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***Prosopis pubescens* (Screw bean mesquite) seedlings are hyper accumulators of copper**

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Abstract

Due to health reasons, toxic metals must be removed from soils contaminated by mine tailings and smelter activities. The phytoremediation potential of *Prosopis pubescens* (screw bean mesquite) was examined by use of inductively-coupled plasma spectroscopy (ICP-OES). Transmission electron microscopy (TEM) was used to observe ultrastructural changes of parenchymal cells of leaves in the presence of copper. Elemental analysis was utilized to localize copper within leaves. A 600 ppm copper sulfate exposure to seedlings for 24 days resulted in 31,000 ppm copper in roots, 17,000 ppm in stems, 11,000 in cotyledons and 20 ppm in the true leaves. In order for a plant to be considered a hyper accumulator, the plant must accumulate a leaf: root ratio of <1. Screw bean mesquite exposed to copper had a leaf: root ratios of 0.355 when cotyledons were included. We showed that *Prosopis pubescens* grown in soil is a hyper accumulator of copper. We recommend that this plant should be field tested.

Keywords

Hyper accumulator; Copper; Nutrients

1. INTRODUCTION

Industrial growth and urban expansion have increased soil contaminants, worldwide. Urban settings such as El Paso, Texas have been affected by heavy metal contamination in soil (Ketterer, 2006). Copper is one of the major heavy metal contaminants in the environment. Soil contamination requires time and cost effective clean-up methods. The background level of copper in soil worldwide is between 2 to 250 ppm (Gardea-Torresdey *et al.*, 2005). The Environmental Protection Agency (EPA, www.epa.gov) has limited copper levels to 1.3 ppm in soil. A recent survey of El Paso's soils has shown that we maintain a copper level of at least 10 ppm (Ketterer, 2006). Copper sulfate is mainly used as a pesticide while copper nitrate is primarily used in electroplating copper on iron, as a catalyst and a nitrating agent in organic reactions. Since seedlings can readily adapt to a changing environment and can

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tolerate various air, water and soil contaminants, they may possess the ability to absorb and remove heavy metal contamination from soil. Copper nitrate (125g/100g water at 20°C) is more soluble in water than copper sulfate (32g/100g water).

Toxic concentrations of copper have negatively affected net photosynthetic rate and Calvin cycle enzymes (Burzynsky and Zurek, 2007), chlorophyll content and photosynthetic parameters (Borghi *et al.*, 2007), CO₂ fixation (Demirevska-Kepova *et al.*, 2004) and carbohydrate metabolism (Roito *et al.*, 2005). Chlorosis has also been suggested to be the result of a reduced number of chloroplasts per parenchymal mesophyll cell of leaves as well as a change in cell size (Baryla *et al.*, 2001). In *Rumex japonicas*, excess copper leads to the substitution of essential micro and macro nutrients such as calcium, magnesium and iron (Ke *et al.*, 2007).

Over 450 plant species have been identified as hyper accumulators of various metals such as zinc, nickel, and cadmium (Brooks *et al.*, 1998; Khan *et al.*, 2000). Metals such as zinc, copper, manganese, nickel and cobalt are essential for plant growth (Marschner, 1995). These metals in excess are detrimental to plant life. Among the local desert shrub species that have been found to accumulate heavy metals is mesquite, *Prosopis sp.* (Aldrich *et al.*, 2004). Desert seedlings are physiologically tolerant to drought, high salt concentrations, and nutrient poor soils (Aldrich *et al.*, 2006). Honey mesquite has been shown to hyperaccumulate arsenic, chromium (III) and (VI), and lead (Aldrich *et al.*, 2004; Mokgalaka-Matlata *et al.*, 2009; Arias *et al.*, 2010).

Two mechanisms of tolerance to heavy metals have been suggested for seedlings: 1) exclusion, transport of metal is restricted to the lower plant parts; and 2) accumulation, metals are accumulated in nontoxic form in the upper plant parts (Baker, 1981). Excluders will have a leaf:root metal ratio of >1 while accumulators have a ratio of <1. Brooks *et al.* (1977) were the first to use the term hyper accumulators to describe seedlings with nickel concentrations higher than 1000µg/g in dried leaves. Thirty seven hyper accumulators of copper are known (Baker and Brooks, 1989).

