Arbuscular mycorrhiza fungi facilitate rapid adaptation of *Elsholtzia splendens* to copper

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HIGHLIGHTS

- Direct evidence supporting that AMF facilitate the rapid adaptation of *Elsholtzia splendens* to Cu stress is first provided.
- The main mechanism is alleviation of the incurred cost by Cu stress.
- The alternative mechanism is the adaptive plasticity in response to Cu stress.

GRAPHICAL ABSTRACT
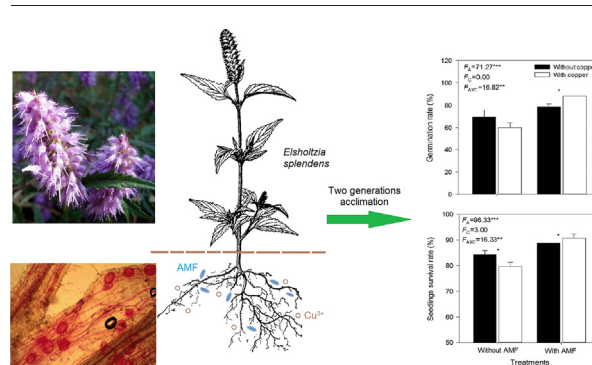
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ABSTRACT

Closely associated microbes have been shown to drive local adaptation of plants. However, few studies provide direct evidence, disclosing the role of arbuscular mycorrhiza fungi (AMF) in their rapid adaptation of plants toward heavy metal tolerance. *Elsholtzia splendens* is a Cu-tolerant plant that was used as a model plant to study seed morphological traits as well as traits related to seed germination and seedling growth. This was achieved after acclimation for two generations with 1000 mg/kg CuSO$_4$ in either absence or presence of AMF. In the absence of AMF, acclimation to Cu for two generations significantly decreased surface area, perimeter length, and perimeter width of *E. splendens* seeds, as well as seedling survival rate and fresh weight of the radicle of seedlings. However, in the presence of AMF, both the germination rate and the germination index of *E. splendens* seeds as well as the fresh weights of hypocotyl and radicle significantly increased. These results revealed that after Cu acclimation treatment, seeds and seedlings that had been inoculated with AMF outperformed those without AMF inoculation under Cu addition, indicating that AMF can facilitate rapid adaptation of *E. splendens* to Cu stress. In addition, two generations of Cu acclimation under AMF absence significantly increased radicle length, while amplitude increased under AMF presence, indicating that the direct adaptive plasticity response of radicle length to Cu stress helps with the Cu stress adaptation of *E. splendens*.

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1. Introduction

Plants are immobile and consequently, to adapt to their local environment, they have to modify their physiological processes (Ernst,

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2006). Through ongoing natural selection, favourable metabolic characteristics are passed to the next generation via modification of the genetic structure of a local population (Ernst, 2006). The genetic adaptation of plant populations to copper (Cu) was reported earlier, and one recent report suggested that *Melandrium rubrum* from a population growing on a Cu mine was more Cu-tolerance than plants growing on normal soil (Chudzińska et al., 2014). Subsequent to these studies, adaptation to heavy metal stress among plants was extensively studied and since then, well-documented examples of rapid evolutionary adaptation have been established (Eränen, 2006, 2008). Recently, it has been reported that heavy metal posed a severe impact on aboveground plant traits as well as belowground microbial communities (Wilke et al., 2005; Li et al., 2011). In contrast, belowground microbes have been reported to be beneficial for plant growth under heavy metal stress (Yang et al., 2013; Meier et al., 2011; Colpaert et al., 2011). However, little research has specifically addressed how belowground microbes influence evolutionary processes in plants (Lau and Lennon, 2011).

Several recent studies have demonstrated local adaptation in plants to be driven by closely associated microbes (Rodriguez and Redman, 2008). As an important component of soil microbiota, arbuscular mycorrhiza fungi (AMF) occur in an estimated 80% of all plant species, where they play a vital role in enhancing the survival of plants in polluted environments, including those contaminated with heavy metals (Wang and Qiu, 2006; Barrow et al., 2008; Hajiboland et al., 2010). Recently, habitat-adapted symbiosis was reported to lead stress tolerance in plants within a single growing season (Rodriguez et al., 2008). For example, Schultz et al. (2001) reported that AMF help *Andropogon gerardii* (Poaceae) to adapt to high- and low-nutrient prairies, while Schechter and Bruns (2008) reported that local AMF can help *Collinsia sparsiflora* adapt to serpentine soils. Although few studies provide direct evidences that reveals the role of AMF in adaptation to heavy metal contaminated habitats, it has been well documented that AMF inoculated plants improve growth and production under Cu stress either by decreasing the resulting oxidative stress, by reducing the transfer of Cu into shoots, or by improving nutrient uptake (Carvalho et al., 2006; Hildebrandt et al., 2007; Ferrol et al., 2009; Meier et al., 2011, 2012, 2015; Wang et al., 2007). Thus, we predicted that AMF inoculation will help plants adapt to heavy metal contaminated habitats.

