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Effects of copper sulfate on seedlings of *Prosopis pubescens* (Screwbean mesquite)

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Abstract

Phytoextraction is an established method of removal of heavy metals from contaminated soils worldwide. Phytoextraction is most efficient if local plants are used in the contaminated site. We propose that *Prosopis pubescens* (Screw bean mesquite) would be a successful phytoextractor of copper in our local soils. In order to determine the feasibility of using Screw bean mesquite, we utilized inductively coupled plasma-optical emission spectroscopy (ICP-OES) and elemental analysis to observe the uptake of copper and the effects on macro and micro nutrients within laboratory-grown seedlings. We have previously shown that *pubescens* is a hyperaccumulator of copper in soil-grown seedlings.

Light and transmission electron microscopy demonstrated death of root cells and ultrastructural changes due to the presence of copper from 50 mg L⁻¹ – 600 mg L⁻¹. Ultrastructural changes included plasmolysis, starch accumulation, increased vacuolation and swollen chloroplasts with disarranged thylakoid membranes in cotyledons.

Inductively coupled plasma-optical emission spectroscopy analyses of macro-and micro-nutrients revealed that the presence of copper sulfate in the growth medium of Petri-dish grown *Prosopis pubescens* seedlings resulted in dramatic decreases of magnesium, potassium and phosphorus. At 500-600 mg L⁻¹ of copper sulfate a substantial increase of sulfur was present in roots.

Keywords

Ultrastructure; ICP-OES; Phytoremediation

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2. INTRODUCTION

In their minimal concentration, heavy metals can be utilized as essential micronutrients for propagating the growth and the development of plants (Tanyolac, Ekmekci, and Unalan, 2006). Copper is needed in small amounts by plants, where at the cellular level it plays a key role in signaling of transcription and protein trafficking machinery, oxidative phosphorylation and iron mobilization (Yruela, 2005). Additionally, copper is also a required key component in both structural components and catalytic enzyme activity cofactors (Tanyolac *et al.*, 2006). Copper excess may result in a toxic induction by altering protein function (Tanyolac *et al.*, 2006). The background copper concentration in soil is between 13 mg L⁻¹ and 24 mg L⁻¹ (Kabata-Pendias and Pendias, 1992). The optimal copper concentration for healthy plant growth ranges from 2-20 mg L⁻¹ (Thompson, Hall, and Meerdink, 1991). Because of the redox-active transition metal nature of copper, copper may bind to sulfhydryl groups in protein which may lead to the inhibition of protein activity and disruption of the protein structure (Tanyolac *et al.*, 2006).

Understanding the effects of copper sulfate on the developing stages of *P. pubescens* will help determine if this plant could be utilized for an environmental restoration of an area highly contaminated with copper. Seedling stage is the most vulnerable phase; therefore, it is crucial to determine if seedlings may tolerate heavy metals for their use in phytoremediation. Cotyledon damage can reduce survival or growth (Bonfil, 1998).

Objectives of this study were to identify ultrastructural changes of cotyledons due to copper sulfate, to determine copper and nutrient concentrations within seedlings, and to measure toxicity of copper sulfate in roots.

3. MATERIAL AND METHODS

3.1. Plant material and Copper treatment

Seeds of *Prosopis pubescens* were obtained from Granite Seed Company (Lehi, UT). Seeds were germinated within two sterile filter paper in Petri dishes for 8 days under the same dark/light cycle (6:18hrs at 20°C). Copper solutions (2ml) (50, 100, 200, 300, 400, 500, and 600 mg L⁻¹) every 2 days were administered for the next 8 days.

3.2. Copper and nutrient content

Sixteen day-old seedlings were rinsed in 2% nitric acid to remove surface traces of copper. Three hundred samples (root, stem and cotyledon) were dried for 72 h at 90°C, weighed, and then digested following the EPA Method 3051. Digested samples were diluted to a 5% sample in solution and read using a Perkin Elmer 4300 DV ICP-OES spectrometer (Shelton, CT). This multi-metal analysis read for copper, as well as, micro nutrients and macro nutrients. One blank (5% nitric acid) and six spiked (1mg L⁻¹ solution of each metal of interest) were prepared.

2.3. Transmission electron microscopy and elemental analysis

After 8 days of copper exposure, cotyledons were prepared for transmission electron microscopy following the method outlined in Zappala, 2012. Seventy five BEEM capsules

were prepared in total. Thin sections were not post-stained. Three hundred twenty five photographs were collected digitally with the ES1000W Gatan camera model 785 (Warrendale, PA) to assess ultrastructural changes. Copper (8.96 g/cm^3) was localized using a Hitachi S-3400N SEM and Noran System Six Energy Dispersive X-Ray Microanalysis System (Thermo Electron, Madison, WI) (New Mexico State University, Las Cruces, NM), totaling 115 spot scans.

