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Arbuscular mycorrhizal fungi alleviate arsenic toxicity to *Medicago sativa* by influencing arsenic speciation and partitioning

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ABSTRACT

In a pot experiment, *Medicago sativa* inoculated with/without arbuscular mycorrhizal (AM) fungus *Rhizophagus irregularis* were grown in four levels (0, 10, 25, and 75 mg/kg) of arsenic (As)-polluted soil to investigate the influences of AM symbiosis on plant As tolerance. The results showed that mycorrhizal inoculation significantly increased plant biomass, while As addition decreased mycorrhizal colonization and hyphal length density. Mycorrhizal inoculation dramatically improved plant phosphorus (P) nutrition, restricted As uptake and retained more As in roots by upregulating the expression of the AM-induced P transporter gene *MsPT4* and the metallothionein gene *MsMT2*. High soil As content downregulated *MsPT4* expression. Dimethylarsenic acid (DMA) was detected only in the shoots of mycorrhizal plants, indicating that AM fungi likely play an essential role in As detoxification by biological methylation. The present investigation allowed deeper insights into the As detoxification mechanisms of AM associations and demonstrated the important role of AM fungi in plant resistance under As-contaminated conditions.

1. Introduction

Arsenic (As), as a documented class 1 human carcinogen, is ubiquitously present in the environment (Valko et al., 2006). Excessive As adversely influences the growth and development of organisms and damages the structures and functions of ecosystems (Barral-Fraga et al., 2016; Sun et al., 2016). As seen in reports, at least 50 million people in 19 countries are under the threat of endemic arsenism, among which most are Asians (Berg et al., 2001). Inorganic As is the main As species in the environment, existing as arsenate [As(V)] and arsenite [As(III)] (Dixit and Hering, 2003). In addition, small amounts of organic As produced by biological metabolism are also present, including monomethylarsenic acid (MMA), dimethylarsenic acid (DMA) and trimethylarsine oxide (TMAO) (Mandal and Suzuki, 2002). Arsenic toxicity largely depends on the chemical form and the oxidation state of the As species. Organic As is considered to be much less toxic than inorganic As. Hence, arsenical biomethylation is generally recognized as a mechanism of arsenic detoxification (Styblo et al., 2000).

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil fungi that establish mycorrhizal symbioses with most of the terrestrial higher plants and are an important “bridge” connecting plants and soil (Schwab et al., 1991). AM can alleviate many aspects of environmental stresses on plants, such as enhancing water uptake and mineral nutrition or mediating heavy metal uptake and accumulation in plants (Li et al., 2013; Wu et al., 2016; Zhang et al., 2015).

As for mycorrhizal effects on As tolerance, what has been well demonstrated is the “growth dilution effect”, which means that AM enhances plant phosphorus (P) nutrition, promotes plant growth and thus dilutes the As absorbed by plants (Chen et al., 2007). Hyphae of AM fungi take up P from a long distance, where plant roots cannot reach, via a high-efficient P transporter, which has been identified as *GiPT* in *Glomus intraradices* and *GvPT* in *Glomus versiforme* (Spagnoletti et al., 2016; Campos-Soriano et al., 2010; Harrison and Buuren, 1995). Subsequently, AM fungi transfer P into plant cortex cells via plant phosphate transporters, which express only on the symbiotic interfaces in cells containing arbuscules. These AM-induced P transporters have been found in many plant species (Javot et al., 2007; Karandashov and Bucher, 2005). Furthermore, Christophersen et al. (2009) revealed that AM fungi downregulated the gene expression of high-affinity inorganic orthophosphate (Pi)-uptake systems in root epidermis and root hairs while upregulating those of AM-induced P transporters in barley, indicating a potential mechanism involving AM-enhanced plant As tolerance. In their research, however, only one As addition level (2.5 mg/kg) was used, and no effect of As addition on the expression of the AM-induced P transporter gene was detected.

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In addition, the mechanisms identified in numerous plant species showed that intracellular arsenic can be complexed with Cys-rich peptides such as metallothioneins (MTs) or phytochelatins and then stored in vacuoles to reduce their mobility (Tsai et al., 2009). Studies have shown that AM exhibits a strong ability to immobilize metals. AM fungi can adsorb and absorb heavy metals, thus inhibiting metal portioning to plant shoots (Chen et al., 2005; Dong et al., 2008; Wu et al., 2016). MTs may play a key role in metal complexation, and genes encoding MTs in AM fungi have been identified in several fungal species (Gonzalez-Guerrero et al., 2007; Lanfranco et al., 2002). As a ubiquitous mechanism of heavy metal detoxification, however, MTs can also be produced by plants themselves, chelating and immobilizing As (Liang et al., 2016). It is clear that heavy metals can induce plants to produce MTs to alleviate the toxicity of heavy metals (Tsai et al., 2009). Few studies, however, have demonstrated whether AM fungi can affect the production of MTs in plants under soil As contamination.

In addition to the mechanism of immobilization at the mycorrhizal level, AM fungi can also produce and secrete glomalin-related soil protein (GRSP) into the soil (Wright and Upadhyaya, 1996), which has been shown to bind heavy metals, such as Cu, Cd, Pb and Zn, in the soil, or even passively adsorb them to the cell walls of extraradical mycelium (Vodnik et al., 2008; González-Chávez et al., 2009; Cornejo et al., 2017). A recent study reported that increasing As dosages in soil improved soil total GRSP concentration, with the potential of AM fungi sequestering soil As by GRSP and lowering As concentration in mycorrhizal plants (Spagnoletti et al., 2017).

