted directly from the field (Gai et al., 2006; de

Oliveira Freitas et al., 2014; Symanczik et al., 2014; Wetzel et al., 2014). A limitation of this method is that spore density assessed in soils does not necessarily reflect the AMF population that is actually colonizing the plant roots (Clapp et al., 1995; Hempel et al., 2007). In fact, some AMF species may not be detected at all because they sporulate only infrequently or not at all in the field (Clapp et al., 1995; Sanders, 2004). Recently developed molecular tools for detection of AMF species directly in plant roots and soils result in a better understanding of the abundance and diversity in this group of fungi (Schüßler et al., 2001; Chen et al., 2014; Krishnamoorthy et al., 2014). Helgason et al. (1998) designed the primer AM1 to match all small subunit ribosomal RNA (SSU) sequences known at that time from the *Glomales*. In combination with the universal eukaryotic primer NS31 (Simon et al., 1992), the AM1 primer can amplify partial 18S rRNA sequences (approximately 550 bp) from the three traditional families of *Glomales*. Compared to other regions, AMF SSU sequences have relatively low variability, which makes phylogenetic analysis more convenient (Kohout et al., 2014). However, the primers for the SSU rRNA gene cannot cover the whole range of AMF, and usually co-amplify DNA from plants (Alguacil et al., 2011; Hazard et al., 2014) or non-target fungal groups (Borriello et al., 2012; Chen et al., 2014).

Nowadays, the use of molecular methods may provide a more detailed analysis of root or soil AMF communities compared with spore-based morphological approach. However, undoubtedly, sporulation is a key part of the AMF life-cycle, and therefore, the analysis of AMF diversity based on spore 18S rRNA can reflect an important life history strategy of AMF for surviving in abiotic environments. In order to obtain a more dynamic and more complete picture of the AMF abundance and diversity actually present in rhizosphere soils and roots, we have combined the sequence analysis based on the SSU region of rRNA genes of AMF in rhizosphere soil, root and spore samples extracted directly from study sites. In the present study, we compared AMF status

and community structure in the root and rhizosphere of a metal tolerant tree, black locust (*Robinia pseudoacacia* L.), among study sites contaminated or uncontaminated with Pb, Zn, Cu or Cd. Black locust is a commonly planted legume tree in China with high drought tolerance (Yang et al., 2014) and has been extensively used for reforestation to control soil erosion in the Loess Plateau. Black locust can form symbiotic associations with both nitrogen-fixing rhizobia and phosphorus-acquiring AMF. As a pioneer species, *R. pseudoacacia* appeared to be a promising woody legume for phytoremediation in heavy metal polluted areas due to its fast growth, deep root system, heavy metal tolerance and ability to fix atmospheric nitrogen (Vlachodimos et al., 2013). In our previous investigation, we reported that *R. pseudoacacia* is frequently found in heavy metal polluted areas and commonly colonized by AMF (will be published separately). However, a detailed knowledge of AMF status in these bacteria-AMF-plant interactions under heavy metal polluted conditions is still lacking. The objectives of the present study was to (1) compare different experimental methods to analyze AMF community structure by molecular analysis of root, soil and spore samples collected directly from study sites; (2) evaluate the toxic effect of heavy metals (Pb, Zn, Cu and Cd) on AMF status, abundance and community structure; and (3) identify environmental factors controlling status, abundance and species compositions of AMF in forest soils. We hypothesize that (1) AMF status, richness and diversity would differ between heavy metal polluted and unpolluted areas due to different disturbance tolerance among AMF species; and (2) AMF community structures present in root, soil and spore samples in different study sites would differ because of different ecological preferences and life history strategies among AMF species.

2. Materials and methods

2.1. Study sites

The study sites are located in Qiandongshan lead and zinc region, Feng County, northwest part of China. Qiandongshan lead and zinc region is the largest and the most typical of five national nonferrous metal planning mines, and accounts for 25% of the total reserves of Feng County (Yao et al., 2004). The predominant pollution sources in this region are mine wastewater, beneficiation wastewater, and mine tailings (Hou et al., 2003). The region has a warm temperate semiarid climate with an annual average temperature of 11.4°C. The annual average rainfall and frost-free period of Feng County are 613.2 mm and 188 days, respectively. The main soil types is cinnamon and brunisolic soil according to the traditional soil genesis classification in China, and the soil texture is from light to heavy (Xu et al., 2012). The mineral resources are very abundant and mining industry has become an important economic mainstay in Shaanxi Province. Specifically, mineral resources of lead and zinc are mostly distributed in this region and its annual output accounts for 72% of the entire provincial output. Feng County, with an annual production capacity of 100,000 tons of zinc (Zn) concentrates, 30,000 tons of lead (Pb) concentrates, 10,000 tons of electrolytic lead, and 5,000 tons of lead alloy, has become one of the four largest Pb-Zn bases in China. However, due to the traditional development model, which focuses more on economic growth using less advanced technologies and neglecting environmental protection, a large amount of waste from factories has caused serious environmental pollution.

The heavy metal polluted area was divided into sites of high (S9, 33°51.288'N, 106°39.429'E; S10, 33°51.290'N, 106°39.438'E), medium (S8, 33°51.034'N, 106°38.647'E), low (S6, 33°51.972'N, 106°39.312'E; S7, 33°49.810'N, 106°39.054'E), and non-polluted (S1-S5, 34°03'54"N-34°04'20"N, 106°46'30"E-106°48'38"E) levels. The separation between polluted

and non-polluted soils was based on recommendation of environmental quality standard (Grade II) in soils of China (GB 15618-1995). The division of polluted soils into levels of high, medium, and low pollution was based on the variations of Pb and Zn concentrations in the study area.

2.2. Sampling and analysis

Sampling was conducted from August to September (rainy season), 2012. At each site, three healthy, similarly-sized *R. pseudoacacia* were randomly selected. The plants were young trees of 7-12 years old with 4-7 m height and 3.5-8.0 cm diameter at breast height (DBH). The soil samples were collected beneath each tree from at least three directions. In each direction, the top 3-cm was removed to eliminate part of the leaf litter and one large hole (diameter, 50 cm; depth, 30 cm) was excavated in order to collect enough root fragments. The large hole was within 100 cm of the trunk to ensure that the collected root samples belonged to the correct species. The roots were carefully excavated and traced from the originating tree to ensure identity. The soils loosely and tightly bound to the surface of roots were removed by clean tweezers and brush, and defined as rhizosphere soil samples for DNA extraction and spore density determination. The zone we studied here was sometimes more than 1 mm wide from the surface of the roots (rhizosphere soil) due to the moist soil during the rainy season, but this zone of soil was still influenced by root secretions and associated soil microorganisms to a certain extent. The remaining extracted soils from the hole at different directions were homogenized and then approximately 500-1000 g soil was placed in plastic bags for determination of soil physical-chemical properties. The soil samples were sieved (2 mm mesh size) to remove stones, coarse roots and other litter. In some sites heavily polluted by heavy metals, additional large-diameter excavations were necessary to collect adequate quantities of fine roots for DNA extraction and measurement of mycorrhizal colonization. For each tree, portions of each composite root sample

were contained in 50 ml centrifuge tubes filled with formaldehyde-acetic acid alcohol (FAA) for determining mycorrhizal colonization; the remaining root samples and rhizosphere soil samples were kept in cooling boxes with ice cartridges during transport to the laboratory and were stored at 4°C prior to DNA extraction.

