Impact of Funneliformis mosseae on the growth, lead uptake, and localization of Sophora viciifolia

Zhouying Xu, Yihui Ban, Ren Yang, Xiangyu Zhang, Hui Chen, and Ming Tang

Abstract: On the basis of a pot experiment under lead (Pb) stress, we investigated the effects of an arbuscular mycorrhizal (AM) fungus (Funneliformis mosseae) on the growth and Pb uptake of Sophora viciifolia L., and explored the Pb localization in AM roots using transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDS). The results showed that high Pb levels (500 and 1000 μg/g) inhibited the growth of S. viciifolia. Compared with the noninoculation treatment, F. mosseae inoculation decreased the Pb concentrations above- and belowground by 61.0% and 15.2%, when exposed to Pb at a concentration of 1000 μg/g. The root length, fork number, tip number, surface area, and volume of mycorrhizal S. viciifolia were higher than those of the corresponding nonmycorrhizal plants. These parameters of mycorrhizal plants increased by 220%, 219%, 157%, 225%, and 278% when plants were exposed to Pb at 1000 μg/g compared with nonmycorrhizal plants. The ratio of root length with diameters between 0–0.2 mm to the total root length significantly increased under Pb stress, and F. mosseae inoculation significantly reduced the ratio. Under Pb stress, F. mosseae increased the ratios of root length with 0.61–0.8 and 0.81–1.0 mm diameters to the total root length, indicating that F. mosseae tended to thicken the roots of S. viciifolia under Pb additions. The combined results of TEM and EDS indicated that Pb deposited in not only plant cells but also the cell walls and vacuoles of the AM fungal intracellular hyphae, thus revealing the subcellular-level mechanism of AM fungi in alleviating the Pb toxicity to the host plant.

Key words: arbuscular mycorrhizal fungi, Sophora viciifolia L., root characteristics, Pb ultrastructural localization, TEM–EDS.

Résumé: D'après une expérience en pot en présence d’un stress au Pb, nous avons examiné les effets de champignons mycorhiziens arbusculaires (MA) (Funneliformis mosseae) sur la croissance et l’assimilation de Pb chez Sophora viciifolia L., et nous avons exploré l’localisation du Pb au niveau des racines MA au moyen de la microscopie électronique à transmission (MET) et de la spectroscopie rayons X à dispersion d’énergie (SDE). Les résultats ont révélé que des taux élevés de Pb (500 et 1000 μg/g) inhibaient la croissance des semis de S. viciifolia. Par rapport au traitement sans inoculation, l’inoculation de F. mosseae a fait baisser les concentrations de Pb de 61 % (à la surface) et 15.2 % (sous la terre) lorsque mis en présence de 1000 μg/g de Pb/g. La longueur des racines, le nombre d’embranchements, le nombre de terminaisons, la superficie et le volume de S. viciifolia en mycorhize étaient supérieurs à ce que l’on observait chez les plantes non mycorhiziennes. Ces paramètres associés aux plantes mycorhiziennes ont augmenté de 220 %, 219 %, 157 %, 225 % et 278 % lorsque les plantes étaient exposées à 1000 μg/g de Pb/g comparativement à des plantes non mycorrhiziennes. Le rapport de la longueur de segments racinaires au diamètre de 0 à 0.2 mm sur la longueur totale des racines a augmenté significativement sous un stress au Pb, et l’inoculation de F. mosseae a fait baisser significativement ce rapport. Sous un stress au Pb, F. mosseae a haussé les rapports de la longueur de segments racinaires au diamètre de 0.61 à 0.8 mm et de 0.81 à 1.0 mm sur la longueur totale des racines, indiquant que F. mosseae avait tendance à épaissir les racines de S. viciifolia des suites de l’ajout de Pb. Les résultats de MET alliés à ceux de SDE portent à croire que le Pb se déposerait non seulement dans les cellules végétales, mais aussi dans les parois cellulaires et les vacuoles des hyphes intracellulaires de champignons AM, levant ainsi le voile sur un mécanisme subcellulaire permettant aux champignons AM d’atténuer la toxicité du Pb chez la plante hôte. [Traduit par la Rédaction]

Mots-clés : champignons mycorhiziens arbusculaires, Sophora viciifolia L., caractéristiques des racines, localisation ultrastructurale, MET–SDE.
Introduction

Arbuscular mycorrhizal (AM) fungi are an important group of soil microorganisms, accounting for 30% of the soil microbial biomass (Olsson et al. 1999) and forming mutualistic symbioses with 80% of vascular plants (Trappe 1987; Glick et al. 1998; Wu et al. 2006). The fungi penetrate the cortical cells of roots and form internal structures, including intercellular or intracellular vesicles and intracellular thin-walled and branched arbuscules. AM fungi can improve plant mineral nutrient uptake and soil quality by transporting nutrients from soil to roots and also by their involvement in bioremediation of heavy metal (HM)-contaminated soil (Punamiya et al. 2014). Therefore, AM fungi are critical for the establishment and vigor of plants in HM-disturbed areas.

