

## Effects of copper on chlorophyll, proline, protein and abscisic acid level of sunflower (*Helianthus annuus* L.) seedlings

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**Abstract:** The effect of copper chloride (CuCl<sub>2</sub>) on the level of chlorophyll (a+b), proline, protein and abscisic acid in sunflower (*Helianthus annuus* L.) seedlings were investigated. Control and copper treated (0.4, 0.5 and 0.6 mM) seedlings were grown for ten days in Hoagland solution. Abscisic acid content was determined in root, shoot and leaf tissues of seedlings by HPLC. Copper stress caused significant increase of the abscisic acid contents in roots, shoots and leaves of seedlings. The increase was dependent on the copper salt concentration. Enhanced accumulation of proline in the leaves of seedlings exposed to copper was determined, as well as a decrease of chlorophyll (a+b) and total protein ( $p < 0.05$  or  $p < 0.01$ ). It was observed that the level of chlorophyll (a+b) and total protein ( $p < 0.05$  or  $p < 0.01$ ) remarkably decreased as copper concentration increased to 0.6 mM, although the levels of proline and abscisic acid in the leaves of plants were increased - a dose-dependent behavior. The same trends were also observed with the level of abscisic acid of stems and roots. Copper has dose-dependent effects on chlorophyll, proline, protein and abscisic acid level of sunflower (*Helianthus annuus* L.) seedlings. Thus, we assumed that copper levels increase above some critical points seedling growth get negative effects. This assumption is in line with previous findings.

**Key words:** Sunflower seedling, Copper, Abscisic acid, Chlorophyll, Protein, Proline  
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### Introduction

Copper is a ubiquitous pollutant in the environment due to the emission and atmospheric deposition of metal dust released by human activities. In addition, soils may contain elevated levels of copper because of its widespread use as a pesticide, land application of sewage sludges as well as mining and smelting activities (Alaoui-Sosse *et al.*, 2004). Copper is an essential micronutrient for normal plant metabolism (Sharma and Agrawal, 2005). Copper is involved in a number of physiological processes such as the photosynthetic and respiratory electron transport chains (Van Assche and Clijsters, 1990) and as a cofactor or as a part of the prosthetic group of many key enzymes involved in different metabolic pathways, including ATP synthesis (Harrison *et al.*, 1999).

Decrease of chlorophyll content in plants after exposure to copper is frequently reported in the literature (Devi and Prasad, 1998; Chettri *et al.*, 1998; Singh *et al.*, 2007). Prasad *et al.* (2001), attributed Cu-induced chlorophyll content decrease to Cu modification of chlorophyll degradation. Copper can also substitute for Mg in the chlorophyll present in both antenna complexes and reaction centers (Kupper *et al.*, 1996).

Abscisic acid (ABA) is a plant hormone that plays important role during many phases of the plant life cycle, including seed development and dormancy and in plant responses to various environmental stresses. Because many of these physiological processes are correlated with endogenous ABA levels, the regulation of ABA biosynthesis is a key element facilitating the elucidation of these physiological characteristics

(Seo and Koshiba, 2002). In some studies that were made, reported that the ABA content increased in plants which were exposed to copper and nickel pollution (Monni *et al.*, 2001). It was found that, by nickel application, the water potential and stomatal conductance the leaves decreased while ABA content increased (Rausser and Dumbroff, 1981). The relative water rate was decreasing, stomal resistance and ABA content increasing at the cadmium application to ten days old bean seedling leaves (Poschenrieder *et al.*, 1989).

Free proline accumulation was observed in response to a wide range of abiotic and biotic stresses in plants. Possibly on osmoticum, synthesis of proline is considered to be one of the first metabolic responses to stress, and is perhaps a second messenger (Delauney *et al.*, 1993; Hare and Cress, 1997). Environmental stimuli including water deprivation, salinization, high and low temperature, heavy metal toxicity, pathogen infection, anaerobiosis, nutrient deficiency, atmospheric pollution and UV-irradiation induce the elevation of the proline level in plants (Hare and Cress, 1997). Proline accumulation in plant tissues has been suggested to result from, a decrease in proline degradation, an increase in proline biosynthesis, a decrease in protein synthesis or proline utilization, hydrolysis of proteins (Charest and Phan, 1990).

For all plants, quality of irrigation water is greatly important. Sunflower is an important field crop, cultivation of which depends on the quality of the soil and water, beside some other factors. One of the important factors which spoil the quality of water and soil are heavy metals. In this study, the effects of the copper on the content



of chlorophyll (a+b), proline, protein and abscisic acid in sunflower seedlings were investigated. It was determined how some physiological and biochemical parameters were changed due to concentration of the copper.

### Materials and Methods

In this study, 7-day old sunflower seedlings (*Helianthus annuus* L.) were used. The stock solutions of copper were prepared at a concentration of 0.4, 0.5 and 0.6 mM CuCl<sub>2</sub>. Seeds of sunflower (*Helianthus annuus* L.) were surface sterilized in 10<sup>-3</sup> M HgCl<sub>2</sub> for 2 min (Sresty and Madhava Rao, 1999), washed in distilled water and germinated between wet paper towels at 25 °C in the dark for 3 days. Subsequently plants were cultivated hydroponically in a growth chamber at the light intensity of 4500 lux (16 hr light/ 8 hr dark). During this period day/night temperatures ranged 25°C/ 25°C. After 7 days, plants were transferred to Hoagland solutions containing 0 (control) and 0.4-0.6 mM Cu (CuCl<sub>2</sub>). After 10 days of copper-treatment, seedlings were used for chlorophyll (Monni *et al.*, 2001), proline (Bates *et al.*, 1973), protein (Lowry *et al.*, 1951) and ABA analysis (Cabot *et al.*, 1986).