Elsholtzia splendens is an annual herb that belongs to the family Labiatae; it is a Cu-tolerant plant that has been used as a metal hyperaccumulator (Jiang et al., 2008). *E. splendens* is widely distributed in Cu-contaminated soils (Tang et al., 1999; Lou et al., 2004). *E. splendens* has been reported to be an obligate symbiont with AMF (Yang et al., 2010) and thus provides an attractive model species to study the role of AMF for the adaptation of plants to copper. Inoculation with soil microbes or AMF has been reported to significantly affect growth, photosynthesis, antioxidant abilities and reproduction of *E. splendens* under Cu stress (Li et al., 2013; Wang et al., 2015; Jin et al., 2015). Here, we report results of *E. splendens* seed germination and seedling growth after two-generation of acclimation to 1000 mg/kg CuSO₄. Our aim was to ascertain the following questions: 1) Is two-generation acclimation sufficient to impact seed morphology and offsprings fitness? 2) Can AMF facilitate rapid evolutionary adaptation of *E. splendens* to Cu? 3) Will this prospective adaptation carry a performance cost in clean soil? The results of our investigation provide basic references for the application of hyperaccumulators in the phytoremediation of Cu-contaminated soils.

2. Materials and methods

2.1. Seeds and soil collection

On December 20th, 2013, *E. splendens* seeds were collected from non-contaminated soil in Tainan village, Hong'an County, Hubei Province, China. The non-contaminated soil had pH 5.47 ± 0.15, organic carbon 2.46 ± 0.09, total Cu 52.6 ± 2.8 mg/kg, and available Cu 8.7 ± 3.4 mg/kg.

On December 21st, 2013, rhizosphere soil of *E. splendens* was collected at Cu mine tailings, which are located on the Chimashan Mountain, Yangxin County, Hubei Province, China. The Cu-contaminated rhizosphere soil had pH 5.78 ± 0.16, organic carbon 0.87 ± 0.07, total Cu 6057.2 ± 96.8 mg/kg, and available Cu 676.3 ± 58.4 mg/kg. All soil was sieved with a 2-mm sieve to remove litter and vegetation, then stored at −20 °C until further use as a resource for soil microbes.

2.2. First generation acclimation

On early May 2014, a mixture composed of peat, sand and vermiculite (6:3:1 v/v/v) were used as the cultural substrate. The substrate was autoclaved for 2 h at 121 °C to neutralize all native microbial populations (Andrade et al., 2009; Jin et al., 2015). After autoclaving, the substrate having pH 6.43 ± 0.05, organic matter 46.33 ± 0.30 mg/g, total nitrogen 5.09 ± 0.12 mg/g, and Cu 18.9 ± 2.1 mg/kg, was filled into plastic boxes (dimensions of 60 cm length, 40 cm width, and 25 cm height) sterilized by 75% ethanol. Four treatments were used in this study: (1) −Cu − AMF (no Cu addition and no AMF inoculation); (2) −Cu + AMF (no Cu addition and AMF inoculation); (3) +Cu − AMF (Cu addition and no AMF inoculation); (4) +Cu + AMF (Cu addition and AMF inoculation). The Cu-contaminated rhizosphere soil was removed from the refrigerator and incubated at room temperature for 48 h. The spore density is 780 per 100 g soil. According to previous publications (Lau and Lennon, 2011), 5% (volume:volume) Cu-contaminated rhizosphere soil was added to the substrate as the inoculation of AMF. In the treatments without AMF inoculation, 50 mL soil leaching solution filtered with 2.0 mm, 1.0 mm, 0.5 mm, 0.1 mm, 0.075 mm, and 11 μm filter membrane was applied to each pot to compensate microbes except for AMF (Walling and Zabinski, 2006). Then, in the treatments with Cu addition, CuSO₄·5H₂O solution were added to each pot until Cu content in substrate reach 1000 mg/kg, while in the treatment without Cu treatment, the equivalent distilled water was added. The plastic pots were balanced in the greenhouse for 1 month till use.