Materials and methods

Experiment design

A pot experiment was conducted in a controlled environment chamber. Treatments were factorial combinations of 2 factors: (i) inoculation: with F. mosseae (M) or with autoclaved F. mosseae (NM), and (ii) Pb (Pb(NO₃)₂) levels: 0, 50, 500, and 1000 µg/g. The 8 treatment combinations were M + 0, M + 50, M + 500, M + 1000, NM + 0, NM + 50, NM + 500, and NM + 1000. There were 20 pots in each treatment and 1 plant per pot. All the parameters were determined with 5 single plants except the Pb localization study.

AM fungi inoculum

Funnelliformis mosseae inoculum, consisting of spores (the spore density was 120–165 spores per 100 g air-dried soil), mycelia, and root fragments with a colonization level of 92%, was obtained by trap culture with Zea mays (Marques et al. 2007). Funnelliformis mosseae (BGC XJ01A) spores were purchased from the Institute of Plant Nutrition and Resource, Beijing Academy of Agriculture and Forestry Sciences, China.

Plant material preparation

Sophora viciifolia seeds collected from Qiandongshan lead and zinc mine were sterilized in a 1% (m/v) KMnO₄ solution for 10 min, rinsed with double-distilled water (ddH₂O) 3 times, and germinated on wet filter papers. The germinated seeds were transferred to vermiculite and cultured for 1 month. At the same time, 160 pots were filled with 140 g of a culture substrate (soil:sand = 2:1, m/g; available N, 44.3 m/g; and available K, 152.8 m/g). All the pots were incubated for 2 weeks in a greenhouse. After that, 160 seedlings of similar size were selected and individually transplanted into separate pots. Half of these pots (80) received 40 g of F. mosseae inoculum, with the other half receiving 40 g of autoclaved (121 °C for 30 min) F. mosseae inoculum before Pb treatments.
transplanting (Wu et al. 2006). The pots were placed in an artificial climate incubator (ZPQ-350, Heilongjiang Dong-Tuo Instrument Manufacture Co., China) with the following condition parameters: daytime — 25 °C, 70% humidity, 14 h light at 4450 lx light intensity; night time — 20 °C, 60% humidity, 10 h at 0 lx light intensity (Ge et al. 2008; Gu et al. 2010). Pot placement was regularly changed to minimize location effect. Hoagland nutrient solution (Hoagland and Arnon 1950) was added to all the pots regularly. The samples were collected and the basic growth indicators were determined 120 days after inoculation.

Growth indicators determination
Plant height and diameter were measured by caliper, and the above- and belowground biomasses were determined after drying in an oven at 105 °C for 30 min, then at 70 °C until constant masses.

Root characteristics and mycorrhizal colonization determination
The cleaned roots were arranged in a water-filled transparent tray and scanned using a transmitted light scanner (Epson Expression 1680, Germany). Total root length, volume, surface area, and number of tips and forks were calculated using the software WinRHIZO 2003b (Regent Instruments Inc., Canada).

Root fragments stored in formalin–acetic acid were stained according to the modified method of Phillips and Hayman (1970). Mycorrhizal coverage of colonized segments was determined using the method modified by McGonigle et al. (1990) under a compound-light microscope (Olympus BX51, Japan) at a magnification × 200. The calculation of vesicular, arbuscular, hyphal, and total colonization of root by F. mosseae were estimated using the following formula (Hemavani and Thippeswamy 2013):

Percentage of colonization = 100%
× (Total No. of AM-positive segments/
Total No. of observed root segments)

Pb distribution in plant tissue
Parts of the roots and leaves were washed with 10 mmol/L EDTA (pH 8.0) and rinsed with ddH2O to remove the metal ions from the root surface (Hoffmann et al. 2004), oven-dried at 105 °C for 30 min followed by 70 °C until constant masses, weighed and ground with a stainless steel mill, then passed through a 0.1 mm nylon sieve. About 0.1 g of the plant sample was digested using the HNO3–HClO4 (3:1) method (Wang and Zhou 2003). The digested solutions were washed with ddH2O in 25 mL flasks. Pb quantification was performed with a Flame Atomic Absorption Spectrometer (Hitachi, Japan).

