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Accumulation, speciation and cellular localization of copper in *Sesbania drummondii*

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Abstract

Growth, accumulation and intracellular speciation and distribution of copper (Cu) in *Sesbania drummondii* was studied using scanning-electron microscopy (SEM), X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS). The growth of seedlings was assessed in terms of biomass accumulation. The growth of the seedling was enhanced by 73.5% at a low Cu concentration (50 mg l^{-1}) compared to the control treatment. Additionally, seedling growth was inhibited by 18% at 300 mg l^{-1} Cu with respect to the control. Copper concentration in roots and shoots was increased with increasing Cu concentration in the growth solution. The accumulation of Cu was found to be higher in roots than in the shoots. At a concentration of 300 mg l^{-1} Cu, the roots accumulated 27,440 mg Cu kg^{-1} dry weight (dw) while shoots accumulated 1282 mg Cu kg^{-1} dw. Seedlings were assessed for photosynthetic activity by measuring chlorophyll a fluorescence parameters: F_v/F_m and F_v/F_0 values. Photosynthetic integrity was not affected by any of the Cu treatments. The X-ray absorption spectroscopic (XAS) studies showed that Cu was predominantly present as Cu(II) in *Sesbania* tissue. In addition, from the XAS studies it was shown that the Cu exists in a mixture of different coordination states consisting of Cu bound to sugars and small organic acids with some possible precipitated copper oxide. From the EXAFS studies, the coordination of Cu was determined to have four equatorial oxygen(nitrogen) ligands at 1.96 Å and two axial oxygen ligands at 2.31 Å. Scanning-electron microscopy studies revealed the distribution of Cu within the seedlings tissues, predominantly accumulated in the cortical and vascular (xylem) regions of root tissues. In the stem, most of the Cu was found within the xylem tissue. However, the deposition of Cu within the leaf tissues was in the parenchyma. The present study demonstrates the mechanisms employed by *S. drummondii* for Cu uptake and its biotransformation.

Keywords: Cu accumulation; Cu speciation; X-ray absorption; Scanning-electron microscopy; *Sesbania drummondii*; Cu toxicity

1. Introduction

Copper (Cu) is an essential element for plant growth and development. It is involved in a wide range of biochemical and physiological processes. Although an essential element Cu becomes phytotoxic at excessive levels. Mining, smelting and land applications of sewage sludge, together with the use of fungicides containing Cu, and other human activities, has lead to widespread soil contamination with Cu (Hong-yun et al., 2005).

Many studies had been carried out on the effect of Cu on the growth, mineral nutrition and metabolism of plants. Copper in excess reduces plant growth (Mocquot et al., 1996), mineral nutrient uptake (Yang et al., 2002) and photosynthetic activity (Lidon et al., 1993). It is also known to damage cell membranes by binding to the sulfhydryl groups of membrane proteins and inducing lipid peroxidation (Kennedy and Gonsalves, 1987; De Vos et al., 1989). Furthermore, Cu in excess induces the generation of hydrogen peroxide (H_2O_2), hydroxyl ($OH^{\cdot}$) radicals, or other

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reactive oxygen species (ROS), which are extremely toxic to living cells (De Vos et al., 1991; Stohs and Bagchi, 1995; Murphy and Taiz, 1997).

Some plant species actively take up and accumulate metals to high levels in the above ground tissues, far exceeding the levels detected in the soil (Brooks, 1998). For a plant species to be efficient in Cu phytoextraction, it should accumulate a Cu concentration of 1% or greater of the shoot dry weight and produce abundant shoot biomass (Baker and Brooks, 1989). Plants that accumulate high level of metals have mechanisms to protect themselves from metal toxicity. To combat metal toxicity, plant cells have antioxidants such as α-tocopherol, β-carotene, glutathione (GSH), ascorbate and antioxidative enzymes (such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR)) that participate in scavenging reactive oxygen species such as O_2, O_2^-, $OH^{\cdot}$ and H_2O_2 (Halliwell, 1982). Metal binding peptides such as phytochelatins and metallothioneins have also been advocated as one of the many mechanisms for coping with elevated metal stress in some plants (Rauser, 1990; Zenk, 1996). Production of the metal binding peptides in response to Cu has been reported in several species (Mehra et al., 1988; Inouhe, 2005). Apparently, the mechanisms through which Cu induces antioxidative responses within different plant species are not yet fully understood (Wang et al., 2004).

