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Role of chelant on Cu distribution and speciation in *Lolium multiflorum* by synchrotron techniques

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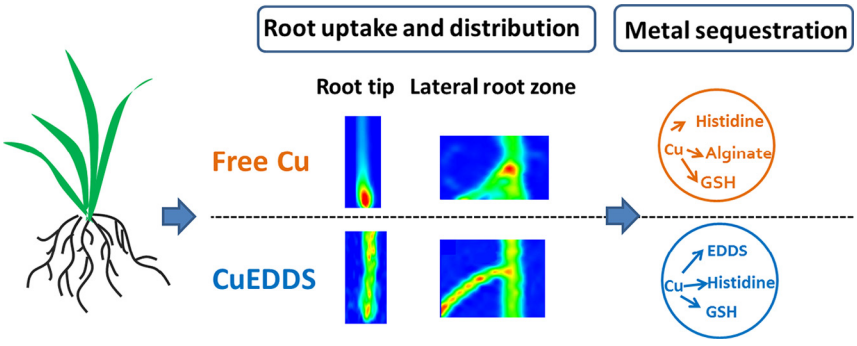
HIGHLIGHTS

- EDDS alleviated the deposition of Cu in the root meristem of root apex and the junction of lateral root zone.
- EDDS formed complex with Cu, decreased the root sequestration of Cu, and facilitated Cu translocation as CuEDDS.
- In spite of CuEDDS, a proportion of Cu-histidine and Cu(I)-glutathione like species was observed in ryegrass tissues.
- A conceptual model was developed on Cu uptake and transport mechanisms in ryegrass.

GRAPHIC ABSTRACT



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ABSTRACT

Chelants are known to enhance metal translocation in plants; however, the underlying mechanisms are still not fully understood. This study aimed to elucidate the distribution and speciation of Cu in ryegrass (*Lolium multiflorum*) in both absence and presence of the biodegradable chelant [*S,S′*]-ethylenediamine disuccinic acid (EDDS). The results showed that EDDS increased the Cu translocation factor from root to shoot by 6–9 folds under CuEDDS in comparison with free Cu (50–250 μM). Synchrotron-based microscopic X-ray fluorescence (μ-XRF) mapping revealed that EDDS alleviated Cu deposition in the root meristem of root apex and the junction of lateral root zone, and facilitated Cu transport to root stele for subsequent translocation upwards. X-ray absorption near edge structure (XANES) analysis found that free Cu was sequestered in plants as a mixture of Cu-organic ligands. In the EDDS treatment, Cu was primarily present as CuEDDS (49–67%) in plants with partial chemical transformation to Cu-histidine (21–36%) and Cu(I)-glutathione (0–24%). These results suggest that EDDS improves internal Cu mobility through forming CuEDDS, thus decreasing the root sequestration of Cu, and ultimately facilitating Cu transport to plant shoots.

1. Introduction

Heavy metals pollution is one of the most serious environmental issues in our planet, mainly derived from the mining activities, industrial emissions, and pesticides application (Luo et al., 2011; Li et al., 2014). Once deposited into soil, they will persist in soil and cannot be degraded

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by microorganisms. Copper (Cu) is an essential element for organisms, but highly toxic at high concentration. Excessive Cu reduces food crop production, inflicts toxicity to aquatic organism, and causes "Wilson disease" to human beings (Adrees et al., 2015; DeForest and Meyer, 2015). Copper pollution in soil primarily results from the use of fungicides, sewage sludge application, wastewater irrigation, mining, and smelting (Brun et al., 1998; Alloway, 2013; Li et al., 2014). The polluted soil requires effective remediation technologies for risk management and future land use. Phytoextraction, as an *in situ*, cost-effective, and environmental friendly biotechnology, has gained much attention for soil remediation over the last three decades (Huang et al., 1997; Vamerali et al., 2010; Attinti et al., 2017).

The efficiency of phytoextraction is typically constrained by low metal availability in contaminated soils. Therefore, synthetic chelants are applied to solubilize metals from soil, and facilitate metal diffusion or convection to roots, which in turn increase the uptake and transport of metals by plants (Huang et al., 1997; Wu et al., 2007; Nowack et al., 2006). During the last 20 years, [S,S′]-ethylenediamine disuccinic acid (EDDS) has been developed as a sound alternative to EDTA due to its rapid biodegradation rate in soil, which substantially reduces the leaching risks of solubilized metals (Meers et al., 2008; Wang et al., 2012). EDDS can effectively mobilize trace metals (especially for Cu) from contaminated soils, enhance metal uptake, and improve metal translocation from roots to shoots of many non-accumulating plant species (Meers et al., 2005; Luo et al., 2005; Cestone et al., 2012a). However, the underlying mechanisms by which EDDS influences Cu uptake and accumulation by plants still remain unclear.

There are two main pathways that proposed for the uptake of metal-EDDS or metal-EDTA complexes by plants. On the one hand, many researchers believe that plants are able to absorb intact complexes through the apoplastic flow, since metal-complexes (*e.g.* CuEDDS, PbEDTA, and CdEDTA) have been detected in some plants (Schaider et al., 2006). The complexes are unlikely to cross the cell membranes due to its large molecular size and lack of transporters (Leštan et al., 2008; Niu et al., 2011a). In addition, metal-complexes can be transported across the root cortex to stele, particularly in root apex or lateral root zone, where Casparian strip has not been developed or has been damaged (Niu et al., 2011b; Tao et al., 2016). On the other hand, some researchers maintain that plants mainly absorb free metals dissociated from metal-complexes (Sarret et al., 2001; Tian et al., 2011). Actually, FeEDTA, PbEDTA and ZnEDTA were found to dissociate either prior to or during absorption by plant roots (Chaney et al., 1972; Sarret et al., 2001). Intriguingly, both mechanisms were suggested on the uptake of CuEDDS by different plants according to recent studies (Cestone et al., 2010; Niu et al., 2011b; Johnson and Singhal, 2013), and the relative importance of two absorption pathways can be related to the supplied concentration of CuEDDS. Therefore, direct evidence is still required to verify the exact pathways.