Pb localization in mycorrhizal roots of S. viciifolia
TEM and EDS were used to detect the location of Pb in mycorrhizal S. viciifolia roots. Briefly, S. viciifolia roots of the “M + 0” and “M + 1000” treatments were washed gently with ddH2O and then cut into 0.5 cm length segments. After being placed into a primary fixative of 4% (v/v) glutaraldehyde for about 6 h at 4 °C, the segments were washed 5 times with time intervals of 5, 10, 15, 20, and 30 min using PBS buffer (0.1 mol/L, pH 6.8), followed by post-fixation in 1% (m/v) osmic acid for 2 h at 4 °C. After being washed by PBS buffer, the samples were dehydrated through an ethanol concentration series, 30% (v/v), 50% (v/v), 70% (v/v), 80% (v/v), 90% (v/v) for 15 min, and 100% (v/v) for 30 min twice. Before going onto the embedding plates, the samples were infiltrated by anhydrous ethanol – epoxy resin EPON-812 (1:1, v/v) overnight. Afterwards, the samples were placed in an oven at 60 °C for 48 h, and finally kept in desiccators (Zhang et al. 2010). Semithin sections were cut on the microtome (LKB-V 2088, Sweden), stained with toluidine blue (Sigma, USA), and then examined under a microscope (Olympus BX51, Japan). The identified semithin sections containing mycorrhizal structures were further chipped to ultrathin sections on an ultramicrotome (Leica RM2016, Germany). The ultrathin sections were stained only with uranyl acetate, set on 300-mesh copper grids, and viewed through TEM (Hitachi H-7650, Japan) at 80 kV. Viewing stained sections permitted the identification of Pb deposits, as they are electron dense. To show the presence of Pb in those deposits, X-ray microanalysis was performed using EDS (EDAX, USA) equipped on the TEM.

Statistical analysis
Data were analyzed using SAS version 6.1 software packages (SAS Institute Inc., USA) and SPSS version 16.0 software packages (SPSS Institute Inc., USA). Comparisons between means were carried out using Duncan’s test, at a significance level of P < 0.05. Graphs were prepared using Sigma Plot version 10.0 (Systat Software, Canada).

Results
The effect of AM fungi inoculation on the growth of S. viciifolia under Pb stress
Typical structures of AM fungi were observed in the roots of inoculated S. viciifolia after harvest. The vesicular, arbuscular, hyphal, and total colonization of roots by F. mosseae decreased with increasing Pb concentrations (Table 1). The symbiotic relationship between F. mosseae and S. viciifolia was well established under Pb stress conditions, and the highest total colonization, 95.46%,

Table 1. Colonization of Funneliformis mosseae in roots of Sophora viciifolia under different Pb levels.

<table>
<thead>
<tr>
<th>Pb (µg/g)</th>
<th>Vesicle</th>
<th>Arbuscule</th>
<th>Hyphae</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>52.20±2.71a</td>
<td>33.48±2.82a</td>
<td>82.45±3.80a</td>
<td>95.46±6.15a</td>
</tr>
<tr>
<td>50</td>
<td>38.45±2.45b</td>
<td>23.52±2.09b</td>
<td>68.48±3.85b</td>
<td>88.24±3.81b</td>
</tr>
<tr>
<td>500</td>
<td>20.82±2.10c</td>
<td>11.60±1.31c</td>
<td>52.26±2.56c</td>
<td>58.50±2.37c</td>
</tr>
<tr>
<td>1000</td>
<td>17.94±1.8c</td>
<td>0.00±0.0d</td>
<td>38.25±1.17d</td>
<td>42.46±2.11d</td>
</tr>
</tbody>
</table>

Note: All the values are means of means ± SD of 120 repetitions. Different letters within a column indicate significant differences, according to Duncan’s test.
Effects of inoculation on *Sophora viciifolia* roots under Pb stress

