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Biological Effects of copper on Prosopis pubescens (Screw bean mesquite)

Marian Nicte Zappala
University of Texas at El Paso, nicte23@hotmail.com

BIOLOGICAL EFFECTS OF COPPER ON *PROSOPIS PUBESCENS* (SCREW BEAN MESQUITE).

MARIAN NICTE ZAPPALA, B.S., B.A., M.S.

Environmental Science and Engineering

APPROVED:

Joanne Tontz Ellzey, Ph.D., Co-Chair

Jorge L. Gardea-Torresdey, Ph.D., Co-Chair

Jianying Zhang, Ph.D.

Jose R. Peralta-Videa, Ph.D.

Benjamin C. Flores, Ph.D.
Dean of the Graduate School

DEDICATION

To God, who gave me talents and continues to guide me towards the right doors. To my loving husband, Mark, whose generous heart committed his life along with mine to the completion of this degree. His appreciation of my intellectual development has refueled my passion for the sciences. To my mother who has given her unconditional support and help. Her love and patience have strengthened my determination and my goals. To my little Juliana Catherine and my future children, for whom I hope to be an example of the importance of a higher education. To my brother Saul and my best friends Erika D. Wilson and Melisa M. Bertaud de la Pena, for loving and encouraging who I am.

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by

MARIAN NICTE ZAPPALA, B.S., B.A., M.S.

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ABSTRACT

Phytoremediation is an emerging technology for cleaning industrial and urban contamination (Peuke and Rennenberg, 2005). Many factors including germination rate, biomass changes, biochemical changes, and nutrient changes must be determined in order to utilize a plant for phytoremediation. Our results showed that *Prosopis pubescens* (Screw bean mesquite) is a hyperaccumulator of copper from copper sulfate and a feasible option to remediate fields with up to 200 ppm of copper sulfate.

Screw bean mesquite seeds are viable even after 30 years of storage. Both copper sulfate and copper nitrate decreased the germination rate of screw bean mesquite seeds. In soil grown screw bean mesquite plants, germination rates were not affected by either copper sulfate or copper nitrate. Physiological studies showed biomass decreased with copper exposure. Root cell area increased with increasing copper exposure. Only after copper nitrate exposure, chlorophyll levels decreased; unmasking the yellow/orange pigments from carotene and xanthophyll and causing chlorosis.

We measured copper and nutrient concentrations within seedlings. Petri dish grown seedlings accumulated 47,000 ppm (27,500 ppm) in roots, 23,000 ppm (21,000 ppm) in stems, and 9,000 ppm (16,000 ppm of copper) in cotyledons from copper sulfate (copper nitrate). Control seedlings grown in soil accumulated 31,000 ppm in roots, 17,000 ppm in stems, 11,000 ppm in cotyledons, and 20 ppm of copper from copper sulfate in true leaves. Copper did not change calcium, iron, manganese and zinc concentrations. Magnesium, potassium, phosphorus decreased while sulfur increased in Petri dish grown seedlings exposed to copper sulfate and copper nitrate.

We identified various ultrastructural changes of cotyledons and true leaves due to copper toxicity including separation of the cell membranes from cell walls, denser cytoplasm with dark aggregates in epidermis, parenchyma, and developing phloem as well as increased vacuolization, swelling of chloroplasts and disarray of thylakoid membranes. Plasmolysis was evident beginning with

100 ppm of both copper sulfate and copper nitrate. Elemental analysis confirmed the presence of copper in experimental samples.

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1.1 Environmental Pollution

Urbanization expansion has increased soil contaminants, worldwide. Urban settings such as El Paso, Texas have been affected by background levels of copper in soil (2 to 250 ppm) (Gardea-Torresdey *et al.*, 2005). Background copper concentrations in soil are from 13 to 24 ppm (Kabata-Pendias and Pendias, 1992). Soils with higher copper concentrations must be remediated. A recent survey of El Paso's soils has shown that we maintain a copper level of 10 ppm (Ketterer, 2006). This increasing soil contamination requires time and cost effective methods to remediate. Since plants can readily adapt to a changing environment and can tolerate various air, water and soil contaminants; plants possess the ability to absorb and remove heavy metal contamination from soil. The removal of specific heavy metals from the soil by a plant is called phytoremediation. Locally, several mesquite species are being studied for their phytoremediation potential.

The Bordeaux fungicide mixture contains copper sulfate and hydrated lime, also known as calcium hydroxide. This fungicide was developed in France to control fungi affecting wheat and oat crops. It was very popular during the 18^{th} and 19^{th} centuries due to its effectiveness. In 1761, seeds were treated with copper sulfate with no evidence of negative effects to the crops (Millardet, 1885). Copper sulfate was subsequently used as an effective fungicide around the world. Copper nitrate is a compound utilized as a catalyst and nitrating agent in organic reactions as well as in fungicides and wood preservatives (Michael and Irene Ash, 2004).

The U.S. Southwest has a vast population of mesquite species due to their adaptation to desert climate. Mesquite species are not identified as crops for food consumption; therefore, no

copper sulfate had been used or tested on mesquite. Even though mesquite is not a food crop, it is vital to the cultural practices of the Southwest region. This project has studied the uptake and effects of copper on screw bean mesquite, specifically. No previous phytoremediation research on this species has been reported.

1.2 Phytoremediation

To reduce the amount of heavy metals in the environment, several techniques have been proposed such as chemical, physical, and biological techniques. However, besides their high cost, the chemical and physical techniques affect soil properties and may make the soil a useless medium for growing plants. Phytoremediation is a biological method in which plants are used to remove, stabilize, or degrade pollutants in soil and water. Some plants have the capacity to grow on heavy metal-containing soils and accumulate the metals in their tissues without showing symptoms of toxicity (Padmavathiamma *et al*., 2007).

Phytoremediation is an emerging technology for cleaning industrial and urban contamination (Peuke and Rennenberg, 2005). This clean, efficient, inexpensive, and non-environmentally disruptive technology uses plants to absorb, degrade and/or eliminate metals, pesticides, solvents, explosives, crude oil and its derivatives, and various other contaminants from soil, water or air (Peuke and Rennenberg, 2005). Over 450 plant species have been identified as hyperaccumulators of various metals such as zinc, nickel, and cadmium (Brooks *et al*., 1998). They can tolerate heavy metal contamination up to certain levels before cellular damage exceeds the plant's ability to live.

Metals in low concentrations such as zinc, copper, manganese, nickel and cobalt are essential for plant growth (Marschner and Dell, 1994). Metals such as cadmium, lead and

mercury are soil pollutants. All metals in excess are pollutants. Since heavy metals cannot be chemically degraded and have to be physically removed or immobilized (Gaur and Adholeya, 2004), secondary management practices are necessary. The use of plants to absorb heavy metals is an example of one of these management practices called phytoextraction.

Phytoextraction can be further divided into two categories: continuous and induced (Peer, *et al.,* 2007). Continuous phytoextraction is the uptake of metals through the lifetime of the plant; whereas, induced phytoextraction uses chelators to enhance the uptake of metals in short periods of time. Chelators such as EDTA have mobilized copper in *Brassica juncea* (Indian mustard) and *Helianthus anuus* (sunflower) (Peer *et al.,* 2007). The use of chelators to enhance uptake and accumulation of various metals has not been clearly understood at the molecular level. Figure 1 (Peer *et al.,* 2007) shows the pathway of metal and nutrient uptake in plants. Soluble metals may enter through the root symplast by crossing the plasma membranes of epidermal cells, parenchymal cells, endodermal cells, and xylem cells. A second pathway is apoplastic, where heavy metals travel between root cells into the xylem of the vascular bundle. If the soluble metals are to be transported up the shoot into the leaves, they must enter the xylem by membrane pumps or channels. The flow of the xylem sap will move the metals up to the leaves where they can be stored in various cell types, depending on the species and the form of the metal. Some of these cell locations include the cell wall, cytosol, vacuole and/or chloroplasts (Peer *et al.*, 2007).

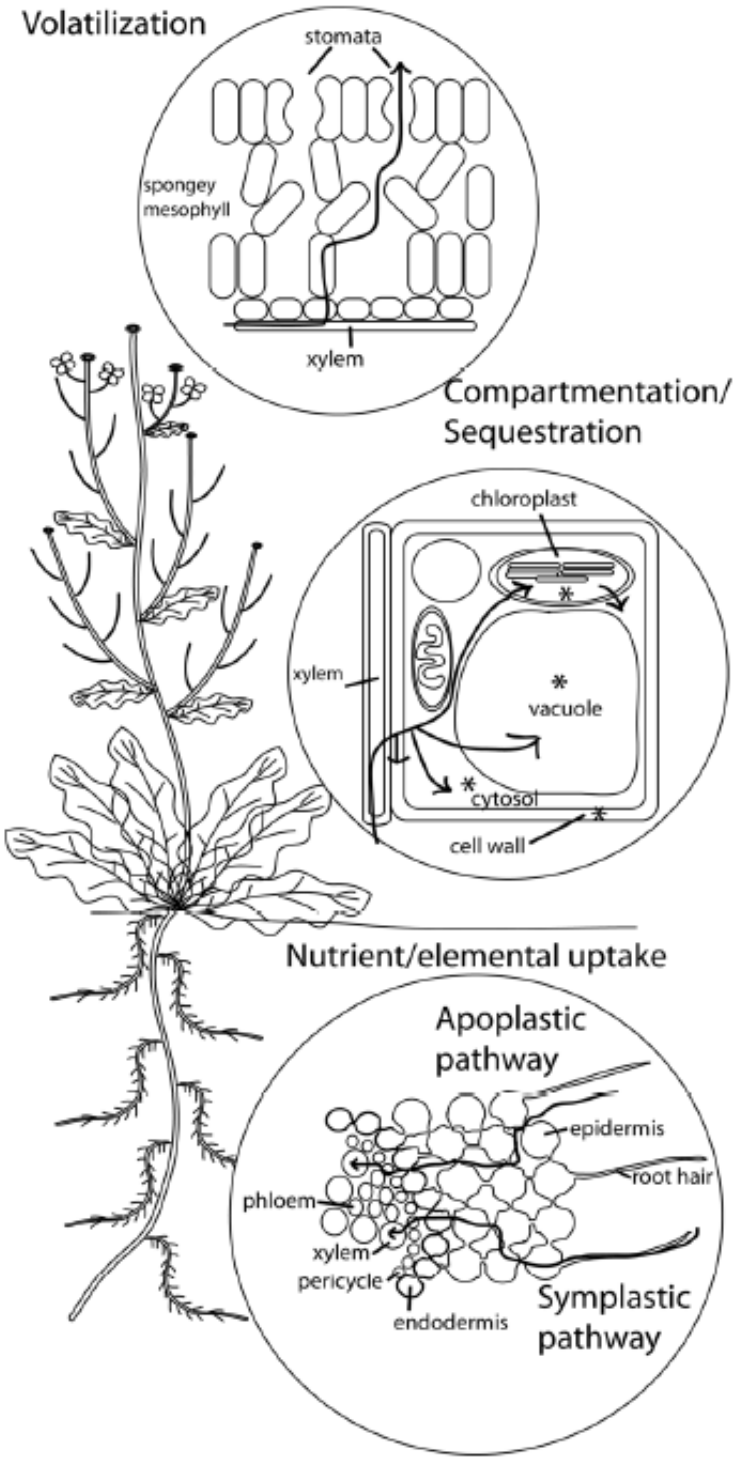


Figure 1. General pathway of soluble metals and nutrients in plants. (Peer *et al.*, 2007)

Current practices of plants in phytoremediation entail the growth of seedlings in the laboratory and then transplantation into the contaminated soils. One recent exception is a phytoremediation project in Arizona mine tailings sowed seeds directly in soils (Haque *et al*., 2009). A large biomass is crucial for the cost-effectiveness of phytoremediation techniques and so many hyperaccumulators have been recommended to remove metals from soil. However, few are adapted to desert environments which promote slow growth due to harsh environmental conditions such as high heat and low water availability. For example, *Thlaspi caerulescens*, a known zinc hyperaccumulator is very small and slow-growing (Puldorf and Watson, 2003). Further, even hyperaccumulators will not remediate contaminated soils if their biomass is limited. Therefore, it is important to identify ways to increase biomass, growth time, or to simplify starting materials.

Two mechanisms have been suggested for plants to tolerate high levels of metals: 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 stem:root metal ratio of<1 while accumulators the ratio of >1 (Boularban *et al.,* 2006). Brooks *et al.* (1977) were the first to use the term hyperaccumulators to describe plants with nickel concentrations higher than 1000 µg/g in dried leaves. Thirty seven hyperaccumulators species of copper are known (Baker and Brooks, 1989).

1.3 Screw Bean Mesquite

Prosopis pubescens (Fig. 2) is a fast proliferating shrub that grows among seemingly harsh conditions, such as heavy metals and in water scarcity. Currently, this plant produces large biomass, and thus, is a source of stock food and a means for erosion control. More importantly,

what has been found by various researchers is that *Prosopis juliflora* readily takes up chromium and lead heavy metals (Arias, *et. al*. 2010). However, it has not been reported if this plant has the ability to readily uptake copper. The effect that copper has on this plant could lead to the use of this plant for restoration of areas that are contaminated with heavy metals. Sufficient information is currently unavailable for this specific mesquite's heavy metal tolerance.

Screw bean mesquite can grow on a wide range of soil textures (Campbell, *et al*., 1964; Martin, 1980) such as rocky clay silt (Everett, 1957); light and sandy to heavy clay (Campbell, *et al.,* 1964); silty clay loam (Goel, *et al.,* 1996); and clay to loam soil textures (Taylor, *et al.,* 1998). As with many of the mesquite species, screw bean mesquite is very tolerant to saline soils (Kearney, *et al.*, 1960; MacMahon, 1988) and alkaline soils (Goel, *et al*., 1996). Screw bean mesquite is a drought-tolerant species; however, it requires between 3 to 20 inches of annual precipitation to be a dominant species (Campbell, *et al.,* 1964; Martin, 1980).

There are forty-four *Prosopis* species (Burkart, 1976). In general, not all *Prosopis* seeds mature and germinate (Pasiecznik, *et al*., 2001). It is estimated that within the American *Prosopis* species, about 17% of seeds are immature and unable to germinate (Solbrig and Cantino, 1975). Seed maturity depends on the ripening of the pod. Seed pods of ten to fifteen years of storage have fifty percent viability (Pasiecznik and Felker, 1992). Seeds may be destroyed from excess water immersion or deep soil deposition (Pasiecznik, *et al*., 2001). Livestock are the primary seed dispersal agents. Cows and rodents are the most effective in scarifying mesquite seeds, therefore improving germination (Campos and Ojeda, 1997).

Figure 2. Screw bean Mesquite (*Prosopis pubescens)*

Among the plants that have been found to accumulate heavy metal are: local desert shrub species, *Prosopis glandulosa* commonly known as honey mesquite (Aldrich *et al*., 2004). Desert plants 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). Aldrich also discovered that mesquite accumulates lead without any outward signs of stress (Aldrich *et al.,* 2004). This indicates that mesquite tolerates certain quantities of toxins very efficiently.

The Mesquite tree grows in depleted, overgrazed areas of former grasslands. It is common in the Southwest. Southwest culture has deemed high importance to the medical uses of Mesquite trees, even though its consumption as a food is limited to their seeds. Mesquite seeds (beans) were used by the Native Americans of Southern New Mexico, Arizona and Mexico as a major food source. The dried beans were ground and made into Pinole, and were fermented by the Pima to make an alcoholic beverage. The gum was used as a black dye for weaving and to

mend pottery. Mesquite was an important source of fuel and building material to the early white settlers of the Southwest (Native American Herbs & Plants of the Southwest, 2009). Mesquite tea is astringent and antibacterial, and is useful internally for diarrhea and GI tract inflammations, ranging from ulcers to colitis. Used externally, the tea makes a good antiseptic wash for any irritated or broken skin injuries.

Senthilkimar *et al*. (2005) showed that a *Prosopis sp*. could accumulate 20 ppm of copper in the shoot from an average 6 ppm soil concentration. This is approximately a three-fold accumulation of copper from the initial soil concentration. Mesquite of the *Prosopis* species, "was found to grow successfully at a copper mine" near Arizona (Haque *et al*., 2009). These plants seemed to adapt to the soil in the mine and grow without symptoms of toxicity (Haque *et al*., 2009) *P. pubescens* was used in this project since it has both constitutive and adaptive mechanisms for coping with elevated metal concentration (Haque *et al.*, 2009). Screwbean mesquite is found across the entire southwest states including California, Utah, Nevada, Arizona, New Mexico, and Texas (Fig.3).

Figure 3. USDA map of Screw bean Mesquite distribution.

1.4 Copper Toxicity

Copper is an essential element in plant nutrition. It is a key component of cytochrome *c* oxidase, plastocyanin, superoxide dismutase, and tyrosinases (Tsukihara *et al.* 1995; Clingeleffer *et al.* 1984; Li *et al*., 1995; Jaenicke *et al*., 2003). In their minimal concentration, heavy metals can be utilized as essential micronutrients for propagating the growth and the development of plants (Tanyolac *et al*., 2006). Amongst the heavy metals, 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, it has been found that copper is also a required key component in biological systems as both structural components and catalytic enzyme activity cofactors (Tanyolac *et al*.,

2006). Copper that is found in concentrations in excess beyond the optimal micronutrient requirements may lead to toxic induction by altering protein function (Tanyolac *et al*., 2006).

From the beginning of mining and smelting activity, copper has become a constant soil contaminant. Presently, copper is one of the major heavy metal contaminants in the environment. Studies done by Visviki and Rachlin (1992) showed that copper increases cell volume, lipid content and decreases pyrenoids in *Dypsis minuta* Beentje, palm tree (Prasad, 2004). Brinkhuis and Chung (1986) found in *Laminaria saccharina* (L) Lam, brown algae, that copper affected the volume of thylakoids, amount of vacuolation, and cell wall structure (Prasad, 2004).

In determining whether screw bean mesquite is appropriate for phytoremediation of copper, it is important to note that the effects of heavy metals are species-specific. To determine the extent of soil toxicity, germination studies are often carried out. No previous germination, seed viability or copper absorption studies had been performed to determine the effects of copper sulfate and copper nitrate on screw bean mesquite seeds of different ages. Copper sulfate (Fig. 4) and copper nitrate (Fig. 5) have different solubility in water; therefore, will be absorbed differently by screw bean mesquite. Copper sulfate (Fig. 4) (32g/100g water) is less soluble than copper nitrate (Fig. 5) (125g/100g water at 20ºC). Copper nitrate will release more ions and possibly having the most affect on screw bean mesquite. The permeability of the seed coat changes with storage conditions of the seeds, therefore, possibly modifying the effects of copper toxicity in older seeds.

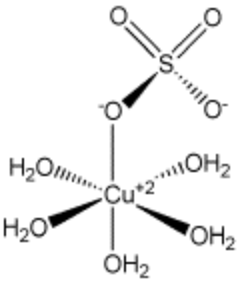


Figure 4. Chemical structure of copper sulfate.

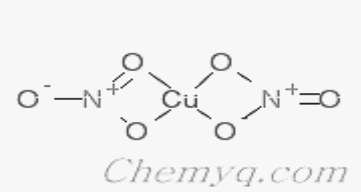


Figure 5. Chemical structure of copper nitrate.

1.5 Tolerance Mechanisms

Plants possess numerous potential cellular/molecular mechanisms that may be involved in the detoxification of heavy metals as well as tolerance to metal stress (Hall, 2002). Molecular mechanisms for the transport of cadmium in plants have been proposed by Prasad (2004). Little is known about the behavior of heavy metal transporters in plants. Copper binds to various organic ligands such as metallothioneins, phytochelatins, and citrate (Gardea-Torresdey *et al*., 2005). Hall (2002) has suggested that "more evidence is needed to establish a relationship between copper sensitivity and metallothionein production". Metallothioneins are produced from glutathione in the presence of copper. However, copper also depletes glutathione due to oxidative stress (Prasad, 2004). Victor, *et al.,* (2007) have documented copper tolerance in *Prosopis juliflora* (Sw.) DC. In 3-8 day seedlings adverse effects of copper were observed from 80-1280 ppm of copper.

Because of the redox-active transition metal nature of copper, it has been shown that the toxicity caused by exposure to a high concentration of copper may result from the binding of the metal to sulfhydryl groups in proteins, leading to the inhibition of enzyme activity and in disruption of protein structure (Tanyolac *et. al*., 2006). Another adverse effect of copper on the plant life cycle is that, in excess, copper has a toxic effect on the primary reactions of photosynthesis and electron transport, therefore, inhibiting photosynthesis (Tanyolac *et. al*., 2006). Major changes that have observed by the decrease of photochemical activities in plant proteins were associated with thylakoid membranes, decrease of biomass, and the prevention of chlorophylls from integrating into the chloroplast photosynthetic membranes (Tanyolac *et. al*., 2006). Copper may substitute for magnesium in chlorophyll, resulting in damage to the function and the structure of the chlorophyll (Tanyolac *et. al*., 2006).

A review of the heavy metal literature by Gardea-Torresdey *et al.,* 2005 listed the effects of several heavy metals primarily on desert plants. Cadmium, nickel, and zinc decreased seed germination. Plant growth was stunted by cadmium, chromium, copper, and lead (Gardea-Torresdey *et al*., 2005). However, plant growth may be increased in the presence of zinc. Cadmium decreases lipid content and induces the production of phytochelatins (Prasad, 2004). Chromium produces membrane abnormalities, chlorosis and root damage (Prasad, 2004). Nickel has been shown to change protein production, decrease chlorophylls and enzymes while it increases free amino acids (Gardea-Torresdey *et al*., 2005). Chloroplasts are destroyed in the presence of mercury (De la Cruz, 2002). Various heavy metals can cause the same physiological, structural, and/or molecular effects which indicate similar transport, sequestration or immobilization mechanisms of those metals. Copper decreases thylakoid surface area that reduces photosynthesis, as well as plant growth and reproductive processes (Tanyolac *et al.*,

2006; Muccifora, 2008). Since all these effects are linked, understanding each step will enable future manipulation of other steps in the mechanisms.

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), CO_2 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). Excess in copper can lead to substitution of other essential micro and macronutrients such as calcium, magnesium, and iron (Ke *et al.,* 2007) in *Rumex japonicus*.

Toxic concentrations of copper have negatively affected chlorophyll content and photosynthetic parameters (Borghi *et al.,* 2007) and carbohydrate metabolism (Roito *et al.,* 2005). Copper can affect structural components of plant cells. Copper deficiency may be expressed as a light overall chlorosis along with the permanent loss of turgor in young leaves. Copper toxicity leads to a decreased number of chloroplasts, membrane damage, and alteration of permeability of cell membranes (Muccifora, 2008). A decrease of photochemical activity by the reduction of thylakoid membrane in chloroplasts will decrease the biomass growth and the prevention of chlorophyll to integrate into chloroplast photosynthetic membranes (Tanyolac *et. al*., 2006). Chlorophyll (a and b) and carotenoid (β-carotene and xanthophyll) pigments in the leaf are responsible for the absorption of energy from the sun's rays used in photosynthesis. Deficiency of nutrients such as iron or magnesium may cause a decrease in chlorophyll pigments leading to yellowing of the leaves or chlorosis. The decrease of chlorophylls unmasks the yellow

and orange pigments of the carotenoids. Copper toxicity in screw bean mesquite may also lead to chlorosis by a decrease in pigments or nutrient deficiencies.

Heavy metal contamination has also produced both ultrastructural and protein changes in plants. In 2000, Monni *et al.* found that a mixture of copper and nickel affected the elongation of shoots, as well as the root dry weight of *Empetrum nigrum* L. (Black crowberry). The visible effects are the result of metabolic changes in the plant. Among the plants that have been found to accumulate heavy metal is a local desert shrub species, *Prosopis glandulosa* Torr. *(*PRGL2), commonly known as honey mesquite (Aldrich *et al*., 2004). Desert plants are physiologically tolerant to drought, high salt concentrations, and nutrient poor soils (Aldrich *et al*., 2006). Mesquite has been shown to hyperaccumulate arsenic, chromium, and lead, respectively (Aldrich *et al.*, 2004). This indicates that mesquite tolerates certain quantities of toxins very efficiently. *Prosopis glandulosa* is a small to medium sized native North American tree found from California to Louisiana. It has deep roots and therefore it is capable of surviving in areas with very low precipitation (3") but with groundwater reserves in lower soil layers. Honey mesquite offers many benefits to the desert ecosystems such as it enhances soil fertility and provides protection and shelter for plants and wildlife (Ansley *et al*., 1996).

In a study done by De la Rosa *et al.,* (2005), tumbleweed responded to cadmium uptake by producing low-molecular weight thiols. Gardea-Torresdey *et al.,* (2004) have not observed any physiological damage on creosote bush after absorbing copper. However, copper has been found to be toxic to many plant species and be a world-wide pollutant (Wenger *et al.,* 2003). Creosote bush, *Larrea tridentata,* is among the plants that have been found to accumulate heavy metals in the Southwest region (Gardea-Torresdey *et al*., 2004). Heavy metal contamination might not be physically or physiologically apparent under its tolerant threshold. It will be

interesting to investigate its physiology at the molecular level to see the subtle physiological changes that creosote bush overcomes.

With the use of X-ray absorption, the atomic structure and speciation of copper in creosote bush has been determined (Polette, *et al.,* 2000). It was revealed that copper is reduced from Cu (II) to Cu (I) as it is transported from the root to the leaves within creosote bush (Polette *et al.,* 1998). The reduction indicates a possible detoxification mechanism rather than a tolerance mechanism involved in creosote bush.

There are two main theories on how plants survive heavy metal contamination: 1) tolerance mechanism and 2) a detoxification mechanism.

Heavy metal tolerance was first reported by the Czech scientist S. Prat *et al.,* (1934), and has since been studied extensively by a number of scientists. The investigation of the evolutionary properties of plants growing in contaminated sites was studied by Bradshaw and McNeilly (1981). Their findings include the following: plants growing in contaminated sites are genetically adapted to be tolerant of heavy metals; metal-tolerant plants do not compete well in non contaminated sites; selection is so strong that genetic adaptation to contaminated sites takes place even though there is potential for gene flow from nearby non tolerant populations.

Heavy metal tolerance of the plants may be due to the evolution of chemical functional groups that reduce the toxic effects of the heavy metals. There are hypotheses on how plants tolerate the presence of heavy metals. However, the mechanisms are still poorly understood. Plants are said to either diminish or neutralize the effects of heavy metals (De la Rosa *et al.,* 2005). The suggested mechanisms include: metal exclusion, synthesis of metal binding peptides, and inactivation of metals via chelation and sequestration (Clemens, 2001). It was noted by Guerinot (2000) that microorganisms transport metals into the cytoplasm using cationic

transporters such as calcium, iron, manganese, and zinc transporters (Guerinot, 2000). Heavy metals are then imported into vacuoles by an ATP-ase P-type pump (Mills *et al.,* 2003). These mechanisms are achieved either by direct metal pumping heavy metal transporters or by coordination with low-molecular weight thiols (Li *et al.,* 1996; Marrs and Walbot, 1997; Thomine *et al.,* 2000; Vande Weghe and Ow, 2001). Little is known about heavy metal transporters' behavior in plants. Metallothioneins and phytochelatins are thought to bio-inactivate heavy metals. Both of these complexes contain sulfide groups that chelate metals (Clemens *et al.,* 2002). Phytochelatins are produced with the help of the enzyme phytochelatins synthetase (γ-glutamyl cysteine dipeptidyl transpeptidase) (Cobbett, 2000). In the presence of cadmium, phytochelatin synthetase enzyme is activated (Marrs and Walbot, 1997; Maier *et al.,* 2003a; Maier *et al.,* 2003b). There is also evidence that cadmium regulates the enzyme's expression (Lee and Korban, 2002).

Phytochelatins transport metal ions such as cadmium and copper into the vacuole (Prasad, 2004). De la Rosa *et al.,* (2005) proposed that cadmium enters into *Salsola kali* (tumbleweed) root cells through calcium channels. The table below (Fig. 6) is a summary of metal and organic complexes formed within plants. Copper binds to various organic ligands such as methallothioniens, phytochelatins, and citrate (Gardea-Torresdey *et al.,* 2005). There is a multitude of information on phytochelatins and other metal-binding complexes induced by cadmium (Prasad, 2004); however, this is not true for copper (Prasad, 2004). Metallothioneins are produced from glutathione and the presence of copper. However, copper also depletes glutathione presence by oxidative stress (Prasad, 2004).

Element	Analogue	Organic ligand
Arsenic	Phosphate	Phytochelatin, thiol, glutathione, ADP-As, ascorbic acid
Cadmium	Zn, Fe	Phytochelatin, glutathione, γ-glutamylcysteine, thiols
Chromium	Mn	Thiols
Copper	Cu	Citrate, metallothioniens, phytochelatin 2, phytochelatin 3
Mercury	Unknown[a]	Thiols
Nickel	Fe	Nicotianamine, histidine, thiols, citrate
Lead	Zn, Fe	Glutathione
Selenium	S	Cystiene, methionine, with and without methylation
Zinc	Zn	Phytochelatin, glutathione, γ-glutamylcysteine, thiols, citrate, malate

[a]Enters cell through passive diffusion.

Figure 6. Element list and their possible organic ligands (Peer *et al.*, 2007).

Plants possess numerous potential cellular/molecular mechanisms that may be involved in the detoxification of heavy metals as well as tolerance to metal stress (Hall, 2002). Some of the mechanisms may involve mycorrhizal fungi, cell wall and plasma membrane binding and efflux pumping, chelation by phytochelatins, repair of stress-damaged proteins, and compartmentalization of metals in vacuoles (Hall, 2002). Hall (2002) concluded his review with the statement, "It appears likely that specific mechanisms are employed for specific metals in particular species." Further investigation of the copper-metallothionein complex is essential in the understanding of copper phytoextraction in various plants.

1.6 Objectives

I hypothesize that *Prosopis pubescens* will have tolerance to some concentrations of copper. It is important to determine at what level of copper death occurs in plant cells. The important factor to look for is at which level the copper begins to degrade the plants' cells to a point that is no longer sustainable. Expected obvious cellular disease was observed as copper concentration increased. Disease is "regarded as any impairment of structure or process of

sufficient intensity or duration to noticeably or permanently affect the normal development of the plant" (Stevens and Stevens, 1952).

To determine the maximum level at which screw bean mesquite can tolerate copper, we have exposed it to several levels of copper sulfate or copper nitrate, in increments of 100 ppm. I hypothesized that the threshold level is approximately at 300 ppm. This project will include concentrations of copper as high as 600 ppm in order to determine ultrastructural changes within cells.

Plant growth is directly linked to its nutrition, structural normality, efficiency of enzymatic and photosynthetic mechanisms, protein production, and CO_2 and water availability. Therefore, investigating the effects of copper on photosynthesis should help to elucidate effects on nutritional, enzymatic, and structural functionality.

This research project also investigated the uptake of copper in mesquite and its effect on ultrastructural and protein changes through the use of microscopy. Cotyledons were chosen because they provide nutrients to the germinating seedlings. Determining the effects of copper on the developing stages of *P. pubescens* will help to determine if this plant could be utilized for a desert restoration of an area highly contaminated with heavy metals. Mature screw bean mesquite (one month old) plants were also tested in soil to determine their hyperaccumulation potential.

This project has four key objectives:

1. To determine if *Prosopis pubescens* is a hyperaccumulator of copper when grown in soil.

2. To report on the effects of copper sulfate and copper nitrate on screw bean mesquite seed germination and to identify the copper absorption of the seed coat as well as assess the importance of seed age.

3. To identify ultrastructural changes of cotyledons due to copper toxicity. Measure copper and nutrient concentrations within seedlings. Localize copper within cotyledons and true leaves of screw bean mesquite. Measure changes in amylase, cell death and photosynthetic pigments after copper exposure.

4. To measure copper uptake in plants grown in soil and identify the nutrient changes due to copper uptake. Observe ultrastructural changes of true leaves due to copper toxicity.

Chapter 2: MATERIALS AND METHODS

2.1 Source of Screw Bean Mesquite

Seeds of *Prosopis pubescens* (Screw bean mesquite) were obtained from Granite Seed Company (Lehi, UT). Two ages of seeds (1980 and 2009) were studied for their applicability in phytoremediation. Seeds were scarified with 2% Chlorox for 15 minutes. Seeds were germinated using sterile paper under complete darkness for the first three days followed by five light days to induce germination. Plants were maintained under controlled laboratory conditions at a temperature of about 24 C. For the germination studies, the seeds were germinated within two sterile filter papers in petri dishes under the same dark/light cycle (6:18hrs at 24 C). Nine hundred seeds were used for each concentration to determine percentage germination. For all other studies, germinated seeds were transferred on the ninth day to Petri dishes. Copper solutions (2ml) every two days were given for the next eight days. The copper concentrations used were 0 ppm, 50 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm, 500 ppm, and 600 ppm of either copper sulfate or copper nitrate. Plants, in triplicate, were grown in autoclaved soil for 30 days in a greenhouse at UTEP. Plants were exposed to 0 ppm-600 ppm of copper sulfate or copper nitrate.

2.2 Microscopy

The microscopic techniques used for this research project were light microscopy and transmission electron microscopy (TEM). These two techniques served to visualize any damaging toxic effects of the copper on the cotyledons and true leaves of the screw bean mesquite.

2.2.1 Sample Preparation for Light and Transmission Electron Microscopy Analyses

- Quickly minced specimens on a piece of dental wax in the bottom of a clean glass Petri dish or mincing dish using an acetone-cleaned single-edge razor. The final pieces were about $1mm^3$.
- Transferred the small pieces of specimen to fixation vials using a toothpick or applicator stick. Filled the vials 1/3 full with modified Karnosvky's fixative: 2% paraformaldehyde-2.5% glutaraldehyde in 0.06M HEPES buffer, pH 7.4. Placed the caps on the vials. Labeled vials and their lids with the fixation number and initials.
- Placed the labeled vials in the rotary agitator at room temperature for 1 hour.
- Using a Pasteur pipette, removed the fixative from the specimen vials into the fixative waste jar, in the biohazard hood. Filled the specimen vials ½ full with fresh cold 0.06M HEPES buffer and rinsed three times (15 min each).
- Using a syringe, under the hood, injected only enough 1% OsO_4 – 0.06M potassium ferrocyanide in 0.12M HEPES buffer to completely cover the specimen in the vial.

