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Unraveling the effects of arbuscular mycorrhizal fungus on uptake, translocation, and distribution of cadmium in *Phragmites australis* (Cav.) Trin. ex Steud

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ABSTRACT

Phragmites australis (Cav.) Trin. ex Steud. has been reported to form a symbiosis with arbuscular mycorrhizal fungus (AMF). However, the tolerance mechanism for AMF symbiosis on cadmium (Cd) phytotoxicity still remains unclear. In this study, we investigated the effects of *Rhizophagus irregularis* inoculation on Cd-stressed (0, 1, and 20 mg L^{-1}) roots, stems, and leaves of *P. australis* with regard to subcellular Cd distribution and chemical forms of Cd. In addition, transmission electron microscopy and Fourier transform infrared spectroscopy were used to investigate variations in subcellular structures and functional groups in plant organs. The results showed that AMF inoculation could induce selective Cd distribution at subcellular levels, depending on different Cd treatments. The investigation of the chemical forms illustrated that AMF inoculation could alleviate Cd toxicity in all organs. Increases were observed in the ratios of undissolved Cd (F_{HAc}) and oxalate Cd (F_{HCl}), while decreases were observed in pectates and protein-integrated Cd (F_{NaCl}) as well as water soluble Cd (F_W). Hydroxyl (-OH), amide (-NH), carboxyl (C=O), and phosphate (P=O) groups as well as C-O and C-N stretching played predominant roles for the enhancement of Cd tolerance in response to AMF inoculation. These results provide instructive evidence for the mechanisms by which AMF inoculation enhances the Cd tolerance of *P. australis* via Cd uptake and distribution.

1. Introduction

Cadmium (Cd) is a non-essential heavy metal (HM), which is often found in plants, where even at low levels, it can induce damage due to its high solubility in water and ready plant intake (DalCorso, 2012). Cd pollution is currently one of the most severe environmental concerns in China due to both rapid industrialization and urbanization (Hu et al., 2013; Tian et al., 2012). In recent years, Cd has become the main pollutant, thus affecting the safety and quality of staple crops such as rice (Li et al., 2016). Excessive concentrations of Cd can passively impact the biological functions of plant cells, such as plant growth, photosynthesis, nutrients uptake, and enzyme activity (Yadav, 2010; Fu et al., 2011; Wang et al., 2017). Even at low Cd concentrations, a negative effect on vegetative and reproductive plant organs can be induced by Cd via uptake and transport of Cd from roots to shoots (DalCorso et al., 2010).

Phragmites australis (Cav.) Trin. ex Steud. is a widespread and dominant macrophyte plant in wetland with a high ability for the accumulation of HMs (including Cd) and has therefore been used for phytoremediation (Fediuc and Erdei, 2002; Rocha et al., 2014; Ye et al., 1997). Obolewski et al. (2011) suggested that Cd treatment induced high Cd concentration in all analyzed samples of shoots, which was indicative of particularly high potential Cd uptake by *P. australis*. Several researchers indicated that high amounts of lignin and cellulose in *P. australis* might help to enhance its capability for accumulating HMs (Lenssen et al., 1999; Srivastava et al., 1994). Although *P. australis* provides an ideal natural resource for the effective phytoremediation of HM contamination (as previously reported), its tolerance to Cd stress is still inhibited by excessive Cd in the substrate (Fediuc and Erdei, 2002; Rocha et al., 2014).

Therefore, to improve HM tolerance, while reducing HM toxicity in plants, one efficient strategy is the inoculation with arbuscular mycorrhizal fungus (AMF), which has been introduced recently (Jiang et al., 2016). It has been reported that AMF could play a significant role

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in protecting host plants from HM toxicity (Göhre and Paszkowski, 2006; Jankong and Visoottiviseth, 2008). In addition, previous studies illustrated that AMF inoculation (*Rhizophagus irregularis* in particular) can alleviate the deleterious effects of excessive Cd stress and as a result, significantly improve plant growth (Wang et al., 2017) and affect the translocation of Cd and nutritive elements (Huang et al., 2017).

However, detailed information on the tolerance mechanism with which AMF inoculation enhances Cd tolerance in root, stem, and leaf cells of *P. australis* still remains unclear, in particular with regard to Cd uptake, translocation, and distribution. In this study, *R. irregularis* was selected in combination with *P. australis* as a model for AMF inoculation to determine the tolerance mechanisms AMF convey in response to Cd stress. Therefore, the aims of this work were to i) assess the role of AMF in Cd uptake and translocation via investigating the subcellular distributions and chemical forms of Cd; ii) investigate the effects of AMF on Cd-stressed *P. australis* organs, applying transmission electron microscope (TEM) and Fourier transform infrared spectroscopy (FTIR). This study provides new insights into the effects of AMF inoculation to remedy Cd-contaminated sites in situ.

2. Methods and materials

2.1. Preparation of materials and pot experiment

In this study, *R. irregularis* (FR717169) was selected as the experimental AMF and was obtained from the Life Science College of Heilongjiang University (Harbin, Heilongjiang Province, China). Prior to the pot experiment, *Trifolium repens* L. was cultivated in a soil-vermiculite-sand substrate mix (volume ratio: 2:3:5) to propagate the original inoculum in a greenhouse for three months. The light/dark circle in the greenhouse was set to 14/10 h, with light time at 28 °C and dark time at 23 °C under a relative humidity of 75%. Subsequently, the belowground parts of *Trifolium repens* L. were collected, cut into small pieces (< 1 cm), and arranged in a dark, well-ventilated, and air-dried environment.