Ambrosia mosseae inoculation of *S. viciifolia* under Pb stress at 50 μg/g increased the root length and number of tips to 1.16 and 1.45 times those of the controls, respectively; but 500 and 1000 μg/g inhibited the root growth of mycorrhizal plants, with the root length, fork number, tip number, surface area, and volume being 57%, 44%, 59%, 41%, and 34% that of controls under 1000 μg/g (Fig. 1). In nonmycorrhizal plants, a Pb concentration of 50 μg/g did not affect the root length, tip number, fork number, and surface area, but it decreased the root volume. Pb stress at 1000 μg/g inhibited root growth, with the root length, tip number, fork number, surface area, and volume being 67%, 46%, 35%, 36%, and 25% that of controls, respectively (Fig. 1). The length, fork number, tip number, surface area, and volume of mycorrhizal roots were 4.20, 4.19, 3.57, 4.35, and 4.78 times the nonmycorrhizal roots, under 1000 μg/g (Fig. 1). As shown in Table 3, both AM fungi and Pb concentration had significant effects on the length, fork number, tip number, surface area, and volume of *S. viciifolia* roots.

Under Pb concentration of 50 μg/g, the percentage of root length in the 0–0.2 mm diameter class of mycorrhizal plants was significantly lower than that of nonmycorrhizal plants (i.e., 23% of the latter), but no significant differences existed between the percentages of root length in the 0.21–0.4 and 0.41–0.6 mm diameter classes of mycorrhizal and nonmycorrhizal plants; the percentages of root length in the 0.61–0.8, 0.81–1.0, and >1.0 mm diameter classes of mycorrhizal plants were significantly higher than those of nonmycorrhizal plants (i.e., 3.21, 3.52, and 1.78 times the latter) (Fig. 2). Under 500 μg/g, the percentages of mycorrhizal root length in the 0–0.2 and 0.21–0.4 mm diameter classes were significantly lower than those of nonmycorrhizal plants (i.e., 19% and 69% of the latter); but the percentages of root length in the 0.41–0.6, 0.61–0.8, and 0.81–1.0 mm diameter classes of mycorrhizal plants were significantly higher than that of nonmycorrhizal plants.

### Table 2. Basic growth indicators of *Sophora viciifolia* seedlings in different treatments.

<table>
<thead>
<tr>
<th>AM fungi*</th>
<th>Pb (μg/g)</th>
<th>Seedling length (cm)</th>
<th>Basal diameter (mm)</th>
<th>Aboveground dry biomass (mg/plant)</th>
<th>Belowground dry biomass (mg/plant)</th>
<th>Root/shoot index</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>0</td>
<td>26.10±2.01ab</td>
<td>2.90±0.17a</td>
<td>1.08±0.12a</td>
<td>0.59±0.19a</td>
<td>0.55±0.12bc</td>
</tr>
<tr>
<td>50</td>
<td>29.13±3.13a</td>
<td>2.37±0.19b</td>
<td>0.88±0.21ab</td>
<td>0.41±0.13b</td>
<td>0.46±0.05c</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>23.33±2.37b</td>
<td>2.58±0.20ab</td>
<td>0.77±0.15bc</td>
<td>0.62±0.06a</td>
<td>0.82±0.09ab</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>16.43±3.32c</td>
<td>2.26±0.21b</td>
<td>0.59±0.12cd</td>
<td>0.22±0.03c</td>
<td>0.86±0.12a</td>
<td></td>
</tr>
<tr>
<td>NM</td>
<td>0</td>
<td>11.87±2.02d</td>
<td>1.58±0.19c</td>
<td>0.42±0.12d</td>
<td>0.19±0.02cd</td>
<td>0.48±0.12c</td>
</tr>
<tr>
<td>50</td>
<td>8.20±0.66e</td>
<td>1.77±0.05c</td>
<td>0.09±0.01e</td>
<td>0.09±0.01d</td>
<td>0.94±0.24a</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>7.17±1.07e</td>
<td>1.57±0.28c</td>
<td>0.05±0.01e</td>
<td>0.04±0.01d</td>
<td>0.92±0.05a</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>5.17±1.14e</td>
<td>1.53±0.13c</td>
<td>0.05±0.01e</td>
<td>0.04±0.01d</td>
<td>0.98±0.32a</td>
<td></td>
</tr>
</tbody>
</table>

Note: All the values are means ± SD of 5 replications. Different letters within a column indicate significant differences, according to Duncan’s test.