2.3. Soil chemical properties

The soil samples were air dried at room temperature, ground to a fine powder in an agate mortar, and then sieved through a 10 mesh (< 2 mm) and an 80 mesh (< 180µm). The finely ground powder (< 180 µm) was used to determine the chemical properties and the coarse powder (< 2 mm) was used to measure pH and electrical conductivity (EC). Soil chemical and physical analysis was conducted in the College of Natural Resources and Environment, Northwest A&F University, Yangling, P.R. China. The soil pH was determined using a combination glass electrode (Leici PHS-3D, Shanghai, China) according to the international analysis method of ISO 10390: 2005 (soil/water, 1:5). The air dried soil samples were shaken with deionized water (soil:solution ratio, 1:5) for 3 min and the EC of the supernatant was determined with a conductivity meter (DDSJ-308A, Zhejiang, China). Soil organic matter (OM) content was measured by dichromate oxidation and titration with ferrous sulfate (Nelson et al, 1982). Total nitrogen (TN) content was determined according to the semi-micro Kjeldahl method (Bremner et al., 1982). Soils were treated with HF-HClO₄, and then the total phosphorus (TP) content was measured colorimetrically after wet digestion with HF-HClO₄ (Jackson, 1958). Available nitrogen (AN) was determined with a micro-diffusion technique after alkaline hydrolysis (Page, 1982). Available phosphorus (AP) was determined by extraction with a buffered alkaline solution of 0.5 M sodium bicarbonate (pH = 8.5). The product was quantified colorimetrically in a spectrophotometer (Hitachi, UV2300) at 660 nm (Page, 1982). The total and DTPA-extractable

heavy metal concentrations were determined by flame atomic absorption spectrometry (FAAS, Hitachi Z-2000, Tokyo, Japan), following the dissolution of a 0.5 g soil sample with aqua regia ($\text{HNO}_3/\text{HCl} = 1:3$) and HClO_4 , DTPA solution (0.005 M diethylene triamine penta-acetic acid (DTPA), 0.01 M CaCl_2 , 0.1 M triethanolamine, pH = 7.3). The blank reagent and standard reference soils were analyzed for quality assurance and quality control. All of the results were calculated from triplicate analytical data.

2.4. Mycorrhizal colonization analysis

To evaluate mycorrhizal colonization, the root samples were washed with tap water, and then cut into about 1-cm length segments. The method modified by Koske and Gemma (1989) was used to clean and stain root samples. The segments were first softened in 2.5% KOH at 90 °C for 1 h, bleached in alkaline hydrogen peroxide at room temperature for 30 min, acidified in 1% HCl at room temperature for 1 h, and then stained with trypan blue (0.05%) at 90 °C for 20 min. The mycorrhizal colonization was estimated according to Trouvelot et al. (1986). More than two hundred root fragments per site were used to measure the mycorrhizal colonization (MC) under a light microscope (Olympus Bx51, Japan) at 200 × magnification. The percentage of mycorrhizal structures in each 1 cm root fragment was assessed as 0, 10, 20...100%. The intensity of MC was measured as follows (Liu and Li, 2000):

$$\text{MC}\% = \frac{\sum(0\% \times N_0 + 10\% \times N_{10} + 20\% \times N_{20} + \dots + 100\% \times N_{100})}{(N_0 + N_{10} + N_{20} + \dots + N_{100})}$$

Where N is the number of root fragments.

2.5. Spore isolation

AMF spores were isolated from soil samples using wet sieving and sucrose density gradient centrifugation (Daniels and Skipper, 1982). Briefly, distilled water was added to 30 g of soil and the solution passed through a sequence of sieves (2000, 500 and 32 μ m). The soil fraction in the last sieve was collected into plastic tubes. Spore suspensions (25 ml) were transferred to 50 mL centrifugation tubes, and another 25 ml of a 70% sucrose solution were added at the bottom of the tubes, which were then centrifuged for 2 min at 3000 rpm. Spores were collected from the water-sucrose interface, washed and transferred to Petri dishes with filter paper for sorting and quantification under a dissection microscope at up to $\times$ 400 magnification. Only spores that appeared to be viable (based on color, shape, surface condition and examination of spore contents) were counted (Eom et al., 2001). AMF spore density was expressed as number of spores in one gram of dry soil. Spores from soil samples from each site were pooled and then grouped according to spore morphology and color using a dissecting microscope. Spore number of each morphotype in each pooled sample was counted, and the spore morphotypes were further identified using the molecular method as described below.

2.6. Molecular analysis

Fine roots and rhizosphere soil were homogenized in liquid nitrogen using ceramic mortar and pestle. Root DNA was extracted from approximately 100 mg lyophilized roots using the Plant DNA Extraction Kit (Tiangen Biotech, China) following the manufacturer's instructions. Soil DNA was extracted from approximately 500 mg lyophilized soils using the E.Z.N.A. Soil DNA Kit (Omega, USA) following the protocol of the manufacturer. A total of 60 samples, consisting of 30 soil samples and 30 root samples, were subjected for DNA extraction. The purity and the concentration of the DNA were measured by SmartspecTM Plus Spectrophotometer (Bio-Rad, USA), and the quality was checked by agarose gel electrophoresis (Invitrogen, USA) with

ethidium bromide staining (Sigma, USA). The DNA samples were then diluted 1: 10 with ddH₂O to be used as PCR template. For spore samples, up to four clean and healthy-looking spores of each morphotype per pooled sample were transferred into microcentrifuge tubes and vortexed at maximum speed. Spores were further rinsed four times with sterilized distilled water. Single spores were transferred into tubes with 10 µL ddH₂O, and crushed with forceps under the dissecting microscope.