Replaced caps on vials, which had been labeled with a fixation number and initials. Placed these vials in an ice bucket (dark, 0-4°C) for four hours.

- Wearing disposable gloves, under the hood, removed the osmium tetroxide from the specimen using a clean Pasteur pipette. Pipetted the osmium into the osmium waste jar. Filled the specimen vial ½ full with cold 0.06M HEPES buffer and repeated for three rinses (5 min. each). Removed the rinse with a Pasteur pipette and placed in the osmium waste jar.
- Added enough 2% aqueous uranyl acetate (pH 5.2) to the specimens to cover them. Wrapped the specimen vials in aluminum foil and incubated at room temperature for one hour.
- Pipetted the uranyl acetate into the uranyl acetate waste jar. Rinsed three times with distilled water (5 min. each). Removed the rinse and placed it in the uranyl acetate waste jar.

Dehydration Series (Replaced cap on specimen each time).

Ethanol (time)	Time
30%	5 min
30%	5 min
50%	5 min
50%	5 min
70%	5 min
70%	5 min
90%	5 min
90%	5 min
100%	15 min
100%	15 min

Pipetted ethanol into ethanol waste jar with Pasteur pipette.

Embedding in Poly/Bed 812 Plastic. Wore disposable gloves.

100% acetone	15 min
100% acetone	15 min

Pipetted acetone into acetone waste jar with Pasteur pipette.

Added 70% acetone: 30% Poly/Bed 812 – 24 hrs with mild agitation on rotary agitator.

Pipetted waste plastic and acetone using Pasteur pipette into plastic waste jar.

Added 50% acetone: 50% Poly/Bed 812 – 24 hrs with mild agitation on rotary agitator.

Pipetted waste plastic and acetone using Pasteur pipette into plastic waste jar.

Added 30% acetone: 70% Poly/Bed 812 – 24hrs with mild agitation on rotary agitator.

Pipetted waste plastic and acetone using Pasteur pipette into plastic waste jar.

Added 100% Poly/Bed 812 to specimen vial. Allowed to infiltrate for 1 hr. Returned lid to 100% plastic. Pipetted 100% plastic from specimens to plastic waste jar.

Placed a drop of fresh 100% plastic in the bottom of a BEEM capsule, which had already been labeled with a fixation number. With an applicator stick or a toothpick, transferred the specimens to the BEEM capsules. Added 100% plastic to fill the BEEM capsule, covered with lid, and placed in the embedding tray for 72 hrs in a 60°C oven.

2.2.2 Sample Processing for Light Microscopy Analyses

Trapezoids were made on the BEEM capsule after locating the embedded sample. A total of 150 BEEM capsules were processed for cotyledons and true leaves. Large trapezoids (2mm by 2mm) were prepared for histology samples, while smaller trapezoids of 0.5mm by 1mm were made for sectioning thin sections.

Thick sections (1 μm) using a Leica Ultracut microtome (Leica Microsystems, Bannockburn, IL) (Fig. 7) were stained with Toluidine Blue and Basic Fuschin to identify any

structural changes. Other stains were also used to screen for changes in starch, lipid, and protein bodies. Slides were analyzed using a Zeiss Axioskop fluorescent microscope. Photographs were obtained using an AxioCam digital camera and quantified using Zeiss Rel 4.5 Software (Carl Zeiss Inc, Peabody, MA) (Fig. 8). About 200 photographs were taken to assess what areas to select for electron micrographs. The area of cotyledon parenchymal cells was compared between the control and the experimental to determine if toxicological effects could be observed at the histological level. Two hundred seventy images were used to compare area changes.

Thick Section Staining Protocol

1. While on a hot plate, added just enough Toluidine Blue O staining solution to cover the sections.
2. When the edge of the stain droplet just began to turn metallic gold color (15 – 30 seconds), removed the slide from the hot plate and held at an angle over the sink. Directed a stream of distilled water from a squeeze bottle just above the stain drop so that the stain was quickly and completely washed from the slide.
3. Wiped excess water from the bottom of the slide and dried slide again on the hot plate.
4. Placed a drop of Fuchsin stock and Sodium Borate stock mixture on the sections and heated briefly, then rinsed with a jet of distilled water and dried.

Figure 7. Leica Ultracut R Ultra Microtome

Figure 8. Carl Zeiss Axioskop Light Microscope

2.2.3 Sample Processing for Transmission Electron Microscopy Analyses

Thin sections (60-90 nm) obtained with a Leica Ultracut microtome (Leica Microsystems, Bannockburn, IL) were post stained with uranyl acetate (6 min, pH 3.7) followed by Reynolds Lead Citrate (6 min, pH 12) (Reynolds, 1963). Grids were examined and photographed in a Zeiss EM-10 transmission electron microscope at an accelerating voltage of 60 or 80 kV (Fig. 9). Photographs were acquired with Kodak SO-163 film and digitized with an Epson Perfection V700 photo scanner. Later images were collected digitally with the ES1000W Gatan camera model 785 (Warrendale, PA). A total of 650 electron micrographs were collected for analysis for both the cotyledon and true leaves studies.

Thin Section Staining Protocol

1. Using jewelers forceps, carefully inserted grids with sections into a staining pad.

2. Placed a piece of filter paper inside a 10 cm Petri dish and moistened the filter paper with distilled water. Placed the cover on the dish and then placed the dish on the heated hot plate until condensation formed (about 1 minute) on the inside cover.
3. Took the staining pad to the hood and placed the staining pad, with grids in place, inside the Petri dish. Used a syringe to draw out a small amount of uranyl acetate, and then attached a micro filter to the syringe. Placed 1 – 3 drops of uranyl acetate on each grid so that both sides of the grid were covered with uranyl acetate. Replaced the cover and returned the dish to the hot plate for 6 minutes. Since uranyl acetate is light sensitive the dish was covered with a box.
4. Took the dish to the hood, uncovered it and allowed it to cool for 3 minutes.
5. Held the pad vertically over a 250 ml beaker so that the grids were also vertical. Using a plastic squeeze bottle, gently allowed a stream of double-distilled water to flow on to the pad and down over the grids into the beaker for 1 minute (Allowing the water to flow too quickly will cause the sections to be washed away). Used a wedge of filter paper to wick the base of each grid.
6. Returned the staining pad to the Petri dish. Placed 1 – 3 drops of Reynold's Lead Citrate onto each grid. Wiped the condensation from the top of the Petri dish and replaced the cover for 6 minutes.
7. Held the pad vertically over a beaker so that the grids were also vertical. Gently allowed a stream of double-distilled water to flow onto the pad and down over the grids into the beaker for 1 minute (Allowing the water to flow too quickly will cause the sections to be washed away). Used a wedge of filter paper to wick the base of each grid.

8. Carefully removed each grid from the staining pad with a pair of jewelers forceps. Wicked the forceps with a wedge of filter paper, blotted the shiny side of the grid on a clean piece of filter paper and placed the grid into the grid box, with the sections on the dull side of the grid.

Figure 9. Carl Zeiss EM-10 Transmission Electron Microscope

2.3 Elemental Analysis from Scanning Electron Microscopy

Copper was localized in the block face of each BEEM capsule using a Hitachi S-3400N (Fig. 10) (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 EDAX software on the spot scan setting for 46 BEEM capsules. Spot scans of cell wall, vacuole space for both parenchymal and epidermal cells were collected of cotyledons and leaves, totaling 230 spot scans.

Figure 10. Hitachi S-3400N SEM with Thermo EDAX Software

2.4 Root Length and Biomass Change

Root length (cm) and weight (g) of each seedling were measured before and after copper exposure. A total of 1000 seedlings were measured to determine the effect of copper on the root length and biomass. Copper exposure of seedlings was for 8 days.

2.5 Soil Preparation

Soil was tested for its water saturation capabilities. Each pot was weighed, 500 grams of soil was added to each pot. Drops of water were added until the soil was saturated and then the pot was weighed. This determined how much water should be administered to the pots of the greenhouse experiments. Soil was mixed with various concentrations of copper (0 ppm-600 ppm) a month prior to planting screw bean mesquite seeds. Every third day, pots were watered with 100 ml of distilled water. Screw bean mesquite seeds were scarified with 15 minutes of 2% Chlorox, as they were previously scarified for laboratory grown seedlings. 150 seeds were planted in each pot of each concentration (triplicates) and harvested 18 days later for ultra-structural, photosynthesis, and copper content analysis.

2.6 Total Protein Survey

Plants were harvested after 16 days of growth, 8 days of which were under copper exposure. One and a half grams of the whole plant was harvested for the protein extraction. Triplicates of each copper treatment were prepared for a total of 150 samples. Reagent A, B, and C were prepared from the Pierce P-PER plant extraction kit (VWR Cat#89803). Reagent A was a preservative-free HEPES buffer at pH 7.0. Reagent B was a protein stabilizer and reagent C was an organic extraction solution. Fresh plant tissue was placed in the mesh screens provided in the kit. The P-PER working solution was added according to the weight of fresh plant sample. For example, for 80 mg of plant tissue, 1.75 ml of the reagent solution was used. The mesh screen with the fresh sample was homogenized in the working solution. The lysate was withdrawn and centrifuged to partition the organic and aqueous layers. The protein extract was recovered from the lower, aqueous layer of the partition.

Serial dilutions of albumin and Bradford reagent were used as protein standards. Samples were read in a spectrometer (Du 640, Beckman Coulter, Brea, CA). Visible mode at 595 nm was used. Bradford reagent was added to the plant extract and incubated for 1 minute before reading.

2.7 Inductively Coupled Plasma (ICP-OES) Spectrometer

Plants were exposed to copper exposure for 8 days within laboratory Petri dishes which emulated miniature green houses. Roots and stems were placed between two sterile filter papers which were then soaked with the corresponding copper solutions. Plants were 8 days old at the start of the experiments and 16 days old at the end of the experiments. At 16 days, plant samples were washed with 3 ml of 0.01M hydrochloric acid (HCl) to remove any external debris. The

plant samples were separated into root, stem, cotyledon, and leaf tissues and dried for 80 hours at 90° C. After weighing the dried samples, 600 samples were digested with 67-70% pure nitric acid (HNO_3) using a microwave (Fig. 11) (CEM Marsx, Matthews, NC). The EPA method used for the digestion was EPA #3051. Samples were read using an ICP-OES spectrometer (Fig. 12) (Perkin Elmer 4300 DV, Waltham, MA) (Chemistry Department) within two weeks of digestion. Samples read were from cotyledon, stem, root, seed, and seed coat, separately. This multi-metal analysis read for copper, as well as, micro nutrients (boron, iron, zinc, manganese, and molybdenium); and macro nutrients (calcium, potassium, sulfur, phosphorus, and magnesium). One blank (5% nitric acid) and six spiked (1 ppm solution of each metal of interest) were prepared.

Figure 11. CEM Marsx Microwave Digestion System

Figure 12. Perkin Elmer Optical Emission Spectrometer (Optima 4300 DV)

2.8 Spectrophotometer Readings

2.8.1 Amylase analysis

Fresh seedlings were weighed (0.07-0.1 g of tissue) and the corresponding volume of 2 mN imidazole buffer pH 7.0 was added to prepare a 10% w/v extract which was ground using a mortar and pestle. Samples were centrifuged for 15 min at 14000 rpm at -4C and 700 µl of 1% starch (in 2 mM imidazole buffer pH 7.0) was added to a 2 ml Eppendorf tube. The iodine reagent (0.0075% Iodine and 0.075% KI) was prepared. Four hundred µl of the enzyme extract was added to the Eppendorf tube containing the 700 µl 1% starch solution. One hundred and fifty µl of the aliquot from the reaction mixture was added to 200 µl of cold trichloroacetic acid (TCA) at time periods 0, 20, 40, 60 and 80 min. The 90 samples were centrifuged for 5 min at 14000 rpm RT and a microplate containing 300 µl of the Iodine reagent was prepared. Thirty

liters from each stopped reaction (TCA tubes) was added to the microplate well containing the Iodine reagent.

A calibration curve was prepared in a microplate using the following standards:

	Iodine reagent (µl)	1% starch (µl)	Imidazole buffer (µl)
Std 1	300	0	30
Std 2	300	2	28
Std 3	300	4	26
Std 4	300	6	24
Std 5	300	8	22
Std 6	300	10	20

Color developed and samples were read at 660 nm in a spectrometer (Fig. 13) (Cary 50 spec UV-Visible, Varian) (Chemistry Department). The enzyme activity was calculated using the calibration curve slope and the sample slopes, as well as, the dilution factors and biomass weight.

Figure 13. Varian Cary 50 Spec UV-Visible Spectrophotometer

2.8.2 Cell death analysis

Cell death was quantified by the Evans blue staining method (Hung, M.C. *et al.*, 2007). This method was initially taken from Baker and Mock (1994). Roots were harvested at various time points (20 and 180 minutes) from various copper concentrations. The triplicate root samples were stained in 0.25% aqueous Evans blue solution for 15 min at room temperature, and then washed twice for 15 min with dH_2O overnight. Dye was extracted from a solution of 50% methanol/1% SDS for 1h at 50ºC and its subsequent quantification by monitoring the A_{595}.

2.8.3 Chlorophyll analyses in cotyledons

Concentrations of chlorophylls and carotenoids were determined in screw bean mesquite cotyledons. Cotyledons (1-2.5 g) were ground up with 5 g of sand, 2.5 g magnesium sulfate, and 12 ml of distilled water. This mixture was centrifuged at 5,000 rpm for 5 min. The supernatant was combined with 8ml of 100 % acetone and left to settle for 10 min. The extraction was centrifuged at 5,000 rpm for 2 min. The supernatant was read at 663 nm and 644 nm for chlorophyll a, chlorophyll b; 436 nm for β-carotene; and 474 nm for xanthophylls in a spectrometer (Cary 50 spec UV-Visible, Varian, Inc., Palo Alto, CA). The following equations were used to determine the concentration of the pigments:

$$C_a = (12.63*OD_{663})-(2.52*OD_{644})$$

$$C_b = (20.47*OD_{644})-(4.73*OD_{663})$$

$$C_c = (OD_{436}*454*(0.46/OD_{436}))/196$$

$$C_x = (OD_{474}*454*(0.561/OD_{474}))/236$$

All concentrations were normalized by sample weight (g) and expressed in mg/ml.

2.9 Statistical Analyses

All statistical data was processed by Julia Bader, Ph.D., Statistician, at the Statistical Consulting Laboratory, UTEP for analysis. All tests were conducted with the 0.05 level of significance, using SAS Version 9.2 software.

2.9.1 Analyses for Germination:

Statistical analyses included both the 2-factor and 3-factor factorial with the Analysis of Variance (ANOVA) and General Linear Mixed Models (GLMM) for repeated measures. If a significant factor effect or interaction was found, it was then followed by a post-hoc procedure such as Tukey's test and simple effect comparisons.

2.9.2 Analyses for Soil Experiment:

Statistical analyses included the General Linear Model and General Linear Mixed Models (GLMM), using SAS Version 9.2 software. If a significant factor or interaction was found with the GLMM, it was followed by a Tukey's test post-hoc procedure.

2.9.3 Analyses for Ultrastructure:

General Linear Model (GLM) and General Linear Mixed Models (GLMM) used. 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.

Chapter 3: RESULTS AND DISCUSSION

LABORATORY GROWN SCREW BEAN MESQUITE SEEDLINGS

Below are images (Fig. 14) of the laboratory design for the studies done with screw bean mesquite seedlings. In this chapter, we will first present the laboratory grown seedlings separated into copper sulfate and then copper nitrate. The second section will present studies done in more mature screw bean plants grown in soil. Each section will end with a comparative discussion of the results.

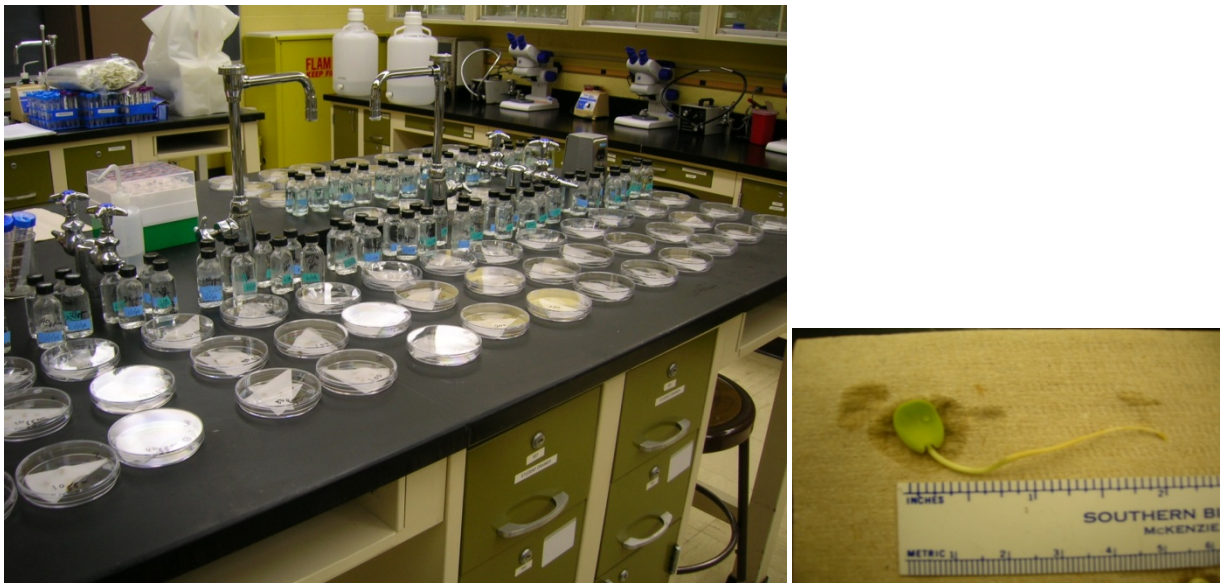


Figure 14: Picture of laboratory grown screw bean mesquite. Petri dishes used to emulate "miniature greenhouses". Subset picture of a seedling's root being measured.

3.1 Germination and Seed Viability

We documented that the age of seeds plays a role in germination by investigating the effects of copper on seeds collected in two different years (1980 vs. 2009). It is important to determine the adequate seed age for use in phytoremediation. *Prosopis* seeds may be viable for

up to 50 years (Glendening and Paulsen, 1955). Freshly-harvested *Prosopis* seeds were found to have a ninety percent germination rate (Ffolliot and Thames, 1983) because freshly-harvested seed coats had not hardened. However, old seed coats degraded over time and could germinate without scarification treatments (Pasiecznik and Felker, 1992) which are usually necessary to break the seed's dormancy and to increase water permeability (Crocker and Barton, 1953). We hypothesized that *Prosopis pubescens* seeds, collected in prior years, may still be used to remediate low contaminated soil or top soils. We chose seeds collected from 1980 (stored seeds) to test how older seeds responded to copper toxicity. Figure 15 shows the germination effects of copper sulfate and copper nitrate on stored and recently harvested seeds after three days. Figure 16 shows the germination percentages of over 300 scarified seeds screened for the germination of seeds collected in 1980 and 2009.

A

Control	50ppm	100ppm	200ppm	300ppm	400ppm	500ppm	600ppm

B

Control	50ppm	100ppm	200ppm	300ppm	400ppm	500ppm	600ppm

Figure 15. Copper sulfate effects on germinated seeds collected in 1980 (A) and in 2009 (B).

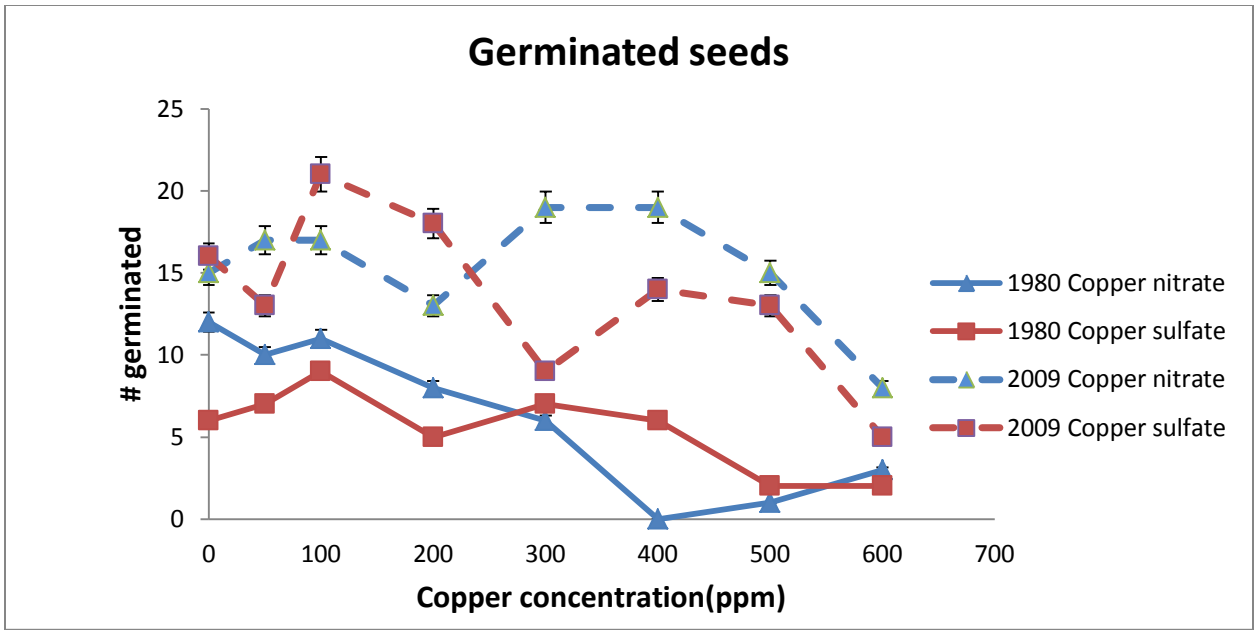


Figure 16. Germination after 8-day copper sulfate (■) and copper nitrate (▲) exposure in seeds collected in 1980 (solid line) and 2009 (dashed line).

The overall germination pattern for 1980 seeds exposed to copper sulfate showed no significant effect ($p > 0.05$). However, for copper nitrate, we observed significant decreases in germination rates with an increase in copper exposure ($p = 0.029$) (Fig. 16). At 400 ppm of copper nitrate, there was no germination due to the high levels of copper toxicity. However, after 400 ppm we observed a recovery of almost 20 % germination. This may be explained by the degradation of the seed coat of 1980 seeds and may not be reflective of copper toxicity.

The overall pattern in the 2009 seeds showed that both copper sulfate and copper nitrate decreased germination percentage ($p<0.0001$ and $p=0.0001$, respectively) (Fig. 16). Seeds collected in 2009 showed a very slow decrease in germination. Fresh seeds have softer seed coats so they are expected to be more sensitive to copper absorption and its toxicity. However, in these experiments we found that the older seeds (1980) were the most affected by copper nitrate.

It was determined that significant differences occurred between copper sulfate and copper nitrate as copper increased across both ages of seeds ($p=0.0454$ with the 3-way interaction), showing that there is no similar trend to compare the effects of copper types.

The viability of seeds must be determined before selection for phytoremediation. Previous viability studies performed on *Prosopis* species have shown that seeds can remain dormant for 50 years (Ffolliot *et al*., 1983). In this project, we chose screw bean mesquite seeds that were 30 years apart to test the effects of copper on germination and on the physiology of seedlings. Our results showed that screw bean mesquite seeds are viable even after 30 years of storage. Germination rates in the laboratory were 9% germination rate of 2009 seeds and 4% germination rate of 1980 seeds under control settings.

We concluded that *P. pubescens* has a similar germination success to honey mesquite. Honey mesquite produces a large amount of seeds; however, only a small percentage produce mature trees (Scifres *et al.*, 1971). Honey mesquite seeds germinated within two days of planting in the green house and their germination was not photoperiod dependent (Scifres *et al.*, 1969). Temperature and soil depth were factors related to success of germination. The seeds have a hard, impermeable seed coat, and so scarification is required for the germination of honey mesquite and it is usually accomplished by their passage through the digestive systems of animals (Fischer *et al.*, 1959). Soil covering is not necessary for germination but it is necessary for the establishment and survival of seedlings (Vilela, *et al*., 2001). Honey mesquite seedlings were most successful when planted 0.5cm deep at a soil temperature of 27C (Scifres *et al*., 1972).

In our project, both copper sulfate and copper nitrate have decreased the germination rate of the younger screw bean mesquite seeds; however, all seeds were more susceptible to copper

nitrate toxicity. There are no specific mechanisms to explain the reduction of germination rate or biomass in any *Prosopis* species. Generally, germination rates on both, old and new seeds are dose-dependent. We concluded that screw bean mesquite seeds can be used as the starting material to remediate copper pollution.

Victor *et al*. (2007) studied the adverse effects of copper on seed germination in *Prosopis juliflora*. They tested copper nitrate from 10-1280 ppm in the first two to eight days of germination. Like our methodology, seeds were continuously exposed to copper and showed similar germination patterns among ranges of concentration. The lower concentration group (10-160ppm) was significantly different in their germination percentage to the higher concentration groups (320-1280ppm). With additional days of copper exposure, the percentage of germination of copper-exposed groups was negatively affected.

To determine if copper was internalized into the embryo, we analyzed copper content in the separated coat (Fig. 17). Figure 17 showed that the increase of copper exposure resulted in the increase in copper accumulation in the seed coat of screw bean mesquite ($p<0.0001$), for both copper sulfate and copper nitrate. For example, an average of 10,000 ppm of copper in the scarified coats of new seeds was accumulated after an 8 day exposure with 600 ppm copper sulfate. Figure 18 also showed that an increase of copper exposure resulted in no accumulation of copper in the seed embryo of screw bean mesquite ($p > 0.05$ for both copper nitrate and copper sulfate).

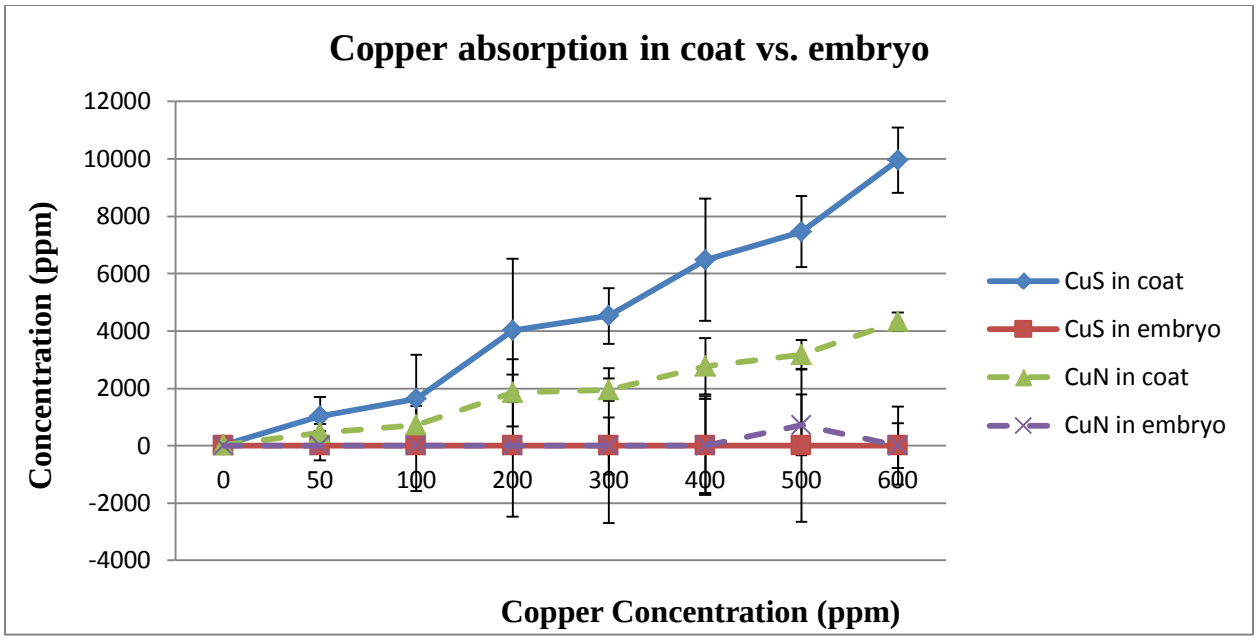


Figure 17. Copper absorption in seed coat vs. embryo of screw bean mesquite

In comparison, copper nitrate effects on seed coat followed a different pattern compared to copper sulfate. There was an average of 4,000 ppm of copper accumulated in the coat after a 600 ppm exposure of copper nitrate, while copper sulfate accumulation in the coat with an average of 10,000 ppm, showing that copper sulfate is absorbed more readily by the seeds.

We concluded that the coat-imposed dormancy may be the first step in defense against metal contamination in screw bean mesquite. We tested the levels of copper absorbed by the seed coat, embryo and whole seed using ICP-OES and recorded the germination rates of scarified seeds exposed to copper. There was a greater copper sulfate accumulation in the coat alone than in the seed, which suggests that the seed coat is the primary protection against metal toxicity of the embryo.

Copper exposure resulted in the increase in copper accumulation in only the seed coat of screw bean mesquite, for both copper sulfate and copper nitrate. These results suggested that seed coats of *P. pubescens* are the first level of protection against copper toxicity and new seeds

are preferable to increase biomass for copper remediation. Previous studies done by Ahsan *et al*. (2007) in the accumulation of copper in rice seeds showed a similar pattern to our results: increasing copper with increasing copper accumulation. Ahsan *et al*. (2007) did not test copper concentrations within coat and embryo separately; however, their results suggested that excess copper could be changing the water absorption of seeds. Kim *et al*. (2005) have measured water content in stress-treated plants showing a change in osmotic processes. No studies have been done in the water absorption and copper in screw bean mesquite.

3.2 Copper Absorption in Screw Bean Mesquite Seedlings

Inductively coupled plasma spectroscopy has been used to identify the amount of copper collected by screw bean mesquite seedlings. We have quantified the amount of copper for roots, stems and cotyledons. We observed a normal distribution in the plant which is very high in the roots and diminished as it progressed through the stem and cotyledons. In Figure 18, we show that increasing amounts of copper exposure to the seedling has dramatically increased the accumulation of copper in the roots. There is a grouping between 0-200 ppm and another from 300-600 ppm of copper sulfate. Lower amounts of copper accumulated in the stems and cotyledons. In 600 ppm of copper sulfate treatment, there was a 47,000 ppm, 23,000 ppm, and 9,000 ppm accumulation, on average, in roots, stems, and cotyledons, respectively.

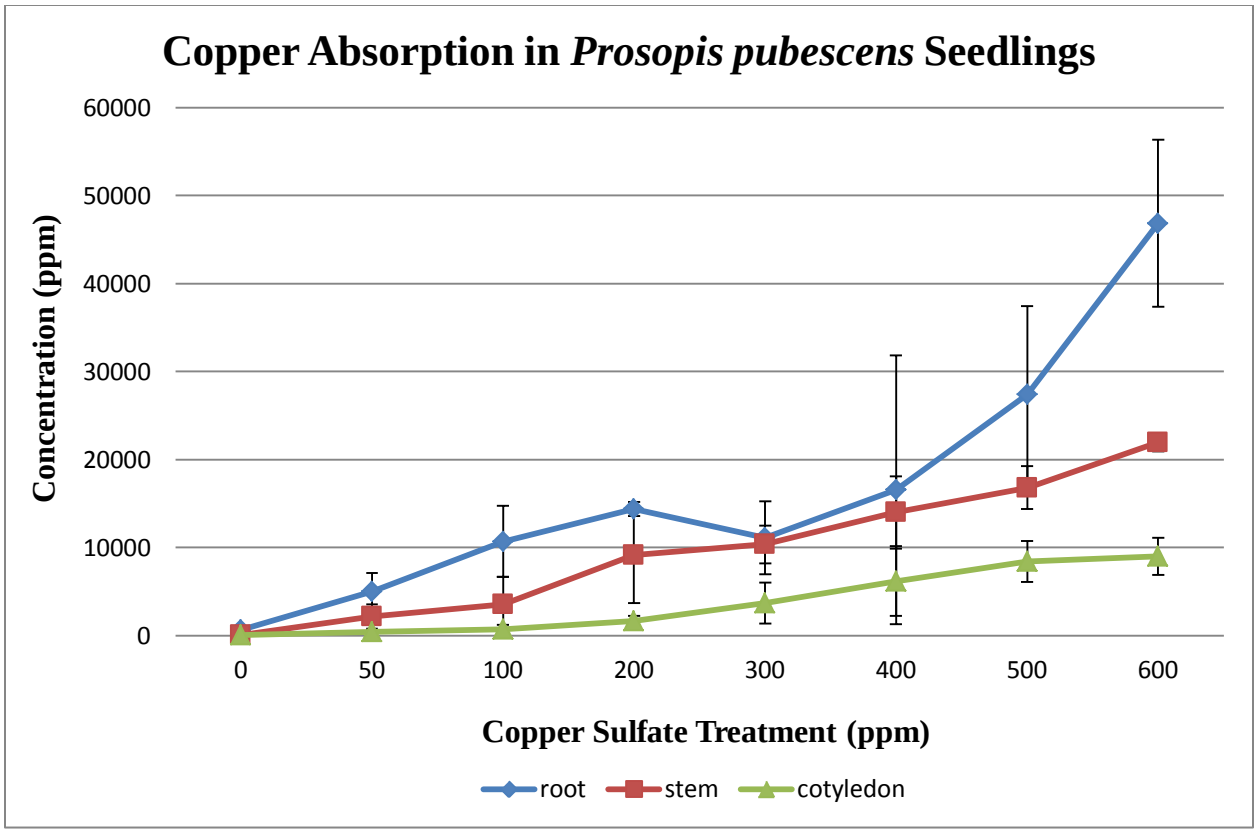


Figure 18. Copper absorption from copper sulfate comparison within screw bean mesquite seedlings.