*AM, arbuscular mycorrhiza. M, with *Funneliformis mosseae* inoculation; NM, without *F. mosseae* inoculation.
Fig. 1. Length (A), fork number (B), tip number (C), surface area (D), and volume (E) of *Sophora viciifolia* roots colonized or not by *Funneliformis mosseae* at different Pb(NO₃)₂ levels. Labels along the x axis are the different treatments: NM, nonmycorrhizal plants; M, mycorrhizal plants; the numerical values are different Pb levels (0, 50, 500, 1000 μg/g). All the values are the means of quintuplicates ± SD. Different letters above bars indicate significant differences according to Duncan’s test.

(i.e., 3.49, 5.03, 6.84, and 3.10 times the latter) (Fig. 2). Under 1000 μg/g, the percentage of root length in the 0.61–0.8 mm diameter class of mycorrhizal plants was significantly higher than that of nonmycorrhizal plants, specifically it was 2.11 times the latter (Fig. 2).

The effect of AM fungi inoculation on Pb distribution in *S. viciifolia*

In nonmycorrhizal plants, shoot and root Pb concentrations rose with the increase of the Pb application, and the shoot and root Pb concentrations, under 1000 μg/g,
Fig. 2. Percentage of root length in different diameter classes is measured in *Sophora viciifolia* roots colonized or not by *Funneliformis mosseae* at different Pb(NO₃)₂ levels. Labels along the x axis are the different treatments: NM, nonmycorrhizal plants; M, mycorrhizal plants; the numerical values are different Pb levels (0, 50, 500, 1000 μg/g). All the values are the means of quintuplicates ± SD. Different letters above bars indicate significant differences according to Duncan’s test.

Table 4. Pb concentrations and uptakes in mycorrhizal and nonmycorrhizal *Sophora viciifolia* under different Pb levels.

<table>
<thead>
<tr>
<th>Pb concn. (μg/g)</th>
<th>Contribution of AM fungi (%)</th>
<th>Pb uptake (μg/pot)</th>
<th>Contribution of AM fungi (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM (μg/g) fungi²</td>
<td>Shoots</td>
<td>Roots</td>
</tr>
<tr>
<td>0</td>
<td>M NDF</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>NM NDF</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>50</td>
<td>M 10.64±1.18f</td>
<td>1.63±0.19f</td>
<td>—66</td>
</tr>
<tr>
<td></td>
<td>NM 31.46±2.51e</td>
<td>11.03±1.40e</td>
<td>—</td>
</tr>
<tr>
<td>500</td>
<td>M 93.08±6.85d</td>
<td>24.18±1.55d</td>
<td>—54</td>
</tr>
<tr>
<td></td>
<td>NM 200.19±18.31b</td>
<td>38.09±5.02c</td>
<td>—</td>
</tr>
<tr>
<td>1000</td>
<td>M 118.48±8.35c</td>
<td>47.49±4.03b</td>
<td>—61</td>
</tr>
<tr>
<td></td>
<td>NM 303.81±13.65a</td>
<td>56.00±8.65a</td>
<td>—</td>
</tr>
</tbody>
</table>

Note: All the values are means ± SD of 5 replications. Different letters within a column indicate significant differences, according to Duncan’s test. ND, not detected.

²AM, arbuscular mycorrhiza. M, with *F. mosseae* inoculation; NM, without *F. mosseae* inoculation.

were 9.66 and 4.96 times that under 50 μg/g. Shoot and root Pb uptake also grew with increasing Pb application, reaching the highest level under 1000 μg/g, i.e., 14.10 and 2.46 μg/pot, respectively. The Pb concentrations and Pb uptakes of the shoots were higher than those of the roots under all the Pb levels (Table 4).

The Pb concentrations of mycorrhizal plants also rose with increasing Pb application, but the shoot and root concentrations were lower than those of nonmycorrhizal plants. The Pb concentrations of mycorrhizal shoots were 66% (under 50 μg/g), 54% (under 500 μg/g), and 61% (under 1000 μg/g) lower than those of the nonmycorrhizal plants, respectively, and the corresponding data of mycorrhizal roots were 85% (under 50 μg/g), 37% (under 500 μg/g), and 15% (under 1000 μg/g), which indicated that *F. mosseae* inoculation reduced the Pb concentrations in *S. viciifolia* and alleviated the Pb toxicity to plants (Table 4). With the Pb application increasing from 50 to 500 μg/g, the shoot and root Pb uptake of mycorrhizal plants increased, and the shoot Pb concentration under 500 μg/g was 7.78 times that under 50 μg/g; no significant difference was found in the shoot Pb uptake of mycorrhizal plants between 500 and 1000 μg/g, but the root Pb uptake under 1000 μg/g was significantly lower than the value under 500 μg/g. Under 500 and 1000 μg/g, the shoot and root Pb uptake of mycorrhizal plants was significantly higher than that of nonmycorrhizal plants (Table 4). As shown in Table 3, both AM fungi and Pb concentration had significant effects on the shoot Pb concentration, root Pb concentration, shoot Pb uptake, and root Pb uptake of *S. viciifolia*. 