Partial AMF 18S ribosomal RNA (about 550 bp) was amplified from soil, root and spore DNA extracts with the universal eukaryotic primer NS31 (Simon et al., 1992) and primer AM1, designed to amplify AMF 18S ribosomal RNA sequences but not plant sequences (Helgason et al., 1998). The PCR reactions were carried out in a final volume of 25 µL, containing 12.5 µL 2 × Taq Master Mix (Beijing CoWin Biotech Co., Ltd), 0.5 µL each primer (10 µM, Invitrogen), 1 µL template and 10.5 µL RNase-Free Water with the following cycling conditions: 94 °C for 4 min, followed by 30 cycles of 94 °C for 50 s, 56 °C for 60 s, 72 °C for 70 s and extension at 72 °C for 10 min. All the PCR reactions were run in BioRad PCR cycler (USA). PCR products were examined on a 1.5% (w/v) agarose gel with ethidium bromide staining in the presence of DL2000 DNA ladder. PCR products were purified using Universal DNA Purification Kit (Tiangen Biotech CO., LTD, Beijing, China). Purified PCR products were cloned into the plasmid pGEM-T vector following the manufacturer's instructions of the pGEM-T Cloning Kit (Tiangen Biotech CO., LTD, Beijing, China), and then the ligation products were transformed into DH5α competent cells. Totally, 60 clone libraries were constructed (30 clone libraries for root samples and soil samples). Within each clone library, 40 positive clones were selected and restriction digested using *Hinf*I, and *Hsp92*II enzymes according to the manufacturer's advice (Takara, Kyoto, Japan). Digested products were examined on a 2.0% (w/v) agarose gel.

1 Representatives of each RFLP type detected were sequenced with primer T7 by GenScript
2 Corporation (Nanjing, China).

3 **2.7. Data analysis**

4 All glomeromycotan sequences were aligned using DNAMAN version 6.0 (Lynnon Biosoft,
5 USA), and clustered to species-level groups according to 97% sequence similarity. Each species-
6 level group was regarded as an AMF group type. The AMF group types found in this study and
7 the GenBank sequences most similar to clone sequences were included in the phylogenetic tree
8 construction. Phylogenetic analysis was performed using MEGA version 5.0, and the neighbor-
9 joining tree was constructed by performing 1000 replicates to produce bootstrap values. The 54
10 sequences obtained in this study had been deposited in the GenBank database with accession
11 numbers KM233891-KM233916 and KM659233-KM659260.

12 The sequence group data were imported into SPSS v16.0 (Chicago, USA) to calculate the
13 Shannon-Wiener index (H'), Margalef (d_{Ma}), Evenness (E_h) and Simpson index (D) as follows:

$$H' = - \sum_{i=1}^S P_i \ln P_i, P_i = \frac{n_i}{N}$$

$$d_{Ma} = \frac{S - 1}{\ln N}$$

$$E_h = \frac{H'}{H_{max}}$$

$$D = 1 - \sum_{i=1}^S P_i^2$$

14 where N is the total number of clones selected from each clone library, S is the total number of
15 AMF sequence groups detected in each site, n_i is the number of the i^{th} sequence group present in
16 each clone library.

Redundancy analyses (RDA) were conducted to determine the multivariate relationship between AMF community compositions and environment variables using the software Canoco (version 4.5, Centre for Biometry, Wageningen, The Netherlands). Pearson's correlation coefficients were calculated to determine the relationships among environmental factors, mycorrhizal colonization, spore density and the biodiversity indices by using SPSS software version 16.0 ($P < 0.05$). Significant differences were detected by employing a one-way analysis of variance (ANOVA) ($P < 0.05$) and significant differences between means were determined by Duncan's test ($P < 0.05$). Principal component analysis (PCA) was performed to compare the AMF communities from root, soil and spore samples at family level. In this analysis, the presence or absence of each family was scored as "1" or "0", respectively.

3. Results

3.1. Soil properties and AMF status

The main characteristics of the soil samples collected from ten study sites are presented in Table 1. The soil samples were all slightly alkaline with pH of the ten study sites ranging from 7.45 (S9) to 8.36 (S2). S10 had the highest EC (0.94 dS/m), TP (0.85 g/kg), TN (1.50 g/kg), AP (15.6mg/kg) and AN (25.5 mg/kg), whereas the highest OM were found at S9 (12.2 g/kg). The values of pH, OM, EC, TP, TN, TP, AP and AN showed small but significant difference between study sites, but no obvious difference of AN could be found among the five study sites located in uncontaminated area ($P > 0.05$). The concentrations of heavy metals showed a large variability among the five study sites selected from contaminated area (Table 1). S10 had the most serious heavy metal pollution and the total concentrations of Pb and Zn at this site were 16.9 and 2.7 times higher than the environmental quality standard (Grade II) in soils of China (GB 15618-

1995), whereas S6 was only slightly polluted by Pb (65.4 mg/kg), Zn (136 mg/kg), Cu (29.0 mg/kg) and Cd (0.32 mg/kg). The DTPA-extractable Pb, Zn, Cu and Cd presented the same pattern as total Pb, Zn, Cu and Cd (Table 1). In our study, the heavy metal concentrations of soils were found to decrease in the order Pb>Zn>Cu>Cd at all study sites. The increasing order of total/DTPA-extractable concentrations of Pb at five study sites located in polluted area was S6<S7<S8<S9<S10, whereas the increasing order of total/DTPA-extractable concentrations of Zn and Cu at these five study sites changed to S7<S6<S9<S8<S10 (Table 1). The concentration of DTPA-extractable heavy metals in relation to total concentration was as follows: Pb: 9.1%, Zn: 2.4%, Cu: 4.5% and Cd: 23.5%, when all soil samples were taken into consideration.

Mycorrhizal colonization (MC) and AMF spores could be detected even at the most heavily contaminated site. The spore density (SD) varied among study sites, ranging from 2.19/g soil (S7) to 5.77/g soil (S5), but no significant correlation could be found between SD and any environmental parameters ($P > 0.05$) (Supplementary Table S1). The MC significantly decreased with increasing total/DTPA-extractable Pb, Zn concentrations ($P < 0.01$), however, no significant correlation could be found between MC and soil EC, TN, AP and AN levels ($P > 0.05$) (Supplementary Table S1). The overall trend was higher mycorrhizal colonization and AMF spore density in uncontaminated areas and lower in heavily contaminated areas, with intermediate values in moderately contaminated areas (Table 1)

3.2. AMF community structure based on molecular analysis

The AMF occurrence was analyzed in 30 root samples and 30 rhizosphere soil samples of *R. pseudoacacia*. Template DNA was successfully amplified with the PCR primers AM1-NS31, yielding PCR products of expected length (about 550 bp). For the 60 clone libraries, a total of 2400 clones were screened, and 2273 clones containing insert of correct size of SSU rRNA gene

1 were investigated by *Hinf*I and *Hsp*92II RFLP typing. Between one and four representatives of
2 each RFLP type was sequenced, yielding 54 AMF and nine non-AMF sequences. Representative
3 sequences of each sequence phylotype were submitted to the NCBI database and were included
4 in the phylogenetic tree shown in Fig 1.