On early May 2014, *E. splendens* seeds were sterilized in a 0.5% hypochlorite solution, then rinsed thoroughly with sterile distilled water. Seeds were germinated in a greenhouse at the Taizhou University in the Zhejiang Province of China. The temperature in the greenhouse was 30.0 ± 5 °C/18.0 ± 2 °C day/night, relative humidity was 70% ± 10.5%, and the light had approximately 80% of the strength of natural sunlight. On early June 2014, seedlings at the six-leaf stage were transplanted into one soil-filled plastic box. Three replicates were used per treatment resulting in a total of 12 plastic boxes. All boxes were randomly placed in the greenhouse and their positions were randomly changed once per week. All boxes were well watered. During December 2014, matured seeds were collected from *E. splendens* that had been acclimated for one generation. Seeds were stored in a low-humidity storage cabinet (HZM-600, Beijing Biofuture Institute of Bioscience

Table 1
The colonization rate of AMF in the fine roots of *Elsholtzia splendens* in different four treatments. −Cu − AMF, indicates no Cu addition and no AMF inoculation; −Cu + AMF, indicates no Cu addition and AMF inoculation; +Cu − AMF, indicates Cu addition and no AMF inoculation; +Cu + AMF, indicates Cu addition and AMF inoculation. Same small letter indicates there is no significant difference between −Cu + AMF and +Cu + AMF treatments.

Generation	−Cu − AMF (%)	+Cu − AMF (%)	−Cu + AMF (%)	+Cu + AMF (%)
First generation	0 ± 0	0 ± 0	46.67 ± 30.55a	73.33 ± 11.54a
Second generation	0 ± 0	0 ± 0	49.77 ± 8.72a	80.00 ± 17.64a

Table 2
Seed morphology of *Elsholtzia splendens* with acclimation to Cu for two generations in both absence and presence of AMF. Two-way ANOVA results show the effects of AMF (A), Cu (C), and the interactive AMF and Cu (A × C) effects. Different small letters within each category indicate a significant difference between two generations of acclimation either with Cu present or without Cu ($p < 0.05$). *, $p < 0.05$; **, $p < 0.01$.

	Without AMF		With AMF		F value		
	Without Cu	With Cu	Without Cu	With Cu	A	C	A × C
Area (mm^2)	0.24 ± 0.03a	0.20 ± 0.03b	0.26 ± 0.03a	0.24 ± 0.02a	10.47**	8.77**	0.76
Perimeter (mm)	1.86 ± 0.11a	1.71 ± 0.12b	1.96 ± 0.09a	1.88 ± 0.07a	10.75**	8.34**	0.66
Length (mm)	0.69 ± 0.04a	0.63 ± 0.04b	0.73 ± 0.04a	0.70 ± 0.03a	13.33**	7.93*	0.75
Width (mm)	0.50 ± 0.02a	0.46 ± 0.03b	0.51 ± 0.20a	0.50 ± 0.02a	7.67*	8.84**	1.50
Length-to-width ratio	1.39 ± 0.04a	1.38 ± 0.04a	1.43 ± 0.03a	1.42 ± 0.05a	6.84*	0.58	0.01
Thousand-grain weight (g)	0.24 ± 0.02a	0.22 ± 0.02a	0.24 ± 0.01a	0.23 ± 0.01a	0.36	8.49**	0.26

and Biotechnology Development) until further use. Soils were left in the greenhouse and watered once a week to make the microbes live. Fine roots were collected and gently washed under tap water to remove soil for the measurement of the colonization rate of AMF.

2.3. Analysis of the root colonization of AMF

Fine roots were cut into 1-cm-long segments and fixed using formalin acetic alcohol fixation solution. Root segments were dissociated with 10% KOH solution at 90 °C for 1 h, rinsed and acidified in 2% HCl for 5 min, rinsed and stained with 0.01% acid fuchsin/lactic acid/glycerol solution at 90 °C for 1 h, and decolorization with lacto glycerol (1:1:1 lactic acid, glycerol and distilled water) (Jin et al., 2015). The presence and absence of vesicles, hyphae, and spores of mycorrhizal arbuscules in fine roots segments were investigated with the conventional pressing plate method using a BA310 digital microscope (Motic Instruments Inc., Xiamen, Fujian, China) at 40 × magnification. Each root segment was checked for three visual field to the root colonization rate of AMF was calculated as the following formulae: root colonization percentage = no. of infected root segments / no. of total root segments × 100 (Thapa et al., 2015).