This paper focuses on the potential of *Prosopis pubescens*, commonly known as screw bean mesquite, as a hyper accumulator of copper. Screw bean mesquite is found along the rivers of the Southwest. This native species has a high tolerance for a dry and hot environment. The aims of this study were to measure copper uptake in seedlings grown in soil and to identify the nutrient changes associated with copper uptake. An understanding of copper effects could lead to the use of this plant for restoration of contaminated desert soils.

2. METHODS AND MATERIALS

2.1 Plant material and Cu treatment

Screw bean mesquite (*Prosopis pubescens*) seedlings in triplicate were grown in 500 g pots for 24 days in soil. Soil was tested for its water saturation capabilities and watered to 40 % saturation every other day (100 ml of distilled water/500 g of potting soil). The average relative humidity and temperature for the greenhouse used were 30% humidity and 30° C (May-June). Local climate affected the relative humidity and temperature of the greenhouse which was not light supplemented and was equipped with an evaporative cooler (no additional misters). The relative humidity may have been as low as 20% and temperature as high as 32° C which is closer to the local environment. Natural light cycles of 12 hours were used in the greenhouse. An estimated 150 seeds were planted per pot after being scarified with 2% Chlorox. Soil containing copper sulfate or copper nitrate was prepared using water saturation and copper weight ratios two months before use. Copper concentrations included 0 ppm, 50 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm, and 600 ppm. Seeds were

planted and allowed to germinate. Twenty-four day-old seedlings were harvested for the various studies.

2.2 Copper and nutrient content

Twenty-four day-old seedlings were rinsed in 2% nitric acid to remove surface traces of copper. Seedlings were separated into root, stem, cotyledons and leaves. Samples were dried for 72 h at 90°C, weighed, and then digested following the EPA Method 3051. Digested samples were diluted with Millipore water for a total solution of 15 ml with 5% sample in solution. Samples were read using a 4300 DV ICP-OES spectrometer (Perkin Elmer, Massachusetts). This multi-metal analysis read for copper, as well as, micro nutrients (boron, iron, zinc, manganese, and molybdenum); and macro nutrients (calcium, potassium, sulfur, phosphorus, and magnesium). One blank (5% nitric acid) and six spiked (1 ppm solution of each metal or nutrient) were prepared.

2.3 Transmission electron microscopy and elemental analysis

After 24 days of Cu exposure, leaves were prepared for transmission electron microscopy. Samples of 1 cubic mm were fixed in 2.0% (wt/vol) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.06 M HEPES buffer, pH 7.4 for 1 h at room temperature with agitation. Specimens were washed 3 times for 15 min each with cold 0.06 M HEPES buffer and then post-fixed with 1% (wt/v) osmium tetroxide, 0.05 M potassium ferrocyanide in 0.12 M HEPES buffer for 1 h in the refrigerator (−4°C). Specimens were then washed in 0.06 M HEPES buffer followed by three 15 min washes with distilled water and then *en bloc* staining in the refrigerator with 0.5% (wt/v) aqueous uranyl acetate for one hour. Specimens were then dehydrated with ethanol at increments of 10% (starting with 30% ethanol) followed by 100% acetone. Specimens were infiltrated with and embedded in Poly Bed 812 (Polysciences, Inc., Warrington, PA). Thick sections (1 µm) were stained with Toluidine Blue and Basic Fuschin to determine suitable areas to be thin sectioned (60–90 nm). The thin sections were not post-stained. Grids were examined and photographed in a Hitachi H-7650 transmission electron microscope at an accelerating voltage of 60 or 80 kV. Copper (8.96g/cm³) was localized in the block face of each BEEM capsule using a Hitachi S-3400N (New Mexico State University, Las Cruces, NM) under the supervision of Peter Cooke, Ph.D. Backscattered images were obtained at 25kV. Elemental analysis was obtained using a Noran System Six Energy Dispersive X-ray Microanalysis System (Thermo Electron, Madison, WI) on the spot scan setting.

2.4 Statistical analyses

Statistical analyses included ANOVA as the SAS General Linear Mixed Models (GLMM). If a significant effect in treatment was found with the GLMM it was followed by a Tukey's test, post-hoc procedure.

3. RESULTS

3.1 Basic physiological studies in soil

Copper did not have an effect on the length of the roots or stems of seedlings after 24 days. No chlorosis was observed. No statistical differences were found in the germination of seeds in soil exposed with copper sulfate or copper nitrate (Fig. 1A). Figures 1B and 1C show no relative differences between control seedlings and the seedlings grown in high concentrations of copper sulfate.