5 The neighbor-joining phylogenetic analyses of 54 representative AMF sequences from
6 rhizosphere zone soil samples (Y1-Y19), root samples (Y23-Y45) and spore samples (Y19-Y22,
7 Y45-54) revealed 28 AMF sequence groups. The sequence groups or phylotypes covered six
8 families of *Glomeromycota*: the *Glomeraceae*, *Claroideoglomeraceae*, *Diversisporaceae*,
9 *Acaulosporaceae*, *Pacisporaceae*, and *Gigasporaceae*. Of all the AMF sequence groups, 17
10 belonged to the *Glomus* group, three to *Acaulospora*, one to *Claroideoglomus*, two to
11 *Diversispora*, two to *Pacispora*, one to *Entrophospora* and two to *Scutellospora*. All the AMF
12 genera in the soil samples were also found in the root samples except for *Acaulospora*, *Pacipora*
13 and *Scutellospora* (Fig. 1). The constructed phylogenetic tree included 38 sequences which were
14 downloaded from GenBank and 54 different AMF sequences recorded in this study. All
15 sequence groups had high similarity to AMF and belonged to the phylum *Glomeromycota*.
16 Eleven AMF sequence groups were related to known AMF species because sequences of the
17 same type were obtained from spores collected in our study. These were Glo10 and Glo11 for
18 *Rhizophagus clarum*, Glo14 for *Glomus constrictum*, Glo15 for *Funneliformis caledonium*, Glo17
19 for *Funneliformis mosseae*, Acau1 for *Acaulospora mellea*, Acau2 for *Acaulospora lacunose*,
20 Dive1 for *Diversispora spurca*, Dive2 for *Diversispora celata*, Scut1 for *Scutellospora*
21 *heterogama* and Scut2 for *Scutellospora dipurpurescens*. Two groups, Glo16 and Paci1, did not
22 seem to be related to any sequences of AMF in the database since these phylotypes did not
23 cluster closely with any sequences of reference AMF isolates in international culture collections.

Thus, they may be unique to this study. The remaining groups clustered with sequences of uncultured AMF species (Fig. 1).

3.2.1. AMF communities in roots of *R. pseudoacacia*

The compositions of AMF communities in roots of *R. pseudoacacia* in all 30 samples were determined based on the percentages of clones of the 28 AMF sequence types (Fig. 1). Of the AMF group types detected by the RFLP analysis of root samples, 15 were found in roots of plants growing on uncontaminated sites, with an average of 1.0 group types per sample, while 14 group types were detected in roots of plants growing on contaminated sites, with an average 0.9 group types per sample. In root samples collected from uncontaminated sites, the most frequent group type was Glo9 (33.2%), followed by Glo17 (27.2%). In addition, five group types of Glo2 (0.67%), Glo4 (0.67%), Glo12 (0.67%), and Glo14 (1.33%) were only recorded in roots from uncontaminated sites. In contrast, the abundant AMF group types of root samples collected from HM contaminated sites were Glo9 (35.8%) and Glo17 (37.7%). The third most common group was Acau3, which could not be assigned to any morphologically described species. Acau3 was present in 16.5% of all clones in uncontaminated sites, but was significantly less common in HM contaminated sites (13.8%). Three group types of Glo6 (0.50%), Glo8 (0.17%) and Dive1 (0.67%) were only recovered from roots in HM contaminated sites. Eleven AMF group types of Glo3, Glo5, Glo7, Glo9, Glo10, Glo13, Glo15, Glo17, Clar1, Acau3 and Entr1 were found in roots from plants growing on both HM contaminated and uncontaminated sites (Fig. 2).

3.2.2. AMF communities in rhizosphere soils of *R. pseudoacacia*

Rhizosphere soils of *R. pseudoacacia* had a characteristic AMF community that was clearly different from that of the roots. Significant differences were found in the relative frequencies of

two sequence groups. Sequence group Glo17 is of particular interest since it was found more frequently in soil compared with root samples, accounting for 41.0% of all clones in uncontaminated sites. However, Glo9 was seldom found in soil samples, and only 4.2% of all clones had this sequence group in uncontaminated sites. Soil samples contained more sequence groups (22 types) than root samples (18 types). In soil, 22 AMF group types were detected in samples from uncontaminated sites, with up to 4.4 group types per site. Nineteen AMF group types were detected in HM contaminated soil samples, with an average of 3.8 group types per site. Glo17 and Acau3 were the two most dominant AMF group types in soils contaminated or uncontaminated by heavy metal (Fig. 2).

3.2.3. AMF community structure based on molecular identification of spores

In our study, only 14 out of 36 single spores were successfully amplified and then subjected to RFLP analysis. One representative of each spore-derived RFLP type was sequenced and a total of 11 sequence groups were detected. A BLAST search showed that five sequence groups belonged to *Glomeromycota*, two groups belonged to *Acaulosporaceae*, two groups belonged to *Diversisporaceae*, and two groups were related to *Gigasporaceae*, partly representing AMF community in spore level (Fig. 1). The two most common AMF sequence groups were Glo17 and Acau1 both in contaminated (44.8% for Glo17 and 16.0% for Acau1) and uncontaminated sites (41.0% for Glo17 and 12.7% for Acau1) when all the spores identified by both molecular and morphological methods were taken into consideration (Fig. 2).

3.3. Ecological measures of diversity

The Species richness (S), Shannon-Wiener index (H'), Margalef (d_{Max}), and Evenness (E_h), Simpson index (D) were calculated for each study site (Supplementary Table S2). Species

richness (S) were 8.5 for root samples, 11.4 for soil samples, and 5.2 for spore samples (molecular identification) collected from contaminated and uncontaminated sites. The diversity indices of S , H' and d_{Max} showed significant difference between uncontaminated and contaminated sites for soil samples, whereas no difference could be found for root and spore samples ($P > 0.05$). Significant differences were showed in diversity indices of E_h and D for root samples between uncontaminated and contaminated sites; however, they did not show differences for soil and spore samples ($P > 0.05$, Fig. 3).

Linear correlation analysis was used to determine the relationship between diversity indices of AMF communities and soil properties (Table 2). For root and soil samples, the results showed that Species Richness index (S) and Margalef index (d_{Max}) had significantly negative correlations with soil available P content, total Pb, Zn, Cd and DTPA-extractable Zn concentrations ($P < 0.05$), while the Shannon-Wiener index (H') index had obviously negative correlation with soil OM content, total and DTPA extractable Pb and Zn concentrations ($P < 0.05$). For root samples, Evenness index (E_h) and Simpson index (D) had significantly positive correlations with soil pH, but negative correlations with soil OM content, total Pb, Zn, Cd and DTPA-extractable Pb, Zn and Cu concentrations ($P < 0.05$). On the contrary, Evenness index (E_h) and Simpson index (D) of soil samples only had significantly negative correlations with soil EC, total Pb, Zn and DTPA-extractable Zn concentrations ($P < 0.05$). For spore samples, the Species Richness index (S) and Margalef index (d_{Max}) showed significantly negative correlations with soil OM content, total Zn, Cu and DTPA-extractable Pb and Zn concentrations ($P < 0.05$), while the Shannon-Wiener index (H') index only had negative correlations with soil total P content ($P < 0.01$). However, no correlation could be found between Evenness index (E_h), Simpson index (D) and soil properties ($P > 0.05$).