P. australis (Panjin Institute of Reed Science, Panjin, China) is a common hydrophyte in the wetland, especially in northeastern China and was used as the model plant. Previous studies by our group have already demonstrated that the inoculum *R. irregularis* could successfully colonize the roots of *P. australis*, which then formed AM symbiosis, which led to improved plant growth compared to a control without inoculation (Wu et al., 2014; Wang et al., 2017). After separating inflorescences of *P. australis*, seeds were obtained and sterilized via absolute ethanol for 10 min, followed by rinsing with de-ionized water. Vermiculite was used as experimental substrate after autoclaving at 121 °C for 2 h.

Prior to the experiment, autoclaved vermiculite (100 g per pot) was transferred into each experimental plastic pot (height: 10 cm; upper diameter: 11 cm; bottom diameter: 10 cm). Each pot received an additional 10 g of unsterilized inoculum, which contained 200–250 *R. irregularis* spores. In addition, the same weight (10 g) of sterilized inoculum was supplied to treatments without *R. irregularis* inoculation to avoid partial loss between pots. The greenhouse experiment was a 3 × 2 complete factorial combination, which was comprised of three Cd concentrations (0, 1, and 20 mg L^{-1}) either with or without *R. irregularis* inoculation.

The experimental plants were irrigated with 1/4-strength Hoagland-modified solution (100 mL $week^{-1}$ pot^{-1}) to avoid nutrient deficiency, which was diluted with de-ionized water. The plants were cultured to the five-leaf stage, which took approximately nine weeks. Ten uniform *P. australis* seedlings were transplanted into each experimental pot. Afterward, Cd solutions 0, 1, and 20 mg L^{-1} were added to the Cd-treatment pots every three days. All three levels of Cd were supplied as $CdCl_2{\cdot}2.5H_2O$ to simulate actual contamination. According to Cd addition and whether they were AMF inoculated (M) or not (NM), the six treatments were denominated as follows: 0-NM (cultured in the absence of Cd and *R. irregularis*), 0-M (cultured in the absence of Cd but inoculated with *R. irregularis*), 1-NM (cultured with 1 mg L^{-1} of Cd but without *R. irregularis* inoculation), 1-M (cultured with 1 mg L^{-1} of Cd and *R. irregularis* inoculation), 20-NM (cultured with 20 mg L^{-1} of Cd but without *R. irregularis* inoculation), and 20-M (cultured with 20 mg L^{-1} of Cd and *R. irregularis* inoculation).

After 21 days, all plants were carefully harvested and then rinsed with de-ionized water for 5 min. Root, stem, and leaf samples were collected separately and pretreated differently for subsequent experiments.

2.2. Determination of Cd concentrations

Root, stem, and leaf samples were separately oven-dried at 70 °C until the weight of each organ was invariant. Digestion of dried samples (300 ± 3 mg) in the polytetrafluoroethylene (PTFE) digestion tank included HNO_3, HCl, H_2O_2, and $HClO_4$ (volume ratio = 5:2:1:3) at 120 °C and 155 °C during the first and second stages of digestion, respectively. Eventually, the residue was diluted with 25 mL of 2% HNO_3 (v/v) after cooling to room temperature. Cd was measured via inductively coupled plasma mass spectrometry (ICP-MS, Agilent Technologies 7700x, Santa Clara, USA). GBW07605 (GSV-4) was obtained from the China Standard Materials Research Center (Beijing, China) and was used as standard reference material to assess reliability and to calculate the detection limit for the Cd concentrations. The recovery rates of Cd ranged between 95.5% and 103.5%.

2.3. Distribution of subcellular Cd fractions

According to previously described methodology (Lai, 2015; Wang et al., 2009), the frozen fresh tissues for the subcellular distribution analysis were prepared in liquid nitrogen (−196 °C), homogenized in solutions (pH = 7.5) that contained 0.25 M sucrose, 50 mM Tris-HCl, and 1.0 mM dithiothreitol ($C_4H_{10}O_2S_2$) using a chilled mortar and pestle. Briefly, the homogenate was centrifuged at 3000 × g for 15 min, and the deposition was labeled cell wall fraction (F_{CW}). The supernatant solution was further centrifuged at 12000 × g for 30 min. The deposition and the supernatant were labeled cell organelle fraction (F_{CO}) and soluble fraction (F_S), respectively. All steps were performed at 4 °C. All three fractions were dried and digested for determination of Cd concentrations via ICP-MS, as described above.

2.4. Extraction of chemical forms of Cd

The extraction of the chemical forms of Cd from different organs of *P. australis* were conducted according to previously published methods (Zeng et al., 2011). Six chemical forms were extracted and their order of extraction solutions is shown in Table S1. The frozen fresh subsamples (0.5 g, FW) of plant organs (roots, stems, and leaves) were collected, frozen, and mixed with extraction solutions (w/v = 1:10), prepared with a grinder with continuous grinding (TL2010S, DHS Technology, Shenzhen, China), and mixed for 22 h at 25 °C. Afterward, the homogenate was centrifuged at 3000 × g for 10 min to obtain the first supernatant solution. The deposition was resuspended twice. using the same extraction solution. The three supernatant solutions were collected in combination and placed in a 50 mL centrifugal tube. The deposition was subsequently extracted in the next step with the next extracting solution. The last deposition was the residue, which was dried at 70 °C to a constant weight; then, digested and measured for the determination of Cd concentrations via ICP-MS, as described above.

2.5. TEM observation

Small root or leaf samples (0.01 cm^2) were fixed with 25% glutaraldehyde (v/v) for 2 h with consecutive vacuuming, rinsing in 0.1 M phosphate butter (PBS, pH = 6.8) for three times (15 min/rinse), fixing