2.4. Cell death analysis in roots

Cell death was quantified by the Evans blue staining method (Hung *et al.* 2007). This method was initially taken from Baker and Mock (1994). Roots were harvested at various time points (20 and 180 min) from various copper concentrations.

2.5. Statistical analyses

Statistical analyses included the General Linear Model (GLM) and General Linear Mixed Models (GLMM). If a significant factor or interaction was found with the GLM or GLMM, it was followed by a Tukey's post-hoc procedure test. Simple linear regression was also fitted for copper sulfate absorption across concentrations. All tests were conducted with the 0.05 level of significance, using SAS Version 9.2 software.

3. RESULTS AND DISCUSSION

3.1. Copper absorption in screw bean mesquite seedlings

Inductively coupled plasma-optical emission spectroscopy was used to identify the amount of copper collected by screw bean mesquite seedlings. Figure 1 shows that increasing amounts of copper exposure to the seedling has dramatically increased the accumulation of copper in the roots. Lower amounts of copper accumulated in the stems and cotyledons.

Copper absorption across concentrations was different for the root, stem and cotyledon ($p < 0.0001$). The control root was significantly different from the rest of the copper treatment. Copper absorption in stems showed statistical differences across concentrations of exposure ($p < 0.0001$). We found significant linear relationships between copper absorption and concentration in the stem ($R^2 = 0.9802$). Copper sulfate absorption in cotyledons showed statistical differences across concentrations of exposure ($p = 0.0214$). We found significant linear relationships between copper absorption and concentration in the cotyledons ($R^2 = 0.9322$).

3.2. Cell death in roots

Evidence of copper toxicity within the screw bean mesquite seedlings was determined by quantifying cell death in the roots. The time dependent analysis shown in Figure 2 demonstrates that a longer exposure of copper increases cell death within young screw bean mesquite roots. This method did not differentiate between necrosis and programmed cell death (PCD); however, it did show that there were an increased number of dead cells after copper exposure to roots. Hung *et al.* (2007) were the first to examine copper-induced cell death in rice roots. Copper produces reactive oxygen species (ROS) which are involved in PCD (Hung *et al.*, 2007). Programmed cell death is known to be initiated by protein tyrosine

kinase activation (Pennell and Lamb, 1997). Evans blue (Baker and Mock, 1994) is incorporated in damaged membranes after only 20 min of copper exposure (Hung *et al.*, 2007). In our studies, quantitative assessment of screw bean mesquite root showed cell death after 10 min of copper sulfate exposure ($p < 0.0001$). Roots exposed to 600 mg L⁻¹ of copper sulfate had 72% more dead cells in comparison to the control with 10 min copper exposure ($p = 0.0041$). The results indicated an early response to copper toxicity followed by a leveling off of cell death suggesting an activation of tolerance mechanisms.

3.3. Nutrient changes due to copper exposure

Copper may also interfere with the uptake or distribution of other elements needed for plant growth. These elements consist of the macronutrients (calcium, magnesium, potassium, phosphorus, and sulfur) and micronutrients (iron, manganese, zinc, boron, and molybdenum). We read 700 samples to determine patterns of nutrients in response to copper exposure. Excess in copper can lead to substitution of other essential micro and macronutrients such as calcium, magnesium, and iron. There were no statistical differences in means across concentration for calcium, iron, manganese, boron and zinc after copper sulfate exposure.

Magnesium concentrations were different across roots, stems, and cotyledons as copper exposure increased ($p < 0.0001$), with the means at 0 mg kg⁻¹ DW significantly higher than the higher concentrations (Fig. 3a). There was an overall total magnesium decrease of 50%. Potassium concentrations in roots, stems, cotyledons of seedlings exposed to copper sulfate decreased as copper increased ($p < 0.0001$) (Fig. 4a). Potassium decreased by 70% in roots, while in stems it decreased by 87%. Finally, cotyledons had a 38% decrease of potassium. Our results showed both magnesium and potassium reductions after copper sulfate exposure. Translocation of nitrate, phosphate, calcium, magnesium, and amino acids was reduced when potassium decreased (Armstrong, 1998). Potassium deficiencies may change the rate of photosynthesis and ATP production, transport of water and nutrients, and the synthesis of starch (Armstrong, 1998). Potassium also activates enzymes by changing their physical shape to expose the active site (Armstrong, 1998); therefore, low potassium will change enzyme activity. Starch synthetase is activated by potassium; therefore, reduction of potassium could prevent starch breakdown. Qualitative ultrastructural analyses showed an increase of starch accumulation as copper sulfate exposure increased.