3.4. Redundancy analysis (RDA)

To further resolve the effect of environmental variables on the AMF communities, fifteen environmental factors were analyzed by using Redundancy analysis (RDA). RDA analysis was conducted on each type of samples separately, due to the differences in AMF community structure between root and soil samples (Fig. 2). Spore samples were removed from the analysis because most of the single spores failed to amplify in this study. Results of the RDA are shown in Fig. 4. The length of the arrows indicate the relative importance of each environmental factor in explaining variation of community structures, while the angle between the arrows or axis indicated the degree to which they were correlated (Fig. 4). In root samples, more than 84.2% of the variance in AMF communities could be explained by the two canonical axes. The first canonical axis explained 71.1% of the detected AMF diversity and was negatively correlated with soil pH. The second axis represented 13.1% of variance and was positively correlated with soil OM content, total/DTPA-extractable Cu and Cd concentrations. Soil properties related to pH, EC, OM content, total/DTPA-extractable Cd and Zn concentrations had a strong influence on the AMF composition structure in root samples as indicated by the length of their arrows in the RDA plots, but still a large proportion of the variance remained unexplained. Soil total Cu and DTPA-extractable Cd concentrations were positively associated with Glo6, Glo8 and Dive1, but showed negative correlation with Glo14 in root samples (Fig. 4a). Glo9 was significantly associated with soil EC, total N and available P contents, while Glo17 was abundant within roots of *R. pseudoacacia* growing in soils polluted by Pb, Cu and Cd. Glo4, Glo10, Glo12 and Glo15 were positively related to soil pH, but negatively correlated with soil OM content, DTPA-extractable Pb and Cu concentrations. AMF groups Glo2, Glo3, Glo5, Acau3 and Clar1 found in the upper left part of the graph were negatively correlated with soil total Pb, Zn and DTPA-extractable Zn

concentrations, and Entr1 was only negatively correlated with EC and available P (Fig. 4a). Eight of the fifteen environmental variables fitted as vectors onto the RDA plot were significantly correlated with the AMF community structure in root samples; of these, soil pH, OM, total/DTPA-extractable Zn concentrations were most strongly related to the AMF community composition (Supplementary Table S3).

In the soil samples, a total of 70.0% of the cumulative variance in the AMF community data set was explained by the first two canonical RDA axes (Fig. 4b). The first canonical axis explained 51.9% of AMF composition in soil samples and was negatively correlated with soil pH, while, the second axis explained 18.1% of variance and was positively correlated with soil pH and EC. Soil available P, DTPA-extractable Zn and total Pb, Zn and Cd concentrations showed positive correlation with Glo7 and Acau3, but were negatively related to Glo2, Glo3, Glo5 and Paci2. Glo4 was associated with soil total Cu concentration, however, AMF group Glo6, Glo8, Glo9, Glo12, Glo15, Clar1, Dive1 and Entr1 were represented in the bottom left part of the graph without clear association with environmental parameters (Fig. 4b). Glo10, Glo13 and Glo14 were negatively associated with Cu and Cd contamination, and AMF groups Glo1, Glo7 and Glo16 found in the upper right part of the graph were influenced by both soil pH and EC. Five of the fifteen environmental variables fitted as vectors onto the RDA plot were significantly correlated with the AMF community structure in soil samples; of these, soil total Pb, Zn and DTPA-extractable Zn concentrations were most strongly related to the AMF community composition (Supplementary Table S4).

4. Discussion

1 Under natural conditions, 80%-90% of plants are colonized by AMF leading to mutualistic
2 associations that have been found in most vegetative systems and climates, including some
3 aquatic ecosystems (Gamalero et al., 2009). The extensive extraradical hyphal network produced
4 by AMF allows the plants to access a greater volume of the soil, resulting in the enhancement of
5 plant nutrient absorption and translocation (Fellbaum et al., 2014). The first step of our current
6 study was to analyze the mycorrhizal structures such as hyphae, arbuscules (*Arum*-type and
7 *Paris*-type) and vesicles of *R. pseudoacacia* because the heavy metals were deposited mainly in
8 the inner root parenchyma cells where most of these fungal structures reside (Hildebrandt et al.,
9 2007). Intracellular hyphae, arbuscules and vesicles were commonly found in all study sites and
10 *Arum*-type was the dominant structure (Supplementary Fig. S1, d and e). A few roots forming
11 mycorrhizal structures of *Paris*-type could be also observed in our investigation (Supplementary
12 Fig. S1, c). Higher percentage of arbuscules was observed in soils that were heavily polluted by
13 Pb, Zn, Cu and Cd (Supplementary Fig. S1, e), which may be attributed to alleviation of heavy
14 metal toxicity in *R. pseudoacacia*. The previous studies conducted by our lab in the Pb and Zn
15 mining area showed a negative effect of Pb concentration on mycorrhizal colonization (Xu et al.,
16 2012). With the present analysis, we extend the focus of our work by taking additional
17 environmental factors into account. The correlation analysis showed that MC had a significantly
18 positive relationship with soil pH ($P = 0.038$), but was negatively associated with soil OM
19 content ($P = 0.019$), total P content ($P = 0.033$) and strongly inhibited by Zn and Pb
20 contamination (Supplementary Table S1). These results are in agreement with Vogel-Mikuš et al.
21 (2005) who reported that mycorrhizal colonization levels were significantly lower or absent on
22 the most Pb, Zn and Cd polluted plots. Wu et al. (2010) indicated that elevated concentrations of
23 As, Pb, Zn, Cd and Cu exerted harmful effects on spore numbers of AMF in abandoned

As/Pb/Zn mines. However, our results did not support that and no significant difference could be found between AMF spore density and Pb, Zn, Cu, Cd concentrations or with any of the other studied environmental factors (Supplementary Table S1). The inhibition of mycorrhizal colonization in the contaminated soil could in part be due to the high availability of metals caused by low pH (Table 1), inhibition of fungal spread in the soil (Pawłowska and Charvat, 2004), disturbance of vegetation and erosion of the locations (Liu et al., 2011). Heavy metals have been reported to reduce, delay, or even eliminate AMF colonization and spore density at heavy metal polluted sites (Lingua et al., 2008; Wei et al., 2014). However, in our study, AMF propagules never disappeared completely even in soils contaminated by high concentrations of heavy metals (Supplementary Fig. S1) and other studies have likewise found positive, negative or neutral effects on mycorrhizal colonization and spore density in Pb-Zn mining area (Zarei et al., 2008a; Khade and Adholeya, 2009; Wu et al., 2010). These conflicting observations indicated that the effects of heavy metals on the extent of AMF status may depend on other environmental factors, such as AMF species, host plants, season of the year, stage of plant development, plant nutritional status and environmental conditions (Bever et al., 2001; Oehl et al., 2003; Pande et al., 2004).