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With increasing Pb application, the root to shoot Pb concentration of mycorrhizal plants rose from 0.15 to 0.40, which indicated that AM fungi inoculation increased the ability of *S. viciifolia* to allocate Pb$^{2+}$ to underground. With the Pb application growing from 50 to 500 μg/g, root to shoot Pb uptake of mycorrhizal plants increased from 0.07 to 0.22, which indicated that AM inoculation promoted the Pb retention in roots, but no significant difference was found between Pb applications of 500 and 1000 μg/g (Table 4).

**The subcellular localization of Pb in mycorrhizal roots of *S. viciifolia***

As shown in Fig. 3, most of the root cells of *S. viciifolia* have a roughly rectangular shape. After staining with toluidine blue, AM fungal structures in the root cells were observed. In the figure, “A” indicates the arbuscule structure of AM fungi in the root fragment, which was further chipped to ultrathin sections.

**Fig. 3.** The semithin section of *Funneliformis mosseae* colonized *Sophora viciifolia* root under Pb(NO$_3$)$_2$ stress at 1000 μg/g. A, arbuscular structure; OCC, outer cortex cell.

Figures 4A and 4B show AM fungal hypha in the intercellular space and duct cells of *S. viciifolia* root in “M + 0” treatment. The layered cell walls of AM fungi (arrow “a”), the compartmental organization inside AM fungi (arrow “b”), and the root cell wall and membrane (arrow “c”) are clearly observed in Fig. 4A. Figure 4C shows the TEM photo of *S. viciifolia* root cells in “NM + 1000” treatment. Figure 4D shows the AM fungal hypha located in the intercellular space of *S. viciifolia* root in “M + 1000” treatment, a highly compartmented zone (arrow “i”) inside the AM fungal hypha, and electron-dense grains (arrow “h”) in the cell wall. Figure 4E shows the intercellular space of *S. viciifolia* root in “NM + 1000” treatment. Figure 4F shows AM fungal hypha inside the root cell of *S. viciifolia* in “M + 1000” treatment, and there are electron-dense grains (arrow “l”) inside the compartmental organizations.
Fig. 4. Transmission electron micrographs of *Sophora viciifolia* root under different soil Pb levels and mycorrhizal treatments. (A) The arbuscular mycorrhizal (AM) fungi structure in the treatment of “M + 0”, arrow “a” shows the AM fungal layered cell walls, arrow “b” shows the compartmental organization inside AM fungi, and arrow “c” shows the plant cell wall and membrane. (B) The duct cell of the “M + 0” treatment, arrow “d” indicates the duct cells of *S. viciifolia*. (C) The root cells of the “NM + 1000” treatment, arrow “f” indicates the suspected Pb complex. (D) The hypha in the intercellular space of the “M + 1000” treatment, arrow “h” indicates the suspected Pb complex located at the AM fungal cell wall, arrow “i” shows the compartmental organization inside AM fungi. (E) The intercellular space of the “NM + 1000” treatment, arrow “j” indicates the suspected Pb complex located at intercellular space. (F) The hypha in the plant cells of the “M + 1000” treatment, arrow “l” indicates the suspected Pb complex located at the vacuole of AM fungal hypha.

The elemental composition of the locations indicated by arrows “f”, “h”, “j”, and “i” in Fig. 4 was analyzed using EDS, and the results are shown in Fig. 5. Pb peaks appeared in the spectra of the 4 locations. The EDS results of arrows “f” and “j” indicated that Pb flowed into root cells and was deposited in the intercellular space of *S. viciifolia* root, and the EDS results of arrows “h” and “i” indicated that Pb in the cytoplasm was sequestered by AM fungi and deposited at the cell wall and vacuole of AM fungal hyphae.