The trends for molybdenum concentrations as copper exposure increased were statistically different across root, stem, and cotyledons ($p = 0.0031$). The most significant change was roots ($p < 0.0001$) where the control sample was 19 mg kg⁻¹ DW of molybdenum and the 600 mg L⁻¹ of copper sulfate sample was about 64 mg kg⁻¹ DW of molybdenum (Fig. 3b). Legumes, which have a symbiotic relationship with nitrogen-fixing bacteria, have more molybdenum in their roots than other plants. High levels of molybdenum in roots will enhance plant growth, protein concentrations and nitrogen fixation. Legumes like screw bean mesquite have more molybdenum in their roots than other plants. Most plants are not sensitive to excess molybdenum (Gupta, Chipman, and MacKay, 1978).

Phosphorus was significantly affected by the presence of copper sulfate ($p = 0.0007$) (Fig. 3c). After 100 mg L⁻¹ of copper sulfate exposure, both roots and stems had no phosphorus.

This is crucial since control roots have almost 4,200 mg kg⁻¹ DW and stems have 8,700 mg kg⁻¹ DW of phosphorus. However, after 300 mg L⁻¹ of copper sulfate exposure there was no phosphorus in cotyledons.

Trends in sulfur concentrations as copper sulfate exposure increased were statistically different across root, stem, and cotyledon ($p=0.0003$). The control for roots had 1,900 mg kg⁻¹ DW of sulfur, while the 600 mg L⁻¹ copper sulfate exposed roots had 9,500 mg kg⁻¹ DW of sulfur (Fig. 4b). Sulfur rapidly increased in the roots beginning at 400 mg L⁻¹ of copper ($p<0.0001$). However for the stem and cotyledon, the increases were not significant ($p=0.3226$ and 0.4696 respectively). Sulfur increase is directly linked with increased production of cysteine (Baird, 1914). Both metallothioneins and phytochelatins are cysteine-rich proteins known to bind copper for sequestration or tolerance mechanisms.

3.4 Ultrastructural changes

At 1,600X-12,500X magnification in a Carl Zeiss EM-10 transmission electron microscope, we observed various ultrastructural changes due to copper (Fig. 5). In the control sample, we observed healthy parenchymal cells with large vacuoles, chloroplasts close to the cell wall, and starch grains within chloroplasts (Fig. 5a). Vacuoles maintain turgidity and osmotic balance in the cells (Gunning and Steer, 1975). It is normal for vacuoles to be large and their tonoplast to push the nucleus, chloroplasts and mitochondria against the cell membrane. If a plant absorbs an excess of ions, the vacuole will draw in additional water and increase turgor pressure. At 50 mg L⁻¹ copper sulfate exposure, parenchymal cells of cotyledons showed denser cytoplasm and dense inclusions within small vacuoles. Our cotyledons showed protein bodies within vacuoles similar to the results of Chrispeels, Baumgartner, and Harris (1975) and Toyooka, Okamoto, and Minamikawa (2001) (Fig. 5b). Protein body digestion is characteristic of cotyledons. We frequently observed protein bodies near the edges of some of the vacuoles. Gunning and Steer (1975) determined that legume cotyledons store many protein bodies in fusing vacuoles. Cotyledons of *Prosopis chilensis* seeds have high protein content (Estevez, Escobar, and Ugarte, 2000). Cotyledon cells of *Vigna mungo* seedlings showed the two distinct autophagic processes for the digestion of starch and protein (Toyooka *et al.*, 2001). Digestion of stored protein bodies leads to the formation of the protein storage vacuoles (PSV). Alpha-amylase is transported to the PSV where it digests starch granules. In relationship to these studies, we believe that our control and experimental seedlings were undergoing normal protein digestion.

At 100 mg L⁻¹ of copper sulfate, the cytoplasm was separated from the cell wall and appeared denser than in the control. Plasmolysis was evident (Fig. 5c). Ultrastructural studies of an embryogenic suspension culture of carrot showed the cytoplasmic structure of dead cells (Pennell and Lamb, 1997). Dead cells contained condensed and shrunken cytoplasm with small, membrane-sealed packets which suggest a type of programmed cell death (Havel and Durzan, 1996; McCabe *et al.* 1997).