The phylogenetic analysis revealed that 17 out of 28 AMF groups belonged to *Glomus*, suggesting that it was the most dominant genus in the AMF community of *R. pseudoacacia* from all study sites. The dominance of *Glomus* species has been commonly found in surveys on AMF diversity in various habitats, such as Mediterranean soils (Alguacil et al., 2014), tropical forest soils (Husband et al., 2002), agricultural soils (Dai et al., 2014), grassland soils (Birgander et al., 2014), and heavy metal contaminated soils (Zarei et al., 2008a; 2008b). Yang et al. (2010) found that all the sequences detected in the roots of *Elsholtzia splendens* growing on Cu-contaminated

soil were related to *Glomus* species. Similar to our results, Hassan et al. (2011) indicated that *Glomus* was one of the most common AMF genus detected in trace metal polluted soils. The *Acaulospora* group was the second most dominant genus in our study (Fig. 2), although only 3 out of 28 AMF groups belonging to this genus were found (Fig. 1). Prevalence of *Acaulospora* species in some Chinese natural ecosystems was widely reported (Li et al., 2004). Our results are also in accordance with a study that detected *Glomus* and *Acaulospora* abundantly distributed in heavy metal polluted soils (Ortega-Larrocea et al., 2001). Three possible reasons could explain why *Glomus* and *Acaulospora* groups became the most dominant genera in our study. Firstly, *Glomus* and *Acaulospora* species have the ability to propagate by mycelial fragments and mycorrhizal root fragments, unlike other AMF species that require spore germination (Klironomos and Hart, 2002; Hassan et al., 2011). Secondly, as predominance genera (Daniell et al., 2001), *Glomus* and *Acaulospora* species already adapted to the local environment, and it became much easier for them to develop their own ability to survive in heavily disturbed environments compared with other AMF species. Thirdly, it is quite common for *Glomus* to form anastomoses between mycelia, which may therefore have the ability to re-establish an interconnected network in a disrupted environment (De La Providencia et al., 2005).

The large difference in the diversity of AMF among root, soil and spore samples collected from ten study sites was obvious in the present investigation (Fig. 2). Of particular note was the distribution of Glo9, Glo17 and Acau3. Glo9, showing a high similarity to *Rhizophagus intraradices*, is known as a generalist fungal species since it has been found in diverse environments (Öpik et al., 2003) and host species (Helgason et al., 2007). In the current study, Glo9 was found primarily in root samples (> 30%), but only 4% abundance of this group type was observed in soil samples and it was almost not detected in spore samples. Glo17, which

1 showed a high homology with *Funneliformis mosseae*, can also be considered a generalist fungal
2 species because it is very frequent in diverse ecosystems (Jansa et al., 2014). However, this
3 group species presented an almost opposite pattern in distribution among root, soil and spore
4 samples compared with Glo9. Glo17 was found in 32% of all clones in root samples, but this
5 value increased to 42% of all clones in soil samples (Fig. 2). AMF group Acau3, showing a high
6 homology with *Acaulospora* sp. has been commonly found in China (Zhang et al., 2004).
7 Similarly, the percentage of all clones for this group increased from 15% in root samples to 30%
8 in soil samples (Fig. 2). Our results were supported by previous studies conducted by Chen et al.
9 (2014) and Hempel et al. (2007) who reported that large differences in the AMF community
10 between the root, soil and even spore samples at the AMF species level. Wilde et al. (2009)
11 observed that *R. intraradices* occurred abundantly in roots of host plants but could hardly be
12 retrieved in batches of spores and not at all in single spores of soil samples by molecular
13 analyses. Hempel et al. (2007) suggested that this type of group may generally extend short
14 distances in soils relative to its abundance in roots. Another important aspect that needs to be
15 considered is the properties of the host plant (*R. pseudoacacia*). Black locust can form symbiotic
16 associations with both nitrogen-fixing rhizobia and phosphorus-acquiring AMF. The fixed
17 nitrogen provides legumes with an additional nitrogen source, but at the same time, they require
18 large amounts of energy and phosphorus to balance the nutrients. Black locust may preferentially
19 associate with specific AMF species that are particularly efficient in supplying phosphorus
20 (Scheublin et al., 2004). It is a reasonable assumption that *R. intraradices*, *F. mosseae* and
21 *Acaulospora* sp. might be good candidates for this role, although research on the differences in P
22 acquisition efficiency among AMF species is lacking.

1 The principal component analysis (PCA) revealed a distinct clustering of the AMF
2 communities in root, soil and spore samples at the family level (Fig. 5). The AMF diversity in
3 soil samples showed a much wider distribution in the PCA plot, suggesting the heterogeneity of
4 the soils in our study sites (Table 1). This result was consistent with Martínez-García et al. (2011)
5 who found much higher AMF genetic diversity in soils compared with that in roots. A strong
6 overlap among the community structure of AMF in root, soil and spore samples was noticed in
7 the current study (Fig. 5), suggesting that the plant, AMF and soil formed a unified whole,
8 interrelated and influencing each other.

9 Although distinct patterns of heavy metal tolerance among the AMF groups were found,
10 redundancy analysis (RDA) showed that heavy metal contamination were not the only soil
11 parameters influencing AMF sequence group type distribution (Fig. 3). In root samples, the
12 multivariate analysis showed that, apart from the total Pb, Zn, Cd and DTPA-extractable Pb and
13 Zn concentrations, soil pH, organic matter content (OM) and electrical conductivity (EC) greatly
14 influenced the AMF community structure (Fig. 3a, Supplementary Table S3). Detailed
15 information showed that, in soil samples, the AMF composition was highly associated with soil
16 EC, AP, total Pb, Zn and DTPA-extractable Zn concentrations (Fig. 3b, Supplementary Table
17 S4). Although factors influencing AMF community structure are varied (Zarei et al., 2010), some
18 studies have found heavy metal contamination (Pb, Zn, Cu and Cd) to be the dominant
19 parameters influencing AMF community structure (Del Val et al., 1999; Zarei et al., 2008b). In
20 addition to understanding how AMF communities are influenced by soil factors, we need to
21 determine the relationships among soil parameters in the local heavy metal polluted area with
22 unique and special geological and climate conditions. Generally, the effects of heavy metals on
23 plants and soil microorganisms are dependent both on the chemical speciation of metal and the