**Discussion**

The effects of AM fungi inoculation on the growth and Pb uptake of *S. viciifolia*

Seeds germination and plant growth can be affected by micromolar levels of Pb (Kopittke et al. 2007). In the present study, regardless of the Pb level, *F. mosseae* inoculation significantly promoted plant above- and below-ground dry biomasses, which indicated that *F. mosseae* inoculation enhanced the growth of *S. viciifolia*. However, *S. viciifolia* growth was inhibited by increasing Pb stress. Pb$^{2+}$ could react with phosphate anions in the soil, which restricted plant P absorption. AM fungi can help the plant to absorb P and alleviate Pb toxicity.

Malcová et al. (2003) found *Rhizophagus intraradices* inoculation significantly reduced the shoot and root Pb concentrations of *Z. mays* under Pb stress at 0.01 mmol/L, and the present results are consistent with this research. Nowadays, there are many contradictory reports on the effects of AM fungi on plant tolerance to HMs. Besides the results we obtained in this study, i.e., AM fungi increased the biomass and reduced the Pb concentration of hosts, it is was also previously reported that AM fungi inoculation improved both the biomass and Zn concentration of *Helianthus annuus* L. under Zn stress at 200 and 400 μg/g (Audet and Charest 2012); in addition, *F. mosseae* inoculation was found to promote the HMs uptake but inhibit the growth of *Z. mays* under mixed HMs stress (Cd, Ni, Zn, Cu, Pb, and Mn) (Weissenhorn and Leyval 1995). However, de Souza et al. (2012) found that the association of *Calopogonium mucunoides* Desv. with *Claroideoglomus etunicatum* promoted biomass production. Nutrient uptake was also positively influenced by mycorrhization, but shoot Pb concentration did not differ between mycorrhizal and nonmycorrhizal plants. There was even one report showing that mixed AM fungi inoculation (*Gigaspora margarita*, *Gigaspora decipens*, *Scutellospora gilmori*, *Acaulospora* spp., and *Glomus* spp.) did not affect the growth of *Z. mays* under HMs stress (Wang et al. 2006). Therefore, the effect of AM fungi on the HM tolerance of plants depended on AM fungi species, plant species, HM species, and soil properties.

AM fungal inoculation had variable effects on the HM uptake and translocation. Zafarian et al. (2013) investigated the influence of *F. mosseae* on the Cd, Co, and Pb uptake of *Medicago sativa* L., and the results showed that mycorrhizal *M. sativa* had higher Co and Pb concentrations, and AM fungal inoculation promoted HMs translocation to shoot. Zhu et al. (2001) found AM fungal inoculation reduced the shoot and root Zn concentrations of white clover under Zn stress. Citterio et al. (2005) studied the influence of *F. mosseae* inoculation on the Cd, Ni, and Cr uptake of *Cannabis sativa* L. under stress and found that soil pH, availability of HM, and concentration of HMs were not affected by inoculation. Hence, the effects of AM fungal inoculation on the uptake and translocation of HMs is associated with AM fungi, HMs, and plant species.

The effect of AM fungi on the root characteristics of *S. viciifolia* under Pb stress

In the present study, a Pb$^{2+}$ concentration of 50 μg/g improved root length and tip number of mycorrhizal *S. viciifolia* seedlings and did not significantly affect the root growth of nonmycorrhizal seedlings; therefore, 50 μg/g caused little or no harm to *S. viciifolia* roots. However, 500 and 1000 μg/g stunted the root growth, regardless of inoculation. Hong et al. (2004) found that Pb$^{2+}$ at ≤1 mg/L promoted root growth in *Brassica chinensis* L., *Apis graveolens* L., and *Capsicum annuum* L.; >2 mg/L reduced the root biomass, length, surface, and volume, which were similar to our results. Root length, tip number, fork number, surface area, and volume are the indicators of root growth and development. In adverse situations, roots are the first to be affected, and the plants acclimatize to stresses by adapting root growth and metabolism (Zhang et al. 2004). Gopal and Rizvi (2008) found low Pb stress (0.1 mmol/L) inhibited root growth in *Raphanus sativus* L., through direct contact with high levels of Pb$^{2+}$. A Pb concentration of 100 mg/L significantly reduced the root length in *Prosopis* sp. to 55% that of the control (Arias et al. 2010). Compared with the control, the root length of *Triticum aestivum* L. treated with Pb$^{2+}$ at 100, 250, and 500 μmol/L was reduced by 21%, 33%,
Fig. 5. The energy dispersive X-ray spectroscopy spectra collected over those regions indicated by arrows f, h, j, and i in Fig. 4.
and 40%, respectively, and a 16% and 34% decline was noticed in the shoot length in response to Pb²⁺ at 250 and 500 μmol/L, respectively (Kaur et al. 2013).