3.2 Copper concentrations in screw bean mesquite

Inductively coupled plasma spectroscopy (ICP-OES) was used to identify the amount of copper collected in screw bean mesquite seedlings grown in soil. We quantified the amount of copper for roots, stems, cotyledons and true leaves, totaling 192 samples. In copper sulfate exposed seedlings, we observed a very high concentration of copper in the roots, which diminished as it traveled up the stem. Copper absorption from copper sulfate in roots, stems, cotyledons, and true leaves (Fig. 2A) showed statistical differences across concentrations of exposure ($P < 0.0001$). The root showed that after 200 ppm copper sulfate exposure, the absorption was different from the lower concentrations and the control.

A general grouping between 50–200 ppm suggests that screw bean mesquite seedlings react statistically differently to copper at 300–600 ppm levels.

A 600 ppm copper sulfate exposure for 24 days resulted in a total of approximately 59,000 ppm of copper within the seedlings. The corresponding copper concentrations were as follows: roots (30,000 ppm), stem (17,000 ppm), cotyledons (11,000 ppm) and true leaves (20 ppm). To be identified as a hyper accumulator of a specific metal, the plant must accumulate leaf: root metal ratio of < 1 . Screw bean mesquite leaf: root ratio, considering cotyledons as part of the leaf ratio, was 0.355, and 0.000 without cotyledons. Therefore, screw bean mesquite seedlings at 24 days of growth have been shown to be hyper accumulators of copper from copper sulfate.

Figure 2B shows the increasing amounts of copper in screw bean mesquite seedlings exposed to increasing amounts of copper nitrate. Copper nitrate absorption in roots showed a statistical difference across concentrations of exposure ($P < 0.0001$). The root showed that after a 200 ppm copper nitrate exposure, copper absorption was different from the lower concentrations and the control. Copper presence from copper nitrate in the control roots was 20 ppm, while in the 600 ppm copper nitrate exposed roots there was about 72 ppm.

The overall pattern showed no translocation of copper from copper nitrate from the roots to the stem, cotyledons, and true leaves suggesting a complete sequestration and compartmentalization of copper within the roots.

Screw bean mesquite leaf: root ratio, considering cotyledons as part of the leaf ratio, was 0.685, and 0.600 without cotyledons, suggesting hyper accumulation of copper.

3.3 Nutrient changes due to copper exposure

Copper may interfere with the uptake or distribution of macro nutrients (calcium, magnesium, potassium, phosphorus, and sulfur) and the micro nutrients (iron, manganese, zinc, boron, and molybdenum). Five-hundred samples were tested to determine patterns of nutrients in response to copper exposure. There was no statistical significance in the 3-way interaction of copper type, part and concentration, and the 2-way part by concentration interaction for calcium, iron, magnesium, molybdenum, and zinc. However, the 2-way compound type by part interaction was significant in boron, manganese, phosphorus, potassium and sulfur.

Boron concentrations were different across root, stem, cotyledons, and true leaves as copper exposure increased ($p = 0.0181$). Similar patterns were observed for copper sulfate and copper nitrate in the effect of boron concentrations ($p = 0.5408$). The highest concentration of boron was found in the true leaves, followed by roots, stems and cotyledons treated with copper sulfate (Fig. 3A). In true leaves, boron decreased as copper sulfate exposure increased. We identified a sharp fluctuation of boron concentration in roots, stems and

cotyledons at 200 ppm suggesting a switch in boron accumulation in the roots and translocation in stems and cotyledons.

In copper nitrate treated seedlings, boron concentration, in descending order, is as follows: cotyledons, true leaves, stems, and roots (Fig. 3B). Boron concentrations in true leaves did not fluctuate; however, they were half the amount seen from copper sulfate exposure.

Manganese concentrations created overall different 3-way interaction patterns between copper sulfate and copper nitrate ($p < 0.0001$). Manganese concentrations did not change within roots and stems during copper sulfate exposure (Fig. 4A). A sharp peak at 200 ppm of copper sulfate was the only high concentration of manganese identified within cotyledons. No changes were observed in copper nitrate exposed stem (Fig. 4B). There was a slight decrease followed by an increase of phosphorus in the roots exposed to copper nitrate. In both, copper sulfate and copper nitrate exposures, manganese concentrations created a unique pattern of a sharp high and low concentrations in leaves. Manganese rapidly increased at 100 ppm, followed by an even greater decrease at 200 ppm, a subsequent increase at 300–400 ppm, and decreased at 500 ppm of copper sulfate. In copper nitrate, the pattern was reversed.