2.4. Second generation acclimation

On early May 2015, seeds and the corresponding soils at four different treatments in the first-generation acclimation were used for the next second-generation acclimation following the above protocols. During December 2015, matured seeds were collected from *E. splendens* that had been acclimated for two generations. Seeds were stored in a low-humidity storage cabinet until further use. Fine roots were collected, gently washed, then the colonization rate of AMF in the fine roots at four different treatments was analyzed following the above protocols.

2.5. Measurement of the seed morphological traits

During April 2016, thousand-grain weights were measured using an electronic balance with an accuracy of 0.00001 g. Ten random seeds per individual were scanned using an Epson Perfection 1670 Photo Scanner (Seiko Epson Corporation, Hino, Tokyo, Japan). Seed morphological traits, including seed surface area, perimeter length, and perimeter width were obtained via SmartGrain software and the seed length to width ratio was calculated. Three replicates were used per treatment.

2.6. Measurement of the traits related to seed germination and seedling growth

During May 2016, seeds were randomly selected. Seeds were immersed in a 0.5% hypochlorite solution and subsequently placed in Petri dishes (11 cm in diameter), containing 10 mL of 250 mg/L $CuSO_4 \cdot 5H_2O$ solution after thorough rinsing with sterilized distilled water for three times. Twenty-five seeds were used per dish and three replicates were used per treatment. The Petri dishes were placed in a climate-controlled incubator at 25 °C (day) and 20 °C (night) following a specific light (16 h) and dark (12 h) cycle. The solution was replaced daily to maintain a constant copper concentration. Germination was recorded daily as soon as the radicle length was 2 mm. Germination-related indices were calculated as following: germination rate at the 7th day (%) = total number of germinated seeds during seven days / total number of seeds × 100%. Germination potential at the 4th day (%) = the total number of germinated seeds within four days / total number of seeds × 100%. Germination index at the 7th day = $\sum_{t=1}^{7} Gt/Dt$, where Gt is the number of germinated seeds during day t and Dt is the number of days (t) from germination.

At the 8th day, the lengths of hypocotyl and radicle of seedlings treated with 125 mg/L $CuSO_4 \cdot 5H_2O$ solution were measured using a vernier caliper with an accuracy of 0.1 mm. The fresh weight of
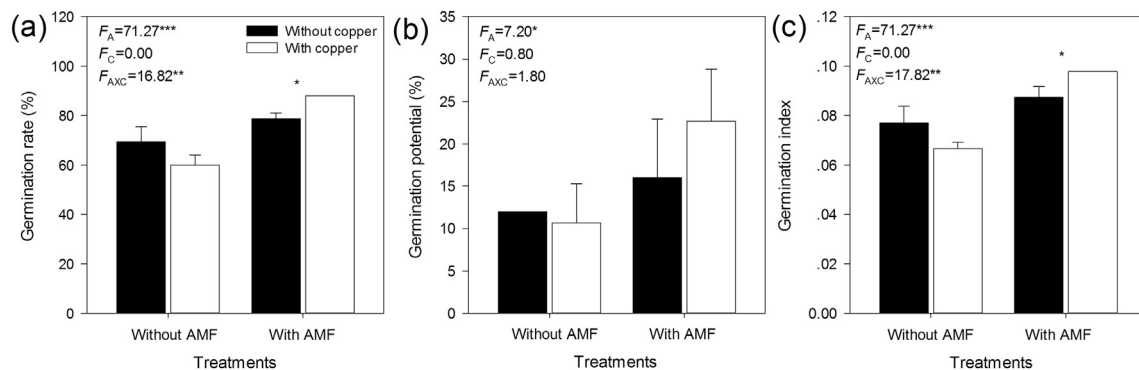