In the present study, *Medicago sativa* inoculated with/without AM fungus *Rhizophagus irregularis* was grown in sterilized soil amended with four different levels of As. The objectives of the experiment are as follows: (1) to investigate the involvement of AM fungi in plant As metabolism and the potential role of AM fungi in alleviating As phytotoxicity; (2) to examine the gene expression of AM-induced P transporters under different soil As levels; and (3) to reveal whether AM influences As accumulation and distribution by regulating the expression of plant metallothionein genes.

2. Materials and methods

2.1. Host plants and AM fungus inocula

M. sativa seeds were surface sterilized by 75% ethanol for 30 s, followed by 30 min in 2% NaOCl. Seeds were then thoroughly washed with sterile water and left to germinate on moist filter paper for 48 h at 25 °C. Uniform seeds with radicles were selected for raising seedlings in sterile soil and river sand mixture until the sprout of 2 new leaves. Seedlings were selected for uniformity before transplanting.

The AM fungus *R. irregularis* BGC BJ09 isolated from non-contaminated soil was obtained from the Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry. The inoculum contained spores, mycelium, sandy soil and root fragments with approximately 1000 spores/100 g soil.

2.2. Cultivation media

The experimental soil was collected from farmland in the Daxing district of Beijing (39°36′N, 116°18′E), China. The soil properties were as follows: pH 8.17 (1:2.5 soil to water), organic matter 30.45 g/kg, extractable P 6.73 mg/kg, extractable As 0.36 mg/kg, available N 8.46 mg/kg, total N 0.17%, and total C 1.78%. The soil was air dried, passed through a 2 mm sieve, sterilized by γ-ray (20 kGy, 10 MeV electron beam) and amended with basal nutrients of 120 mg/kg N (NH_4NO_3), 30 mg/kg P (KH_2PO_4) and 120 mg/kg K (KH_2PO_4, K_2SO_4).

2.3. Experimental procedure

Cuboid plastic pots (8 cm × 8 cm × 10 cm) were separated by a 37 μm mesh into two compartments, named the mycorrhizal compartment and hyphal compartment, respectively. Hyphae can grow through the mesh, but roots cannot. Four As addition levels (0 mg/kg, 10 mg/kg, 25 mg/kg, and 75 mg/kg) were arranged with sodium arsenate solution thoroughly mixed into the soil and then stabilized for 20 days. Mycorrhizal treatments received 50 g of the fungal inoculum, which was carefully mixed into the 650 g growth medium. Non-mycorrhizal treatments received an equivalent amount of autoclaved soil-sand mixture together with a filtrate of the fungal inoculum to provide a similar microflora except for AM fungus. Three seedlings were transplanted to each pot. There were a total of 8 treatments with four replicates, resulting in a total of 32 pots in a randomized block design. The soil moisture content of the 75% water holding capacity was maintained by regularly weighing and adding de-ionized water throughout the experimental period. Plants grew for a total of 60 days. The experiment was carried out in a phytotron with 16 h/25 °C day, 8 h/18 °C night, and a light intensity of 700 μmol·m^{-2} s^{-1}.

2.4. Harvest and sample collection

Plant shoots and roots were separately harvested and washed thoroughly with de-ionized water. After being dried by blotting paper and weighed, 0.1 g of fresh roots was collected for mycorrhizal colonization determination. Fresh roots (0.2 g) were frozen in liquid nitrogen and stored at −80 °C for RNA extraction. The remaining samples were weighed and stored at −80 °C after freeze-drying at −50 °C for 72 h. Soil in the hyphal compartment was collected to measure hyphal length density and GRSP concentrations.

2.5. Mycorrhizal dependency, mycorrhizal colonization and hyphal length density

The mycorrhizal dependency (MD) of *M. sativa* was determined by expressing the dry weights of mycorrhizal plants as a percentage of the dry weights of non-mycorrhizal plants, following the procedure of Menge et al. (1978).

Mycorrhizal colonization was determined and calculated following the procedure of Phillips and Hayman (1970). The hyphal length density was determined and calculated following the procedure of Jakobsen et al. (1992).

2.6. Glomalin concentration

Easily extractable glomalin-related soil protein (EE-GRSP) and total glomalin-related soil protein (T-GRSP) were extracted following the procedure of Wright and Upadhyaya (1996). Air-dried soil (0.5 g) was mixed with 4 ml of 20 mmol/L sodium citrate at pH 7.0 in 50 ml centrifuge tubes, which were then autoclaved at 121 °C for 30 min to extract EE-GRSP. T-GRSP from 0.5 g of soil was extracted by 4 ml of 50 mmol/L sodium citrate at pH 8.0 in 50 ml centrifuge tubes, which were then autoclaved at 121 °C for 60 min. The samples were centrifuged at 10,000 × *g* for 5 min and stored. Each soil sample was extracted three times using the same procedure, and all the supernatants were mixed together. The protein concentrations were measured following the method by Sedmak and Grossberg (1977). Then, 0.5 ml supernatant was mixed with 5 ml 0.01% Coomassie brilliant blue G250, and the absorbance at 595 nm was determined using bovine serum albumin as a standard.

2.7. Chemical analysis

The available soil As concentration was measured following the methods of Woolson et al. (1971). After being stabilized, the soil samples were air dried for 3 days. The extracting solution was 0.5 mol/L $NaHCO_3$ solution at pH 8.5, and the soil: solution ratio was 1:20. Soil at each As level was measured four times. The mixture was sonicated at