Copper sulfate absorption across concentrations was different for the root, stem and cotyledon ($p<0.0001$). The root showed that the control is significantly different from the rest of the copper treatment. A relatively linear correlation ($R^2=0.8077$) can be seen in the root. Copper sulfate absorption in stems shows statistical differences across concentrations of exposure ($p<0.0001$). A relatively linear correlation ($R^2=0.9802$) can be seen in the stem. Copper sulfate absorption in cotyledons shows statistical differences across concentrations of exposure ($p=0.0214$). A relatively linear correlation ($R^2=0.9322$) can be seen in the cotyledons. There is more copper accumulation in the roots, then the stems and finally the lowest accumulation in the cotyledons. At 300 ppm of copper sulfate, we noted that both root and stem copper

accumulations are the same. The linear correlations between copper exposure and copper absorptions are indicative of an active transport mechanism with gradient limitations rather than a simple diffusion uptake.

We also quantified the amount of copper nitrate in root, stem and cotyledons. We observed a normal copper distribution in the plants which was low in the lower copper concentration and increased with increasing copper exposure (Fig. 19). Copper nitrate absorption on all parts of the plant shows statistical differences across concentrations of exposure ($p=0.0004$). The control roots, stems, and cotyledons did not have any copper. There was a linear increase in all plant parts ($R^2=0.7288$, $R^2=0.8833$, $R^2=0.8806$). From 50-400 ppm of copper, accumulation of copper was greater in roots, stems and cotyledons, in descending order. However, after 400 ppm, copper accumulated in stems, cotyledons and roots, in descending order. At 600 ppm of copper nitrate, roots absorbed 27,500 ppm, stems absorbed 21,000 ppm, and cotyledons absorbed 16,000 ppm.

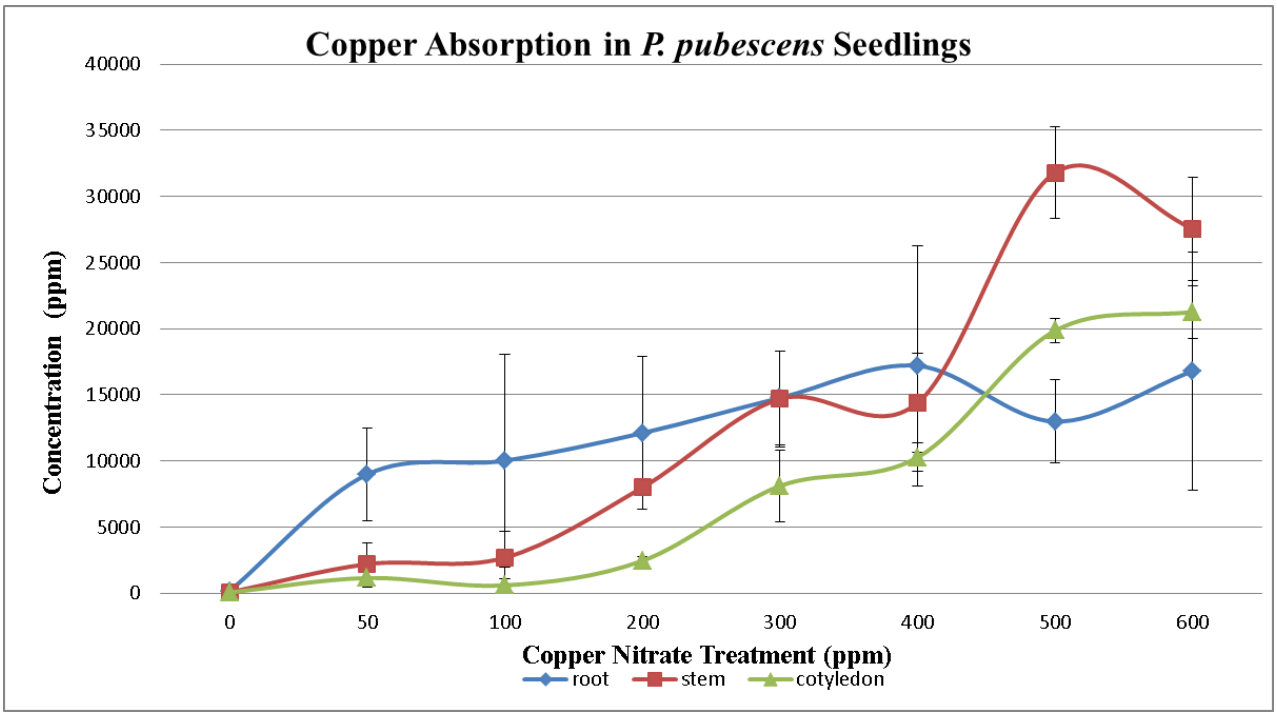


Figure 19. Copper absorption from copper nitrate comparison within screw bean mesquite.

These results show the limit of uptake by these 16 day old seedlings in the root by the leveling off pattern. Previous studies have shown that heavy metal uptake is related to the biomass of a plant (Moynier *et al*., 2009). Overall, there was a consistent rate of copper increase of the seedlings when the control and the highest copper concentration (600 ppm) were compared, showing that copper uptake is mainly dependent on copper availability. There does not appear to be a limiting factor to copper uptake or transport in screw bean mesquite. These results demonstrate that the uptake of copper by *Prosopis pubescens* seedlings was influenced by the amount of available copper provided.

3.3 Root Length and Biomass Changes

Chlorosis was observed in the majority of the seedlings after 200 ppm of copper. Increased copper concentrations increased structural damage of the root and stem, which were more susceptible to fungal growth. Measurements of the initial root length showed variance of growth among the seedlings, and final root lengths were measured for each seedling in a set of nine per group. There was no measureable difference among the different concentrations, therefore, we measured the biomass of seedlings and how it was affected by copper exposure (Fig. 20).

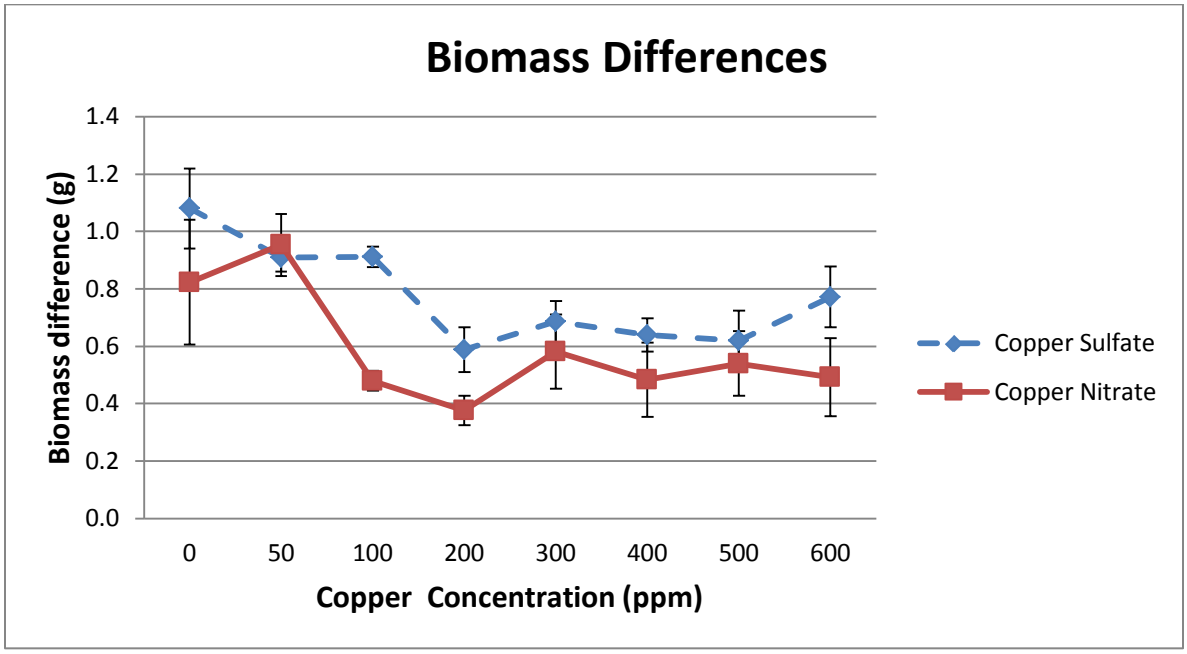


Figure 20. Biomass changes in 2009 seeds due to copper exposure.

Initial biomass (in grams) of 648 seedlings was measured. After eight days of copper exposure, the final biomass of each seedling was obtained. Statistical significance of the copper type by concentration interaction ($p = 0.001$) showed the different effects of copper nitrate and

copper sulfate on the growth of screw bean mesquite ($p<0.0001$). In addition, seedlings from new seeds were more sensitive to the effects of both copper compounds (Fig. 21).

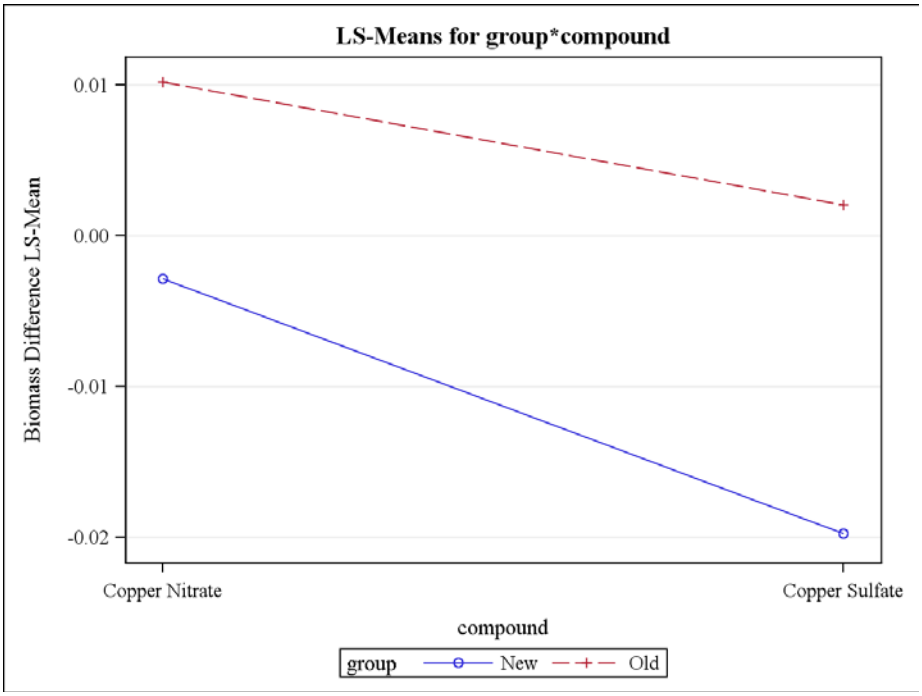


Figure 21. Mean biomass difference between copper nitrate and copper sulfate for old and new seeds.

Our results align with previous studies on low concentration of copper on alfalfa, showing that copper was beneficial to root growth up to 156% (Peralta *et al.*, 2000). At 20-40 ppm of copper, alfalfa demonstrated a concentration-dependent inhibition of root growth (Peralta *et al.*, 2000). In our study we expected the root length to follow the same pattern; however, the seedlings were exposed to copper for shorter periods. Copper nitrate stunted growth in *P. juliflora* seedlings exposed to 80-1280 ppm (Victor *et al.*, 2007) for eight days with a non-linear relationship between the increase of copper and the height of seedlings. In our results, seedling height did not show any significant difference after an eight day exposure. Unlike Victor *et al.* (2007) our seedling experiments started with eight day old seedlings and we tested the copper

effect on healthy eight day old seedlings. No height was stunted at any copper concentration; no chlorosis was seen in the higher copper concentrations (>300 ppm). This suggests that healthy, established seedlings were very tolerant to copper and may be used to phyto remediate highly contaminated fields. We concluded that seedlings may have a tolerance mechanism for the first eight days for copper contamination up to 300 ppm. It would be interesting to test how long healthy seedlings can tolerate copper without outward signs of toxicity.

Chatterjee *et al.* (2000) studied the effects of copper in three month old cauliflower. He found that two month old cauliflower exposed to copper showed signs of copper exposure after only 10-12 days. Cauliflower's growth was affected by its decreased biomass. There was no mention of stunted root length. Our results are comparable to Chatterjee *et al.*'s study (2000), which concluded that the visible, structural symptoms of copper toxicity were very similar to those of iron deficiency. Daniels *et al*. (1972) showed that copper displaced iron in *P. vulgaris*. Iron and copper exchange in *P. pubescens* may occur after an eight day exposure when the threshold of copper has been reached. We conclude that an eight day copper exposure did not adversely affect screw bean mesquite seedlings exposed to less than 300 ppm of copper.

3.4 Chlorophyll Changes in Cotyledons

All four pigments showed no statistical differences when exposed to increasing copper sulfate concentrations (Fig. 22). Chlorophyll a, b, β -carotene and xanthophyll overall did not change with different copper treatments (p=0.8919, 0.9876, 0.9923, 0.9923, respectively). The control sample had a sum of 14.5 mg/ml of chlorophyll a and b while the 600 ppm copper sulfate sample had increased to 18.5 mg/ml. The control sample had a sum of 15 mg/ml of β -carotene and xanthophyll while the 600 ppm copper sulfate sample had increased to 16 mg/ml. The larger concentration of chlorophylls would not unmask the yellow coloration of the carotenoids to create chlorosis. Chlorosis did not occur due to changes in pigments in screw bean mesquite cotyledons.

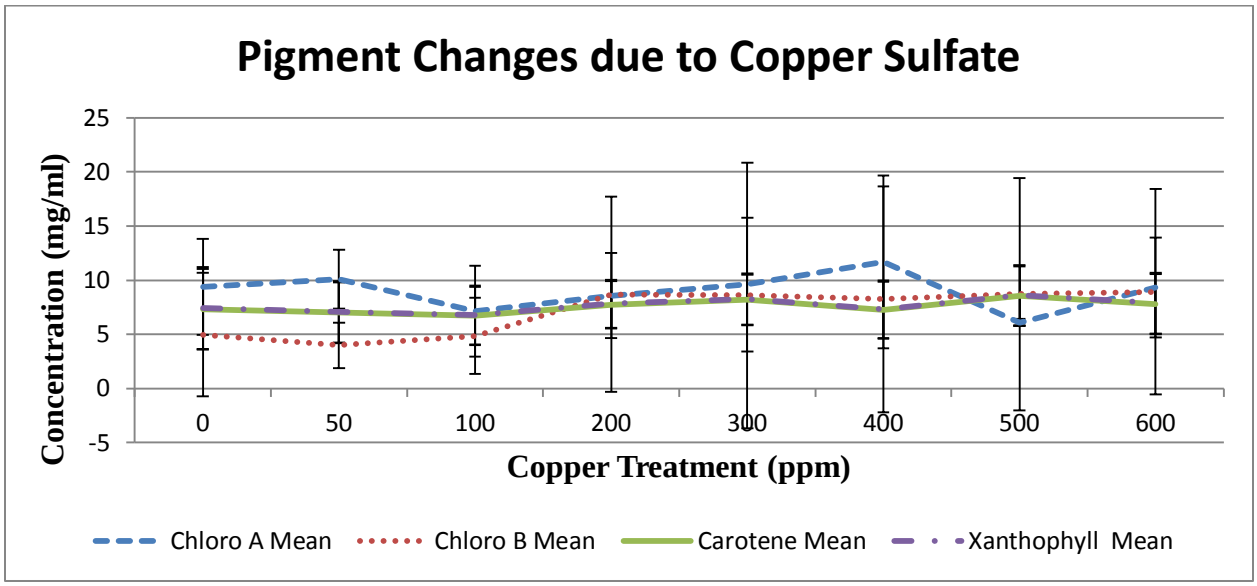


Figure 22. Photosynthetic pigment comparison after copper sulfate exposure.

Chlorophyll b, β-carotene, and xanthophyll did not show any statistical difference when exposed to increasing copper nitrate concentrations ($p > 0.95$) (Fig. 23). However, chlorophyll a

decreased with increasing copper nitrate concentration (p=0.0029). Control cotyledons had 10.5 mg of chlorophyll a while cotyledons exposed to 600 ppm of copper nitrate had 3 mg of chlorophyll a.

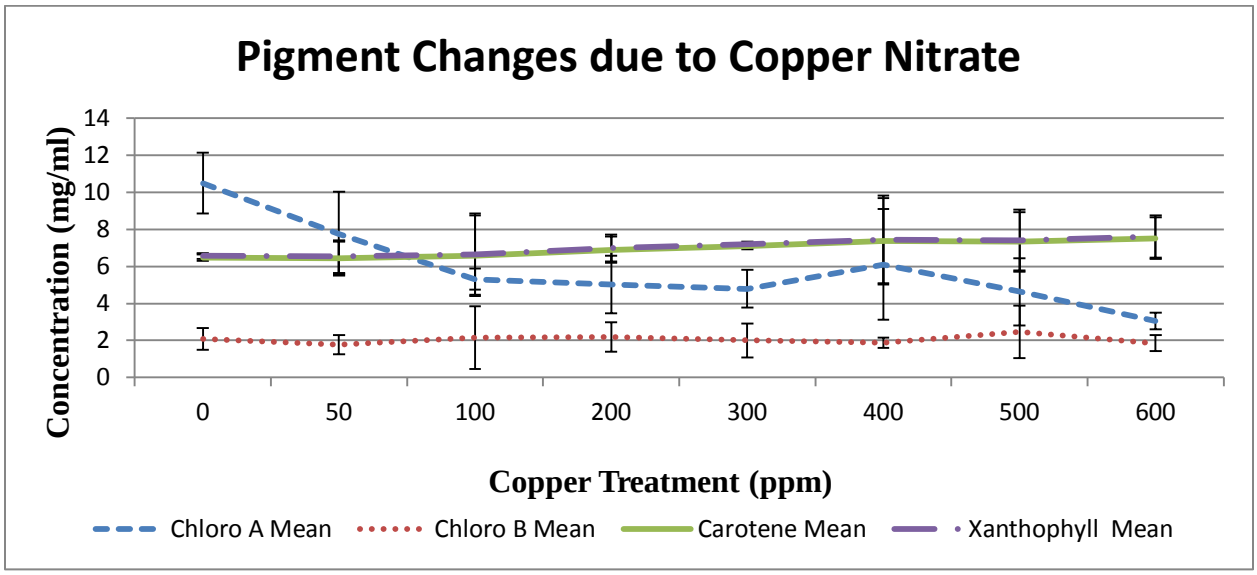


Figure 23. Photosynthetic pigment comparison after copper nitrate exposure.

Chlorophyll a and b absorb red light while carotene and xanthophylls absorb blue light. Chlorophyll a and b will appear green while the carotene and xanthophylls are yellow/orange in appearance. Our results showed no changes in pigment concentrations as copper sulfate exposure increased. We concluded that in copper nitrate samples, chlorosis was caused by the decrease of chlorophyll a which unmasks the yellow/orange pigments from carotene and xanthophyll.

3.5 Total Protein

Statistical analysis showed different effects of copper nitrate and copper sulfate on total protein (Fig. 24) (p=0.0002). Only copper sulfate exposure significantly decreased total protein content ($p<0.0001$), while there was no significant effect of copper nitrate exposure ($p > 0.05$).

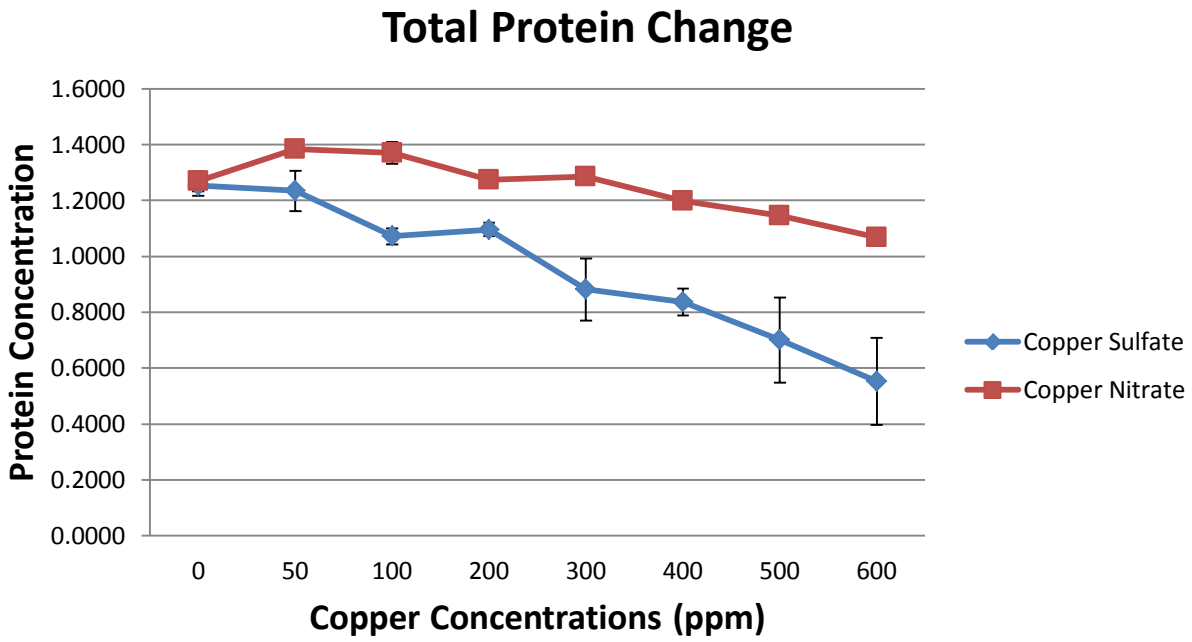


Figure 24. Effects of total protein content in seedlings due to copper.

There is no data on total protein on screw bean mesquite and no studies on how copper affects protein. Statistical analysis showed a linear decrease of total protein with an increase of copper exposure. Total protein is modified and degraded after heavy metal exposure in other species (Davies *et al.*, 1987). The decrease of total proteins may be related to copper effects on the structural integrity of screw bean mesquite seedlings, if it follows the same pattern as that observed by Davies *et al.* (1987). Other important proteins affected may be metabolic and feedback mechanism proteins. Copper sulfate caused negligible effects on the amount of total protein. We concluded that screw bean mesquite may be prepared for copper sulfate toxicity but not copper nitrate. When exposed to copper nitrate screw bean mesquite proteins were degraded.

Gupta *et al.* (2006) studied the levels of total proteins in chickpea after exposure to fly-ash amendments highly contaminated with various heavy metals, including copper, and they identified the decrease of total protein as a result of heavy metal stress, which induced

modification and degradation of proteins. Similarly, Ahsan *et al*. (2007) identified up-regulated proteins after copper exposure which were identified as stress-related proteins such as aldose reductase. Our project also determined that copper sulfate exposure slightly increased total protein content in screw bean mesquite seedlings at copper concentrations of 50 and 100 ppm which may indicate an up-regulation of stress-related proteins.

3.6 Cell Death in Roots after Copper Sulfate Exposure

The time dependent analysis shown on the figure below (Fig. 25) shows that longer exposure of copper increases cell death. Quantitative assessment of screw bean mesquite root showed cell death after 10 minutes of exposure ($p<0.0001$). There was a step-wise increase of cell death as roots were exposed to longer periods of 600 ppm copper sulfate, with the same trend for the control ($p=0.0115$). Roots exposed to 600 ppm 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 immediate response to copper toxicity followed by a leveling off of cell death beginning at 10 min suggesting an activation of tolerance mechanisms.

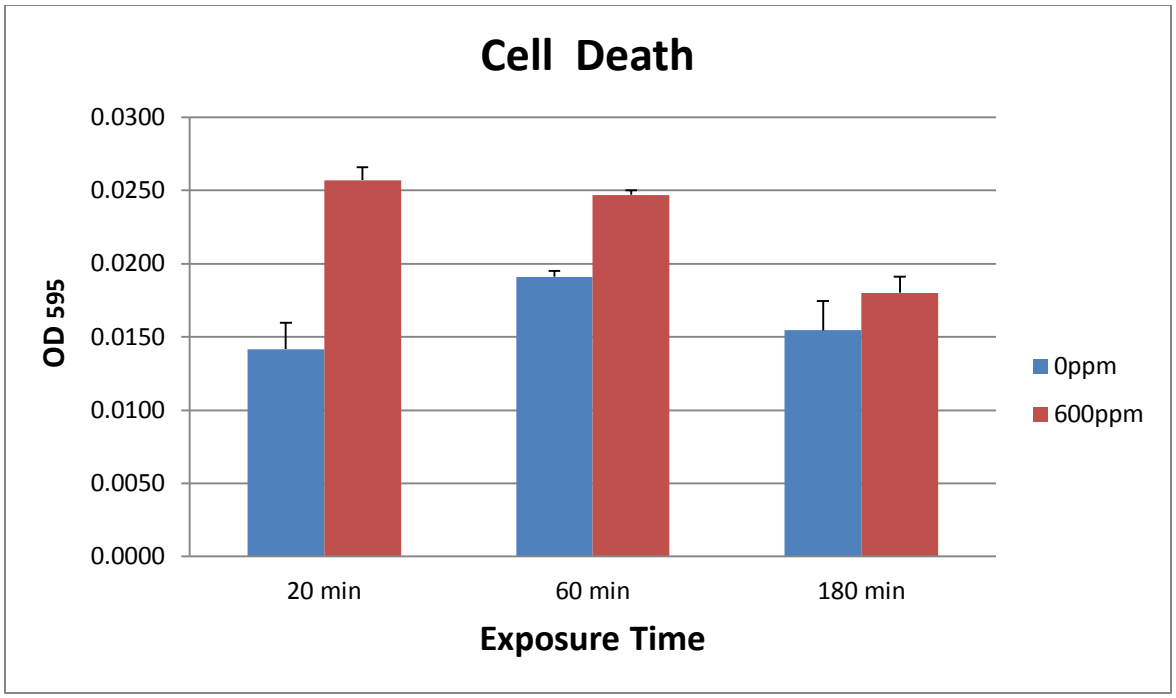


Figure 25. Cell death time-dependent analysis after copper exposure.

Evidence of copper toxicity within the screw bean mesquite seedlings was determined by quantifying cell death in the roots. This method did not differentiate between necrosis and programmed cell death (PCD); however, it did show that there was 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. The objective of their study was to identify a direct link between phosphorylation of tyrosine and copper-induced cell death. They began with a quantitative assessment of cell death using the Evans blue staining method (Baker and Mock, 1994). Evans blue is incorporated in damaged membranes after only 20 minutes of copper exposure (Hung *et al.*, 2007). In our studies, quantitative assessment of screw bean mesquite root showed cell death after 10 minutes of copper sulfate exposure ($p<0.0001$). There was a step-wise increase of cell

death as roots were exposed to longer periods of 600 ppm copper sulfate ($p=0.0115$). Roots exposed to 600 ppm of copper sulfate had 72% more dead cells in comparison to the control with a 10 min copper exposure ($p<0.0001$). The results indicated a quick response to copper toxicity followed by a leveling off of cell death beginning at 10 min suggesting an activation of tolerance mechanisms.

3.7 Amylase Analysis after Copper Nitrate Exposure

We measured the activity of alpha amylase after an 8 day exposure of copper nitrate to germinating seedlings (Fig. 26). The overall pattern for the starch enzyme, alpha-amylase, is as follows: up to 100 ppm of copper nitrate, amylase levels are similar to control showing no effect. At 200-300 ppm, we observed what is expected and similar to other peoples' results which are the inhibition of alpha-amylase. After 400 ppm, alpha-amylase activity increased suggesting an effort of the plant to compensate for the copper toxicity. More energy is needed for compartmentalization, sequestration, or toleration mechanisms. Alpha-amylase showed statistical differences in means among the concentrations ($p=0.0012$), with significantly higher alpha-amylase activity means for the 600 ppm, compared to the other concentrations.

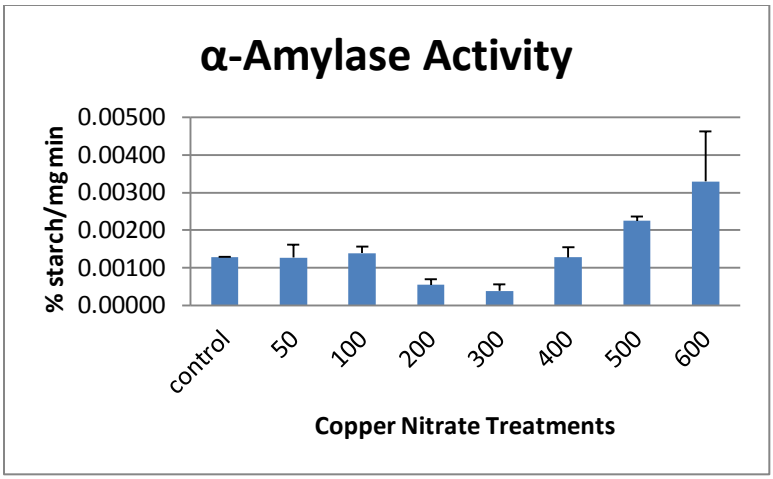


Figure 26. Alpha-amylase activity after copper nitrate exposure.

Alpha-amylase activity was inhibited by copper nitrate beginning at 200 ppm. Starch grain accumulation increased with increasing copper exposure beginning at 200 ppm. Heavy metals have inhibited alpha-amylase in other plants (Mihoub *et al*., 2005; Sfaxi-Bousbih *et al*., 2010) which leads to starch grain accumulation. Alpha-amylase synthesis is initiated by gibberellic acid and it is begun in the embryo (Paleg, 1960; MacGregor *et al*., 1978). Seedlings grown in soil have nutrient sources to compensate for starch sources. We expect more alpha-amylase activity in seedlings grown in Petri dishes which lack soil nutrients. Alpha-amylase needs calcium ions for its activity and stabilization (Yamamoto, 1988). Calcium decreased in the roots of screw bean mesquite, which would reduce the activity of alpha-amylase.

3.8 Histology

Four hundred thirty two pictures were taken and 1296 measurements were acquired to determine if significant cell surface area changes occurred due to exposure of copper sulfate or copper nitrate in screw bean mesquite seedlings. The photographs were taken from parenchymal cells around the vascular bundles from cotyledons. The mean average area of parenchymal cells is 750 μm^2. Figure 27 shows the mean cell areas across all copper concentrations, showing different trends for copper nitrate and copper sulfate ($p < 0.0001$).

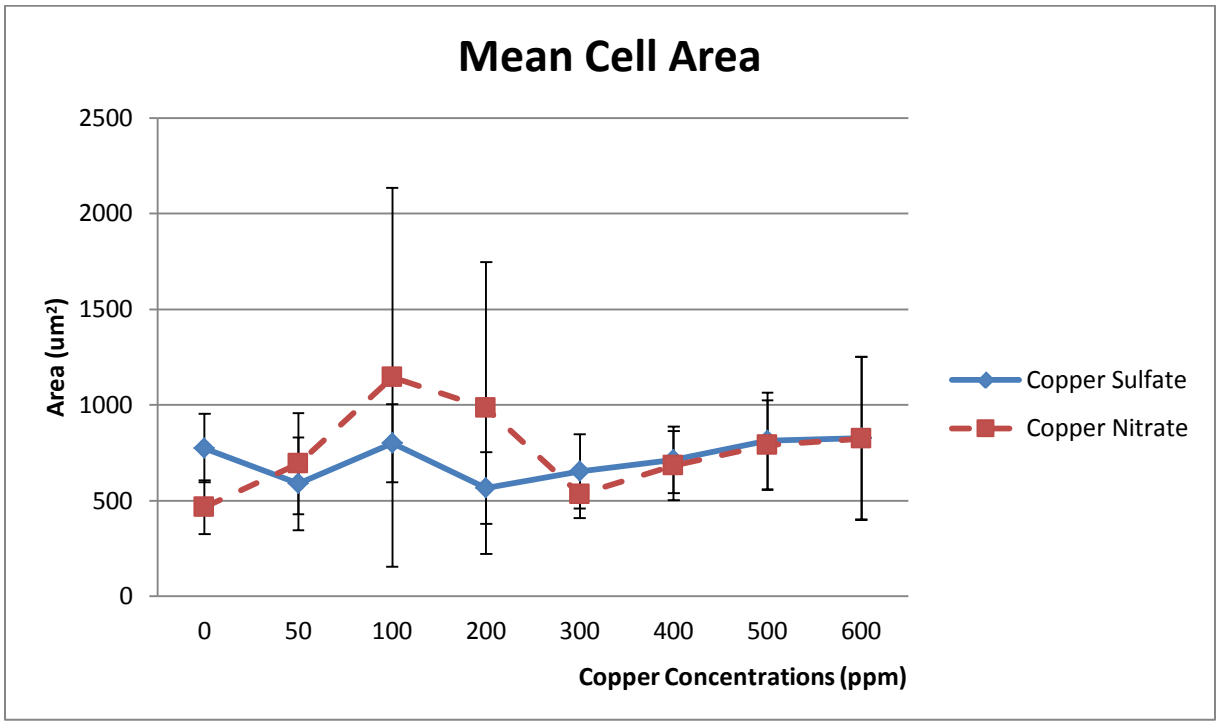


Figure 27. Mean parenchymal cell area changes due to copper toxicity in cotyledons.

Figure 28 shows the surface area measurements taken from one control sample and one 200 ppm of copper sulfate sample. However, similar trends showed cell area increased at 100, 400, 500, and 600 ppm. Swelling is a common response to toxicity. At 200 and 300 ppm, we observed a decrease in cell area suggesting that this is a crucial toxicity threshold in screw bean

mesquite and the plant was responding to the copper toxicity. When unable to counteract the toxicity, cells began to swell again.