In the present study, the percentages of root length in the 0–0.2 mm diameter class significantly increased under Pb stress (50, 500, and 1000 μg/g), and AM fungal inoculation significantly reduced these percentages. Kopittke et al. (2007) found that Pb²⁺ at 0.06 μmol/L increased secondary root number per unit length of main root of Vigna unguiculata L. Sheng et al. (2009) observed that F. mosseae reduced the percentages of Z. mays root length in the 0–0.2 mm diameter class both in the absence and presence of NaCl stress. Our results also show that F. mosseae mainly promotes the percentages of root length in the 0.61–0.8 and 0.81–1.0 mm diameter classes under Pb stress, which indicates that F. mosseae thickens the roots of S. viciifolia. The phenomenon could be explained by the extensive AM fungal hypha network in the root zone of S. viciifolia replacing the function of fine roots. In conclusion, the inhibitory effect of Pb on S. viciifolia root alleviated by AM fungi might be attributed to the change in root exudates, rhizosphere pH, and microbial community structure, all of which influence the availability and mobility of Pb, thus improving the living environment of roots.

**Pb localization**

In a HMs-polluted environment, the HMs-related gene and protein expressions of AM fungi will change to adapt to the environment (Lanfranco et al. 2002; González-Guerrero et al. 2005, 2006; Manceau et al. 2008). In an environment where HM ions enter plant cells and accumulate to a high level, how do the AM fungi inside the roots respond to the stress? As far back as 1993, Turnau and colleagues studied HM distribution in mycorrhizal roots of Pteridium aquilinum L. collected from experimental plots treated with Cd dust and found that the cytoplasm of the mycorrhizal fungi contained more Cd than host cells. Cd was bound to polyphosphate granules. Using a combination of PIXE (particle-induced X-ray emission), proton back-scattering spectrometry, and confocal laser scanning microscopy, Weiersbye et al. (1999) determined in situ elemental concentrations in AM Cynodon dactylon L. roots from gold and uranium mine tailings in South Africa and found that vesicles in the root cells accumulated Mn, Cu, U, Ni, Fe, and Zn. This sequestration of excess metals in the vesicles limited metals availability, and thus toxicity, to the host. Turnau et al. (2013) investigated elemental distribution within mycothecial and nonmycothecial (Glomus tenue) gametophytes of Pellaea viridis Prantl (Pteridaceae) using micro-PIXE. Increased levels of Ni, Cr, Fe, Co, and Ti were found in the part of the gametophyte that hosted in G. tenue. This finding suggested that the fungus might immobilize certain potentially toxic metals that were taken up from the soil by the plant. The studies mentioned above state clearly that AM fungi inside plant roots can accumulate HM ions. However, Marques et al. (2007) studied Zn localization in mycorrhizal (Claroideoglomus claroideum and Rhizophagus intraradices) Solanum nigrum L. roots using the method of autometallography, and the main deposits of Zn were found in the intercellular spaces and in the cell walls of the root tissues. Inoculation with different AM fungal species caused no differences in the location of Zn accumulation. Arias et al. (2010) treated mycorrhizal (Glomus deserticola) Prosopis sp. with different Pb (0, 10, 50, and 100 mg/L) and Cr (0, 20, 40, 75, and 125 mg/L) levels, and X-ray mapping demonstrated high Cr and Pb deposition in xylem and phloem cells. From the above, it can be seen that whether the AM fungal hyphae can sequester HM ions or not depends on the HMs and the plant and AM fungi species. In the present study, the TEM and EDS results suggested deposition of Pb in the intercellular spaces and in the AM fungal structures inside the root, indicating that F. mosseae alleviated the Pb toxicity to S. viciifolia by sequestering the Pb ions in the cytoplasm of root cells.

In conclusion, S. viciifolia is the dominant plant species growing widely in the Qiandongshan lead and zinc mine of northwest China. On the basis of pot experiments under Pb stress, we found that F. mosseae promoted the growth and increased the Pb uptake of S. viciifolia. One of the mechanisms through which F. mosseae improves the Pb resistance ability of S. viciifolia is the sequestration of Pb ions inside their intraradical mycelia.

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