1 mobilization of the metal available for uptake (Krishnamurti et al., 2013). It has been widely
2 noted that the availability and toxicity of heavy metals to plants and AMF were associated with
3 soil P content, soil pH and organic matter content (OM) (Cao et al., 2003; Meharg, 2003;
4 Antoniadis et al., 2008). Among these factors, soil pH plays the most important role in
5 determining metal speciation, solubility from mineral surfaces, movement, and eventual
6 bioavailability of metals, due to its strong effects on solubility and speciation of metals both in
7 the soil as a whole and particularly in the soil solution (Zhao et al., 2010). Our data also provide
8 evidence that significantly negative correlations were observed between soil pH and DTPA-
9 extractable Pb ($P < 0.01$), Zn ($P < 0.05$), Cu ($P < 0.01$), Cd ($P < 0.01$) concentrations, but no
10 correlation was found between soil pH and total Pb or Zn concentrations ($P > 0.05$)
11 (Supplementary Table S5). Apart from soil pH, organic matter content (OM) in soil is also one of
12 the most important soil properties affecting heavy metal availability. The current study found
13 that organic matter content (OM) was positively correlated with soil DTPA-extractable Pb ($P <$
14 0.01), Zn ($P < 0.05$), Cu ($P < 0.01$) and Cd ($P < 0.01$) (Supplementary Table S5). Similar results
15 were demonstrated by Dai et al. (2004) who estimated DTPA-extractable Pb, Zn and Cd
16 concentrations in heavy metal contaminated soils and found that the concentrations of these
17 metals were positively correlated with organic matter content (OM) in soils. Bending et al. (2002)
18 reported that soil organic matter content could influence the functioning of the soil microbial
19 community, because OM affected the soil structural properties and the types of nutritional
20 substrates available. Soil EC provides an evaluation of soil stress level and is typically correlated
21 with pH. In agreement with our findings, Bainard et al. (2014) reported that soil pH or pH-driven
22 changes in soil chemistry and EC were highly correlated with the composition of the AMF
23 community in the soil, and impacted the composition of the AMF community that colonized crop

1 roots. In natural and agricultural ecosystems, the variability in soil pH has been shown to be one
2 of the most important factors influencing the structure and composition of AMF communities
3 (An et al., 2008; Dumbrell et al., 2011). A high P level is another factor that is known to lead to
4 reduced AMF diversity (Gosling et al., 2013). The explanation for this is that the relationship
5 between AMF and host plant was greatly regulated by soil P level, which could directly or
6 indirectly affect AMF status in plant roots and soils (Sheng et al., 2013). However, more studies
7 are still required to get a better understanding of the detailed relationship among soil factors and
8 their specific impacts on *R. pseudoacacia* growth and AMF community structure in heavy metal
9 contaminated areas.

10 **5. Conclusion**

11 The current study described the local AMF community composition and structure in heavy
12 metal contaminated and uncontaminated regions. The results showed that *Rhizophagus*
13 *intraradices*, *Funneliformis mosseae* and *Acaulospora* sp. were the three most dominant AMF
14 group types colonizing *R. pseudoacacia* roots in the study sites. Soil Pb and Zn concentrations
15 were found to be the most important soil factors, which severely affects AMF abundance and
16 community structure. However, other soil chemical parameters, such as soil pH, OM, EC and P
17 level, which showed variation between the sampling sites in the study area, also greatly
18 contributed to soil feedbacks on AMF diversity, as detected by redundancy analysis (RDA). It
19 can be concluded from the current study that several AMF group types presented in the lead-zinc
20 mining region were able to survive and overcome heavy metal polluted conditions and possessed
21 some degree of adaptation to heavy metal stress. Further studies are required to explore the
22 potential of isolates of *F. mosseae*, *R. intraradices* and *Acaulospora* sp. from heavy metal
23 polluted areas in phytoremediation by *R. pseudoacacia*.

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1 **Table legends**

2 **Table 1.** Chemical properties of the rhizosphere soil samples and AMF colonization of *Robinia*

3 *pseudoacacia* growing in uncontaminated area (S1-S5) and lead and zinc mining area (S6-S10).

4 **Table 2.** Correlation coefficients among soil properties and biodiversity indices.

Table 1. Chemical properties of the rhizosphere soil samples and AMF colonization of *Robinia pseudoacacia* growing in uncontaminated area (S1-S5) and lead and zinc mining area (S6-S10).

	Uncontaminated area					HM contaminated area				
Parameters	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
pH	8.16±0.13bc	8.36±0.13a	8.17±0.15bc	8.21±0.09b	8.18±0.09bc	8.24±0.15ab	8.06±0.11c	8.13±0.20bc	7.45±0.17e	7.88±0.16d
OM (g/kg)	7.29±0.28e	7.53±0.38e	7.54±0.33e	8.73±0.43c	7.32±0.43e	7.39±0.53e	8.29±0.29cd	8.15±0.32d	12.2±0.8a	10.3±0.77b
EC (dS/m)	0.74±0.05cd	0.74±0.05cd	0.80±0.04b	0.71±0.03cde	0.75±0.05c	0.73±0.04cde	0.70±0.04de	0.81±0.04b	0.68±0.04e	0.94±0.07a
TP (g/kg)	0.64±0.05cd	0.44±0.05g	0.25±0.05h	0.43±0.07g	0.55±0.10ef	0.72±0.09b	0.59±0.05de	0.67±0.10bc	0.52±0.05f	0.85±0.07a
TN (g/kg)	1.08±0.05cde	1.11±0.07cde	1.16±0.05bc	1.05±0.05ef	1.02±0.05f	1.12±0.06cd	0.82±0.10h	0.85±0.06h	1.23±0.20b	1.50±0.07a
AP (mg/kg)	11.4±0.9c	9.7±1.0f	13.3±1.4b	10.9±0.6cde	11.1±1.4cd	13.2±2.2b	10.0±0.9ef	14.1±1.3b	11.5±1.3c	15.6±1.4a
AN (mg/kg)	16.7±1.6cd	16.4±2.8cd	15.6±1.3d	17.1±1.3cd	17.9±1.9bc	19.4±2.3b	12.1±1.3e	10.2±1.0f	15.6±2.1d	25.5±2.8a
TPb (mg/kg)	20.8±3.7e	26.2±3.9e	20.8±3.6e	21.3±5.4e	20.5±4.1e	65.4±9.3e	181±32d	364±38c	1982±212b	5899±250a
TZn (mg/kg)	62.0±7.4g	79.0±8.9fg	58.2±9.7g	65.9±16.3g	105±17e	136±14e	94.1±20.7ef	611±41b	508±34c	812±21a
TCu (mg/kg)	29.4±2.9c	20.0±2.3e	18.1±3.1e	24.5±6.2d	24.2±6.4d	29.0±4.5c	36.6±3.5b	26.0±3.7cd	74.3±5.3a	37.1±4.5b
TCd (mg/kg)	0.27±0.09de	0.18±0.07e	0.23±0.03de	0.20±0.04e	0.22±0.07de	0.32±0.04d	0.77±0.11c	1.51±0.07a	1.31±0.17b	0.80±0.23c
DPb (mg/kg)	1.92±0.22e	1.62±0.33e	2.10±0.25e	2.70±0.62e	2.69±0.24e	5.56±0.46e	14.9±1.5d	29.3±2.8c	245±13a	161±11b
DZn (mg/kg)	1.47±0.15f	1.87±0.48ef	2.21±0.34de	1.84±0.38ef	1.69±0.26ef	2.58±0.41d	2.13±0.33def	13.5±1.2b	12.3±1.0c	18.6±0.7a
DCu (mg/kg)	0.81±0.09e	1.04±0.19d	0.93±0.16de	0.89±0.20de	0.88±0.11de	1.64±0.21c	0.96±0.16de	2.08±0.21b	2.99±0.21a	1.71±0.23c
DCd (mg/kg)	0.03±0.01e	0.03±0.00e	0.04±0.01e	0.14±0.01bc	0.03±0.00e	0.15±0.01b	0.12±0.02c	0.09±0.01d	0.25±0.04a	0.15±0.02b
MC (%)	38.6±2.6a	34.8±4.7ab	39.3±2.1a	40.0±4.8a	38.8±3.1a	29.3±1.8bc	31.7±3.6bc	27.6±3.6c	21.7±2.8d	12.6±3.3e
SD (g ⁻¹ soil)	3.90±0.38cd	4.93±0.31b	4.13±0.22c	2.95±0.39ef	5.77±0.46a	4.84±0.33b	2.19±0.33g	3.78±0.23cd	3.32±0.38de	2.48±0.76fg