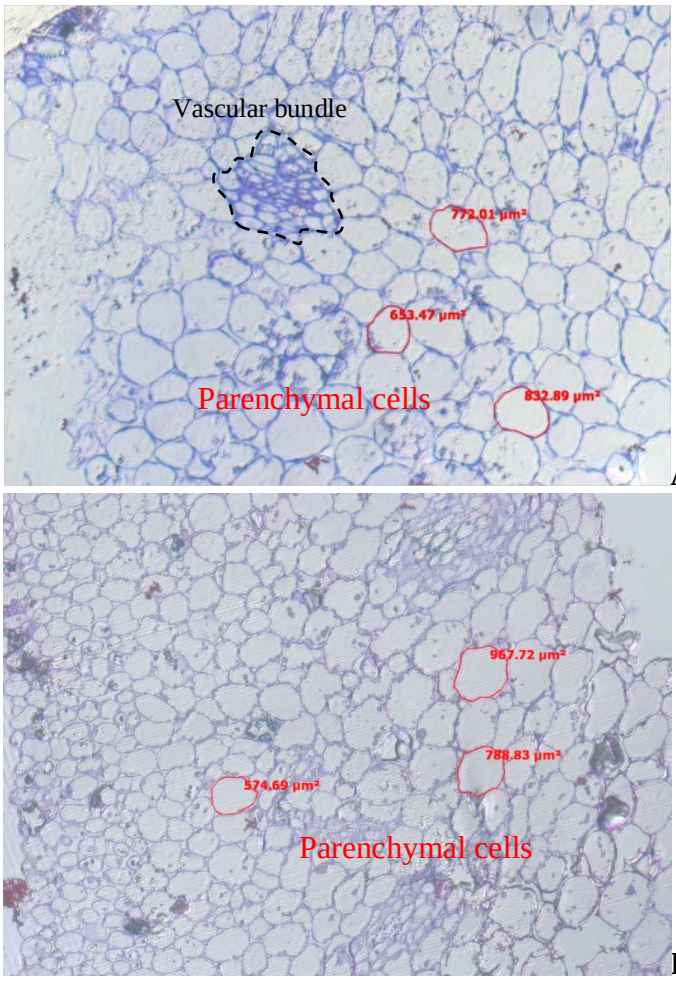


Figure 28. Light Micrograph of cotyledon parenchymal cells of *Prosopis pubescens* stained with Toluidine Blue-Basic Fuchsin that served as a control (0 ppm) (A) and 200 ppm of copper sulfate (B) (200X).

3.9 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. 29). In the control

sample, we observed healthy parenchymal cells with large vacuoles, chloroplasts close to the cell wall, and starch grains within chloroplasts (Fig. 29a). After 50 ppm copper sulfate exposure, cotyledon parenchymal cells showed denser cytoplasm and dense inclusions within small vacuoles. These vacuoles were characteristic of protein degradation within cotyledons (Chrispeels *et al*., 1976) (Fig. 29b). At 100 ppm of copper sulfate, the cytoplasm was separated from the cell wall and appeared denser than in the control (Fig. 29c). At 200 ppm protein degradation characteristic of cotyledons was observed (Fig. 29d, e). Unlike the control, a dark precipitate was observed near the cell walls possibly as copper aggregates. Starting at 200 ppm copper sulfate, there was an increase in swelling of developing chloroplasts and disarray of thylakoid membranes (Fig. 29g, h). In Fig. 29h, the cotyledon parenchymal cells were dying. The chloroplasts were highly enlarged with larger starch granules after exposure to 400 ppm of copper sulfate (Fig. 29h). After an exposure of 500 ppm of copper sulfate (Fig. 29i), a dark precipitate was observed at the edge of the central vacuoles, chloroplasts were swollen and thylakoid membranes were disarrayed. After 600 ppm copper sulfate exposure plasmolysis of cotyledon parenchymal cells was common and the small amount of cytoplasm was very dense (Fig. 29j).

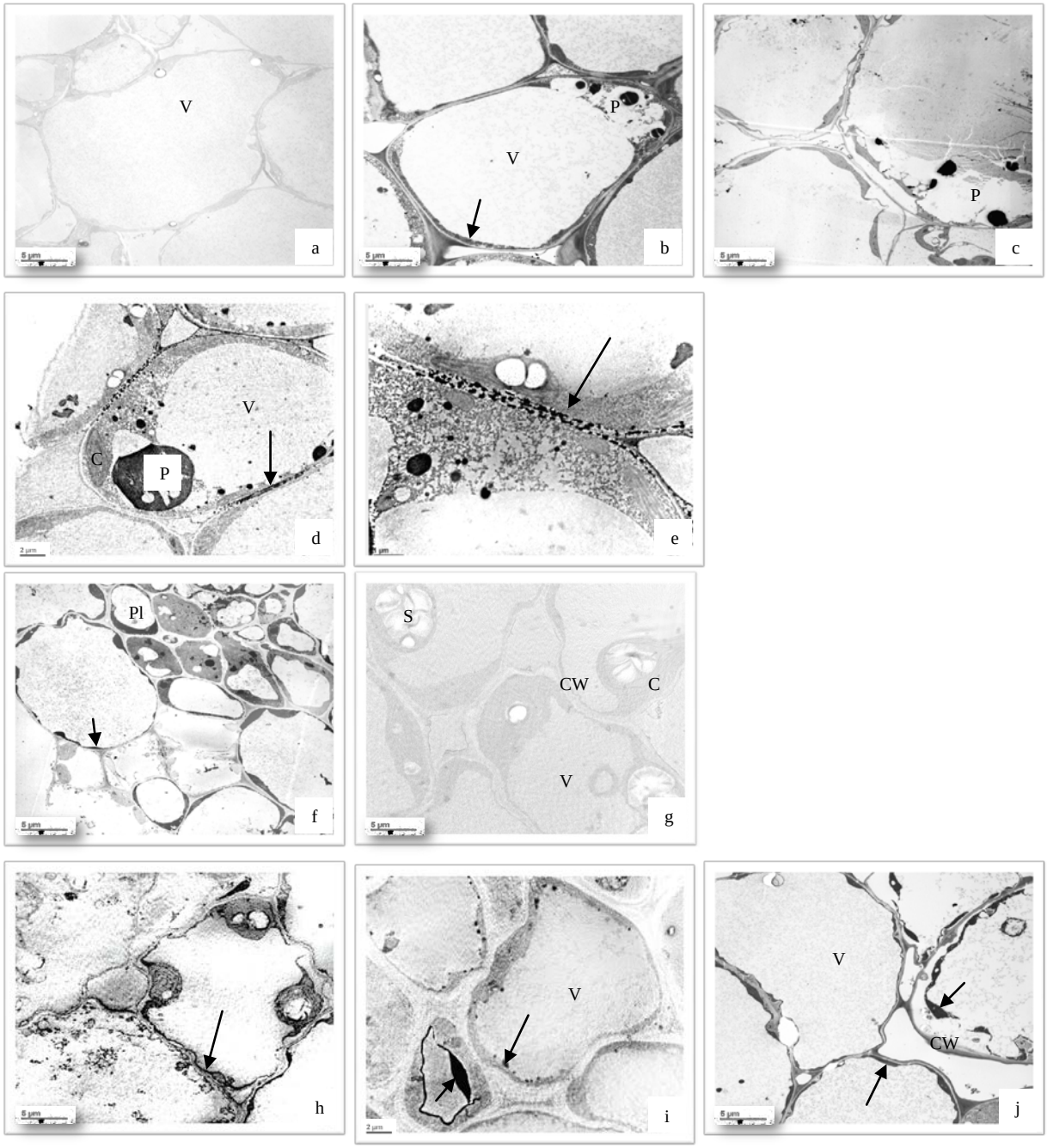


Figure 29. Electron micrographs showing the ultrastructural changes in cotyledons due to copper sulfate: (a) Control section of parenchymal cells (1,600X); (b) 50 ppm of $CuSO_4$ section of parenchymal cells (1,600X); (c) 100 ppm of $CuSO_4$ section of parenchymal cells with plasmolysis; (d) 200 ppm of $CuSO_4$ section of parenchymal cells (1,600X); (e) 200 ppm of $CuSO_4$ section of parenchymal cells (12,500X); (f) 300 ppm of $CuSO_4$ section of parenchymal cells and a vascular bundle (1,600X); (g) 300 ppm of $CuSO_4$ section of parenchymal cells (1,600X); (h) 400 ppm of $CuSO_4$ section of parenchymal cells showing plasmolysis (1,600X); (i) 500 ppm of $CuSO_4$ section of parenchymal cells (5,000X); (j) 600 ppm of $CuSO_4$ section of parenchymal cells (1,600X) (P-Protein bodies; C–Chloroplast; Pl-Phloem; V–Vacuole; S–Starch Grain; and CW–Cell Wall) (Arrows to dense aggregates).

The elemental analysis of in block spot scans of our BEEM samples (with a Hitachi 9500 STEM) have shown the presence of copper in cotyledons of screw bean mesquite plants (Fig. 30). These charged locations coincide with the dark aggregates we observed at low and high magnification transmission electron micrographs. No copper was found in the control and 50 ppm copper sulfate. Copper sulfate was identified in the interior of a cotyledon parenchymal cell in the 100 ppm, 200 ppm, and 300 ppm sample. In 400 ppm of copper sulfate, copper was localized in the cell wall and interior of a parenchymal cell. Copper was identified in the interior of epidermal cell of the 500 ppm copper sample. Finally, 600 ppm copper sulfate contained copper in the cell wall of a cotyledon parenchymal cell. The elemental analysis of copper nitrate samples showed the presence of copper along cell walls of cotyledon parenchymal and epidermal cells (Figs. 31b, 31d, 31f, 3h, 31j, 31l, 31n, and 31p). Dense precipitates were also observed in developing phloem cells.

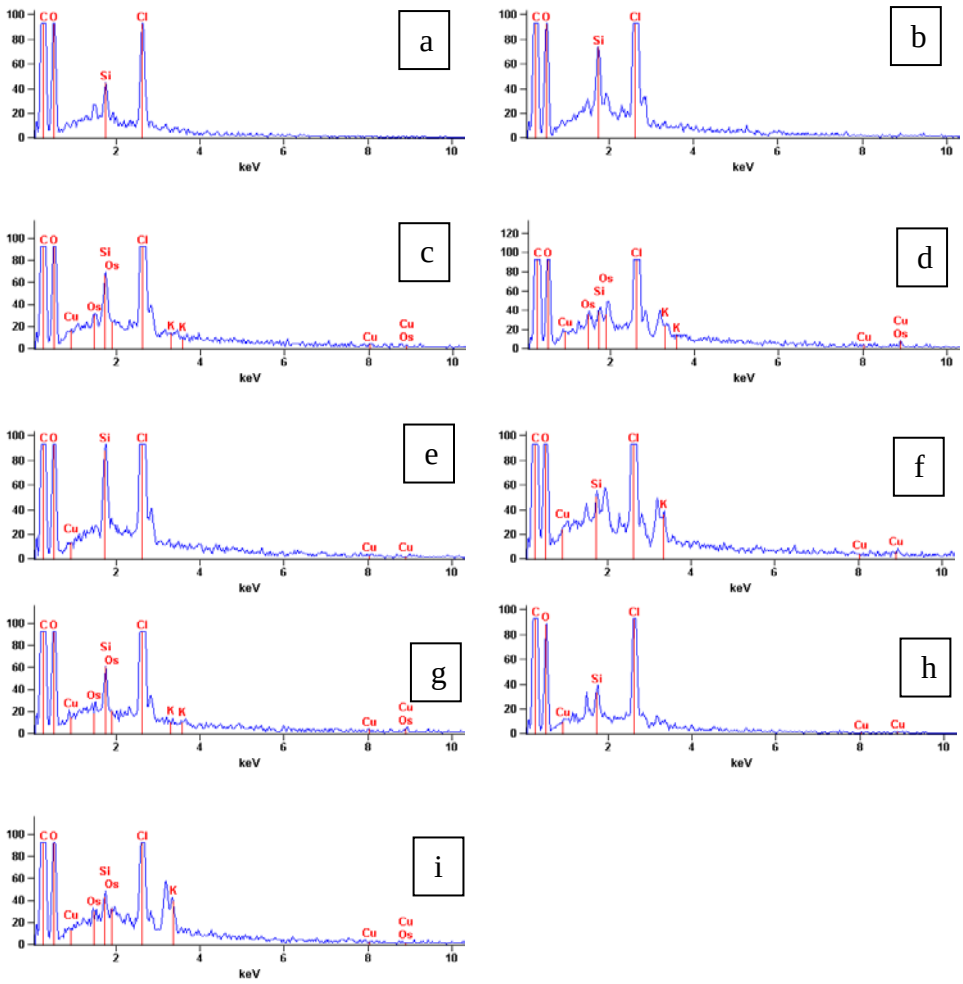
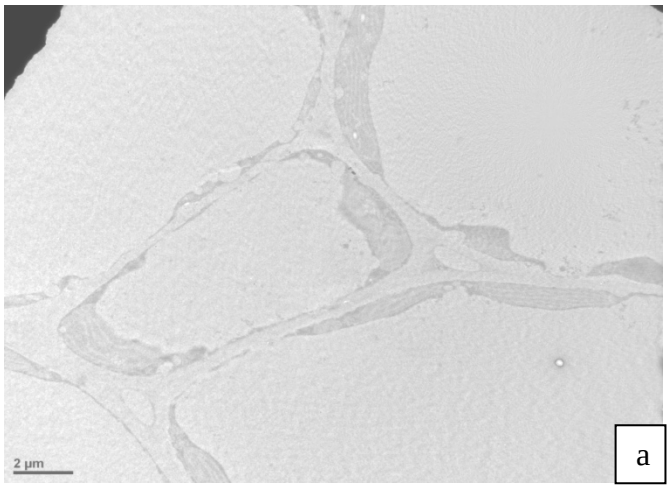


Figure 30. Elemental analysis of the (a) interior of a cotyledon parenchymal cell in control; (b) interior of a cotyledon parenchymal cell in 50 ppm copper sulfate; (c) interior of a cotyledon parenchymal cell in 100 ppm copper sulfate; (d) interior of a cotyledon parenchymal cell in 200 ppm copper sulfate; (e) interior of a cotyledon parenchymal cell in 300 ppm copper sulfate; (f) cell wall in 400 ppm copper sulfate; (g) interior of a cotyledon parenchymal cell in 400 ppm copper sulfate; (h) interior of an epidermal cell in 500 ppm copper sulfate; (i) cell wall in 600 ppm copper sulfate.

At 2,500X-12,500X magnification in a Carl Zeiss EM-10 transmission electron microscope, we observed various ultrastructural changes due to copper nitrate (Fig. 31). In the control sample, we observed large, clear vacuoles in cotyledon parenchymal cells with a small amount of cytoplasm near the cell walls (Fig. 31a). However, as copper nitrate was added more

pronounced ultrastructural changes were observed. After 50 ppm copper nitrate exposure, cotyledon epidermal cells showed a large increase of degrading material within their vacuoles (Fig. 31c). This is similar to protein degradation images from cotyledons. Denser vacuoles and enlarged chloroplasts were representative of parenchymal cells exposed to 100 ppm of copper nitrate (Fig. 31e). In 200 ppm of copper nitrate, exposed epidermal cells were observed. Plasmolysis and breakage of the vacuole tonoplast were evident. A dense precipitate was seen near the tonoplast (Fig. 31g), possibly copper aggregates. Developing phloem cells have very dense precipitates within their cytoplasm and vacuoles after 300 ppm of copper nitrate exposure (Fig. 31i). With 400 ppm copper nitrate exposure (Fig. 31k), the plasma membrane was no longer attached to the cell wall and starch grain accumulation obscures the presence of chloroplasts. Starch grain accumulation increased as the concentration of copper increased. Qualitative observations showed an accumulation of starch grains after 200 ppm of copper treatment. Plasmolysis was evident in cells exposed to 500 ppm copper nitrate (Fig. 31m) and 600 ppm of copper nitrate (Fig. 31o).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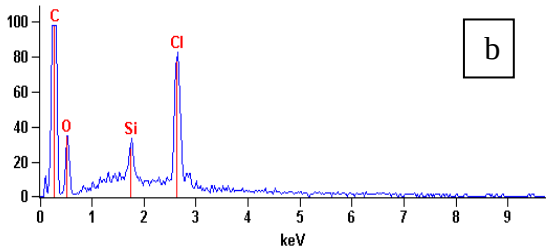
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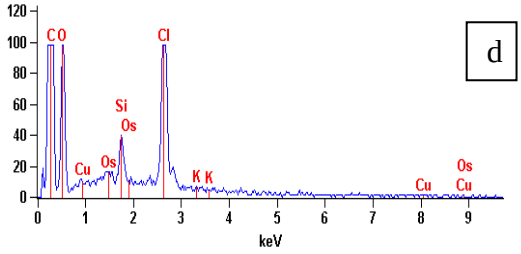
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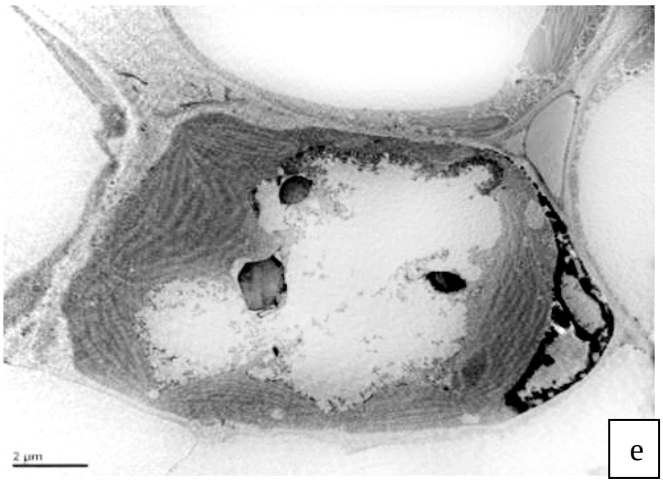
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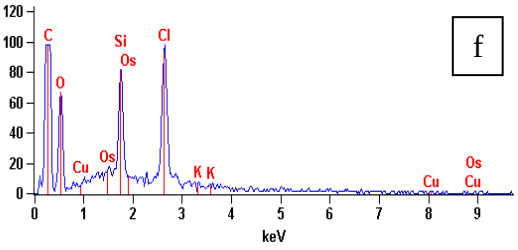
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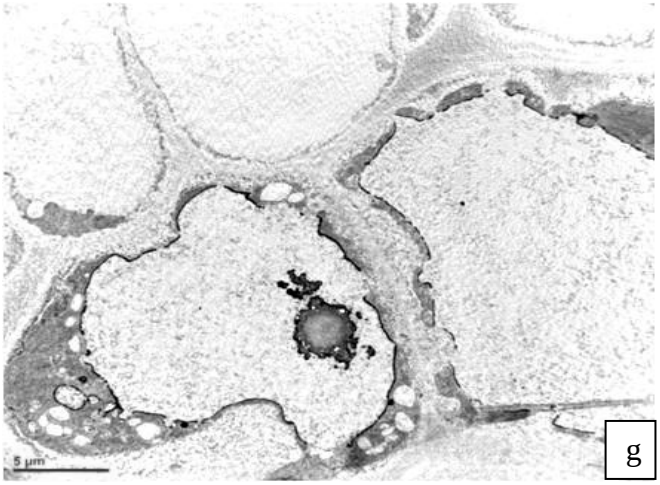
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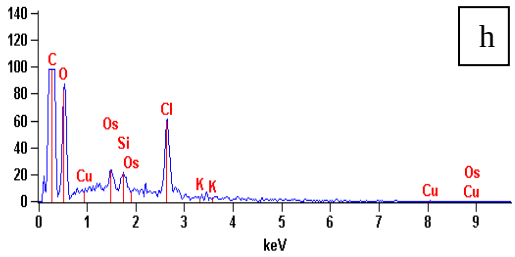
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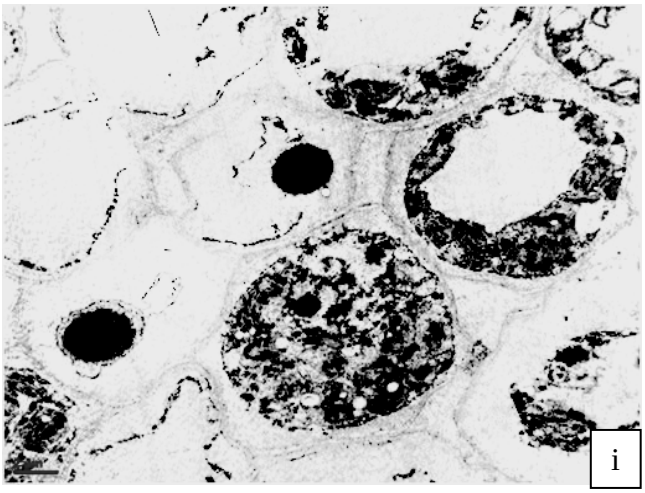
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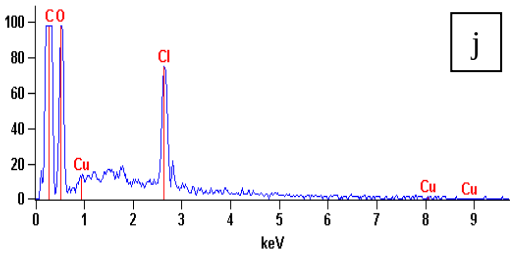
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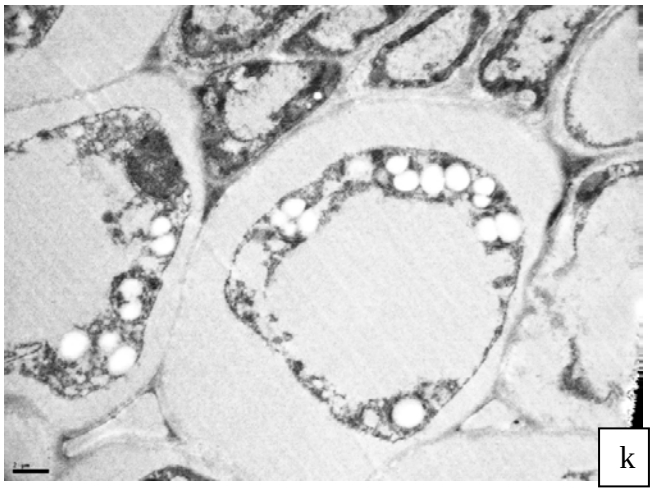
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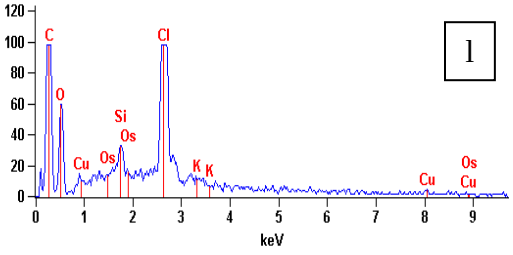
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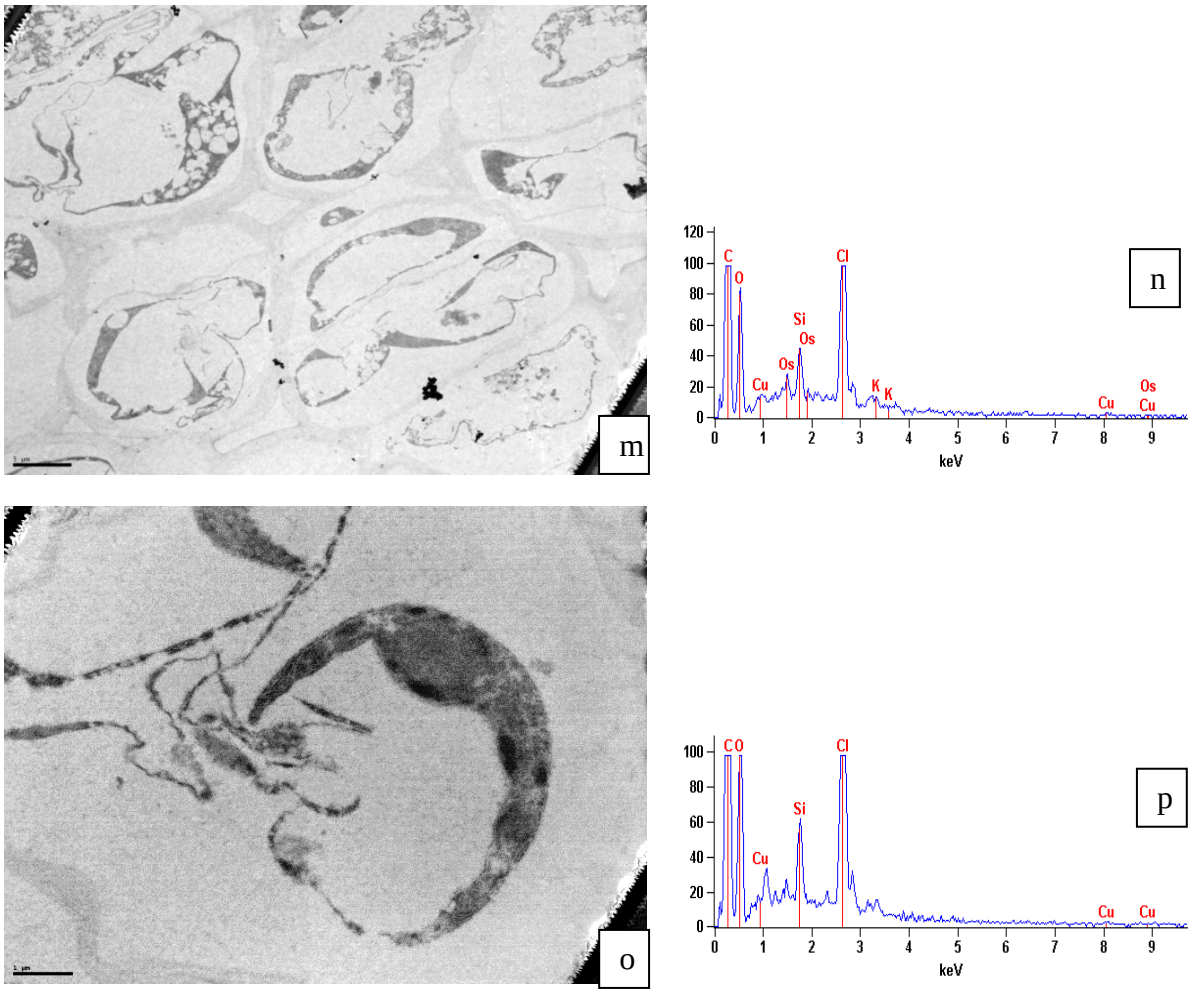


Figure 31. (a) Electron micrograph of control section of cotyledon parenchymal cells (6,000X); (b) elemental analysis in the vacuole of a parenchymal cell; (c) Electron micrograph of 50 ppm of $CuNO_3$ section of cotyledon epidermal cells (3,150X); (d) Elemental analysis in the vacuole of a parenchymal cell; (e) Electron micrograph of 100 ppm of $CuNO_3$ section of cotyledon parenchymal cells (4,000X); (f) Elemental analysis in the vacuole of a parenchymal cell; (g) Electron micrograph of 200 ppm of $CuNO_3$ section of cotyledon epidermal cells (2,500X); (h) Elemental analysis in the vacuole of a parenchymal cell; (i) Electron micrograph of 300 ppm of $CuNO_3$ section of developing phloem cells (12,500X); (j) Elemental analysis in the vacuole of a parenchymal cell; (k) Electron micrograph of 400 ppm of $CuNO_3$ section of cotyledon parenchymal cells (8,000X); (l) Elemental analysis in the vacuole of a parenchymal cell; (m) Electron micrograph of 500 ppm of $CuNO_3$ section of cotyledon parenchymal cells (4,000X); (n) Elemental analysis in the vacuole of a parenchymal cell; (o) Electron micrograph of 600 ppm of $CuNO_3$ section of cotyledon parenchymal cells (4,000X); (p) Elemental analysis in the vacuole of a parenchymal cell.

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). We observed healthy cotyledon parenchymal cells with large vacuoles in the control samples. 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. Cells would be forced to swell to compensate for the increase in vacuole size.

In the control and various concentrations of copper sulfate and copper nitrate, cotyledons showed protein bodies within vacuoles similar to the results of Chrispeels and Harris (1975) and Toyooka *et al.*, (2001). 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 a high protein content (Estevez, Escobar, and Ugarte, 2000) which suggests that screw bean mesquite cotyledons may also digest large amounts of protein in their vacuoles. Digested protein bodies merge to form a central, large vacuole (Chrispeels *et al.*, 1976). 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 awaits the addition of a starch granule to hydrolyze it. In relationship to these studies, we believe that our control and experimental seedlings are undergoing normal protein digestion in the cotyledons.

Starting at 100 ppm of copper exposure, the cytoplasm separated from the cell wall and appeared denser than in the control. Plasmolysis was evident. 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*., 1996). Screw bean mesquite cotyledon parenchymal cells undergoing plasmolysis also showed condensed, shrunken cytoplasms.

Starting at 200 ppm copper exposure, 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* Rich. (Stoyanova and Tchakalova, 1993). At 400 ppm copper 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 is thought to affect this enzyme; therefore, starch grains would be expected to accumulate if amylase was inhibited.

Cells are irregular shaped, the plasma membrane has completely separated from the cell wall and there is a decrease of chloroplasts within 500-600 ppm of copper treated seedlings. The cytoplasm including the 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).

3.10 Nutrient Changes Due To Copper Sulfate Exposure

Copper may also interfere with the uptake or distribution of other elements needed for the growth of plants. 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. There were no statistical differences in means across concentration for calcium, iron, manganese, boron, and zinc after copper sulfate exposure.

Magnesium concentrations decreased in roots, stems, and cotyledons as copper exposure increased ($p<0.0001$), with the means at 0 ppm significantly higher than the higher concentrations (Fig. 32). There was an overall total magnesium decrease of 50%. The sum concentration of magnesium in control seedlings was 8,800 ppm while seedlings exposed with 600 ppm of copper sulfate contained 4,400 ppm of magnesium.

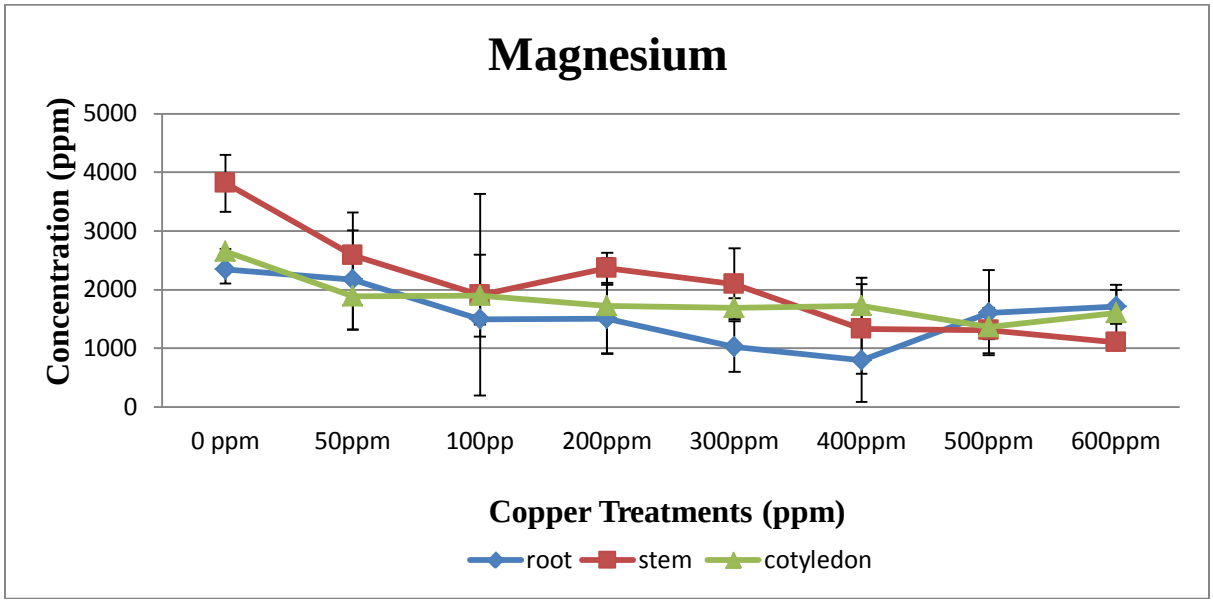


Figure 32. Magnesium changes in root (blue), stem (red), and cotyledons (green) due to copper sulfate exposure.

Magnesium concentrations decreased in screw bean mesquite seedlings exposed to increasing copper sulfate concentrations. Translocation of nitrate, phosphate, calcium, magnesium, and amino acids was reduced when potassium decreased (Armstrong, 1998). Our results showed both magnesium and potassium reductions after copper sulfate exposure. Magnesium is the main element in the structure of chlorophylls. It is also involved in sugar synthesis and translocation, enzyme activation, lipid formation, nitrogen fixation, phosphorus transfer and iron utilization. A decrease in magnesium may reduce starch production or starch breakdown, damage the cell membrane by the reduction of phospholipids, reduce phosphorus and reduce iron utilization. Ultrastructural changes were observed in screw bean mesquite seedlings, including plasmolysis of cotyledon parenchymal cells. Phosphorus was significantly reduced within the roots, stems, and cotyledons. Iron concentration did not change, however, chlorosis was present. Chlorosis occurs by a reduction of either iron or magnesium concentrations. We concluded that chlorosis may be the result of a reduction of magnesium and a reduction of iron utilization.

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 ppm of molybdenum and the 600 ppm of copper sulfate sample was about 64 ppm of molybdenum (Fig. 33). These changes were not seen for the stem and cotyledon ($p> 0.99$).