Sesbania drummondii is a fabaceous shrub, which has been studied extensively in relation to the accumulation of Pb and Hg in hydroponics and soil conditions (Sahi et al., 2002; Ruley, 2004; Israr et al., 2006). In this study we examined the mechanisms used by *Sesbania* to accumulate and tolerate high levels of Cu using scanning-electron microscopy (SEM) and X-ray absorption spectroscopy (XAS) techniques. While microscopy techniques have long been employed for bulk analysis of biological specimens, XAS has emerged as a powerful analytical tool that allows one to give an accurate chemical description of trace level elements including oxidation states, coordination numbers and near neighbor atoms in complex plant and soil samples (Polette et al., 1998). A detailed description of the application of XAS to natural sediments has been previously described by O'Day and Carroll (1998). In order to investigate the internal distribution of metals in tissues, scanning-electron microscopy technique has been used in several studies (MacFarlane and Burchett, 2000; Arru et al., 2004; Sharma et al., 2004; Hong-yun et al., 2005). In view of these facts, the objectives of the present study were: (1) to investigate the effects of Cu on the growth and photosynthesis of *S. drummondii*, (2) to determine the uptake of Cu by *S. drummondii*, (3) to evaluate the pattern of Cu localization in *Sesbania* cells using scanning-electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX), and (4) to investigate the speciation and coordination of Cu in the tissues of *Sesbania* using X-ray absorption spectroscopy (XAS). The results should aid in understanding the nature of Cu in *Sesbania* cells and can be helpful in further manipulation of this plant for the purpose of Cu phytoextraction.

2. Materials and methods

2.1. Plant growth and metal treatment

Seeds of *S. drummondii* were scarified in 85% H_2SO_4 for 35 min, then rinsed in running water for 2 h and washed with deionized (DI) water. Scarified seeds were sown in pro-mix (Premier Horticulture INC., Quakertown, PA, USA). Twelve days old seedlings were transferred to different flasks containing Hoagland's solution (half strength). After 2 d, seedlings were transferred to fresh Hoagland's solution (half strength) supplemented with different concentrations (25, 50, 100, 150 and 300 mg l^{-1}) of $CuSO_4$. Each treatment had four replicates. Seedlings without $CuSO_4$ treatment served as controls. Seedlings were grown at 25 °C using 16/8 h light/dark cycle in a growth room under 200 μmol m^{-2} s^{-1} fluorescent light for 10 d. The metal solutions in all the treatments were changed every 3 d to prevent depletion of metals, nutrients and oxygen. After 10 d, seedlings were used to analyze: biomass accumulation, Cu content in plant tissues and photosynthetic activities as described below.

2.2. Estimation of Cu accumulation and biomass

Seedlings were harvested after 10 d of growth and thoroughly washed with tap water and then DI water for desorption of surface bound Cu. Plant samples were weighed and transferred to capped tubes. One mL of concentrated (16 N) HNO_3 was added to the samples and heated at a temperature of 100 °C overnight. The digested samples were transferred to clean polythene tubes and diluted to 10 ml with DI water. Copper analysis was carried out using inductively coupled plasma atomic emission spectroscopy (ICP-AES).

For the biomass analyses, seedlings were dried in a hot air oven at 68 °C ± 2 °C for 2 d. This determination was performed on the basis of quadruplicate samples. Subsequent to drying the plants were weighed to determine the dry weight. The dry weight was expressed as mg per seedling.

2.3. Photosynthetic activities

Photosynthetic activities of *S. drummondii* seedlings were assessed by measuring chlorophyll a fluorescence parameters as described by Oquist and Wass (1988). Fully expanded intact leaves were used to determine chlorophyll a fluorescence. Two leaves per plant and four plants from each treatment were used. *Sesbania* leaves were dark adapted for 30 min with a Hansatech clip. The fluorescence parameters were measured using Handy-PEA instrument (Hansatech Instruments, UK) at 3000 μmol m^{-2} s^{-1} light (650 nm) for 5 s. The F_v/F_m and F_v/F_0 ratios were calculated from measurements of F_v, F_m and F_0 values.