Fig. 1. Germination rate (a), germination potential (b), and germination index (c) of *Elsholtzia splendens* seeds with Cu acclimation for two generations in both absence and presence of AMF. F_A, F_C, and $F_{A \times C}$ indicate the F values of AMF, Cu, and interactive AMF and Cu, respectively. *, $p < 0.05$.
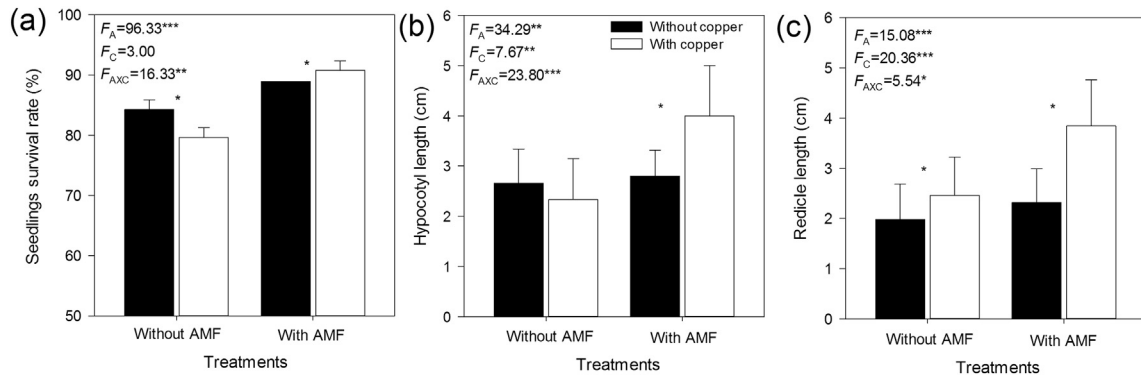
Fig. 2. Survival rate (a), length of hypocotyl (b), and radicle (c) of *Elsholtzia splendens* seedlings with acclimation for two generations with or without Cu in both absence and presence of AMF. F_A, F_C, and $F_{A \times C}$ indicate the F values of AMF, Cu, and interactive AMF and Cu, respectively. *, $p < 0.05$.

hypocotyl and radicle of seedlings were measured with an electronic balance with an accuracy of 0.00001 g.

2.7. Determination of Cu content

The seedlings were continuously growing under sterile hydroponics cultivation with 125 mg/L $CuSO_4 \cdot 5H_2O$ for 2 weeks. The seedlings were separately into root, stem, and leaf, and dried at 70 °C for >24 h to constant weight after 30 min green-killing treatment at 105 °C. The dried tissues were ground to a homogeneous powder. The powder (0.25 g) were digested in 8 mL of HNO_3-H_2O_2 (3:1, v:v) mixture using a microwave digestion method (MARS 240/50, CEM, Matthews, NC, USA). The Cu content in the root, stem, leaf of the seedling was determined using inductively coupled plasma optical emission spectrometer (Optima 2100DV, PerkinElmer, Waltham, Massachusetts, USA). The Cu content was expressed as μg Cu g^{-1} DW (dry weight). The bioaccumulation factor (BF) was calculated as the ratio of Cu concentration in root, stem, or leaf to Cu concentration in hydroponics solution (Wang et al., 2012). The tolerance index (Ti) was calculated as the root, stem, or leaf biomass on hydroponics solution divided by the corresponding root, stem, or leaf biomass on uncontaminated hydroponics solution.

2.8. Statistical analysis

Data are expressed as means with standard deviations (SD). The differences of AMF inoculation, acclimation for two-generations with Cu or without Cu, and their interactive effects on parameters of plant photosynthetic characteristics were determined using two-way ANOVA. Difference between acclimation for two generations with Cu or without

Cu was tested with independent sample *t*-tests. SigmaPlot (version 13.0) was used to create all figures, and the SPSS software package (version 17.0) was used for all statistical analyses.

3. Results

3.1. Effects on root AMF colonization

In the absence of AMF, no colonization of AMF in the fine roots was detected. In the presence of AMF, significant colonization of AMF in the fine roots of *E. splendens* were detected no matter in the first-generation acclimation or the second-generation acclimation (Table 1, Fig. S1). Although the AMF colonization rate in +Cu + AMF treatment was higher than that in −Cu + AMF treatment, no significant difference was found between two treatments no matter in the first-generation acclimation or the second-generation acclimation (Table 1).