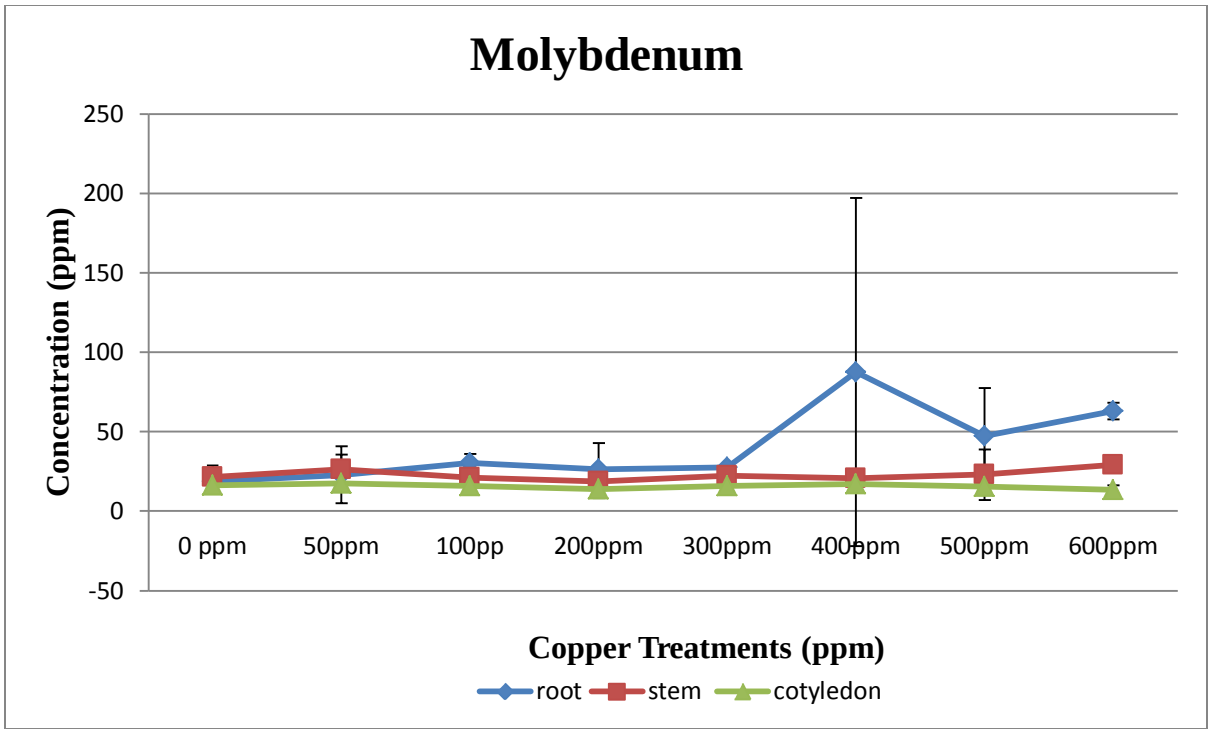


Figure 33. Molybdenum changes in root (blue), stem (red), and cotyledons (green) due to copper sulfate exposure.

Molybdenum is a key component of the enzymes nitrate reductase and nitrogenase. 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. Screw bean mesquite is a legume. Davis *et al*. (1978) found that excess molybdenum is toxic to barley (*Hordeum vulgare L.);* however, crops like cauliflower and onion (*Allium cepa L.*) are able to accumulate up to 600 mg Mo kg^{-1} without exhibiting symptoms of toxicity (Gupta *et al.*, 1978). Most plants are not sensitive to excess molybdenum. Symptoms of toxicity include malformation of the leaves (Gupta *et al.*, 1997), inhibition of roots and stems (Kevresan *et al.*, 2001), and golden-yellow discoloration of the stems (Marschner, 1995). Gupta and Lipsett (1981) determined that excess molybdenum inhibits iron metabolism resulting in chlorosis. We did not have changes in iron concentrations. We concluded that chlorosis may be due to a decrease in magnesium.

Phosphorus concentrations were different across root, stem, and cotyledons as copper exposure increased (p=0.0007). Phosphorus was significantly affected by the presence of copper sulfate (Fig. 34). With a low copper sulfate treatment of 50 ppm, roots and stems dropped dramatically to 0 ppm and 4,000 ppm, respectively. After 100 ppm of copper sulfate, both roots and stems had no phosphorus. This is crucial since control roots have almost 4,200 ppm and stems have 8,700 ppm of phosphorus. For cotyledons, phosphorus fluctuates from 5,000-6,500 ppm between the 50-200 ppm copper sulfate exposures. However, after 300 ppm of copper sulfate exposure there was no phosphorus.

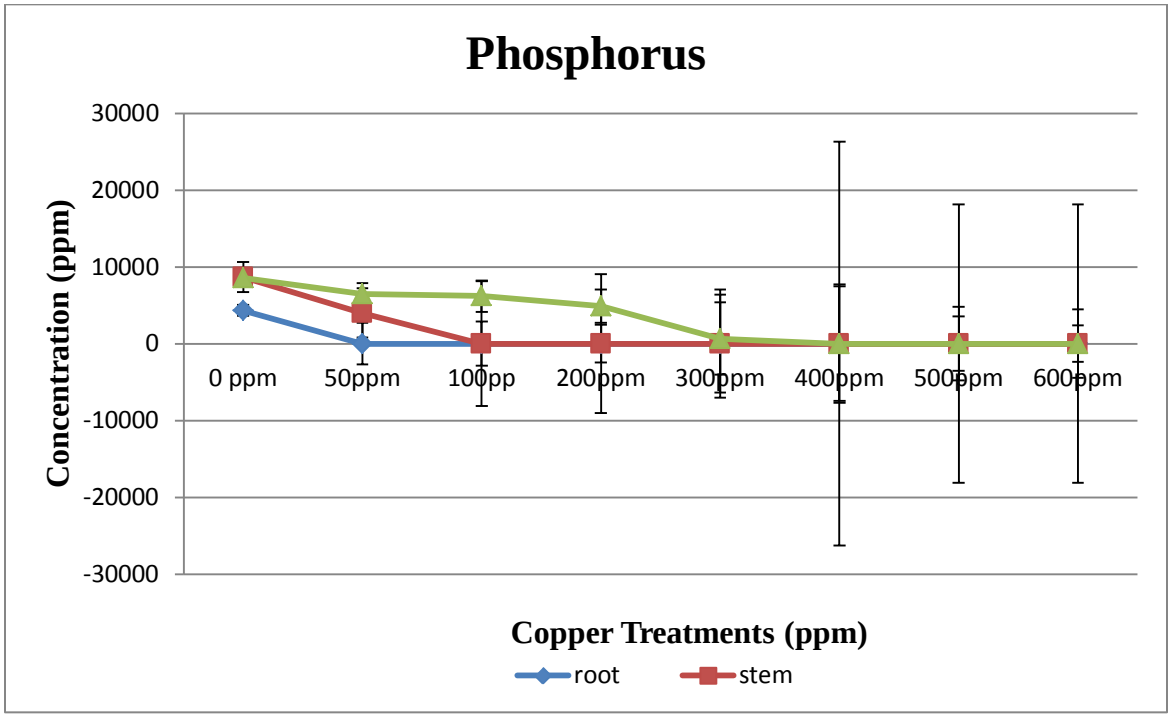


Figure 34. Phosphorus changes in root (blue), stem (red), and cotyledons (green) due to copper sulfate exposure.

Phosphorus is involved in energy carrying phosphate compounds (ATP and ADP), nucleic acids, coenzymes, and phospholipids. Phosphorus was significantly affected by the

presence of copper sulfate within the roots, stems, and cotyledons. This suggests loss of energy and may have produced damage to cell membranes. We observed ultrastructural damage to cell membranes in the cotyledon parenchymal cells.

Potassium concentrations in roots, stems and cotyledons of seedlings exposed to copper sulfate decreased as copper increased ($p<0.0001$) (Fig. 35). Control root contained about 13,500 ppm of potassium, while 600 ppm copper sulfate exposed seedlings had decreased to about 4,000 ppm of potassium. This was a 70% decrease. A greater decrease was seen in stems, where control had 23,500 ppm of potassium and the 600 ppm copper sulfate sample had 4,000 ppm of potassium. This was an 87% decrease. Finally, cotyledons had a 38% decrease with the control containing 8500 ppm of potassium and the 600 ppm copper sulfate sample having 5,250 ppm of potassium.

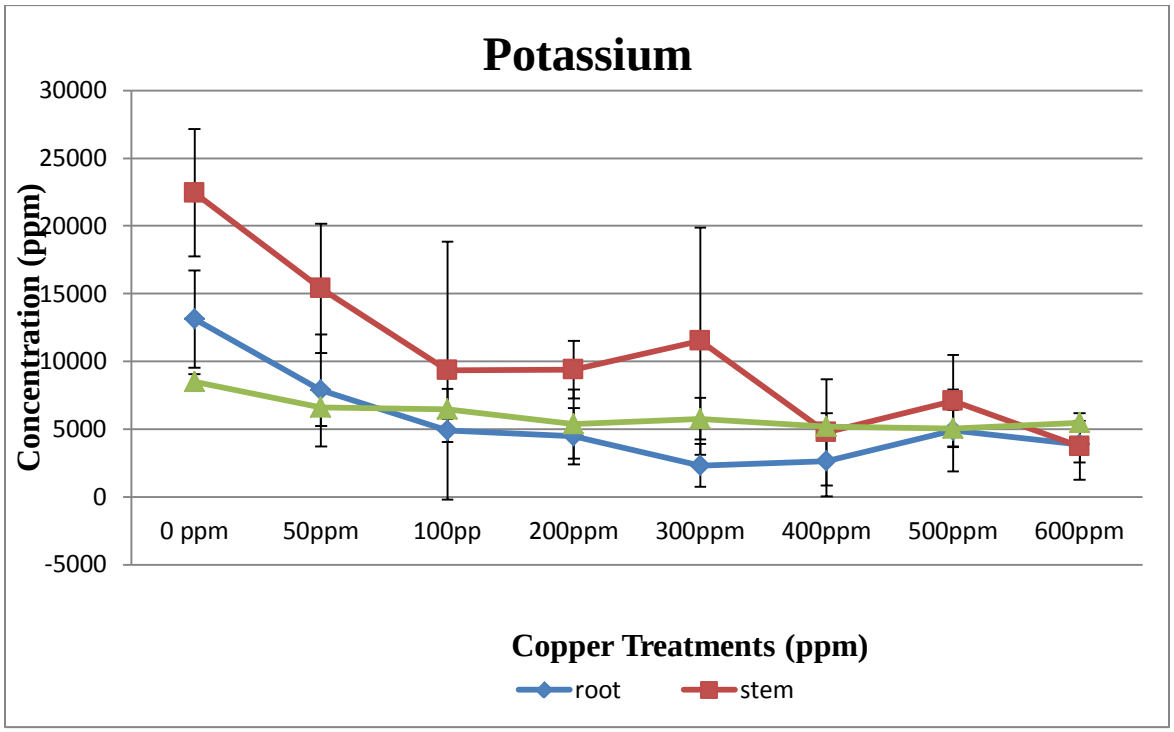


Figure 35. Potassium changes in root (blue), stem (red), and cotyledons (green) due to copper sulfate exposure.

Potassium is involved in enzyme activation, stomatal activity, photosynthesis, transport of sugar, water and nutrients, as well as, starch and protein synthesis. As expected, potassium concentrations changed within screw bean mesquite seedlings exposed to copper sulfate. Potassium concentrations in roots, stems, cotyledons and true leaves of seedlings exposed to copper sulfate decreased as copper increased. 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.

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 ppm of sulfur, while the 600 ppm copper sulfate exposed roots had 9,500 ppm of sulfur (Fig. 36). Sulfur rapidly increased in the roots beginning at 400 ppm of copper ($p<0.0001$). The control for stems had 1,800 ppm of sulfur, while the 600 ppm copper sulfate exposed stems had 4,400 ppm of sulfur. The control for cotyledons had 2,000 ppm of sulfur, while the 600 ppm copper sulfate exposed cotyledons had 4,500 ppm of sulfur. However for the stem and cotyledon, the increases were not significant ($p=0.3226$ and 0.4696 respectively).

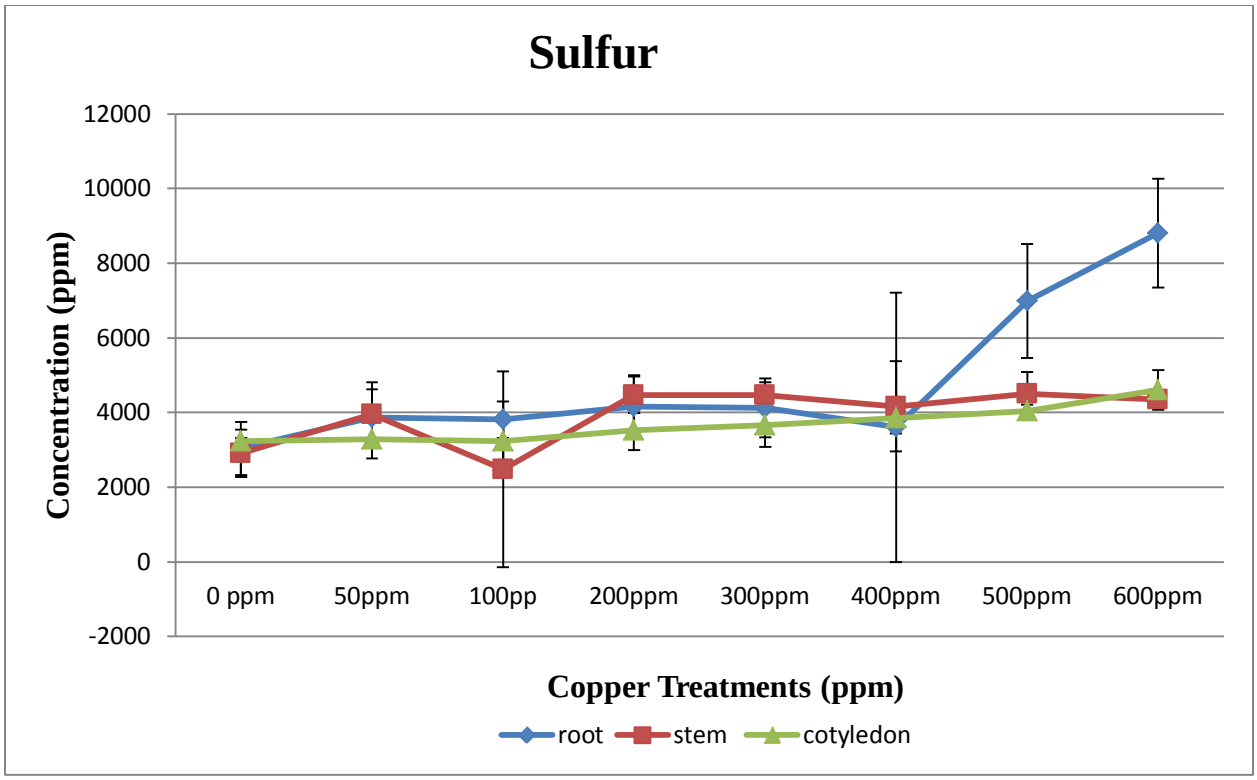


Figure 36. Sulfur changes in root (blue), stem (red), and cotyledons (green) due to copper sulfate exposure.

Cystine, cysteine, and methionine contain sulfur (Baird, 1914). Significant sulfur increases, within seedlings exposed to copper sulfate, were seen in roots, stems and cotyledons. Sulfur increase is directly linked with increased production of cysteine. Both metallothioneins and phytochelatins are cysteine-rich proteins known to bind copper for sequestration or tolerance mechanisms. Screw bean mesquite roots exposed to 600 ppm of copper sulfate accumulated 8,800 ppm of sulfur. Screw bean mesquite stems exposed to 600 ppm of copper sulfate accumulated 4,400 ppm of sulfur. Screw bean mesquite cotyledons exposed to 600 ppm of copper sulfate accumulated 4,600 ppm of sulfur.

3.11 Nutrient Changes Due To Copper Nitrate Exposure

There were no statistical differences for manganese, iron and molybdenum after copper nitrate exposure. Boron concentrations were different across roots, and stems as copper exposure increased (p=0.0016). The highest concentration of boron was found in roots, followed by stems and cotyledons (Fig. 37). In roots, boron decreased 51% as copper nitrate exposure increased to 600 ppm. Control roots had 108.38 ppm of boron, while the 600 ppm of copper nitrate sample had about 53.35 ppm of boron. Calcium concentrations were different across roots and stems as copper exposure increased (p=0.0043). Calcium concentrations fluctuated from 3,915.69 to 5,556.44 ppm in the roots, while in the stems calcium ranges were from 2,892.97 to 4071.05 ppm. There was no apparent pattern for either roots or stems (Fig. 38).

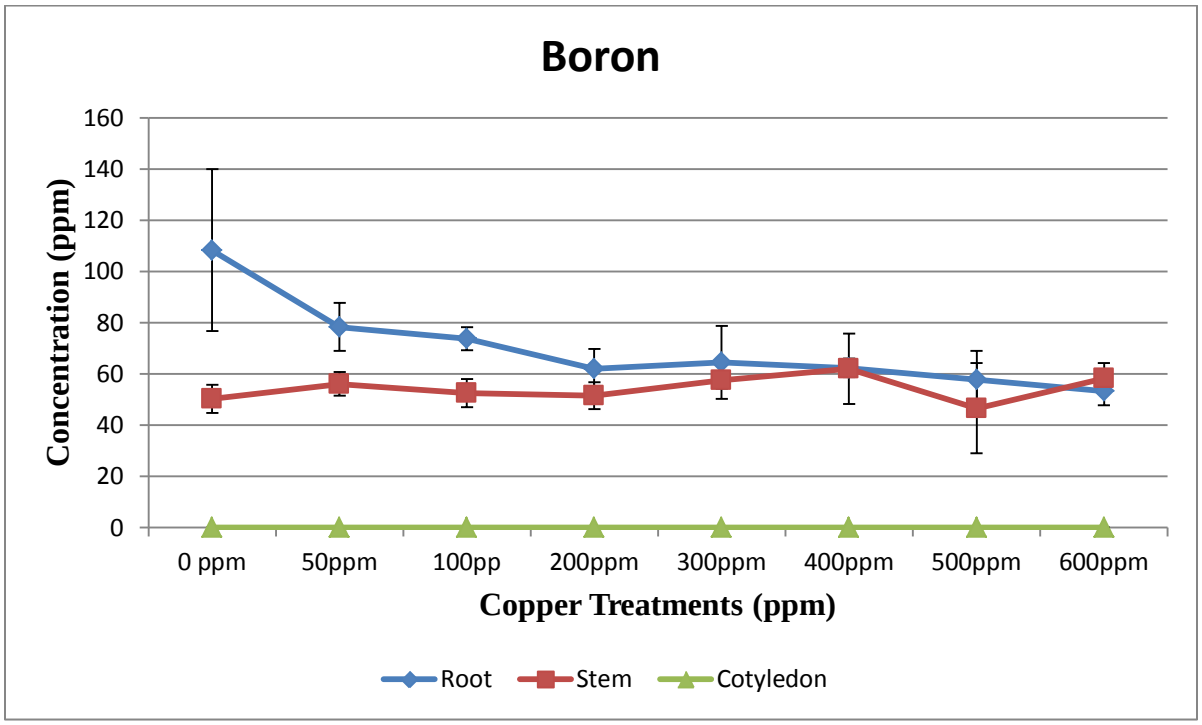


Figure 37. Boron changes in root (blue), stem (red) and cotyledons (green) due to copper nitrate exposure.

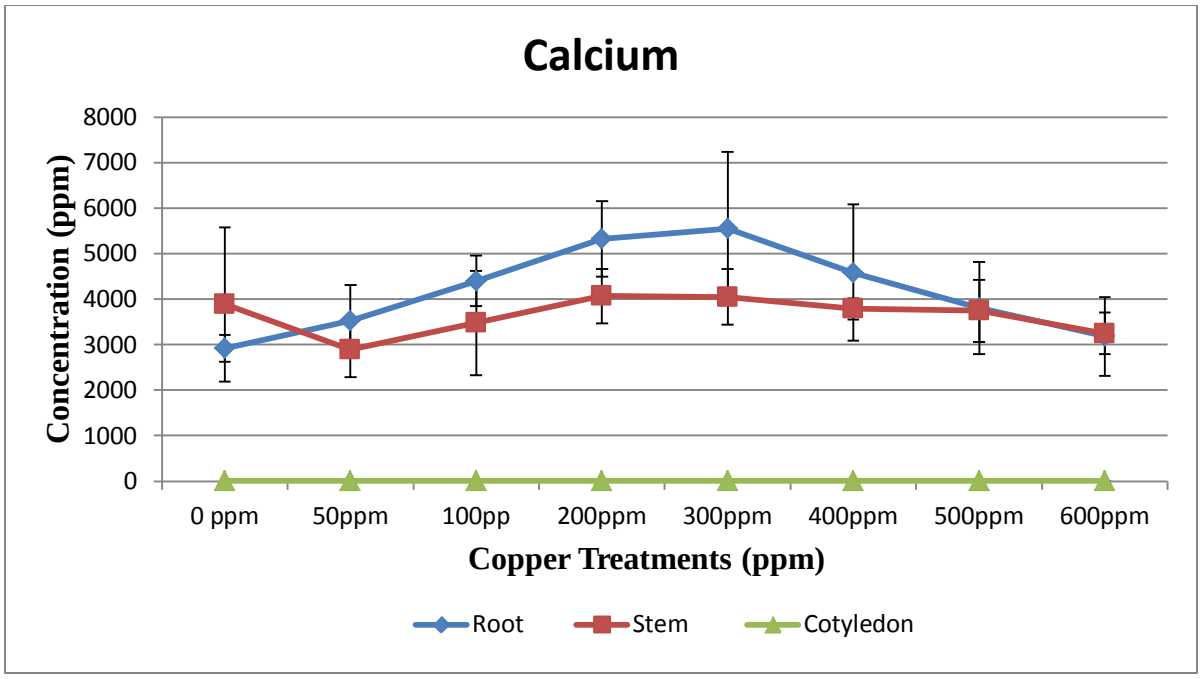


Figure 38. Calcium changes in root (blue), stem (red) and cotyledons (green) due to copper nitrate exposure.

As in the presence of copper sulfate, copper nitrate decreased boron concentrations which may lead to ultrastructural changes in the cell wall. Boron also protects calcium levels in the cell wall (Clarkson and Hanson, 1980). Calcium levels fluctuated in the presence of copper nitrate. We observed an increase of calcium in the roots starting at 50 ppm of copper nitrate. A subsequent decrease, starting at 300 ppm of copper nitrate, was observed in the roots that may lead to cell separation at the ultrastructural level. Yamonouchi (1971) and Yamauchi *et al.* (1986) confirmed that tomatoes with boron deficiency also contained less calcium.

Magnesium concentrations were different across roots and stems as copper exposure increased ($p<0.0001$). Magnesium decreased in roots, and stems as copper nitrate increased (Fig. 39). There was an overall total magnesium decrease of 71%, where roots decreased from 3,005.15 to 674.06 ppm and stems decreased from 3,470.32 to 1,263.23 ppm of magnesium.

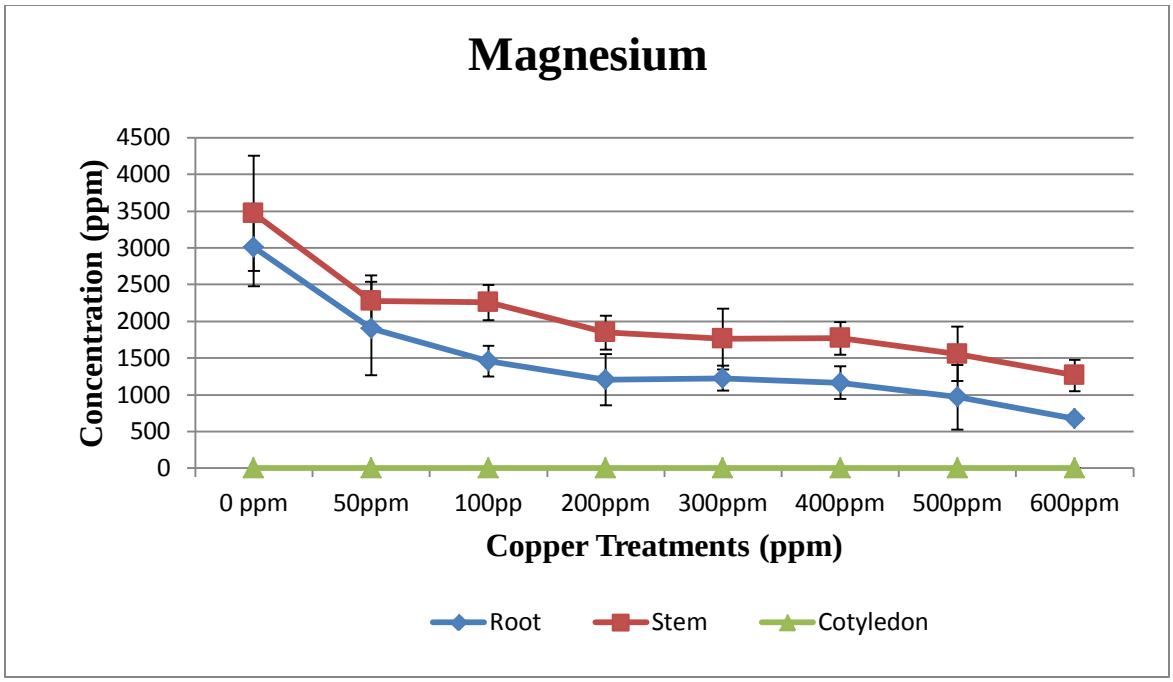


Figure 39. Magnesium changes in root (blue), stem (red) and cotyledons (green) due to copper nitrate exposure.

Our results also showed both magnesium and potassium reductions after copper nitrate exposure. Reduced starch production or starch breakdown and damage to the cell membrane due to loss of phospholipids was observed in screw bean mesquite seedlings exposed to copper nitrate. Chlorosis may be due to the reduction of magnesium and a reduction of iron utilization.

Phosphorus concentrations were different across roots, and stems as copper exposure increased ($p=0.0014$). Phosphorus was significantly affected by the presence of copper nitrate (Fig. 40). With a low copper sulfate treatment of 50 ppm, roots and stems dropped to 0 ppm of phosphorus. This was crucial since control roots have almost 7,678.86 ppm and stems have 9,322.88 ppm of phosphorus. Boron-deficiency also reduces the uptake of phosphorus (Goldbach, 1984). We expect as in copper sulfate samples, an alteration of cell membrane.

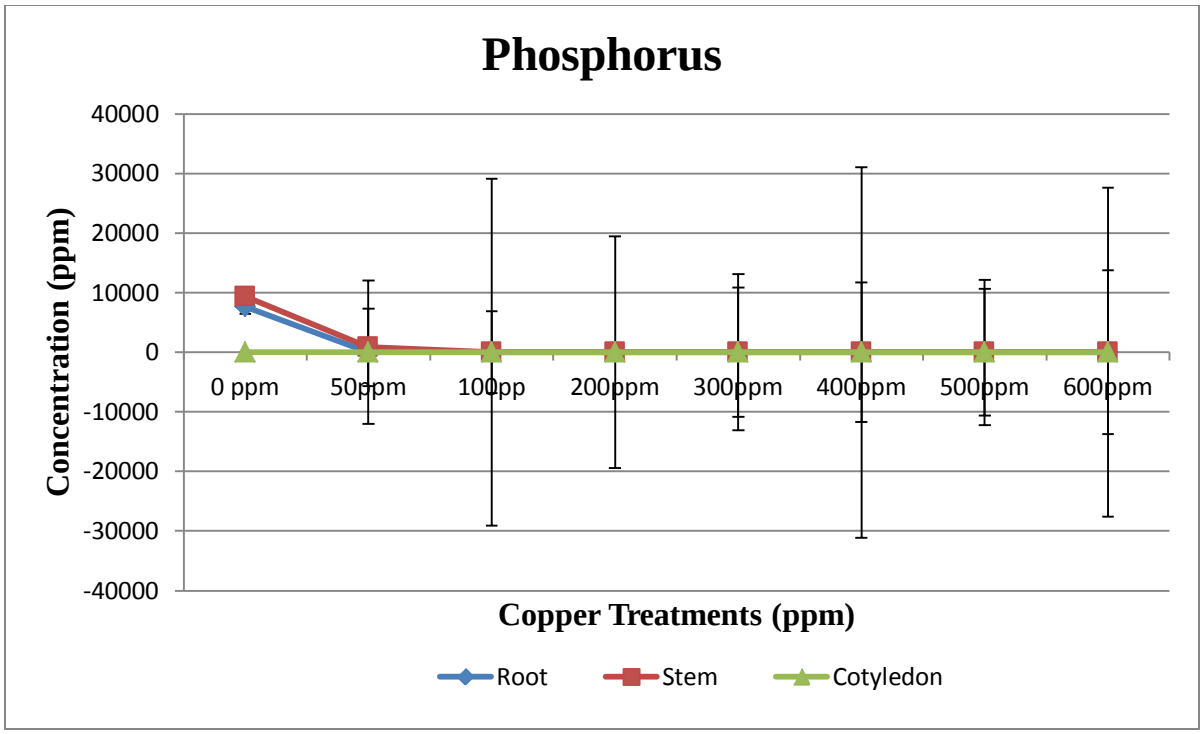


Figure 40. Phosphorus changes in root (blue), stem (red) and cotyledons (green) due to copper nitrate exposure.

Phosphorus was also significantly affected by the presence of copper nitrate within the roots, stems and cotyledons, which suggests loss of energy and may have produced damage to cell membranes. We observed ultrastructural damage to cell membranes in the cotyledon parenchymal cells.

Potassium concentrations were different across roots and stems as copper exposure increased ($p<0.0001$). Potassium concentrations in roots and stems decreased as copper increased (Fig. 41). Control root contained about 19,207.38 ppm of potassium, while the 600 ppm copper nitrate exposed seedlings had decreased to about 1,443.30 ppm of potassium. This is a 92% decrease. A lesser decrease was seen in stems where control had 20,483.86 ppm of potassium and the 600 ppm copper nitrate sample had 4,242.36 ppm of potassium. This was a 69% decrease.

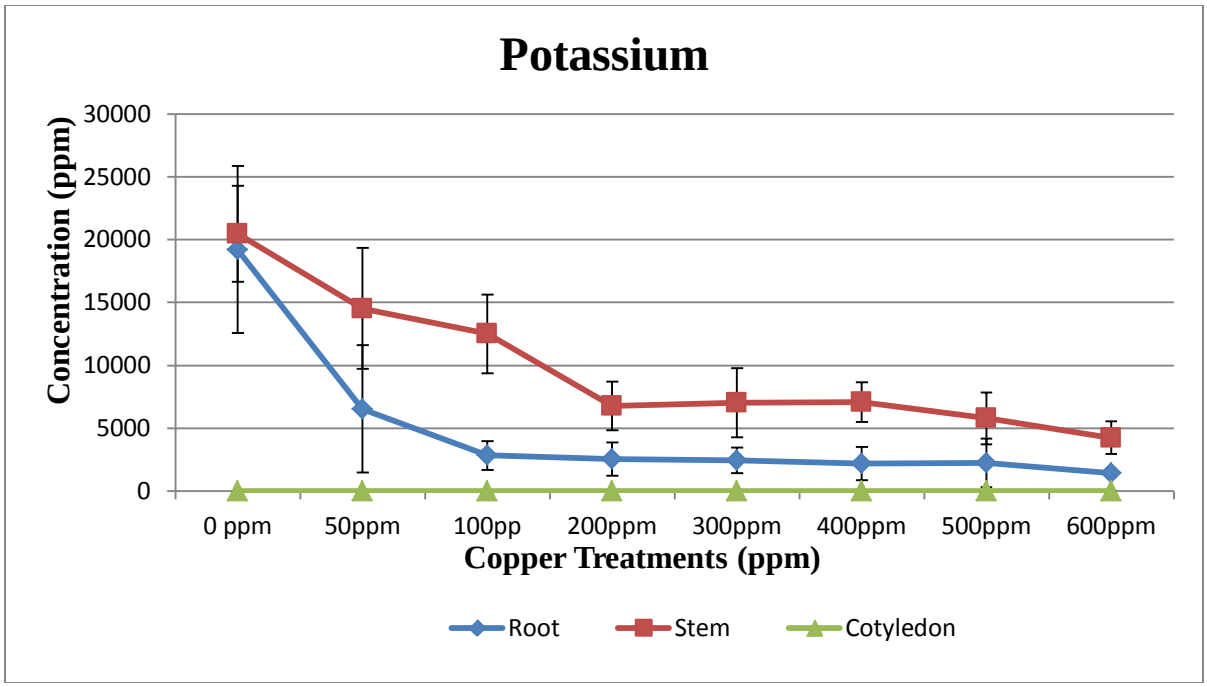


Figure 41. Potassium changes in root (blue), stem (red) and cotyledons (green) due to copper nitrate exposure.

Potassium concentrations in roots, stems, and cotyledons of seedlings exposed to copper nitrate decreased as copper increased. 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.

Sulfur concentrations were different across root, stem, and cotyledons as copper sulfate exposure increased ($p=0.0089$). The control for roots had 3,462.13 ppm of sulfur, while the 600 ppm copper nitrate exposed roots had 2,911.32 ppm of sulfur (Fig. 42). The control for stems had 3,810.54 ppm of sulfur, while the 600 ppm copper nitrate exposed stems had 2,829.52 ppm of sulfur. Sulfur slightly increased to 4,213.73 ppm at 400 ppm of copper nitrate exposure. After 300 ppm of copper nitrate exposure, sulfur rapidly decreased.