Assay values are presented in means of three replicates ± standard deviations. Different letters indicate significant differences according to Duncan's test. Abbreviations: OM, organic matter; EC, electrical conductivity; TP, total phosphorus; TN, total nitrogen; AP, available phosphorus; AN, available nitrogen; TPb, total Pb; TZn, total Zn; TCu, total Cu; TCd, total Cd; DPb, DTPA-extractable Pb; DZn, DTPA-extractable Zn; DCu, DTPA-extractable Cu; DCd, DTPA-extractable Cd; MC, mycorrhizal colonization; SD, spore density.

1 **Table 2.** Correlation coefficients among soil properties and biodiversity indices.

Biodiversity index		pH	OM	EC	TP	TN	AP	AN	TPb	TZn	TCu	TCd	DPb	DZn	DCu	DCd
Root samples	<i>S</i>	NS	NS	(-) *	NS	NS	(-) *	NS	(-) *	(-) **	NS	(-) *	NS	(-) **	NS	NS
	<i>H'</i>	(+) *	(-) *	NS	NS	NS	NS	NS	(-) *	(-) **	NS	(-) **	(-) *	(-) **	(-) *	NS
	<i>dMax</i>	NS	NS	(-) *	NS	NS	(-) *	NS	(-) *	(-) **	NS	(-) *	NS	(-) **	NS	NS
	<i>Eh</i>	(+) *	(-) **	NS	NS	NS	NS	NS	(-) **	(-) **	(-) *	(-) **	(-) **	(-) **	(-) **	(-) *
	<i>D</i>	(+) *	(-) *	NS	NS	NS	NS	NS	(-) **	(-) **	NS	(-) **	(-) *	(-) **	(-) *	NS
Soil samples	<i>S</i>	(+) *	(-) *	NS	NS	NS	(-) *	NS	(-) **	(-) **	NS	(-) *	(-) **	(-) **	(-) *	NS
	<i>H'</i>	NS	(-) *	(-) *	NS	NS	NS	NS	(-) **	(-) **	NS	(-) *	(-) *	(-) **	NS	NS
	<i>dMax</i>	(+) *	(-) *	NS	NS	NS	(-) *	NS	(-) **	(-) **	NS	(-) *	(-) **	(-) **	(-) *	NS
	<i>Eh</i>	NS	NS	(-) **	NS	NS	NS	NS	(-) **	(-) **	NS	NS	NS	(-) **	NS	NS
	<i>D</i>	NS	(-) *	(-) *	NS	NS	(-) *	NS	(-) **	(-) **	NS	NS	(-) *	(-) **	NS	NS
Spore samples	<i>S</i>	NS	(-) *	NS	NS	NS	NS	NS	NS	(-) *	(-) *	NS	(-) *	(-) *	NS	NS
	<i>H'</i>	NS	NS	NS	(-) *	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	<i>dMax</i>	NS	(-) *	NS	NS	NS	NS	NS	NS	(-) *	(-) *	NS	(-) *	(-) *	NS	NS
	<i>Eh</i>	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	<i>D</i>	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

2 Abbreviations: *S*, Species richness; *H'*, Shannon-Wiener index; *dMax*, Margalef; *E_h*, Evenness; *D*, Simpson index;

3 OM, organic matter; EC, electrical conductivity; TP, total phosphorus; TN, total nitrogen; AP, available phosphorus;

4 AN, available nitrogen; TPb, total Pb; TZn, total Zn; TCu, total Cu; TCd, total Cd; DPb, DTPA-extractable Pb; DZn,

5 DTPA-extractable Zn; DCu, DTPA-extractable Cu; DCd, DTPA-extractable Cd; (-), negative correlation; (+),

6 positive correlation; * $P < 0.05$, ** $P < 0.01$, NS, no significance.

1 **Figure legends**

2 **Fig. 1.** Phylogenetic tree showing AMF sequences isolated from roots, spores and rhizosphere
3 soils of *Robinia pseudoacacia* based on partial small subunit sequences (SSU). The tree was
4 obtained by neighbor joining method. Numbers above branches denote bootstrap values from
5 1000 replications. Bootstrap values > 70% were shown. Sequences obtained in the present study
6 were labeled with the database accession number and the internal identification number.

7 **Fig. 2.** AMF communities in root, soil and spore samples collected from different study sites.
8 AMF communities are represented by the percentages of total clones belonging to each AMF
9 sequence group. Each color corresponds to a sequence group.

10 **Fig 3.** Species richness (S), Shannon-Wiener index (H'), Margalef (d_{Max}), and Evenness (E_h),
11 Simpson index (D) of AMF community in ten sampling sites. Different letters indicate
12 significant differences according to Duncan's test ($P < 0.05$).

13 **Fig. 4.** Redundancy analysis (RDA) of AMF group types, their distribution among the root
14 samples (a) and soil samples (b) collected from ten study sites, and correlation with soil chemical
15 parameters. Blue arrows indicated the AMF group types and red arrows represented soil
16 chemical parameters. Abbreviations: OM, organic matter; EC, electrical conductivity; TP, total
17 phosphorus; TN, total nitrogen; AP, available phosphorus; AN, available nitrogen; TPb, total Pb;
18 TZn, total Zn; TCu, total Cu; TCd, total Cd; DPb, DTPA-extractable Pb; DZn, DTPA-extractable
19 Zn; DCu, DTPA-extractable Cu; DCd, DTPA-extractable Cd.

20 **Fig. 5.** Principal component analysis (PCA) of AMF communities in root (R, red symbols), soil
21 (S, green symbols) and spore (SP, blue symbols) samples in response to different environmental
22 factors. The percentages in the axes show the percentage of variation explained by the analysis.

- 1 Triangle represents uncontaminated soils (N); diamond represents heavy metal contaminated
- 2 soils (H).

- 1 **Fig. 1.** Phylogenetic tree showing AMF sequences isolated from roots, spores and rhizosphere soils of *Robinia*
- 2 *pseudoacacia* based on partial small subunit sequences (SSU). The tree was obtained by neighbour joining method.
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