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# Phosphate application enhances the resistance of arbuscular mycorrhizae in clover plants to cadmium *via* polyphosphate accumulation in fungal hyphae

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ABSTRACT

Both phosphorus (P) application and arbuscular mycorrhizal (AM) fungal inoculation can enhance plant resistance to Cd toxicity, however, their interaction and the underlying mechanism are still unclear. The aims of this study were to investigate the effect of P application on AM resistance to Cd and to reveal the corresponding mechanism. We designed a compartmented system to separate extraradical hyphae (EH) of *Rhizophagus irregularis* from the roots of clover (*Trifolium ripense* L.). EH was applied with different levels of P and cadmium (Cd). The content and uptake of P and Cd were monitored. Polyphosphate (polyP) accumulation in EH was quantified. The localization of polyP and Cd was visualized using fluorescent staining. Results indicated that Cd greatly decreased EH biomass and polyP accumulation while P increased polyP accumulation. PolyP accumulation in EH enhanced AM resistance to Cd, and also reduced Cd uptake by plants. Fluorescent images indicated the overlapping of Cd signals and polyP signals. These data suggest that P application can enhance the resistance of AM to Cd *via* polyP chelation, and this represents another mechanism underlying the detoxification of Cd by AM hyphae other than the absorption by hyphae.



## 1. Introduction

Heavy metal (HM) pollution has increasingly become the main public concern due to their long-term and hidden toxicity posed on public health via food chains (Nabulo et al., 2012; Zhao et al., 2012). Among several HM species representing realistic threats to environments, cadmium (Cd) is of great significance nowadays, especially in China (He et al., 2013; Niu et al., 2013). For example, Cd (0.082–1.31 mg/kg) had the highest pollution index (PI) of 5.28, while other investigated HMs (Pb, Zn, Cu) in Chinese arable soils are comparatively safe (Niu et al., 2013). Cd is released into the environment through several passages, *e.g.* heating systems, metallurgic industries, waste incinerators, urban traffic, cement factories, and as a contaminant of phosphate fertilizers (Gallego et al., 2012). It is estimated that approximately 30,000 t of Cd are released into the atmosphere each year, with 4000–13,000 t coming from industrial activities (ATSDR, 2005).

In response to the Cd accumulation in the topsoil in diverse ecosystems, plants have developed some mechanisms to cope with this contaminant, involving those physiological processes from uptake, translocation to phytochelation, extrudation (Gallego et al., 2012). For example, a symplastic uptake of Cd bypassing the Casparian strips was proposed for eggplant (*Solanum melongena*), exhibiting higher rates of root-to-shoot Cd translocation in comparison to *Solanum torvum* (Yamaguchi et al., 2011). The latter was confirmed to be resistant to Cd stress (Arao et al., 2008; Mori et al., 2009). Broadhurst et al. (2013) reported that Si may participate in the detoxicification of Cd in a Cd/Zn hyperaccumulator *Picris divaricata*, while the synthesis of thiol functional groups, as phytochelatins, enhanced the resistance of *Oenothera odorata* to Cd toxicity (Son et al., 2012). Despite these physiological resistances, plants can also acquire additional resistance transferred by soil microbes (Andreazza et al., 2010; Long et al., 2010), *e.g.* arbuscular mycorrhizal (AM) fungi. AM fungi are ubiquitous symbiotic soil fungi, taxonomically belonging to Glomeromycota (Smith and Read, 2008). Once establishing symbiotic relation with plant

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roots, AM fungi improve plant resistance to many biotic and abiotic stresses (Smith et al., 2010), including HM toxicity (Göhre and Paszkowski, 2006).

A large body of research demonstrates that AM fungi can effectively enhance plant resistance to HM toxicity (Göhre and Paszkowski, 2006; Meier et al., 2012), such as Cd (Wang et al., 2012), lead (de Souza et al., 2012), mercury (Yu et al., 2010), and arsenate (Chen et al., 2013). Furthermore, phytoremediation in combination with AM fungi has been proposed as the most potential strategy to alleviate HM pollution in soil environments (Schützendübel and Polle, 2002; Dong et al., 2008). Göhre and Paszkowski (2006) put forward several mechanisms underlying the enhanced tolerance to HMs by AM fungi. While some mechanisms have been demonstrated, *e.g.* the retardation of the transportation of HMs from roots to shoots, the decrease in the mobility of HMs in soils, the inactivation of HMs by compounds excreted from hyphae, the absorption of HMs by hyphal surface (Valko et al., 2005; Bellion et al., 2006; Hildebrandt et al., 2007), others are still to be clarified, *e.g.* the chelation or precipitation of HMs by the polyphosphate (polyP) inside hyphae. PolyP is the largest P storage and a mediator of long-distance P translocation in AM (Hijikata et al., 2010), and thus represents a good indicator of the P-supplying activity of AM fungi (Ohtomo and Saito, 2005). Its role in decreasing HM toxicity in AM has also been suggested (Cornejo et al., 2013), however, no evidence is available till now.

In this study, with clover plants (*Trifolium ripense* L.) as host plants and *Rhizophagus irregularis* as AM fungus, two experiments using tri-compartmented system were designed to address the following questions: (1) whether polyP is involved in the chelation of HMs in AM, and (2) how does the polyP affect the uptake, distribution and transportation of Cd in AM. The tri-compartmented system was built up for the convenient collection of extraradical hyphae, and fluorescent probes in conjunction with confocal laser microscopy system (CLMS) were employed to localize polyP and Cd in AM fungal hyphae in symbiosis with clover plants.