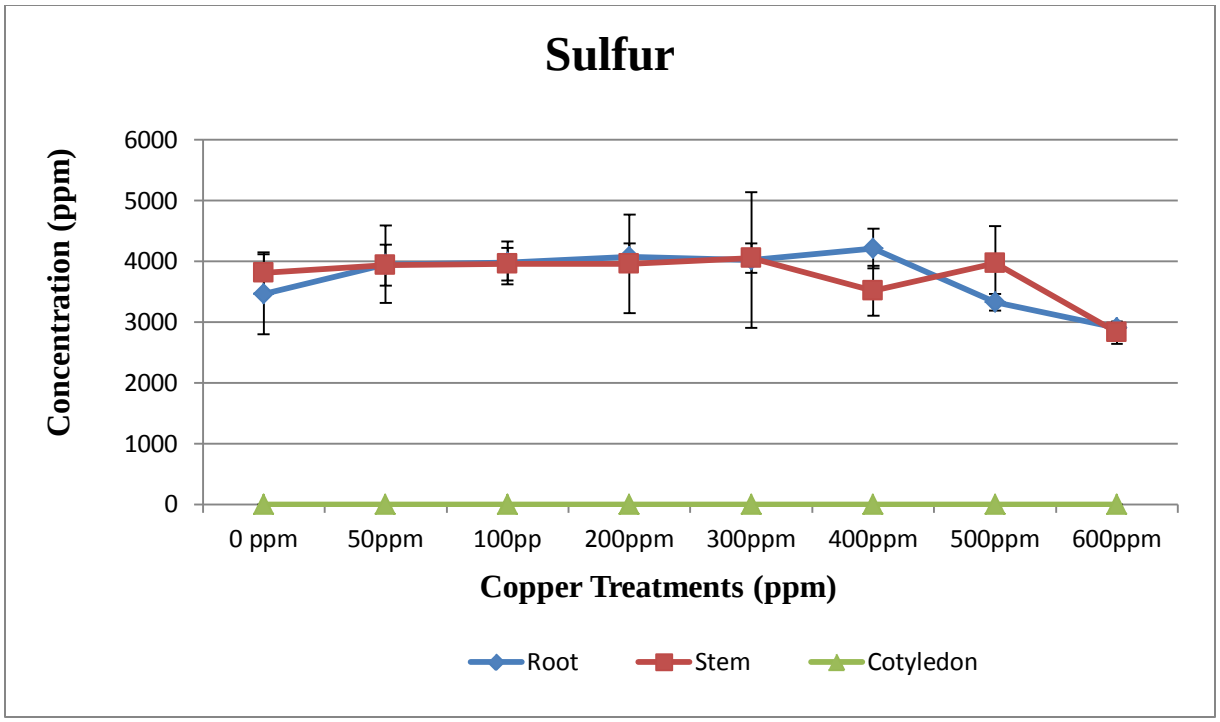


Figure 42. Sulfur changes in root (blue), stem (red) and cotyledons (green) due to copper nitrate exposure.

Significant sulfur increases within seedlings exposed to copper sulfate were seen in roots and cotyledons. Sulfur increase is directly linked with increased production of cysteine proteins such as phytochelatins and metallothioneins.

Zinc concentrations were different across roots, and stems as copper nitrate exposure increased ($p=0.0109$). Zinc concentrations fluctuated between 127.47-242.22 ppm in the roots while in the stems it ranged between 76.22-116.45 ppm (Fig. 43). Zinc decreased slightly with increasing copper nitrate exposure in the stems (78.63 ppm).

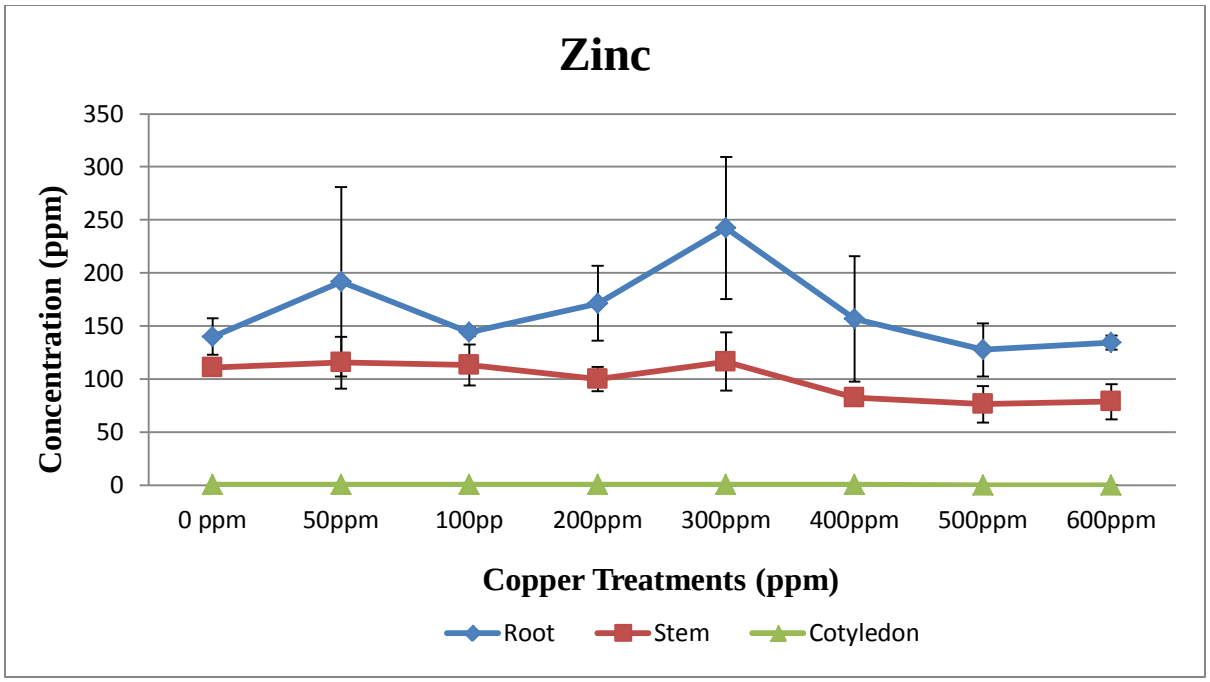


Figure 43. Zinc changes in root (blue), stem (red) and cotyledons (green) due to copper nitrate exposure.

Zinc deficiencies stunt growth and are most evident during secondary and third plant growth (McCauley *et al.*, 2009). Zinc is involved with hormone production and internode elongation (McCauley *et al.*, 2009). Our results showed that zinc absorption fluctuated across copper treatments suggesting an uneven availability of zinc from seed reserves in Petri dish grown seedlings.

GREEN HOUSE GROWN SCREW BEAN MESQUITE SEEDLINGS

3.12 Copper Sulfate Effects on Soil-Grown Screw Bean Mesquite Plants

The figure (Fig. 44) below shows the greenhouse set up of the soil-grown 24-day-old screw bean mesquite plants. Visually, copper sulfate (Fig. 45) or copper nitrate (Fig. 46) does not show an effect on the growth of the plants. There is no chlorosis or death shown within 24 days of growth.

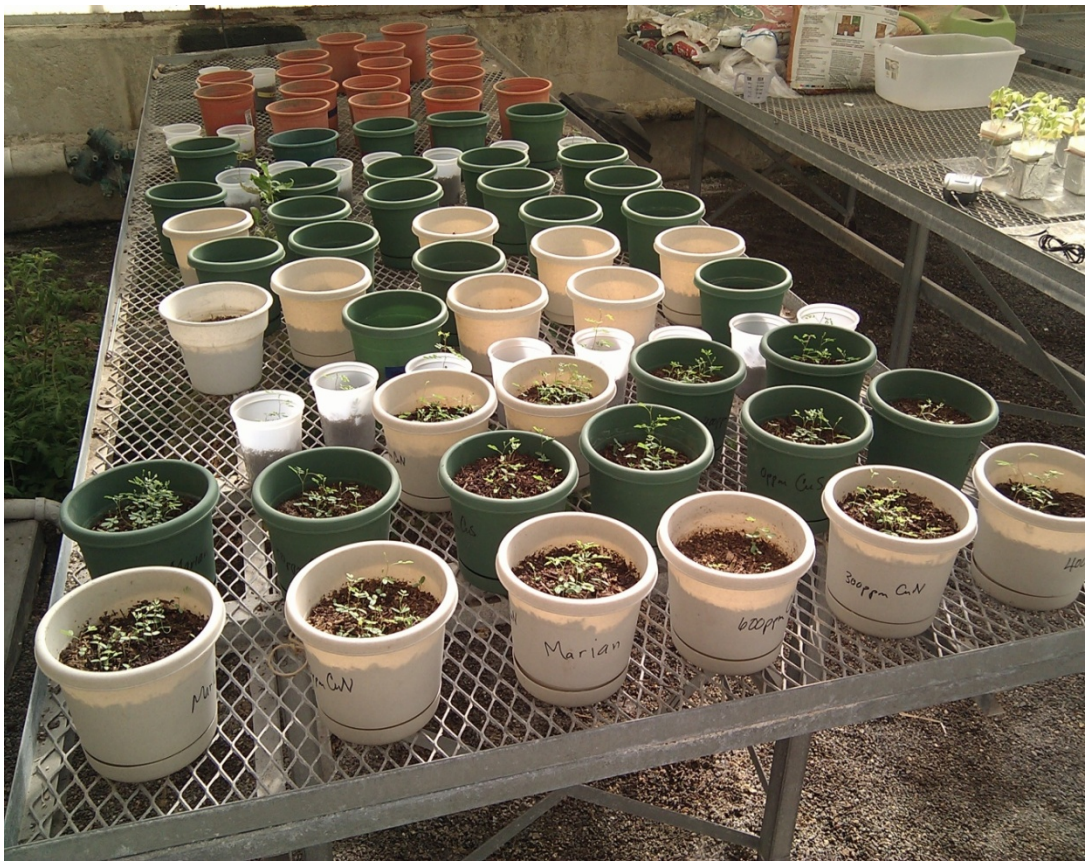


Figure 44. Screw bean mesquite plants in the greenhouse.

Figure 45. Individual pots with different copper sulfate concentrations.

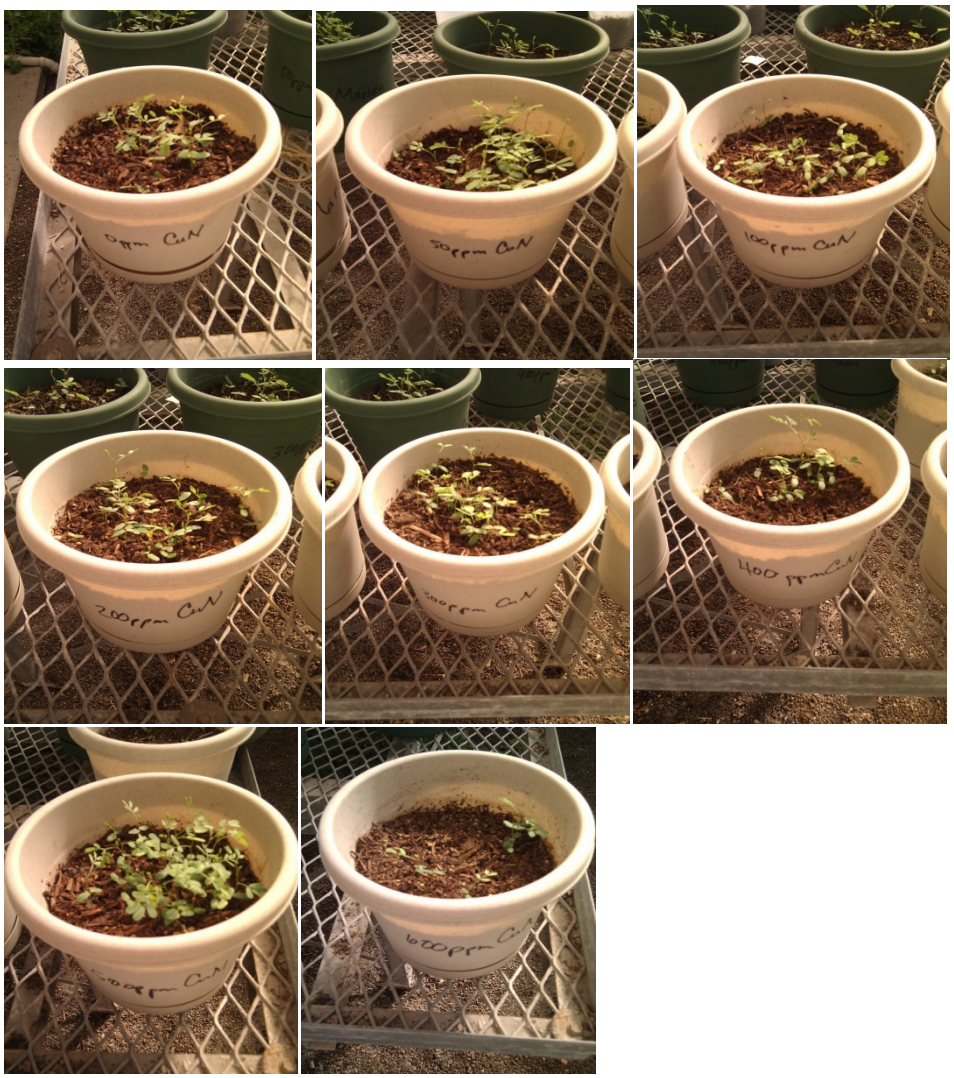


Figure 46. Individual pots with different copper nitrate concentrations.

Figure 47 and 48 show the root growth of screw bean mesquite plants in soil. The soil-grown mesquite plants were also grown for 24 days while lab-grown mesquite seedlings were only grown for 16 days. This growth was substantial since in Petri dishes, screw bean mesquite root measured less than 4 cm. In soil-grown mesquite, roots measured 30 cm after 50 ppm of copper sulfate (Fig. 47) and 25 cm after 50 ppm of copper nitrate exposure (Fig. 48).

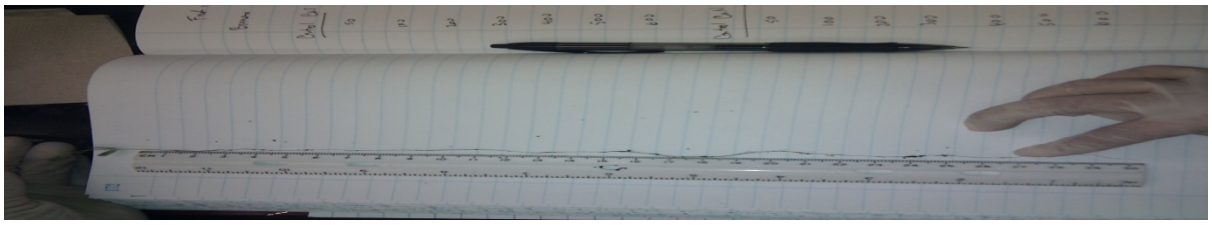


Figure 47. Root measurement (cm) of soil-grown screw bean mesquite plants exposed to 50 ppm of copper sulfate.

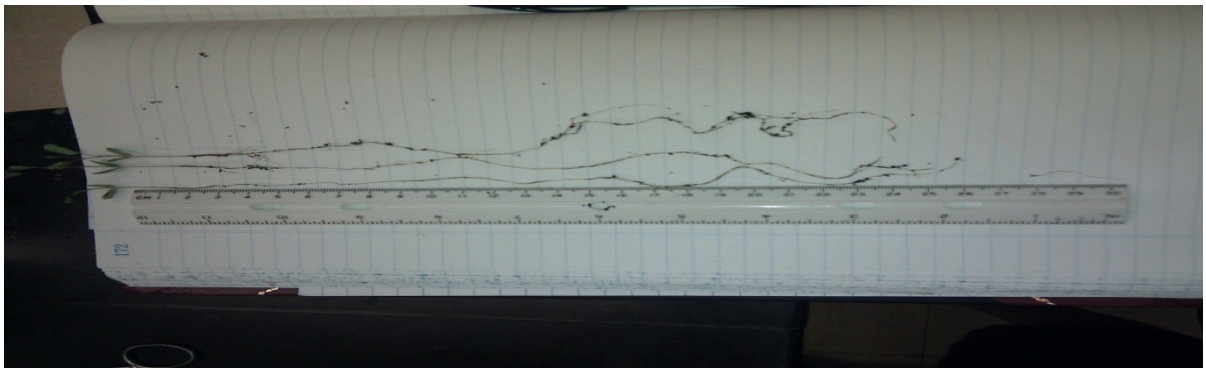


Figure 48. Root measurement (cm) of soil-grown screw bean mesquiteplants exposed to 50 ppm of copper nitrate.

3.13 Germination in Soil

Visually, copper did not have an effect on the growth of the plants within soil for 24 days showing no chlorosis or death. No statistical difference was observed in the germination of seeds in soil exposed with either copper sulfate or copper nitrate (Fig. 49). Germination differences may have been due to individual seed viability. Comparing our soil results to laboratory grown seedlings, we observed more germination in soil than in Petri dishes ($p<0.0001$). However, the 3-way interaction among growth medium, copper type and copper treatment was statistically significant ($p=0.03$), indicating different trends for the comparison of Petri dish vs. soil-grown screw bean mesquite plants across copper type and concentration.

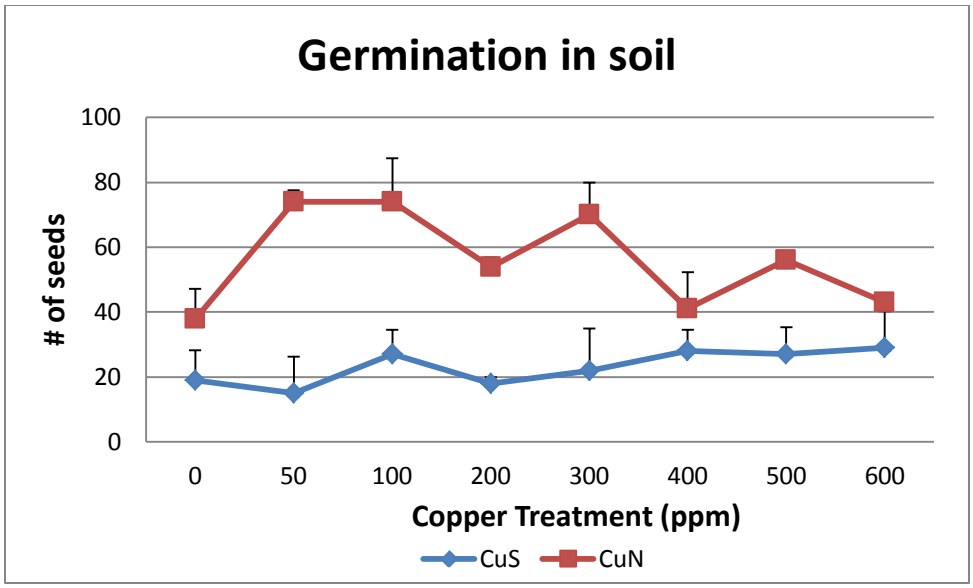


Figure 49. Germination of screw bean mesquite seedlings in soil after copper exposure.

Similar germination rates were observed between copper nitrate and copper sulfate. No statistical difference showed that copper is not affecting germination of screw bean mesquite in soil of young plants. 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 up take 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 plants. Copper concentrations were not measured in germinated seeds.

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. No chlorosis was observed in the 24-day-old seedlings.

3.14 Copper Concentrations in Screw Bean Mesquite

Inductively coupled plasma-optical emission spectroscopy was used to identify the amount of copper collected in screw bean mesquite seeds grown in soil. We quantified the amount of copper for roots, stems, cotyledons and true leaves, totaling 192 samples. We observed a normal copper distribution in the plants, which was very high in the roots and diminished as it traveled up the stem. Figure 50 shows that increasing amounts of copper exposure to the seedling increases the accumulation of copper in the roots. This is true of the pattern observed in stems and cotyledons. Copper sulfate absorption in roots, stems, cotyledons, and true leaves (Fig. 50) showed statistical differences across concentrations of exposure ($P<0.0001$). The roots showed that after 200 ppm of copper sulfate exposure, the absorption was different from the lower concentrations and the control. The linear correlations between copper exposure and copper absorptions are indicative of an active transport mechanism with gradient limitations rather than a simple diffusion uptake.

Copper concentration decreased as it moved up to the stem. We suspect specific compartmentalization of copper via copper-transporters or binding proteins. A general grouping between 50-200 ppm suggests that screw bean mesquite reacts statistically different to copper toxicity at 300-600 ppm levels. We suggest that after 200 ppm of copper, screw bean mesquite's defense mechanisms have become compromised and can no longer regulate the amount of copper translocated.

A 600 ppm copper sulfate exposure for 24 days resulted in a total accumulation of 58,000 ppm of copper. The corresponding copper concentrations were as follows: roots (30,000 ppm), stem (17,000 ppm), cotyledons (11,000 ppm), and true leaves (0 ppm).

Copper sulfate exposure increased the accumulation of copper in the roots, stems, cotyledons, and true leaves. We concluded that the uptake was active transport with gradient limitations due to copper bioavailability. Copper translocation decreased as it moved up to the stem. We suggest that after 200 ppm of copper, screw bean mesquite's defense mechanisms were compromised and could no longer regulate the amount of copper translocated. However, for copper nitrate we identified no translocation of copper to stem, cotyledon, or leaves, which may be indicative of a protective mechanism. We suspect specific compartmentalization of copper via copper-transporters or binding proteins. Copper nitrate (125g/100g water at 20ºC) is more soluble than copper sulfate (32g/100g water), which suggests that copper nitrate would be more readily absorbed. However, in screw bean mesquite we observed more copper sulfate absorption, which leads us to believe that specific cell wall proteins in the roots are facilitating the uptake of copper sulfate, specifically.

Several criteria must be met to be identified as a hyper accumulator of a specific metal: 1) the plant must accumulate stem:root metal ratio of >1; 2) the extraction coefficient (stem/soil) must be >1 (Harrison and Chirgawi, 1989; Rotkittikhun *et al*., 2006); 3) plants must accumulate 10-500 times more metal than in control plants (Allen, 1989; Fifield and Haines, 2000); 4) more than 1000µg/g of copper up taken (Shen and Liu, 1998; Ginocchio and Baker, 2004; Yanqun *et al*., 2004; Bourlarban *et al*., 2006; Rotkittikhun *et al*., 2006). Screw bean mesquite stem:root ratio (16,800/30,774) was 0.55. The extraction coefficient was 28. Experimental plants (600 ppm of copper sulfate) accumulated a total of 58,811 ppm of copper while control accumulated only 1,702 ppm of copper. This is 34.6 times more accumulation than in the control. Screw bean mesquite fulfills three of the criteria to be considered a copper hyper accumulator when grown in soil.

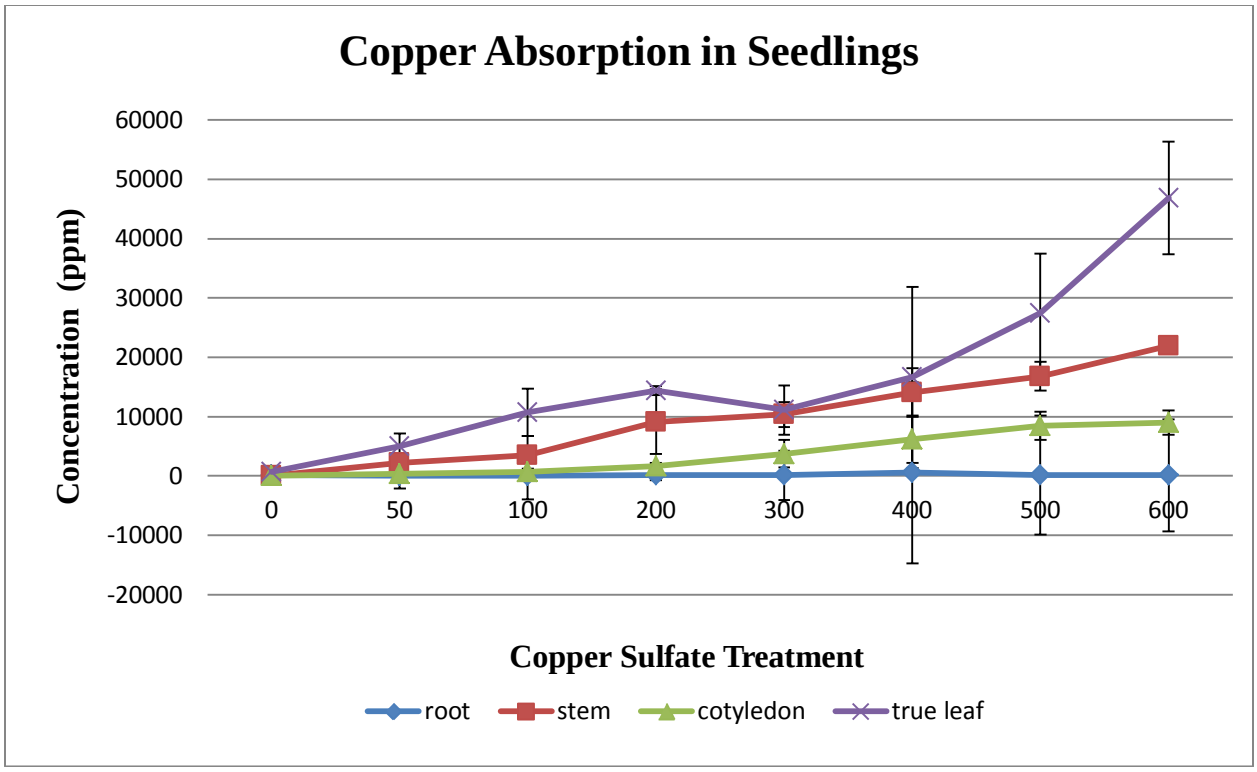


Figure 50. Copper sulfate absorption comparison within screw bean mesquite seedlings.

Figure 51 shows the increasing amounts of copper nitrate in screw bean mesquite seedlings exposed to increasing amounts of copper. 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, absorption was different from the lower concentrations and the control. Copper nitrate in the control roots was 20 ppm, while in the 600 ppm copper nitrate exposed roots there was about 72 ppm. The linear correlations between copper exposure and copper absorptions in the roots were indicative of an active transport mechanism with gradient limitations rather than a simple diffusion uptake.

The overall pattern showed no translocation of copper nitrate in the stem, cotyledons, and true leaves suggesting a complete sequestration and compartmentalization of copper nitrate

within the roots. Immobilization of copper in the roots may be a protective mechanism of screw bean mesquite to reduce toxicity in the photosynthetic mechanism of leaves.

A 600 ppm copper nitrate exposure for 24 days, allowed screw bean mesquite to accumulate only 123 ppm of copper. Screw bean mesquite stem:root ratio was 0.27. The extraction coefficient was 0.33, which is less than 1. Experimental plants (600 ppm of copper nitrate) accumulated a total of 146 ppm of copper while control accumulated only 63 ppm of copper. This is only two times more accumulation than in the control. We concluded that screw bean mesquite is not a hyperaccumulator of copper from copper nitrate.

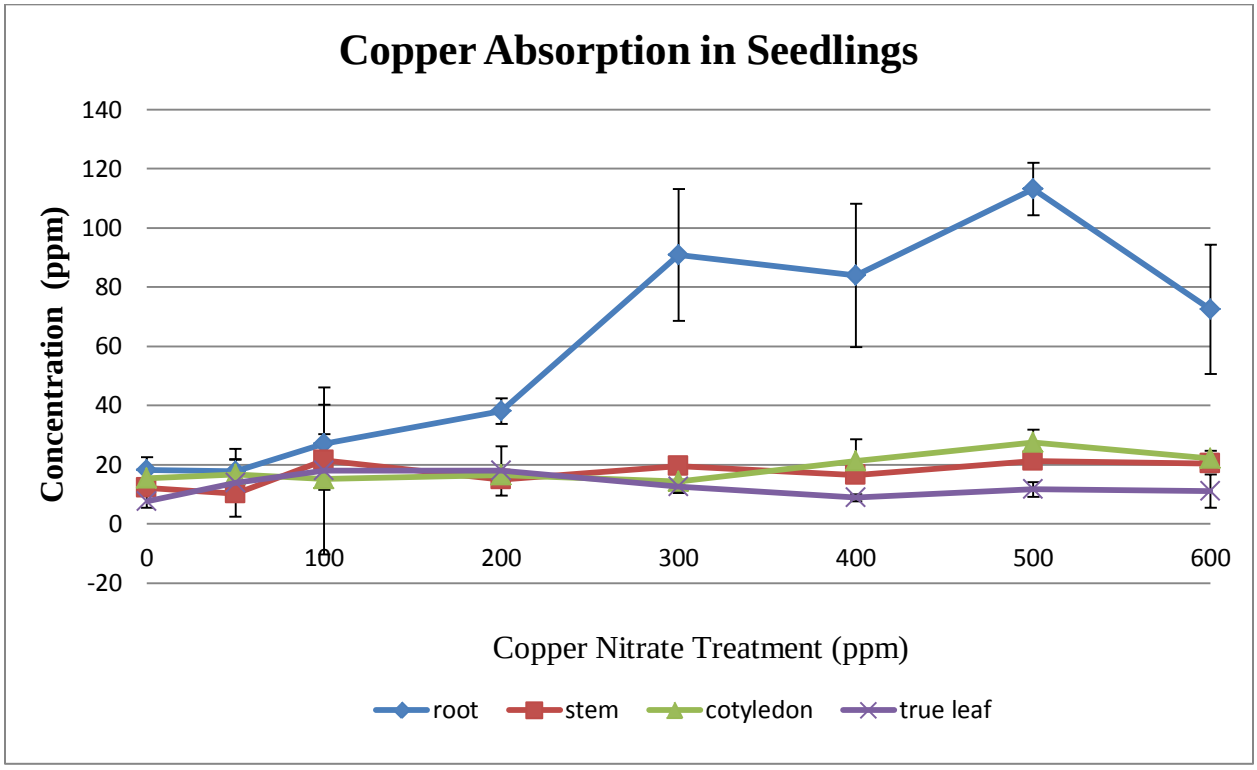


Figure 51. Copper nitrate absorption comparison within screw bean mesquite seedlings.

Screw bean mesquite plants at 24 days of growth were shown to be hyperaccumulators of copper from copper sulfate. Copper uptake is limited by its bioavailability from the soil and the

transport mechanism within the plant (Prasad, 2004). Our results suggest that screw bean mesquite may have evolved a tolerance response to copper sulfate, which may include cysteine-rich proteins for transport.

Screw bean mesquite is not only a hyperaccumulator of copper, but is also tolerant to copper. There is no direct correlation between hyperaccumulation and tolerance (Baker *et al.*, 2000). In general, hyperaccumulators are a minority in metal contaminated regions, where excluders are more widespread (Baker *et al.*, 2000). It is crucial to identify local desert plants that hyperaccumulate metals to have viable clean-up options. We concluded that both copper sulfate and copper nitrate are bioavailable to screw bean mesquite roots. However, phytoextraction of copper from copper sulfate is more successful. More experiments are needed to identify the difference in uptake between copper sulfate and copper nitrate. Phytoextraction of copper in the field will depend on various factors including: growth season, temperature, humidity, soil pH and total organic content of soil (Louma, 1983). All of these factors are interrelated and, therefore, difficult to test in the laboratory. Screw bean mesquite will need to be tested in the field with variant seasons to determine its overall efficiency in cleaning local soils. We predict that the high level of tolerance along with hyperaccumulation of copper sulfate makes screw bean mesquite a viable option for phytoremediation.

Watanabe (1997) determined that phytoremediation costs $0.05 per cubic meter (m3), while removal of soil may cost as high as $30-300/m3. Phytoremediation is slower since plants must grow at their natural rates; however, it is cheaper and more ecological friendly. McGrath (1998) calculated the removal of zinc from crops growing in contaminated soil. Soil contained 100ppm of zinc and *Thlapsi* (pennycress) took up more than 30,000 ppm of zinc, which hypothetically removes 268 pounds and it would take 8.2 years to clean up completely. This is

crucial for hyperaccumulators since plants that do not hyper accumulate metals would only remove approximately 0.9 pounds per year; therefore, taking 2470 years to clean up the same crop area (McGrath, 1998). Copper uptake is different from zinc uptake. Growth rate for pennycress is different from screw bean mesquite. McGrath's study did not test for different zinc compounds. Growth rate in pennycress would not change depending on different zinc compound exposures; however, the rate and amount of uptake does depend on the compound. In our study, we tested two copper compounds: copper sulfate and copper nitrate. Assuming the same as McGrath's study, screw bean mesquite growth rate would not depend on a difference of copper compounds. No data is available in the precise growth rate of screw bean mesquite grown in greenhouses. With the annual biomass accumulation and the growth rate, we could formulate a calculation for pounds per year copper remediation of screw bean mesquite. In conclusion, copper uptake from copper sulfate and copper nitrate is very different. This shows the importance of studying various copper compounds to determine the potential of phytoextraction by the same plant.