3.2. Effects on seed morphology

In the absence of AMF, acclimation to Cu for two generations significantly decreased surface area, perimeter length, and perimeter width of *E. splendens*; however, this treatment had no significant effect on *E. splendens* in the presence of AMF (Table 2). Two-way ANOVA results revealed that acclimation to Cu for two generations had a significant effect on surface area, perimeter length, perimeter width, and the length-to-width ratio, while AMF presence had a significant effect on area, perimeter length, perimeter width, and thousand-grain weight but no significant interactive effects were found (Table 2).
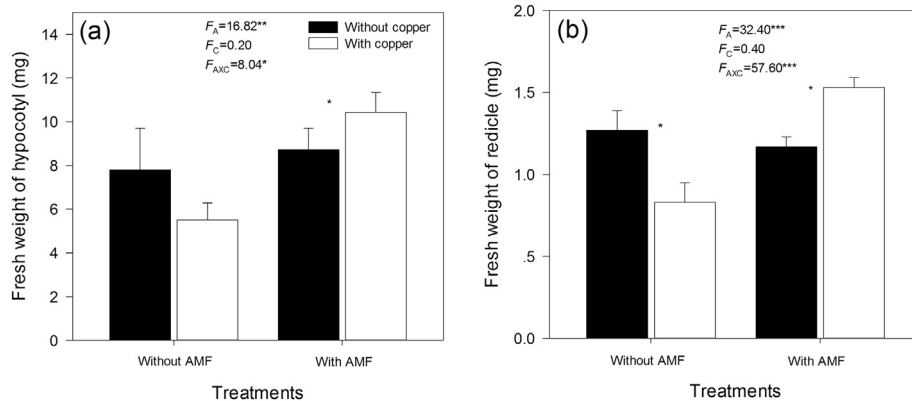

Fig. 3. Fresh weight of hypocotyl (a) and radicle (b) of seedlings of *Elsholtzia splendens* with Cu acclimation for two generations with or without Cu in absence and presence of AMF. F_A, F_C, and $F_{A \times C}$ indicate the F values of AMF, Cu, and interactive AMF and Cu, respectively. *, $p < 0.05$.
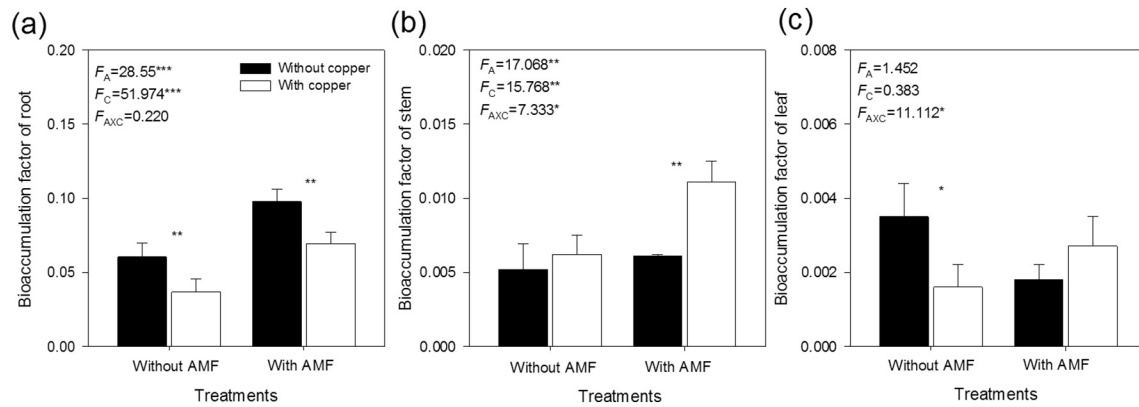
Fig. 4. The bioaccumulation factor of root (a), stem (b) and leaf (c) of seedlings of *Elsholtzia splendens* with Cu acclimation for two generations with or without Cu in absence and presence of AMF. F_A, F_C, and $F_{A \times C}$ indicate the F values of AMF, Cu, and interactive AMF and Cu, respectively. *, $p < 0.05$; **, $p < 0.01$.

3.3. Effects on seed germination

In the absence of AMF, acclimation to Cu for two generations had no significant effect on germination rate, germination potential, and germination index. However, the presence of AMF resulted in a significant increase of the germination rate and the germination index of *E. splendens* seeds (Fig. 1a, b, and c). Two-way ANOVA results showed that AMF and the interactive AMF × Cu had a significant effect on germination rate and index, while AMF had a significant effect on germination potential.