At 200 mg L⁻¹ copper sulfate exposure noticeable damage to the plasma membrane (plasmolysis) was observed (Fig. 5d, e) a dark precipitate was observed near the cell walls which could be protein bodies characteristic of protein degradation. Swelling of developing chloroplasts and disarray of thylakoid membranes (Fig. 5d, e). There was an increase in

swelling of developing chloroplasts and disarray of thylakoid membranes. Excess copper has disorganized the inner membrane of thylakoids in *Elodea canadensis* (Stoyanova and Tchakalova, 1993). In Figure 4h, the cotyledon parenchymal cells were dying. The chloroplasts were highly enlarged with larger starch granules after exposure to 400 mg L⁻¹ of copper sulfate. Starting at 400 mg L⁻¹ copper sulfate exposure, starch grain accumulation increased as the concentration of copper increased. Starch is an energy source produced in chloroplasts and digested for use by alpha-amylase. Copper sulfate is thought to affect amylase; therefore, starch grains would be expected to accumulate. After an exposure of 500 mg L⁻¹ of copper sulfate chloroplasts were swollen and thylakoid membranes were disarrayed. After 600 mg L⁻¹ copper sulfate exposure plasmolysis of cotyledon parenchymal cells was common and the small amount of cytoplasm was very dense (Fig. 5h). Cells are irregular shaped; the plasma membrane has completely separated from the cell wall. The cytoplasm, organelles and vacuoles separated from the cell wall in every single cell observed and moved towards the center of the cell. The chloroplasts were swollen with a disarray of thylakoid membranes. Ultrastructural studies in *Laminaria saccharina* (L.) Lamour showed that copper denatured chloroplasts by the swelling of thylakoid membranes, detachment of lamellae and diffused matrices (Brinkhuis and Chung, 1986).

3.5 Elemental Analysis of cotyledons

The elemental analysis showed the presence of copper in cotyledon parenchymal cells of screw bean mesquite (Fig. 6). No copper was found in the control and 50 mg L⁻¹ copper sulfate. Copper sulfate was identified in the interior of cotyledon parenchymal cells in the 100 mg L⁻¹, 200 mg L⁻¹, and 300 mg L⁻¹ sample. In 400 mg L⁻¹ of copper sulfate, copper was localized in the cell wall and interior of a parenchymal cell. Copper was identified in the interior of an epidermal cell of the 500 mg L⁻¹ copper sample. Finally, 600 mg L⁻¹ copper sulfate contained copper in the cell wall of a cotyledon parenchymal cell.

4. CONCLUSIONS

In comparing Petri-dish grown seedlings with soil grown seedlings (Zappala et al 2013a), we concluded that soil grown seedlings are the best model for phytoextraction research. In comparing the results of copper sulfate exposed seedlings to copper nitrate Petri-dish grown seedlings, we observed that only small amounts of copper were taken-up from copper nitrate. Nevertheless, copper nitrate was more toxic to the seedlings than copper sulfate (Zappala et al 2013b.)

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Figure 1.
Copper sulfate absorption comparison within screw bean mesquite seedlings.

Figure 2.
Cell death time-dependent analysis after copper sulfate exposure.

Figure 3. Nutrient changes in root (blue), stem (red), and cotyledons (green) due to copper sulfate exposure: (a) Magnesium, (b) Molybdenum, (c) Phosphorus

Figure 4.
Nutrient changes in root (blue), stem (red), and cotyledons (green) due to copper sulfate exposure: (a) Potassium, and (b) Sulfur

Figure 5.

Electron micrographs showing the ultrastructural changes in cotyledons due to copper sulfate: (a) Control section of parenchymal cells (1,600X); (b) 50 ppm of CuSO_4 section of parenchymal cells with protein degradation (1,600X); (c) 100 ppm of CuSO_4 section of parenchymal cells with plasmolysis; (d) 200 ppm of CuSO_4 section of parenchymal cells with dense precipitate (arrow) (5,000X); (e) 200 ppm of CuSO_4 section of parenchymal cells with dense precipitate (arrow) (12,500X); (f) 300 ppm of CuSO_4 section of parenchymal cells with dense precipitate (arrow) and a vascular bundle (1,600X); (g) 400 ppm of CuSO_4 section of parenchymal cells showing cell death (1,600X); (h) 600 ppm of CuSO_4 section of

parenchymal cells showing plasmolysis(1,600X). (P-Protein bodies; C-Chloroplast; N-Nucleus; V-Vacuole; S-Starch Grain; M-Mitochondrion; Cy-Cytoplasm; CW-Cell Wall; and Arrow-Dense Precipitates.)

Figure 6.

Elemental analysis of the (a) interior of a cotyledon parenchymal cell in the control; (b) interior of a cotyledon parenchymal cell in 50 ppm copper sulfate; (c) interior of a cotyledon parenchymal cell in 100 ppm copper sulfate; (d) interior of a cotyledon parenchymal cell in 200 ppm copper sulfate; (e) interior of a cotyledon parenchymal cell in 300 ppm copper sulfate; (f) cell wall in 400 ppm copper sulfate; (g) interior of an epidermal cell in 400 ppm copper sulfate