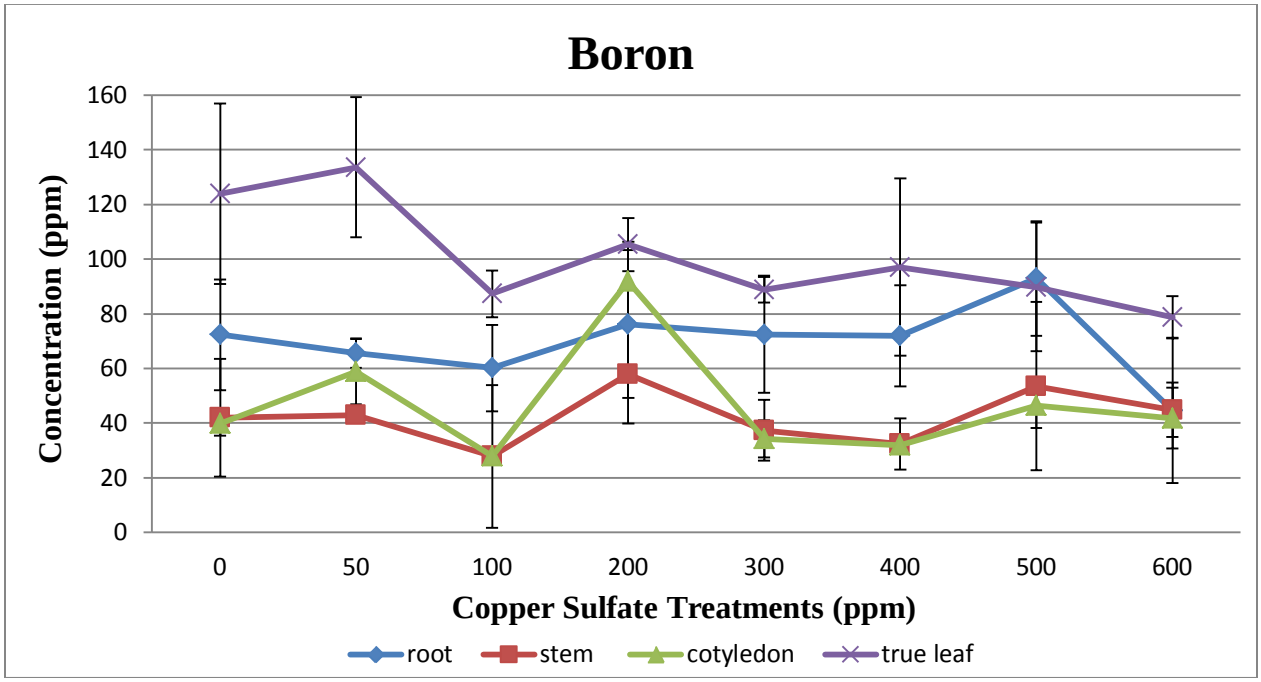
3.15 Nutrient Changes Due To Copper Exposure in Soil-Grown Mesquite Plants

Copper may also interfere with the uptake or distribution of other elements needed for a plant's growth. These elements consist of the macronutrients (calcium, magnesium, potassium, phosphorus, and sulfur) and micronutrients (iron, manganese, zinc, boron, and molybdenum). We read 500 samples 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.

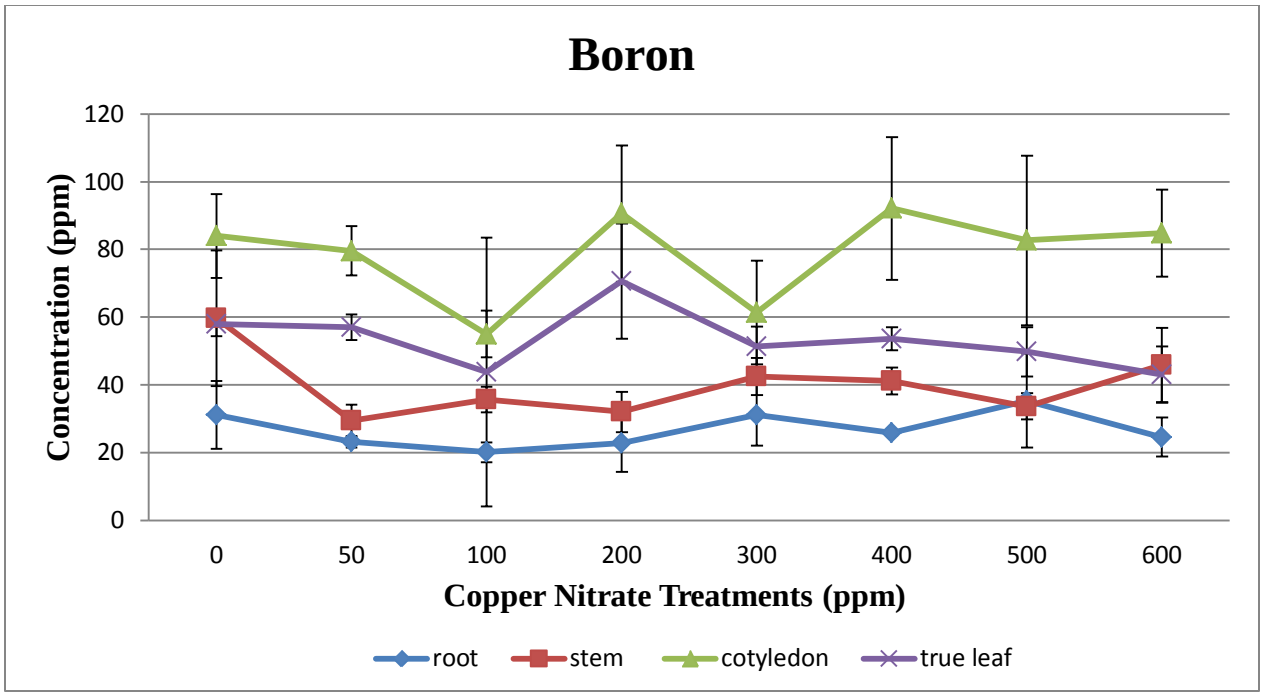
Boron concentrations were different across root, stem, cotyledons and true leaves as copper exposure increased ($p=0.0181$). Similar patterns were observed by copper sulfate and copper nitrate in the effect to boron concentrations ($p=0.5408$). Here we show both copper sulfate and copper nitrate are affecting boron in the same manner. The highest concentration of boron was found in the true leaves followed by roots, stems and cotyledons treated by copper sulfate (Fig. 52). 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 due to copper toxicity.

In copper nitrate treated seedlings, boron concentration, in descending order, is as follows: cotyledons, true leaves, stems and roots (Fig. 53). Unlike copper sulfate, boron concentrations in true leaves did not fluctuate; however, they were half the amount seen in copper sulfate.

Boron decreased with increasing copper sulfate and copper nitrate exposures. 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 suggesting different transportation or tolerance mechanisms. 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 deficiency leads to ultrastructural changes of the cell wall. We observed cell membrane separation after copper sulfate in screw bean mesquite leaves. No ultrastructural changes were observed in leaves exposed to copper nitrate.



A

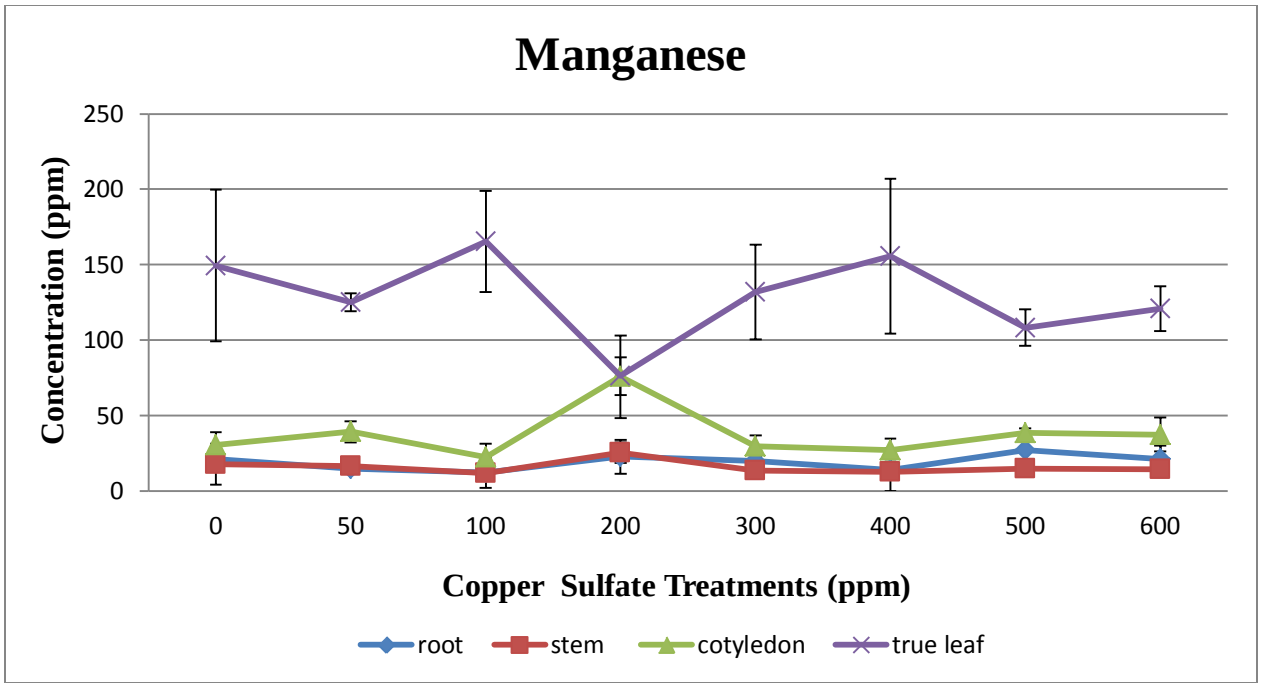


B

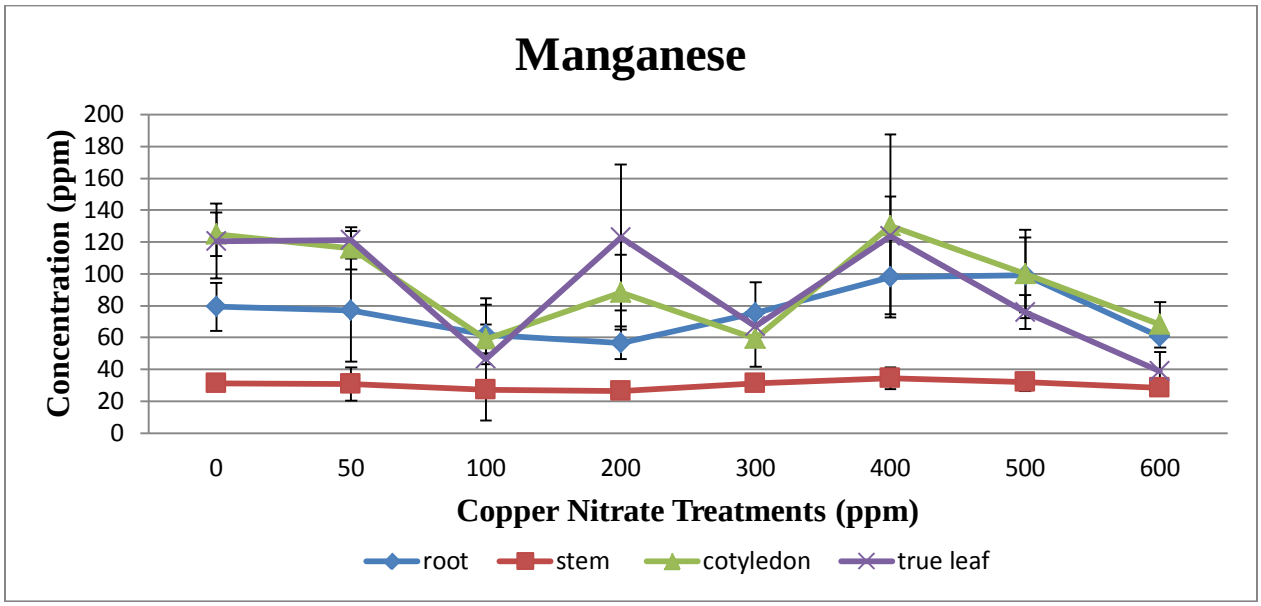
Figure 52. Boron changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper sulfate (A) and copper nitrate (B) exposure.

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. 54). 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. 55). There was a slight decrease, followed by an increase of phosphorus in the roots exposed with copper nitrate. In both copper sulfate and copper nitrate, 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 and subsequent increase at 300-400 ppm, and decreased at 500 ppm of copper sulfate. In copper nitrate, the pattern was reversed. From 100-300 ppm of copper sulfate or copper nitrate something significant is occurring to change the levels of manganese.

Chloroplasts in young plants are the most affected organelles from manganese deficiency (Mengel and Kirkby, 2001). Interveinal chlorosis will be observed with manganese deficiency. Our screw bean mesquite plants did not show any chlorosis after 24 days of growth. Manganese levels, after both copper sulfate and copper nitrate, showed a unique pattern of sharp high and low concentration in leaves. 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 plants. Decreased manganese concentrations result in chlorosis due to limited chlorophyll and poor root development. We did not have an iron decrease in the screw bean mesquite seedlings. We did not test for chlorophyll content due to the absence of chlorosis. Our results show that manganese uptake at the root was normal for both copper sulfate and copper nitrate, which may indicate that limited translocation might have been what caused the low manganese in the true leaves. Rengel (2001) showed that manganese has a poor mobility in the phloem of mature wheat grains. Therefore, we conclude that manganese may have a poor mobility in screw bean mesquite without visible damage to the plant.



A



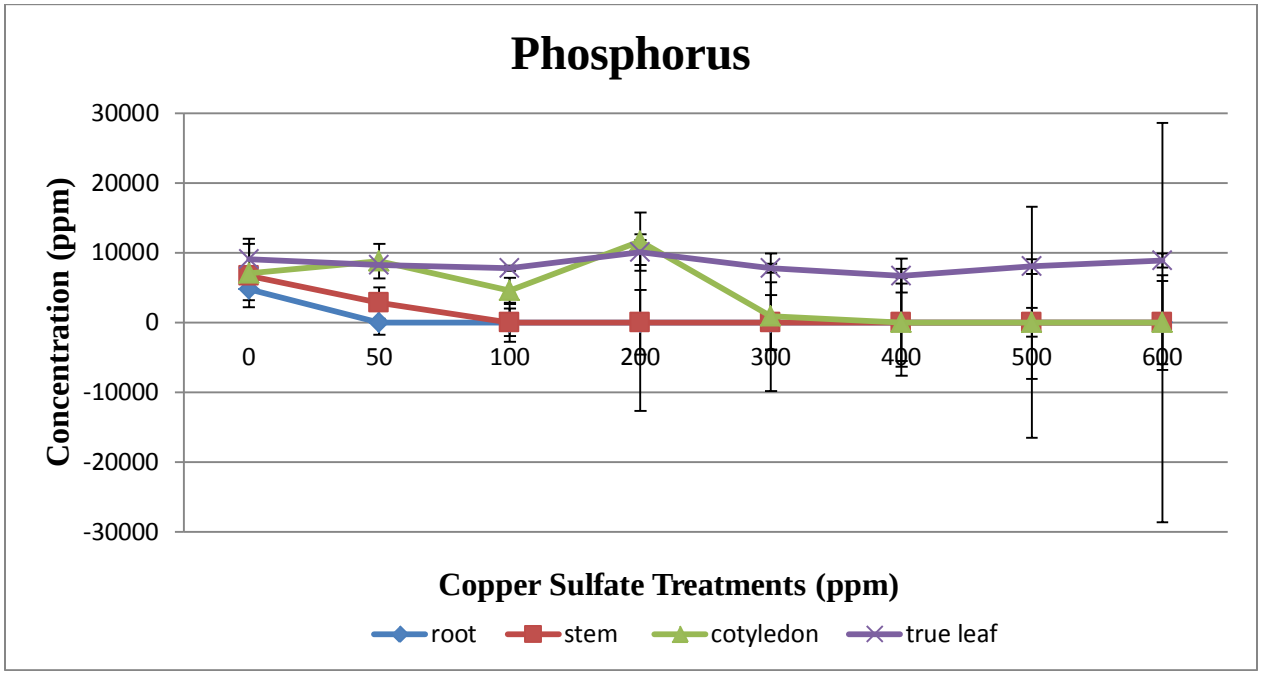
B

Figure 53. Manganese changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper sulfate (A) and copper nitrate (B) exposure.

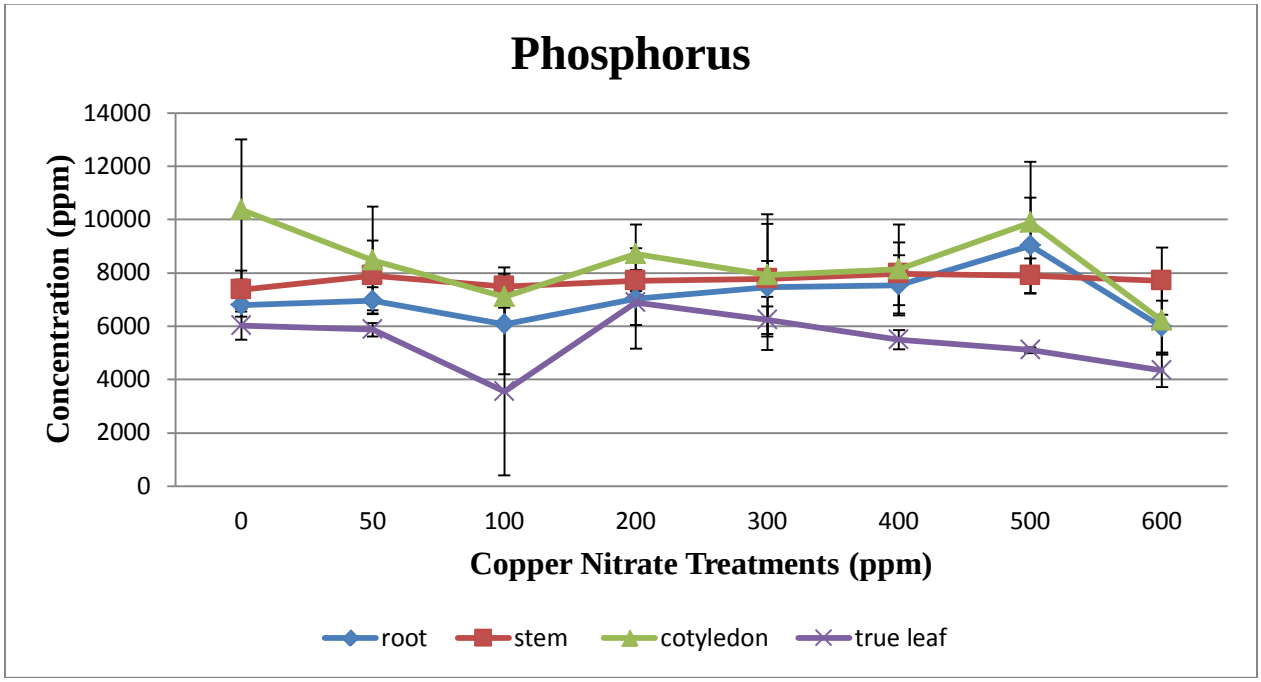
Different 3-way interaction patterns were observed by copper sulfate and copper nitrate in the effect for phosphorus concentrations ($p<0.0001$). Phosphorus was significantly affected by the presence of copper sulfate (Fig. 56). With a low copper sulfate treatment of 50 ppm copper in both roots and stems, phosphorous dropped dramatically to 0 ppm and 3,000 ppm, respectively. After 100 ppm of copper sulfate, both roots and stems had no phosphorus. This is crucial since control roots had almost 5,000 ppm and stems had 6,500 ppm of phosphorus. For cotyledons, phosphorus fluctuated 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 maintains at almost control levels (9,000 ppm) within all copper sulfate exposures of leaves.

Significant changes within seedlings exposed to copper nitrate were seen in cotyledons and true leaves (Fig. 57). 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 ($p<0.0001$). Phosphorus is involved in energy carrying phosphate compounds (ATP and ADP), nucleic acids, coenzymes, and phospholipids. Phosphorus decreased with increasing copper sulfate exposures suggesting loss of energy. We observed ultrastructural damage to cell membranes in the leaf parenchymal cells. Sodium transporters across plasma membranes facilitate the uptake of phosphate and sulfate in the alga *Dunaliella salina* (Meira *et al.*, 2001). This suggests that there is a competition of uptake between phosphate and sulfate uptake. If screw bean mesquite uses sodium channels to transport both

phosphates and sulfates as in *D. salina*, then this may explain the reduction of phosphorus and the increase of sulfur.



A



B

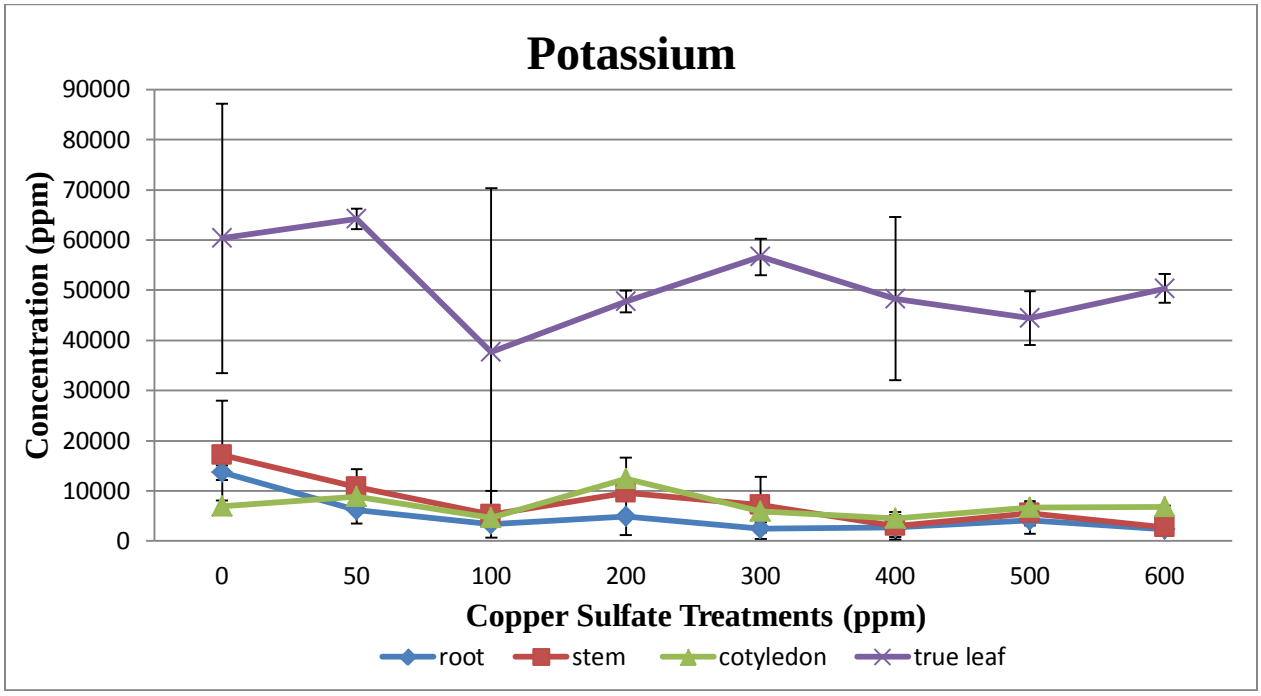
Figure 54. Phosphorus changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper sulfate (A) and copper nitrate (B) exposure.

Similar 3-way interaction patterns were observed by copper sulfate and copper nitrate in the effect 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. 58). Control roots contained about 15,000 ppm of potassium, while the 600 ppm copper sulfate exposed roots had decreased to about 4,000 ppm of potassium. This is a 66% decrease. Similar decrease 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 quickly to 57,000 ppm, an almost 100% recovery.

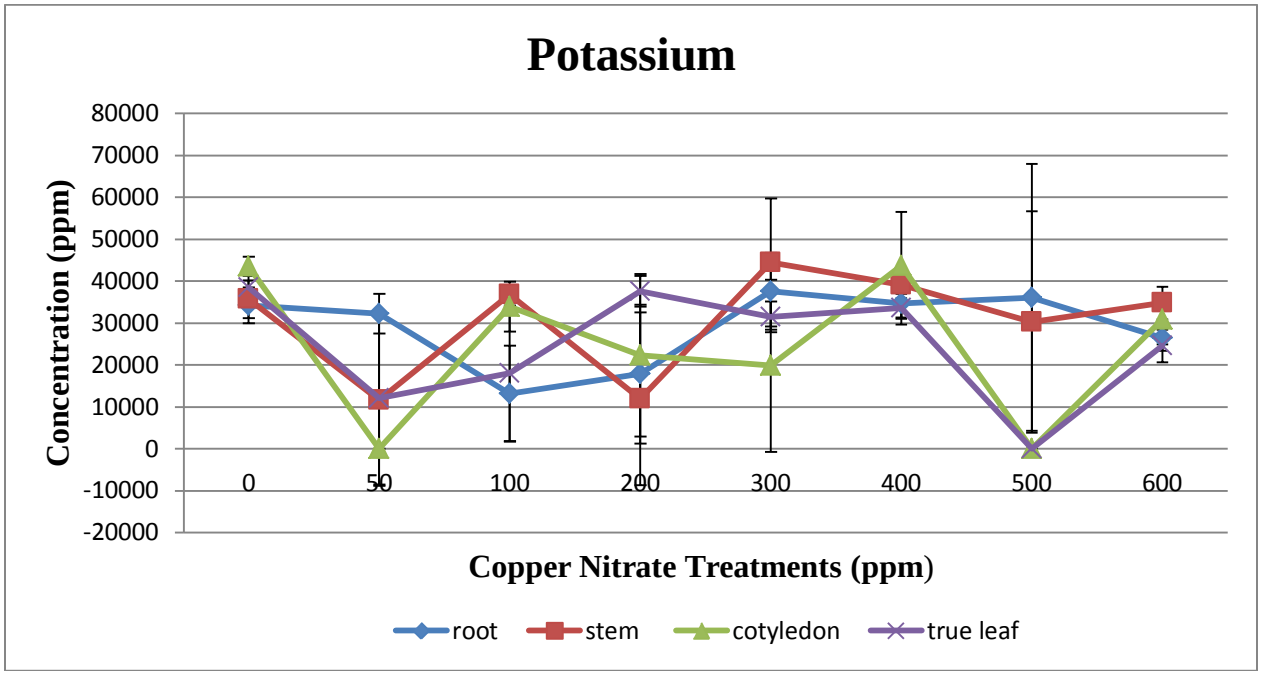
In copper nitrate treated seedlings, potassium fluctuated between 1-45,000 ppm within all parts of the plant (Fig. 59). Control concentration in roots, stems, cotyledons, and true leaves were as follows: 35,000 ppm, 35,000 ppm, 43,000 ppm and 39,000 ppm. At 600 ppm of copper nitrate, potassium was at 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 ($p=0.0144$).

Potassium concentrations slightly decreased after copper sulfate exposure, while potassium concentrations fluctuated with copper nitrate exposure. 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.



A



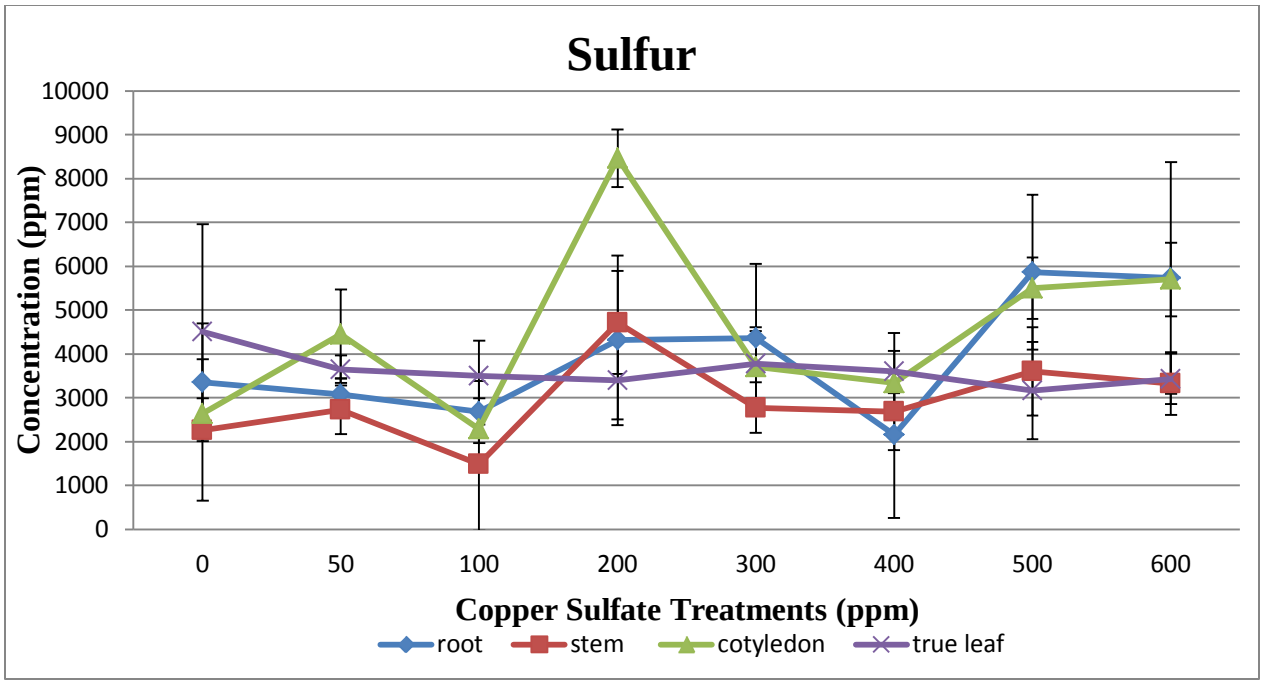
B

Figure 55. Potassium changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper sulfate (A) and copper nitrate (B) exposure.

Similar 3-way interaction patterns were observed by copper sulfate and copper nitrate in the effect to sulfur concentrations ($p=0.0011$). Significant changes within seedlings exposed to copper sulfate were seen in roots, stems, cotyledons and true leaves (Fig. 60). 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 seen only in cotyledons (Fig. 61). 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 ($p=0.0080$).

Cystine, cysteine, and methionine contain sulfur (Baird, 1914). Sulfur concentrations fluctuated in both copper sulfate and copper nitrate exposed screw bean mesquite plants. Overall, there was a slight increase of sulfur after copper sulfate exposures, which may indicate an increase in production of cysteine. Both metallothioneins and phytochelatins are cysteine-rich proteins known to bind copper for sequestration or tolerance mechanisms. Screw bean mesquite seedlings exposed to 600 ppm of copper sulfate accumulated 323% more copper than the controls. This data is comparable to sulfur patterns in screw bean mesquite seedlings grown in soil where seedlings exposed to 600 ppm of copper sulfate accumulated 128% more than the control (Zappala, 2012). This suggests that cysteine production is controlled by variables involved in soil.



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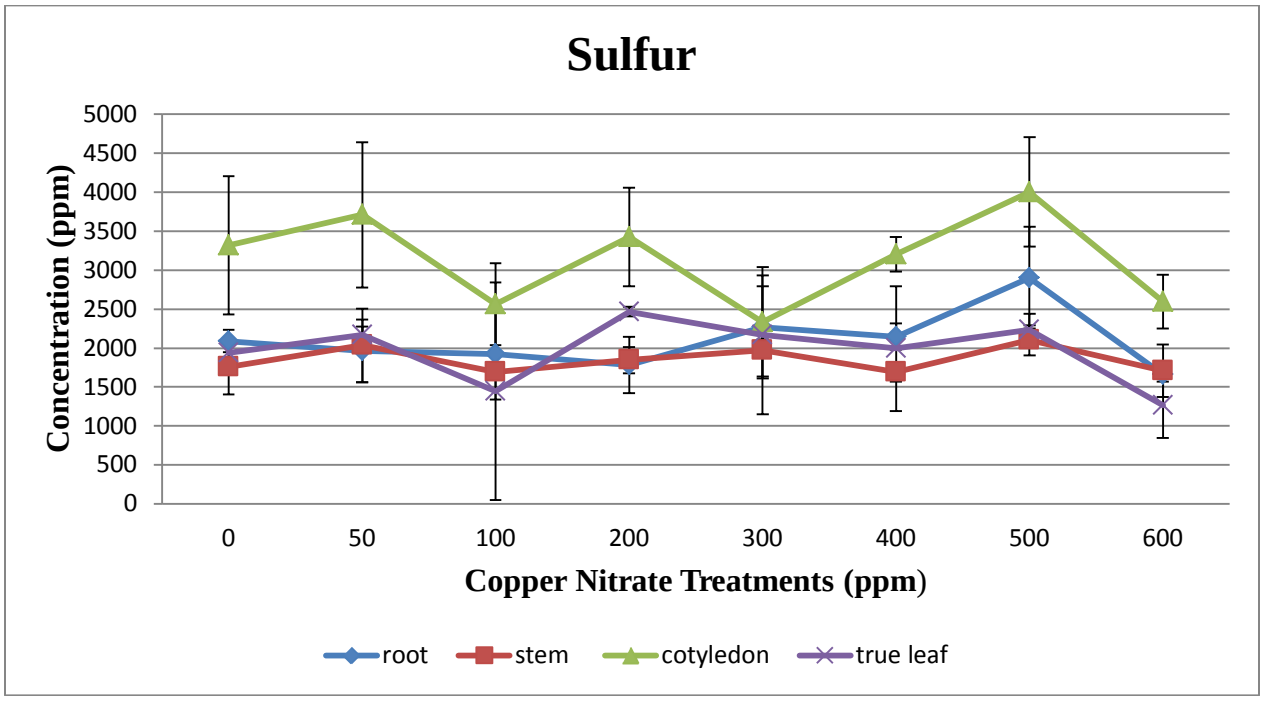


B

Figure 56. Sulfur changes in root (blue), stem (red), cotyledons (green) and true leaves (purple) due to copper sulfate (A) and copper nitrate (B) exposure.

3.16 Ultrastructural and Elemental Analyses

We analyzed the ultrastructural changes of true leaves at higher magnifications using transmission electron microscopy. Figure 57a shows the growing apical tip, while Fig. 57b is a central portion of a mature leaf (200X). Cross sections of a control screw bean mesquite leaf showed a healthy array of parenchymal cells (Fig. 57a). Chloroplasts and vacuoles were uniformly the same shape and size. No visible dark aggregates were observed in the controls. No copper was found in the vacuole or cell wall of the control samples by elemental analysis (Fig. 57b).