3.4. Effects on seedling survival and growth

In the absence of AMF, Cu acclimation for two generations significantly decreased seedling survival rate, while it significantly increased the survival rate in the presence of AMF (Fig. 2a). With AMF present, Cu acclimation significantly increased both hypocotyl length and radicle length of *E. splendens* seedlings, compared to a lack of effect on hypocotyl length and a lower significant increase of radicle length in the absence of AMF (Fig. 2b). Two-way ANOVA results showed that Cu, AMF, and the interactive AMF × Cu had a significant effect on the length of both hypocotyl and radicle.

In the absence of AMF, Cu acclimation significantly decreased the fresh weight of the radicle of *E. splendens* seedlings, while the fresh weights of hypocotyl and radicle significantly increased in the presence of AMF (Fig. 3a, b). Two-way ANOVA results showed that Cu and the

interactive AMF × Cu posed a significant effect on the fresh weight of hypocotyl and radicle.

3.5. Effects on the accumulation of Cu

In the absence of AMF, Cu acclimation for two generations significantly decreased the bioaccumulation factor and tolerance index of root and leaf, but had no significant effect on that of stem (Figs. 4 and 5). In the presence of AMF, Cu acclimation significantly decreased the bioaccumulation factor and tolerance index of root, but significantly increased that of stem while had no significant effect on that of leaf (Figs. 4 and 5). Two-way ANOVA results showed that Cu and AMF had a significant effect on the bioaccumulation of root and stem, the tolerance index of root, stem and leaf, but had no significant effect on that of leaf (Figs. 4 and 5). And the interactive AMF × Cu had a significant effect on the bioaccumulation and tolerance index of stem and leaf, but had no significant effect on those of root (Figs. 4 and 5).

4. Discussion

The rapid evolutionary adaption of plants to heavy metal stress has been well documented, including short-lived herbaceous species as well as long-lived species such as trees (Eränen, 2006, 2008). Gartside and McNeily (1974) suggested that fully Cu tolerant individuals have emerged after just one cycle of selection, and found fully Cu tolerant individuals of *Agrostis tenuis* and *Dactylis glomerata* after six and four
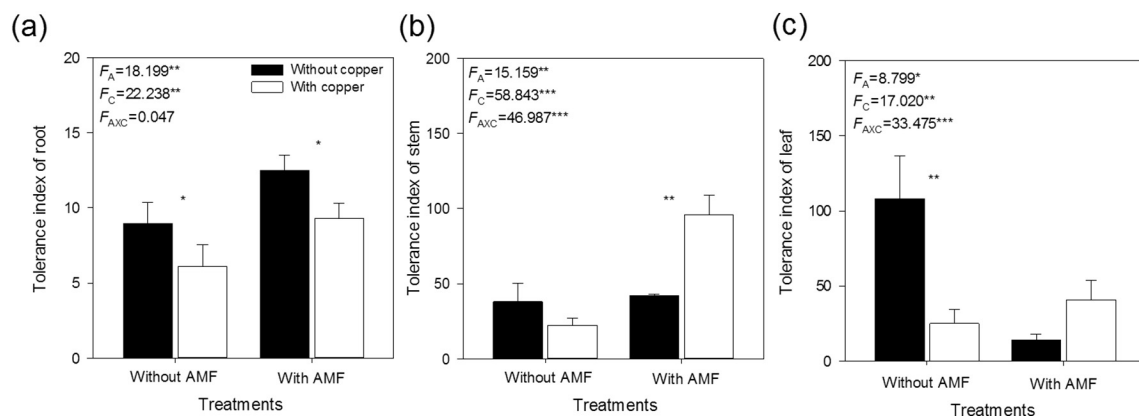

Fig. 5. The tolerance index of root (a), stem (b) and leaf (c) of seedlings of *Elsholtzia splendens* with Cu acclimation for two generations with or without Cu in absence and presence of AMF. F_A, F_C, and $F_{A \times C}$ indicate the F values of AMF, Cu, and interactive AMF and Cu, respectively. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