Different 3-way interaction patterns were observed for copper sulfate and copper nitrate in the effect on phosphorus concentrations ($p < 0.0001$). Phosphorus was significantly affected by the presence of copper sulfate (Fig. 5A). With a low copper sulfate treatment of 50 ppm, copper in both roots and stems dropped dramatically to 0 ppm and 3,000 ppm, respectively. After 100 ppm of copper sulfate, no phosphorus was present in roots or stems. Control roots had almost 5,000 ppm and stems had 6,500 ppm of phosphorus. For cotyledons, phosphorus fluctuates from 8,000–12,000 ppm between 50–200 ppm of copper sulfate exposure. However, after 300 ppm of copper sulfate exposure there was no phosphorus in cotyledons. Phosphorus concentration was similar to control levels (9,000 ppm) within all copper sulfate exposures of leaves.

Significant micronutrient changes within seedlings exposed to copper nitrate were seen in cotyledons and true leaves (Fig. 5B). The control for cotyledons had 10,000 ppm of phosphorus, while the 600 ppm copper nitrate exposed cotyledons had 6,000 ppm of phosphorus. The control for true leaves had 6,000 ppm of phosphorus, while the 600 ppm copper nitrate exposed true leaves had 4,500 ppm of phosphorus. There is a significant phosphorus difference in response to copper nitrate vs. copper sulfate ($p < 0.0001$).

Similar 3-way interaction patterns were observed with copper sulfate and copper nitrate in respect to potassium concentrations ($p = 0.0013$). Potassium concentrations in roots, stems, cotyledons, and true leaves of seedlings exposed to copper sulfate decreased as copper increased (Fig. 6A). Control root contained about 15,000 ppm of potassium, while 600 ppm copper sulfate exposed roots had approximately 4,000 ppm of potassium. This is a 66 % decrease. Similar decreased percentages were seen in stems. In true leaves, potassium decreased from 60,000 ppm to 50,000 ppm. At 100 ppm of copper sulfate, there was a sharp decrease to 38,000 ppm which is a 33% decrease from the control. At 300 ppm, potassium levels increased to 57,000 ppm, a near 100% recovery.

In copper nitrate treated seedlings, potassium fluctuated between 1–45,000 ppm within all parts of the plant (Fig. 6B). Control concentrations in roots, stems, cotyledons, and true leaves were as follows: 35,000 ppm, 35,000 ppm, 43,000 ppm, and 39,000 ppm. At 600 ppm of copper nitrate, potassium was at 29,000 ppm, 36,000 ppm, 30,000 ppm, and 27,000 ppm for roots, stems, cotyledons, and true leaves, respectively. The largest potassium changes were in cotyledons and true leaves. A statistical three-way interaction showed that potassium patterns in copper sulfate and copper nitrate were different ($p = 0.0144$).

Similar 3-way interaction patterns were observed by copper sulfate and copper nitrate in sulfur concentrations ($p=0.0011$). Significant changes within seedlings exposed to copper sulfate were seen in roots, stems, cotyledons and true leaves (Fig. 6A). The control for roots had 3,200 ppm of sulfur, while the 600 ppm copper sulfate exposed roots had 5,900 ppm of sulfur. The control for stems had 3,200 ppm of sulfur, while the 600 ppm copper sulfate exposed stems had 3,200 ppm of sulfur. The control for cotyledons had 2,700 ppm of sulfur, while the 600 ppm copper sulfate exposed cotyledons had 5,800 ppm of sulfur. The control for true leaves had 4,500 ppm of sulfur, while the 600 ppm copper sulfate exposed true leaves had 3,200 ppm of sulfur.

Significant changes within seedlings exposed to copper nitrate were only observed in cotyledons (Fig. 6B). There was a fluctuation of sulfur as copper nitrate increased. There were differences in the sulfur patterns of copper sulfate and copper nitrate across their different copper treatments ($p=0.0080$).

3.4 Histological, Ultrastructural and Elemental Analyses

Figure 8A shows the growing apical tip while Fig. 8B shows a central portion of a mature leaf (200X). We analyzed the ultrastructural changes of true leaves using transmission electron microscopy. Cross sections of control screw bean mesquite leaves showed a normal appearance of parenchymal cells with chloroplasts and large vacuoles (Fig. 9A). No copper was found in the vacuole or cell wall of the control samples by elemental analysis of leaves (Fig. 9C).