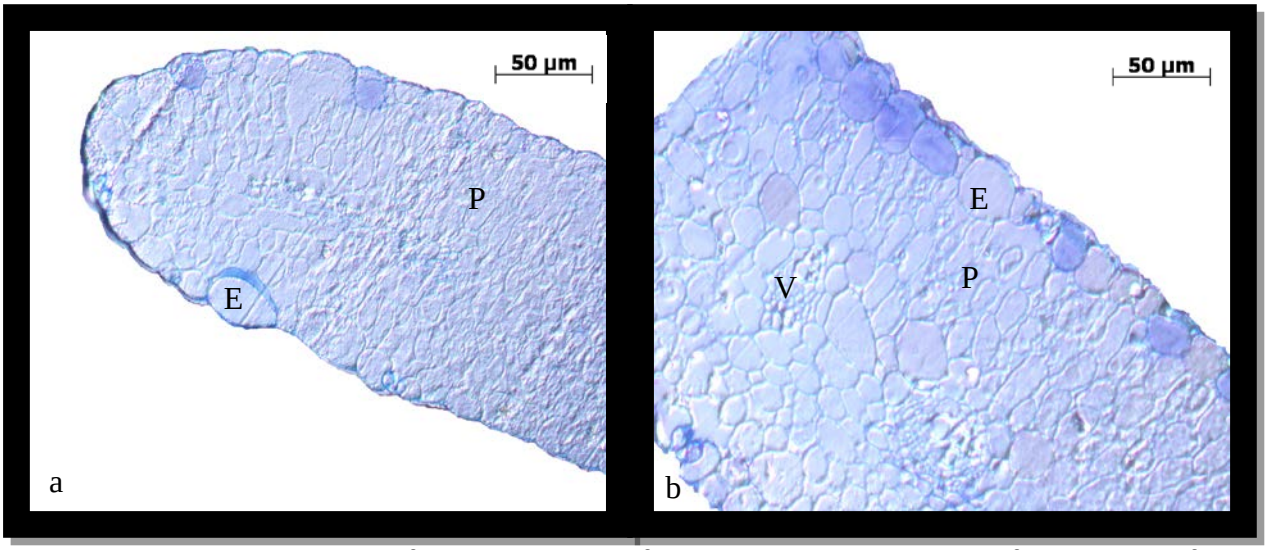
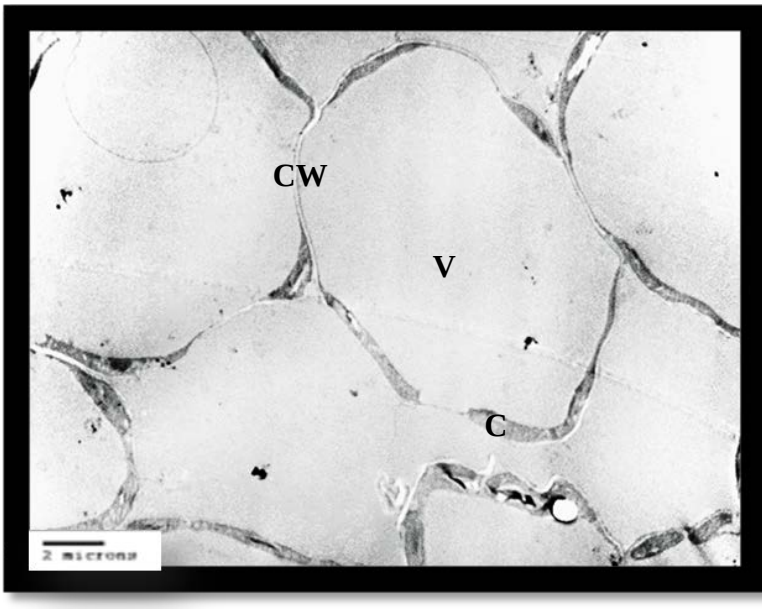
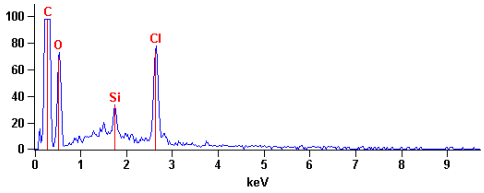


Figure 57. (a) Cross section of a control true leaf (200X); (b) Cross section of a 500 ppm of copper sulfate exposed true leaf (200X) (E:Epidermal; P:Parenchymal;V:Vascular bundle).

Higher magnifications enabled us to see ultrastructural changes that were not visible at the histological level (Fig 58-60). Electron micrographs of soil-grown screw bean mesquite showed dense aggregates along the interior and exterior of cell walls of leaf parenchymal cells. Plasmolysis was evident beginning with 100 ppm of copper sulfate exposure. Starch content increased as the copper exposure increased. At 200 ppm of copper sulfate, a cross section of a leaf showed a dark aggregate accumulating between parenchymal cells (Fig. 59a). 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. 59b). 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. 60a). Chloroplasts were enlarged with abnormal thylakoid membranes. Dark aggregates, between cells and attached to the exterior of cell walls, were present. A higher magnification of another 500 ppm copper sulfate exposed leaf showed swollen chloroplasts along with disarrangement of thylakoid membranes (Fig. 60b). Elemental analysis found copper in the cell wall of a leaf parenchymal cell in screw bean mesquite exposed to 500 ppm copper sulfate (Fig. 60c).

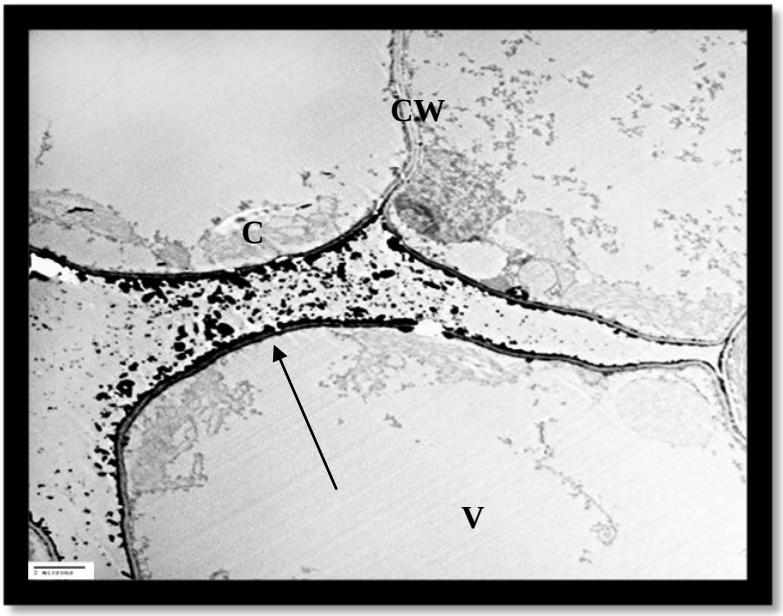
Electron micrographs of soil-grown screw bean mesquite showed dense aggregates along the exterior of the cell walls of leaf parenchymal cells beginning at 200 ppm of copper. Copper does not accumulate in chloroplasts, but in the apoplast and in the vacuole (Quartacci *et al.*, 2000; Baryla *et al*., 2001; Patsikka *et al.*, 2002). Both plasmolysis and increasing starch content were observed in both copper sulfate and copper nitrate exposed seedlings. Toxic concentrations of copper have negatively affected carbohydrate metabolism (Roito et al., 2005) in *Pinus sylvestris*.

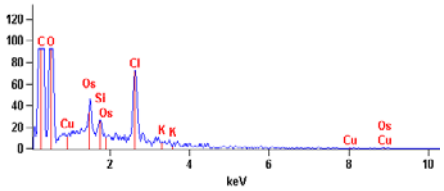




a. **b.**

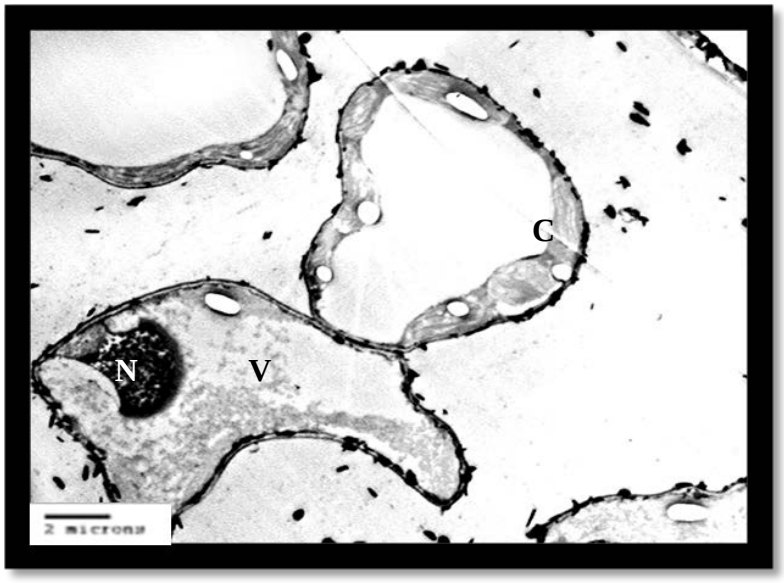
Figure 58. (a) Electron micrograph of control section of leaf parenchymal cells (4000X); (b) Elemental analysis of the cell wall of a control leaf parenchymal cell (C:Chloroplast; V:Vacuole; and CW:Cell Wall).



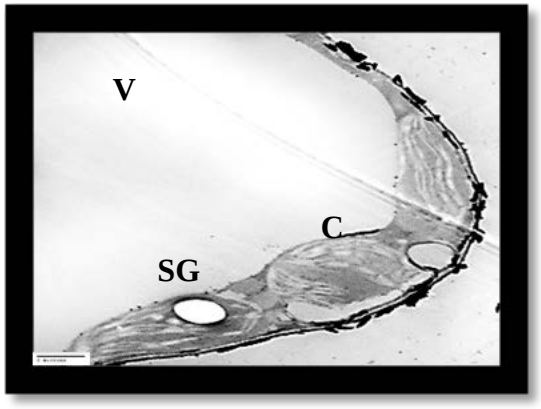


a. **b.**

Figure 59. (a) 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); (b) Elemental analysis of the cell wall of a leaf parenchymal cell exposed to 200 ppm of copper sulfate.



a.



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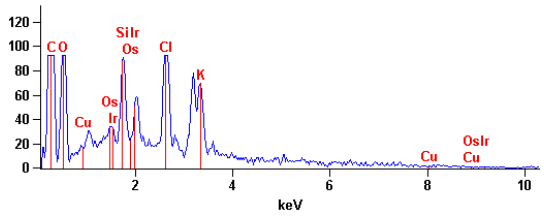


c.

Figure 60. (a) Electron micrograph of 500 ppm of copper sulfate section of leaf parenchymal cells (5000X) (C:Chloroplast; and V:Vacuole); (b) Electron micrograph of 500 ppm of copper sulfate section of leaf parenchymal cell (12,000 X) (C:Chloroplast; V:Vacuole; and SG:Starch Grain); (c) Elemental analysis of the cell wall of a leaf parenchymal cell exposed to 500 ppm of copper sulfate.

In copper nitrate exposed seedlings, we did not find dark aggregates within parenchymal cells in the control leaves or in the experimental. ICP-OES results confirmed the absorption of copper nitrate by the roots; however, no copper nitrate was translocated to the leaves.

Chapter 4: CONCLUSIONS

Many factors must be determined in order to utilize a plant for phytoremediation. We have determined that screw bean mesquite (*Prosopis pubescens*) seeds are viable even after 30 years of storage. This enables their use in large phytoremediation projects that require more seeds than the screw bean mesquite can produce annually. Screw bean mesquite is similar in its seed production and germination rates as Honey mesquite (*Prosopis glandulosa*). Honey mesquite produces a large amount of seeds; however, only a small percentage produce mature trees (Scifres *et al*., 1971). This limits the amount of viable seeds per year. Stored seeds would increase the germination percentage for the phytoremediation of a field.

Both copper sulfate and copper nitrate decreased the germination rate of screw bean mesquite seeds in Petri dish "greenhouses". However, copper nitrate toxicity most affected germination rate. There is no information available to explain the reduction of germination rates by copper in screw bean mesquite. Generally, germination rates decreased with increasing copper concentrations, which shows a dose-dependency.

We also identified that the seed coat of screw bean mesquite is the first barrier against copper toxicity. There was a greater copper accumulation in the seed coat vs. the embryo suggesting that copper was sequestered preventing damage to the germinating seed. This exemplifies the importance of hard coats in desert plants and adds another advantage to dormancy mechanisms of hard coats. In soil grown screw bean mesquite, germination rates were not affected by either copper sulfate or copper nitrate. Seedlings did not take up nutrients from the soil rather nutrients were taken from their reserves, which may suggest no uptake of copper by seeds within soil perhaps due to the protective value of the seed coat. Screw bean mesquite in soil had a 20-25% germination rate.

Physiological studies showed that root length did not decrease with copper exposure in soil samples. However, biomass decreased with increasing copper exposure suggesting that size of root, stem and cotyledons may have been reduced. Histological studies of the cotyledons showed that cell areas increased with copper exposure. In conclusion, physiological and germination studies determined that copper nitrate was more harmful than copper sulfate to screw bean mesquite seedlings.

We also determined the threshold of toxicity. Screw bean mesquite exposed to eight days of copper sulfate or copper nitrate showed no signs of toxicity under 200 ppm of copper exposure, suggesting that fields with copper contamination of less than 200 ppm can be phytoremediated with screw bean mesquite seedlings.

We examined the effects of copper on the photosynthetic pigments of screw bean mesquite cotyledons. Previous studies have shown a decrease of chlorophyll pigments in cotyledons exposed to heavy metals (Mihoub *et al*., 2005; Sfaxi-Bousbih *et al*., 2010). We hypothesized that chlorosis may be due to the changes in chlorophyll a and b levels. Copper sulfate did not affect any of the four photosynthetic pigments suggesting that chlorosis may be due to the decrease in magnesium content. After copper nitrate exposure, chlorophyll a levels decreased, unmasking the yellow/orange pigments from carotene and xanthophyll and causing chlorosis in Petri dish seedlings.

We measured copper and nutrient concentrations within seedlings. Below, there is a table (Table 1) summarizing our results from the copper absorption of screw bean mesquite seedlings. It is evident that screw bean mesquites, in general, uptake more copper sulfate and copper nitrate in laboratory Petri dish “greenhouses”. In soil, the bioavailability of copper is crucial and determines how much copper may be available for uptake. Our soil results show that

copper sulfate is easily absorbed by screw bean mesquite roots. Solubility differences between copper from copper sulfate and copper nitrate may be involved. Copper nitrate (125g/100g water at 20ºC) is more soluble than copper sulfate (32g/100g water), which suggests that copper nitrate would be more readily absorbed. In this case, we concluded that copper uptake is not driven by simple diffusion, but active uptake mediated by transporters (Prasad, 2004). Screw bean mesquite may have evolved copper transporters specific to copper sulfate and not to copper nitrate. Further verification of these observations is needed to determine if there are different phytoextraction potentials within the same plants. Screw bean mesquite phytoextracted only 0.21% of copper from copper nitrate compared to copper sulfate. We demonstrated that *Prosopis pubescens* is a hyperaccumulator of copper from copper sulfate and possibly feasible option to remediate fields with up to 200 ppm of copper sulfate.

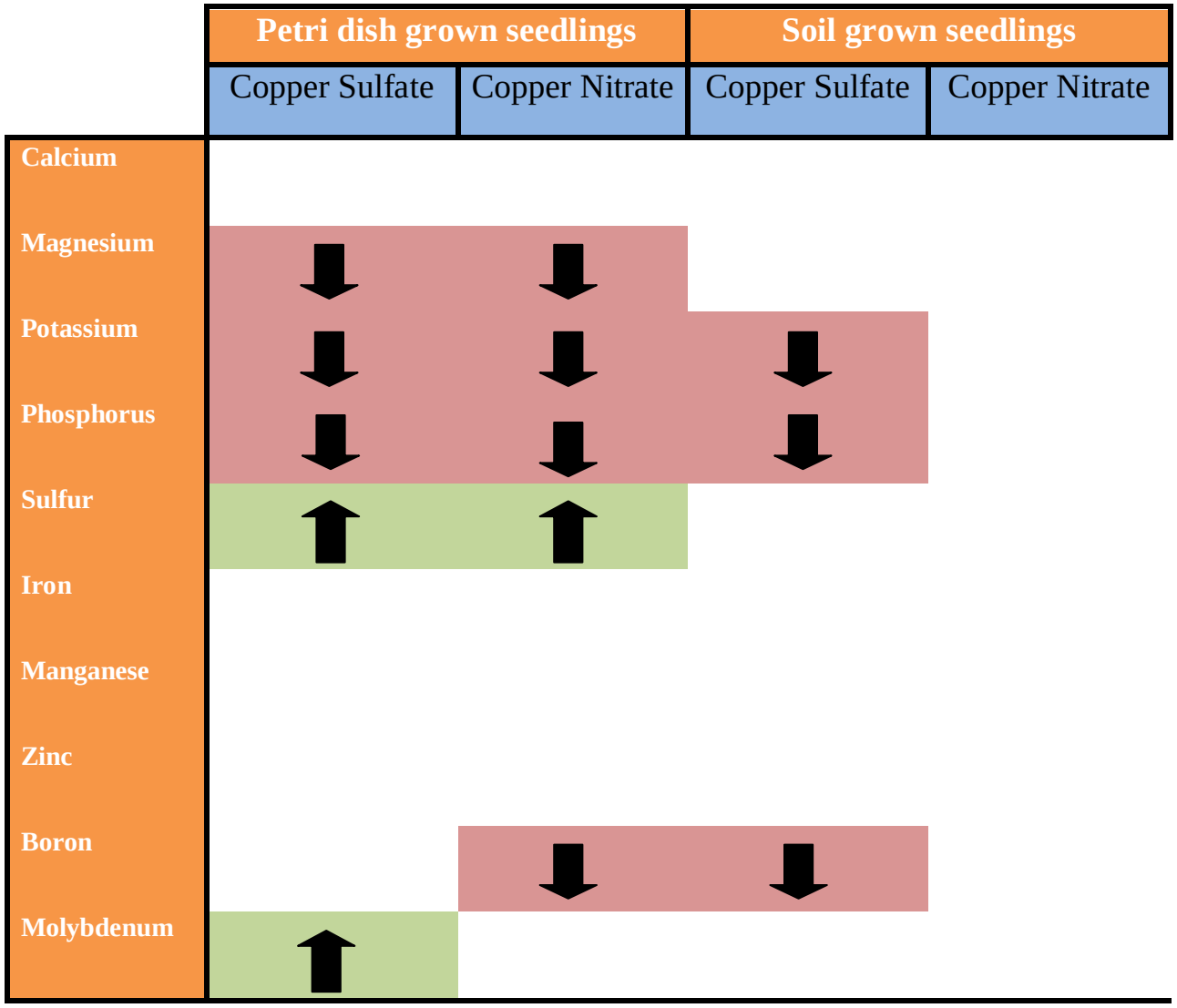
Table 1. Copper absorption in Petri dish grown and soil grown screw bean mesquite seedlings.

	Petri dish grown seedlings		Soil grown seedlings	
	Copper Sulfate	Copper Nitrate	Copper Sulfate	Copper Nitrate
Roots	47,000 ppm	27,500 ppm	31,000 ppm	70 ppm
Stems	23,000 ppm	21,000 ppm	17,000 ppm	20 ppm
Cotyledons	9,000 ppm	16,000 ppm	11,000 ppm	20 ppm
True leaves	---	---	20 ppm	11 ppm

We also quantified micro and macro nutrient concentrations and their changes due to copper presence. Table 2 summarizes the nutrient changes observed in screw bean mesquite seedlings. Copper did not change calcium, iron, manganese and zinc concentrations. Magnesium, potassium, phosphorus decreased, while sulfur increased in Petri dish grown seedlings exposed

to copper sulfate and copper nitrate. Copper sulfate in Petri dish grown seedlings decreased molybdenum, which is involved in nitrogen fixation. This did not occur in soil grown seedlings. Sulfur is a key component of cysteine–rich proteins like metallothioneins and phytochelatins, which are known to be increased in copper presence (Baird, 1914). Soil dynamics are complex; however, we can suggest that soil grown seedlings may be more tolerant and more equipped to tolerate copper than laboratory grown seedlings.

Table 2. Micro and Macro Nutrient concentrations after copper presence in Petri dish and soil grown screw bean mesquite seedlings.



Finally, we identified various ultrastructural changes of cotyledons and true leaves due to the presence of 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). We observed ultrastructural changes including: membranes separating from cell walls, denser cytoplasm with dark aggregates in epidermis, parenchyma and developing phloem cells of cotyledons, as well as, swollen chloroplasts with a disarray of thylakoid membranes. Plasmolysis was evident starting at 100 ppm of both copper sulfate and copper nitrate in cotyledons of Petri dish "greenhouses". Elemental analysis confirmed the presence of copper in experimental samples.

In conclusion, we identified screw bean mesquite as a hyperaccumulator of copper from copper sulfate. Screw bean mesquite is able to tolerate up to 200 ppm of copper without any outward symptoms of toxicity. Seedlings are a viable option in the application for phytoremediation. Screw bean mesquite grown in soil may compensate for high copper concentrations. If seedlings remediate the contaminated field and are consequently removed, screw bean mesquite will not mature and produce seeds. Without seeds, screw bean mesquite will not be genetically modified and produce new hybrids.

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APPENDIX

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Copper isotope fractionation by desert shrubs

Jesica U. Navarrete [a,*], Marian Viveros [b], Joanne T. Ellzey [b], David M. Borrok [a]

[a] University of Texas at El Paso, Department of Geological Sciences, 500 W. University Ave, El Paso, TX 79968, USA
[b] University of Texas at El Paso, Department of Biological Sciences, El Paso, TX 79968, USA

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ABSTRACT

Copper has two naturally occurring stable isotopes of masses 63 and 65 which can undergo mass dependent fractionation during various biotic and abiotic chemical reactions. These interactions and their resulting Cu isotope fractionations can be used to determine the mechanisms involved in the cycling of Cu in natural systems. In this study, Cu isotope changes were investigated at the organismal level in the metal-accumulating desert plant, *Prosopis pubescens*. Initial results suggest that the lighter Cu isotope was preferentially incorporated into the leaves of the plant, which may suggest that Cu was actively transported via intracellular proteins. The roots and stems show a smaller degree of Cu isotope fractionation and the direction and magnitude of the fractionations was dependent upon the levels of Cu exposure. Based on this and previous work with bacteria and yeast, a trend is emerging that suggests the lighter Cu isotope is preferentially incorporated into biological components, while the heavier Cu isotope tends to become enriched in aqueous solutions. In bacteria, plants and animals, intracellular Cu concentrations are strictly regulated via dozens of enzymes that can bind, transport, and store Cu. Many of these enzymes reduce Cu(II) to Cu(I). These initial results seem to fit into a broader picture of Cu isotope cycling in natural systems where oxidation/reduction reactions are fundamental in controlling the distributions of Cu isotopes.

1. Introduction

Copper has emerged as an element of central importance in the biogeosciences due to its ubiquity in both biological and geologic systems and its ability to transfer from one to the other. Copper is a trace metal nutrient for prokaryotes and eukaryotes, as it is needed in trace amounts for a number of metabolic functions and serves as a prosthetic group in metalloproteins. Copper is present in the Earth's crust at ppm concentrations (Parker, 1967), and is found mainly in its oxidized form, Cu(II), in natural waters. Under the circumneutral pH of most natural systems, dissolved Cu(II) is present in organic or inorganic ligand-bound complexes. In biological systems both Cu(II) and Cu(I) are bound, in a variety of coordination states, to specific proteins and enzymes (Bertini et al., 2010).

Cu has two naturally occurring stable isotopes of masses 63 and 65 which can undergo mass dependent fractionation during chemical reactions like surface adsorption, aqueous complexation, mineral precipitation/dissolution, and redox reactions in biologic and geologic systems. For example, adsorption onto mineral surfaces and complexation with dissolved humic acids has been shown to result in an enrichment of the heavier Cu isotope onto the surface or dissolved complex (Balistrieri et al., 2008; Pokrovsky et al., 2008; Bigalke et al., 2010). Conversely, internalization of Cu by bacteria and other microorganisms has been found to preferentially incorporate the lighter Cu isotope into the biologic component (Zhu et al., 2002; Navarrete et al., 2011). These interactions and their resulting Cu isotope fractionations can be used as biogeochemical tools to determine the biological and/or geochemical mechanisms involved in the cycling of Cu in natural systems. These interactions, however, may not be the only mechanisms by which Cu isotopes are re-distributed in the environment. Copper isotopes may be further fractionated in plant systems during active Cu transport from roots to leaves and as isotopic changes are transferred up trophic levels through compartmentalization into higher eukaryotes.

Here some initial Cu isotope results are presented from a study focused on tracking Cu isotope changes at the organismal level in plants. Copper isotopes were measured in roots, stems and leaves in the early stages of growth of *Prosopis pubescens* (Screwbean mesquite), a common desert plant known to accumulate metals (Assadian and Fenn, 1999). These results are compared to previous work and the mechanisms of Cu isotopic fractionation in natural systems discussed.

2. Methods

Plant experiments with *P. pubescens* were conducted in triplicate. Seeds were germinated for 8 days in Petri dishes, which

* Corresponding author.
E-mail address: jnavarrete2@miners.utep.edu (J.U. Navarrete).

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contained filter paper moistened with nutrient solution. Seedlings were then transferred to another Petri dish where they rested on a moistened filter paper with nutrient solution amended with Cu-nitrate. After a period of 8 days, roots, stems and leaves (cotyledon) were harvested and washed with a weak HNO_3 solution (2%) to remove any surface-bound, residual Cu. Roots, stems and leaves were then dried in a drying oven, weighed for dry mass, and digested with a solution of ultrapure 70% HNO_3 and ultrapure 30% H_2O_2. Digests were analyzed using an ICP–OES instrument to measure Cu concentrations, and splits of these samples were prepared for isotopic analysis through ion-exchange chromatography (Borrok et al., 2007; Pribil et al., 2010).

Detailed procedures for preparation of all samples for isotopic analysis have been published previously (Navarrete et al., 2011) and are only briefly outlined here. All isotopic analyses were performed at the Center for Earth and Environmental Isotope Research at the University of Texas at El Paso using a Nu Instruments™ MC–ICP–MS. Each sample was bracketed by the SRM 976 NIST Cu standard and corrections for mass bias were made through standard–sample–standard bracketing. Results are presented in delta notation (parts per thousand) relative to the average of the two bracketing standards as presented in Eq. (1):

$$\delta^{65}Cu = \left(\frac{^{65/63}Cu_{sample} - {}^{65/63}Cu_{ave\ std}}{^{65/63}Cu_{ave\ std}} \right) \quad (1)$$

The average 2σ uncertainty based on replicate measurements of unknowns was 0.15‰.

3. Results and discussion

The initial Cu isotope data for the roots shows a modest enrichment in the lighter Cu isotopes at lower concentrations (50–100 mg/L addition of Cu on the filter paper) while no substantial fractionation was observed (largely within analytical uncertainty) when higher concentrations of Cu were added to the filter paper. A similar trend was observed in the stem. The stem incorporated the lighter Cu isotope below a starting concentration of ~400 mg/L of Cu. Furthermore, there appears to be a roughly linear trend of increasing $\delta^{65}Cu$ over the range of 0 to 400 mg/L Cu. However, above this threshold, the $\delta^{65}Cu$ of the stem became slightly heavier than the starting solution. The leaves of the plant show enrichment of the lighter Cu isotope over the entire range of Cu concentration; however, the magnitude of the fractionations are more pronounced at starting Cu concentrations below ~400 mg/L.

These results demonstrate that uptake of Cu by *P. pubescens* results in substantial fractionation of Cu isotopes and that the direction and magnitude of the fractionations are influenced by the amount of available Cu. Additionally, the germination rate of the seeds dropped off dramatically at a starting concentration of ~400 mg/L Cu, suggesting this might be a toxic threshold for the plant. Because there are key differences in $\delta^{65}Cu$ above and below this threshold in the roots, stems, and leaves, it seems likely that fundamental changes in Cu transport and/or Cu detoxification pathways are reflected in the results. Further work (and data collection) will be necessary to link the isotopic data to these changes.

4. Broader implications

Although only a few investigations exist, a trend appears to be emerging where the lighter Cu isotope tends to be preferentially incorporated into biological components, while the heavier Cu isotope tends to become enriched in aqueous solutions. Fig. 1 presents ranges of Cu isotope data from selected studies to illustrate this point. Although kinetic fractionation of Cu isotopes during incorporation into biological components is probably an important factor controlling this trend, it is also likely that reduction of Cu(II) to Cu(I) plays a major role (Zhu et al., 2002; Navarrete et al., 2011). In bacteria, plants, and animals, intracellular Cu concentrations are strictly regulated via dozens of enzymes that can bind, chaperone, transport, and store Cu. Many of these enzymes reduce Cu(II) to Cu(I) prior to incorporation by proteins (Bertini et al., 2010). Because there is a large amount of structural and functional

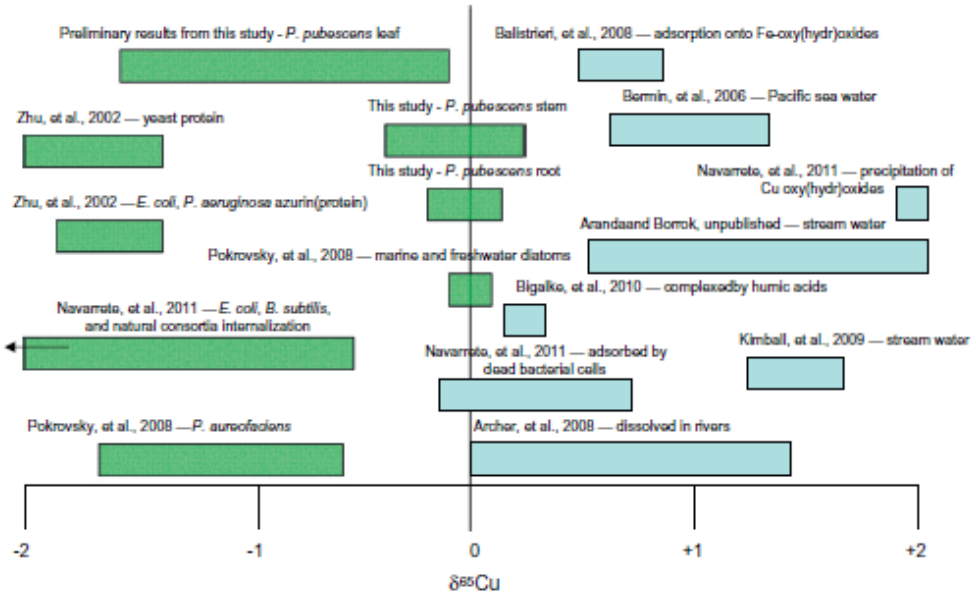

Fig. 1. Reported $\delta^{65}Cu$ for aqueous and biological Cu from selected studies. For experimental systems, the data are normalized to a starting composition of 0‰ $\delta^{65}Cu$. For natural systems, the starting $\delta^{65}Cu$ is likely near 0‰, the approximate crustal average.

conservation among metal proteins in bacteria, yeast, plants, and humans (Peña et al., 1999), reduction of Cu(II) to Cu(I) is probably a universal factor in controlling Cu isotopes in biologic systems. Conversely, others have demonstrated that the oxidation of Cu(I) to Cu(II) during sulfide mineral weathering is an important factor in controlling the heavy δ^{65}Cu of some natural waters (Fernandez and Borrok, 2009; Kimball et al., 2009).

5. Conclusions

The investigation of Cu isotopes during uptake by plants, suggests that in general the lighter Cu isotope is preferentially incorporated into the leaves, probably during active transport by intracellular proteins. In comparison, Cu associated with the roots is not substantially fractionated while the Cu isotope fractionation associated with the stems was variable. These initial results may fit into a broader picture of Cu isotope cycling in natural systems (Fig. 1) where oxidation reactions associated with sulfide weathering and reduction reactions associated with biologic incorporation appear to be fundamental in controlling the distributions of Cu isotopes.

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CURRICULUM VITAE

Marian Nicte Viveros was born on February 27th, 1979 in Mexico City, Mexico to Ezequiel Viveros Maza and Maria Guadalupe Gomez Perez. She graduated within the top 10% of the El Paso High School class of 1997. She obtained a Bachelor of Arts in Anthropology and a Bachelor of Science in Biology with minors in Chemistry, Biochemistry, Human Biology, and Molecular Biology in 2002 from New Mexico State University, Las Cruces, NM. She obtained a Master of Science in Cell and Organismal Biology in 2004 from New Mexico State University. Her research experience began early in her sophomore year as an undergraduate. She has been awarded several fellowships including MBRS, RISE, AGEP (at NMSU and UTEP), which provided research and tuition support. She has also been the recipient of several scholarships and honors at her university and at national conferences. She supervised the Analytical Cytology Core Facility, UTEP-BBRC, from September 2004 to September 2012. She married Mark Matthew Zappala on December 4th, 2010 and gave birth to Juliana Catherine Zappala on January 20th, 2012.

This is a list of articles published, submitted, or in preparation during her doctoral work:

1. Arias, A., J., Peralta-Videa, J.R., Ellzey, J.T., Minghua Ren' Viveros, M.N., Gardea-Torresdey, J.L. 2010. Effects of *Glomus desertícola* inoculation on *Prosopis*: Enhancing chromium and lead uptake and translocation as confirmed by x-ray mapping, ICP-OES and TEM techniques. *Environmental and Experimental Botany* (68: 139-148).
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9. Post Doc Institute Conference, Howard University, Washington D.C. (2008)
10. Preparing Future Faculty Institute, University of Texas at El Paso, El Paso, TX. (2008)
11. HUTEP AGEP Retreat, Mescalero, NM. (2007)
12. North Carolina Opportunity through Education Alliance Day, Greensboro, NM. (2007)

Permanent address: 636 Esmeralda Armendariz

El Paso, Texas 79932

This dissertation was typed by Marian Nicte Zappala (Maiden name Viveros)