## 2. Material and methods

### 2.1. Biological material and experimental design

Seeds of clover (*T. ripense* L.) were commercially got from the market. *R. irregularis* BGC BJ09 (formerly known as *Glomus intraradices* BGC BJ09) were commercially got from Beijing Academy of Agricultural and Forestry. AM fungal inoculum was propagated using *T. ripense* and *Sorghum bicolor* as host plants, and the inoculum was the mixture of spores, hyphae, colonized root fragments and substrates.

Tri-compartmented system was constructed using PVC tube (8 cm in diameter). The tube was longitudinally separated into three compartments including one hyphal compartment (HC, 5 cm in length) in the center and two root compartments (RC, 8 cm in length) at the two sides. The narrower hyphal compartment was specially planned for the concentration of extraradical hyphae. Nylon mesh (30 μm pore size) between RC and HC was used to barrier the root growth into HC, while hyphae can freely grow into HC. Two ends of PVC tube were sealed with plastic membrane. For each compartment, an open (2 cm in width) was cut for filling the substrate, seeding and irrigation. Acid-washed, clean river sands were used as substrate. Each RC and HC was filled with 350 g and 240 g substrate, respectively. In each RC, substrates were inoculated with AMF at the rate of 10% (w/w) (about 910 spores in each RC). Seeds of clover were surface-sterilized with 10% (v/v) $NaClO_3$ for 30 min and washed with double deionized water ($ddH_2O$) for 10 times. Ten seeds were sown in each RC. All PVC tubes were incubated in a growth chamber with a 16/8 h light/dark (26 °C/19 °C) cycle. After seedling emergence, five seedlings were left in each RC. Hoagland nutrient solution of 1/4 strength was applied to both RCs while only $ddH_2O$ was applied to HC to moist the substrate. Direct weighing method was used to maintain the water content at 16% (v/w).

Eight weeks later, plants in each RC showed similar vigor and plenty of hyphae were checked to be present in HC. Then HCs were subject to various treatments involving different application levels of inorganic phosphate (Pi) and Cd. For Pi application, three levels (0, 0.1 and 1 mmol/L) were set up, and for Cd application, two levels (0 and 1 mmol/L) were set up, producing six treatments. For each treatment, six replicates were prepared. Twenty ml of Pi and/or Cd at corresponding levels was applied to HC each day, and totally 300 ml of Pi and/or Cd at corresponding levels was applied at 15 times (two weeks). Two weeks later, extraradical hyphae of *R. irregularis* were carefully collected from HC with pre-cooled $ddH_2O$, filtered on a filter membrane, and then picked up with fine forceps (Model 113SA, OHM-Werkzeuge, Germany) for fluorescence observation. Plants were also harvested for analysis.

### 2.2. Chemicals

4′,6-Diamidino-2-phenylindole (DAPI) was purchased from Sigma–Aldrich. *Measure-iT*™ Lead and Cadmium Assay Kit (M36353) was purchased from Invitrogen. This kit has been used to fluorescently quantify the dissolved $Cd^{2+}$ in cytoplasm (Priester et al., 2009) and was used in this study as Cd probe to observe the localization and distribution of $Cd^{2+}$ in extraradical hyphae.

### 2.3. Plant biomass and root colonization

At harvest, roots were carefully cleared off sands with tap water, and an aliquot of roots was cut into fragments about 1 cm long for the determination of mycorrhizal colonization and alkaline phosphatase (ALP) activity. The biomass of shoots and roots were recorded in dry weight after oven-drying at 70 °C for 72 h, then the contents of Cd and phosphorus (P) in plant tissue were measured using AAS (Hitachi Z-5300).

Root fragments were stained with 0.05% trypan blue in lactoglycerol (Phillips and Hayman, 1970), then mounted on glass slides (20 fragments per slide) for examination. For each sample, one hundred and twenty root fragments were examined to estimate the mycorrhizal colonization (Blal et al., 1990). For the ALP determination, more root fragments were incubated in cellulose–pectinase mixture solution buffered with tris/citric acid (pH 9.2) at 25 °C for 2 h. After enzyme solution was discarded, root fragments were blotted dry with tissue paper and then incubated in BCIP/NBT phosphatase substrate (KPL product, Gaithersburg, MD, USA) at 25 °C for 4 h after vacuumed for 30 min. Incubated root fragments were mounted onto slides and ALP activity of fungal structures in root fragments was detected by recording the purple-black stained parts under a microscope (Vierheilig et al., 2005; Zhu et al., 2007).

### 2.4. Microscopic observation of polyP with TBO staining

Freshly harvested extraradical hyphae were rinsed in ethanol for 15 min, then blotted on filter paper and stained with 0.05% Toluidine blue O (TBO) in 50 mmol/L KCl–HCl (pH 1.0) buffer solution for 15 min at room temperature. Stained hyphae were further rinsed with 50 mmol/L KCl–HCl (pH 1.0) buffer solution and mounted in the same buffer solution on glass slide. Polyphosphate was quantified using the percentage of hyphae with metachromatic granules using a grid line intersect method under microscope (Ezawa et al., 2004).