Plasmolysis was evident beginning with 100 ppm of copper sulfate exposure. Starch content appeared to increase as the copper exposure increased. At 200 ppm of copper sulfate exposure, a cross section of a leaf showed a dark aggregate accumulating between parenchymal cells (Fig. 9B). The elemental analysis showed the presence of copper in the cell wall and the interior of a leaf parenchymal cell exposed to 200 ppm of copper sulfate (Fig. 9D). The cross section of a leaf exposed to 500 ppm of copper sulfate showed a dense nucleus and cell cytoplasmic disarrangement characteristic of programmed cell death (Fig. 9E). Chloroplasts were enlarged with abnormal thylakoid membranes. Dark aggregates between cells and attached to the exterior of cell walls were present. Elemental analysis suggested the presence of copper in the cell wall of a leaf parenchymal cell in screw bean mesquite exposed to 500 ppm of copper sulfate (Fig. 9F).

ICP-OES results confirmed the absorption of copper from copper nitrate by the roots with no translocation.

4. DISCUSSION

Our studies showed that copper is not affecting the germination of screw bean mesquite seedlings in soil. Ahsan *et al.* (2007) reported that seed germination may be affected by heavy metal pollution; however, the mechanism of inhibition of germination is not known. Chaignon and Hinsinger (2003) stated that germination is relatively insensitive to many toxic substances. They concluded that at this very early stage, seedlings do not uptake nutrients from the soil rather they use their seed reserves. This may explain the lack of an effect on germination rate on these screw bean mesquite seedlings.

Rhodes and Felker (1988) stated that screw bean mesquite was hard to grow in a greenhouse, with only 68% survival rates. Seedling survival rates of screw bean mesquite in the field are not available. Our results showed a 20–25% survival rate of screw bean mesquite with no physiological changes due to copper exposure.

Copper sulfate exposure increased the accumulation of copper in the roots, stems, cotyledons, and true leaves. We suggest that the uptake was active transport with gradient limitations due to copper bioavailability up to 200 ppm of copper sulfate exposure. Copper from copper nitrate absorbed into the roots but did not translocate up the stem.

Screw bean mesquite seedlings at 24 days of growth were shown to be hyper accumulators of copper from copper sulfate. Copper uptake is limited by its bioavailability from the soil and the transport mechanisms within the plant (Prasad, 2004). We suggest that screw bean mesquite may have evolved a tolerance response to copper sulfate, which may include cysteine-rich transport proteins.

Our results showed that boron, manganese, phosphorus, potassium and sulfur were affected by copper presence. Boron is involved in the structure of cell walls and membrane function (Blevins and Lukaszewski, 1998). Boron ions within the screw bean mesquite seedlings were transported differently depending on the presence of copper sulfate or copper nitrate. In true leaves, boron decreased as copper sulfate exposure increased. This is significant because boron deficiency causes abnormalities in the cell wall and middle lamella organization (Hu and Brown, 1994). Boron forms cross links in pectin protecting levels of calcium in the cell wall (Clarkson and Hanson, 1980). Yamanouchi (1971) and Yamauchi *et al.*, (1986) confirmed that tomatoes with boron deficiency also contained less calcium. Calcium uptake did not decrease in the copper exposed seedlings. Boron-deficiency may reduce the uptake of phosphorus (Goldbach, 1984). A decrease in phosphorus could alter the membrane function since phosphorus is part of the structure of phospholipids.

Manganese concentrations changed within true leaves during copper sulfate exposure. There was a slight decrease, followed by an increase of phosphorus in the roots exposed with copper nitrate. Manganese concentration decreased in cotyledons and true leaves exposed to increasing copper nitrate concentrations. Manganese is involved in enzyme activation and chlorophyll structure (Millaleo *et al.*, 2010). Manganese decreases the solubility of iron; therefore, an abundance of manganese may lead to iron deficiencies in seedlings. Decreased manganese concentrations may result in chlorosis due to limited chlorophyll and poor root development. We did not have an iron decrease in the screw bean mesquite seedlings. Our results showed manganese uptake at the root was normal for both copper sulfate and copper nitrate. Rengel (2001) showed that manganese has a poor mobility in the phloem of mature wheat grains. From our results, it appears that manganese is not well translocated within screw bean mesquite exposed to copper in the soil.

Phosphorus is involved in the energy carrying phosphate compounds (ATP and ADP), nucleic acids, coenzymes, and phospholipids. Phosphorus is essential for the process of photosynthesis. Phosphorus was significantly affected by the presence of copper from copper sulfate within the roots, stems, and cotyledons.