generations of selection, respectively. Ernst (1999) reported that the evolution to metal tolerance could be realized within less than a decade. Metal adaptation has been achieved within few generations under laboratory conditions in several species (Posthuma and van Straalen, 1993). Furthermore, Eränen (2008) studied the rapid evolution of the copper-nickel resistance of the mountain birch (*Betula pubescens* subsp. *czerepanovii*) and suggested that the particularly strong natural selection eliminated all sensitive genotypes within one or two generations, while only the most tolerant individuals would persist and would be able to produce adapted seeds in individual barrens. In this study, we found Cu acclimation for two generations significantly increase the germination rate of seeds, as well as the germination index, seedling survival rate, length of hypocotyl and radicle, and fresh weigh of hypocotyl and radicle of *E. splendens* in the presence of AMF. However, all these parameters were decreased in the absence of AMF, indicating that the rapidly adapted seeds of *E. splendens* were produced after Cu acclimation for two generations when AMF were present. These results suggest that seeds and seedlings resulting from the Cu acclimation treatment had a relatively short generation time combined with a high degree of genetic diversity, which enabled rapid evolution in response to novel selective pressures, and in turn altered patterns of natural selection within plant populations (Lau and Lennon, 2011). Jeffries et al. (2003) suggested that the positive influence of mycorrhiza for the improvement of seedling survival during the second year of cultivation would not be rare. To our knowledge, our study is the first to directly suggest that AMF benefits the fitness of plant offspring, facilitating the rapid adaptation of plants to Cu stress.

It has been well documented that AMF can help plant adaptation to drought, high temperature, salt, and heavy metal stress (Rodriguez et al., 2008; Wu et al., 2010; Zhu et al., 2011; Birhane et al., 2012). Costs incurred by stress condition can result in reduced performance of adapted seedlings (Eränen, 2008). In the present study, in the absence of AMF, acclimation to Cu for two generations significantly decreased the surface area, perimeter length, and perimeter width of seeds, also decreased the seedling survival rate and the fresh weight of *E. splendens* seedling radicles, indicating the incurred cost of acclimation for two generations: seeds from plants with two generations of acclimation with Cu were smaller having more time to germinate, and seedlings grew slower compared to those acclimated with Cu. AMF colonization always showed no or less effect on plant growth because the economical cost of produce mycelium is lesser than or equal with that produces roots (Brundrett, 1991). Jankong and Visoottiviseth (2008) found that AMF had no significant effect on the growth of *Pityrogramma calomelanos* and *Tagetes erectan* under arsenic stress. However, Jankong and Visoottiviseth (2008) also found that AMF colonization can also promote the growth of *Melastoma malabathricum* under arsenic stress, when the plant's root structure has low branching frequency and no hairy roots. In this study, we indeed found that AMF significantly increased the germination rate and germination index of seeds, as well as the length and fresh weight of both hypocotyl and radicle of *E. splendens* seedlings of *E. splendens* after two-generation acclimation. These results indicated that the cost incurred by Cu acclimation for two generations might be compensated by the benefit produced by the association of AMF. In this study, we found that AMF significantly decreased the bioaccumulation factor and tolerance index of root, but significantly increased that of stem in adapt to Cu stress. This might be the main mechanism for AMF facilitated adaption of *E. splendens* to Cu stress.

Alternatively, Via et al. (1995) indicated that the resistance of seedlings originating from polluted sites could be a sign of adaptive phenotypic plasticity. We also found that Cu acclimation for two generations significantly increased radicle length in the absence of AMF. However, in the presence of AMF, the increasing amplitude indicates that the direct adaptive plasticity response of the *E. splendens* radicle length to Cu stress would benefit the adaptation of *E. splendens* to Cu stress. Both of these mechanisms (cost compensation and adaptive plasticity) have previously been reported by Schultz et al. (2001) in the adaptation

of *Andropogon gerardii* to high- and low-nutrient prairies in both absence and presence of AMF.

5. Conclusions

In the present study, we could only draw the conclusion that AMF facilitates the adaptation of *E. splendens* to Cu stress and suggest the elimination of cost incurred by Cu stress and the adaptive plasticity of *E. splendens* in response to Cu stress as possible mechanisms. However, we could not distinguish whether the evolution of tolerance (involving genetic changes in populations) happened as part of the adaptation after two generations of acclimation. Bahrndorff et al. (2006) reported that both evolution and plasticity changes have important implications for developing bioindicators. To discriminate the evolutionary significance or adaptive phenotypic plasticity of rapid *E. splendens* adaptation under Cu stress, further studies should focus on the phenotypic selection of Cu acclimation in *E. splendens* and the evolutionary selection of plant mutants depending on AMF. We predict that adaptation to, and hence evolution toward the tolerance of the population to higher Cu levels would rapidly happen in *E. splendens* through the assistance of AMF.

Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.scitotenv.2017.05.063.

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