The molecular localization and speciation of Cu in plants can provide valuable insights on the uptake and transport pathways of Cu. Current advanced synchrotron-based microscopic X-ray fluorescence (μ-XRF) and X-ray absorption near-edge structure spectroscopy (XANES) provide a viable opportunity to acquire the information (Majumdar et al., 2012). To our knowledge, the effect of EDDS on the coordination environment of redox-active Cu in high-biomass grasses has not been studied yet. Because Cu can be reduced during plant absorption and translocation (Ryan et al., 2013; Collin et al., 2014), it is still not known whether the reduction process occurs and poses an influence on the dissociation of CuEDDS.

The aim of the present study is to establish a comprehensive understanding of the Cu uptake and transport mechanisms with EDDS in ryegrass (*Lolium multiflorum*). Hydroponic culture was conducted in the current study, due to the convenience in controlling the supplied amount of free Cu or CuEDDS and keeping stable conditions for plant growth. The molecular distribution and speciation of Cu in plants with or without EDDS was investigated using μ-XRF and XANES, respectively.

2. Materials and methods

2.1. Hydroponic cultivation

Seeds of ryegrass (*Lolium multiflorum* Lam. cv. Tetragold) were germinated on moist filter papers for 10 d after sterilization with 95% ethanol and water soaking. Plant seedlings were cultured with modified Hoagland's nutrient solution (HNS). The compositions of HNS include 1 mM KH_2PO_4, 5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 2 mM $MgSO_4$, 0.02 mM $FeSO_4$, 0.05 mM HBO_3, 0.01 mM $MnSO_4$, 0.77 μM $ZnSO_4$, 0.32 μM $CuSO_4$, and 0.02 μM $(NH_4)_6Mo_7O_{24}$. The nutrient solution was adjusted to pH 6.0 before use and replaced weekly. Plant seedlings were cultivated in controlled environment by a climate chamber with the relative humidity of 60%, 16 h day (25 °C) and 8 h night (20 °C) cycle, and light illumination of 325 μmol photons m^{-2} s^{-1}.

After two weeks, plant seedlings were transferred to 100 mL beakers for treatment with free Cu or CuEDDS. Beakers were wrapped with aluminum foils to exclude lights and avoid algae growth in solutions. Each beaker contained about 80 mL HNS with free Cu (0, 50, 150, and 150 μM $CuSO_4$) or CuEDDS (0, 50, 150, 250, 500, 1500, and 3000 μM). Each treatment had three replicates in separate hydroponic containers. K_2HPO_4 was omitted from HNS to avoid precipitation of copper phosphate (Mohtadi et al., 2013). Aliquots of concentrated $CuSO_4$ or CuEDDS solution were added to HNS to create the designed exposure range. CuEDDS was prepared in solution by dissolving equivalent concentration of $CuSO_4$ and EDDS-Na_3 ($C_{10}H_{13}N_2Na_3O_8$, Sigma Aldrich). Specifically, the concentration range of free Cu was designed based on the reported level of Cu (1 to 300 μM) in soil solution from contaminated fields (Zhang et al., 2001; Song et al., 2004; Forsberg et al., 2009). The higher concentration range for CuEDDS was designed since values as high as 3000 μM were recorded in EDDS-assisted phytoextraction sites (Meers et al., 2005).

Plant seedlings were harvested 3 d after treatment with free Cu or CuEDDS (Cestone et al., 2012b). Shoots and roots of ryegrass were washed with deionized water prior to harvest, after which they were separated, oven-dried, weighed, and ground. Plant Cu was subsequently determined using ICP-OES (Agilent 700 series) after acid digestion with $HNO_3/HClO_4$ (4:1) (Luo et al., 2005). The standard references SRM 1515 (apple leaves) and SRM 2711a (Montana II soil) were used for quality control, and the Cu recovery rate reached 92 ± 6% and 87 ± 4%, respectively. Mean values and standard deviations were obtained based on three replicates. Statistical analysis was performed with one-way analysis of variance (ANOVA) using SPSS software version 22.0. Differences between treatment means were tested using the Duncan Test at a significance level of 0.05.

2.2. Micro-distribution assay of copper in root by μ-XRF

The 2-week-old seedlings were exposed to nutrient solutions either containing free Cu (150 μM) or CuEDDS (150 and 1500 μM) as described above. After incubation for 3 d, roots of ryegrass were rinsed with deionized water. Root segments, including root tips (0–1 cm) and mature root segments (1–3 cm, 3–5 cm, and >5 cm), were cut off (Fig. 3), quickly frozen in liquid N_2, and lyophilized. The total Cu concentration in root segments was measured by ICP-OES after acid digestion. Root tips (0–1 cm) and mature root segments (>5 cm) were selected for μ-XRF.

Lyophilized root segments were mounted on 3 M tapes and analyzed by μ-XRF at beamline 4W1B of the Beijing Synchrotron Radiation Facility (BSRF), Institute of High Energy Physics, Chinese Academy of Sciences. The storage ring was operated at energy of 2.5 GeV with a current intensity ranging from 200 to 300 mA. Typical root zones, including the root apex from root tips (0–1 cm) and the lateral root