Potassium is involved in enzyme activation, stomatal activity, photosynthesis, transport of sugar, water and nutrients, as well as, starch and protein synthesis. Potassium concentrations in roots, stems, cotyledons, and true leaves of seedlings exposed to copper sulfate and copper nitrate decreased, as copper increased. Potassium deficiencies may alter activation of enzymes, rate of photosynthesis and ATP production, transport of water and nutrients, and the synthesis of starch (Armstrong, 1998). Nitrates, phosphates, calcium (Ca), magnesium (Mg), and amino acids translocation is reduced when potassium decreases (Armstrong, 1998). Ultrastructural changes were observed in screw bean mesquite seedlings such as, increased vacuolization and damage to cell membranes. Starch synthetase may be activated by potassium; therefore, reduction of potassium could interfere with starch breakdown.

Qualitative ultrastructural analyses showed an increase of starch accumulation as copper sulfate exposure increased.

Sulfur is part of cystine, cysteine, methionine, and amino acids of other plant proteins (Baird, 1914). Sulfur increased within seedlings exposed to copper sulfate in roots, and cotyledons. Sulfur increase may be directly linked to the increased production of cysteine. Both metallothioneins and phytochelatins are cysteine-rich proteins known to bind copper in tolerance mechanisms. Screw bean mesquite seedlings exposed to copper sulfate accumulated 128 % more sulfur than seedlings exposed to copper nitrate. This suggests that screw bean mesquite may have a mechanism that increases cysteine-rich proteins in the presence of copper sulfate.

Copper does not accumulate in chloroplasts, but in the apoplast and the vacuole (Quartacci *et al.*, 2001; Baryla *et al.*, 2001; Patsikka *et al.*, 2002). Toxic concentrations of copper have negatively affected carbohydrate metabolism (Roito *et al.*, 2005) in *Pinus sylvestris*.

Screw bean mesquite is not only a hyper accumulator of copper, but is also tolerant to copper. There is no direct correlation between hyperaccumulation and tolerance (Baker *et al.*, 2000). In general, hyper accumulators are in the minority in metal contaminated regions where excluders are more widespread (Baker *et al.*, 2000). Screw bean mesquite needs to be tested in the field to determine its overall efficiency in cleaning local soils.

In conclusion, we identified screw bean mesquite as a hyper accumulator of copper. Screw bean mesquite has a low germination rate, but large biomass once established. Hypothetically, screw bean mesquite could potentially clean up 4.5 tons per acre of copper sulfate contaminated soil in 4.1 years.

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A

B

C

Fig. 1.

A Germination of screw bean mesquite seedlings (grown in triplicate) in soil with either copper sulfate or copper nitrate

B Photograph of seedlings grown in 0 ppm of copper sulfate (control)

C Photograph of seedlings grown in 600 ppm of copper sulfate

A

B

Fig. 2.

A Copper sulfate absorption comparison within screw bean mesquite seedlings

B Copper nitrate absorption comparison within screw bean mesquite seedlings

A

B

Fig. 3.

A Boron changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper sulfate exposure

B Boron changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper nitrate exposure

A **B****Fig. 4.**

A Manganese changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper sulfate exposure

B Manganese changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper nitrate exposure

Fig. 5.
A Phosphorus changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper sulfate exposure
B Phosphorus changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper nitrate exposure

Fig. 6.

A Potassium changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper sulfate exposure

B Potassium changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper nitrate exposure

A

B

Fig. 7.

A Sulfur changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper sulfate exposure

B Sulfur changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper nitrate exposure

Fig. 8. Cross section of apex of a control true leaf (200X) (a); Cross section of 500 ppm of copper sulfate exposed true leaf (200X) (b)

Fig. 9.

A Electron micrograph of control section of leaf parenchymal cells (4000X) (C: Chloroplast; V: Vacuole; CW: Cell Wall)

B Electron micrograph of 200 ppm of copper sulfate section of leaf parenchymal cells (7000X) (C: Chloroplast; V: Vacuole; CW: Cell Wall; and (→) for dark inclusion)

C Elemental analysis of the cell wall of a control leaf parenchymal cell

D Elemental analysis of the cell wall of a leaf parenchymal cell exposed to 200 ppm of copper sulfate

E Electron micrograph of 500 ppm of copper sulfate section of leaf parenchymal cells (5000X) (C: Chloroplast; N: Nucleus and V: Vacuole)

F Elemental analysis of the cell wall of a leaf parenchymal cell exposed to 500 ppm of copper sulfate