For the chlorophyll analysis leaves were ground with a mortar and pestle (Monni *et al.*, 2001). Aliquots of 10 mg were extracted in 3 ml of 80% acetone over night at 4°C, centrifuged at 3000x g for 3 min, and the absorbance 647 and 664 nm wavelength was measured on a spectrophotometer (CE-5502 Scanning Double Beam UV).

Proteins from seedling's primer leaves were extracted according to Larson and Beevers (1965). The proteins content was determined according to Lowry *et al.* (1951). To estimate proline content of leaf, tissues were homogenized in sulphosalicylic acid and the homogenate was filtered through Whatman's No. 1 filter paper. The filtrate was reacted with acid-ninhydrin and acetic acid for 1 hr in a test tube placed in a water-bath at 100°C. The reaction mixture was extracted with toluene, and the absorbance was measured at 520 nm wavelength. The amount of proline was calculated from a standard curve as µmol g<sup>-1</sup> fresh weight following the method of Bates *et al.* (1973).

The extraction of ABA from the primer roots and leaves of the sunflower seedlings, which were grown in the heavy metal solution, was made according to Cabot *et al.* (1986). Dried extract were solubilized in 2 ml methylene chloride for HPLC. Each sample was injected twice. The quantification was made according to Cabot *et al.* (1986) utilizing absorption spectrum of 254 nm for ABA. HPLC separations were accomplished at room temperature with a Perkin-Elmer liquid chromatograph system (Series 1100) consisting of a sample injection valve (Cotati 7125) with a 20 µl sample loop, an ultra-violet (UV) spectrophotometer detector (Cecil 68174), integrator (HP 3395) and a Hi-Chrom ODS-1 packed (5 µm particle) column (250 x 4.6 ID) with a methanol mobile phase at 1.5 ml min<sup>-1</sup> flow rate.

The content of ABA was determined in the tissues from certain concentration of standard ABA (+/- cis, trans, Sigma), which was prepared in methylene chloride, (20-500 ng) 20 µl was injected to HPLC and then measurements were taken. The retention time for ABA was approximately 1.5 minute. A standard curved was drawn from one of the peak heights at the end of these measurements. The ABA content in the samples was calculated from a standard curved.

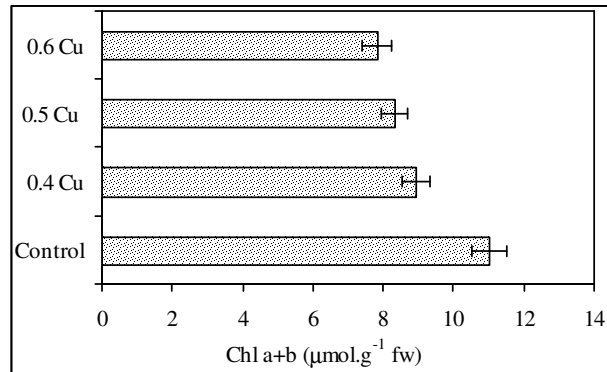
All the analyses were made in three replicates. For each copper concentration and control groups 20 seedlings were used. Statistical analysis was based on SPSS (version 10.0) program. In order a one-way ANOVA test was carried out to detect the significance of differences (p<0.05 or p<0.01) of variables to detect the significance of differences (p<0.05 or p<0.01) of variables.

### Results and Discussion

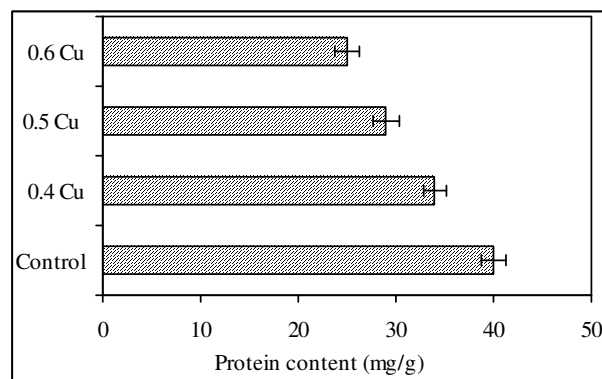
Fig. 1-4 summarize the results of the effects of copper on chlorophyll (a+b), proline, total protein and abscisic acid contents of the sunflower seedlings. Chlorophyll (a+b) and total protein contents (p<0.05 or p<0.01) declined progressively with increasing concentrations of copper in comparison with controls. Significant increases of the contents of proline (p<0.05 or p<0.01) and abscisic acid (p<0.05 or p<0.01) were detected after ten-day exposure to the heavy metals.

The primary leaves of sunflower were significantly affected by copper, which resulted in a decline of chlorophyll (a+b) content (Fig. 1). Presence of 0.4 mM of copper resulted in a significant decrease of chlorophyll (a+b). Beyond that concentration, decrease of chlorophyll (a+b) content was observed with elevated concentration level of copper. When sunflower leaves were exposed to 0.6 mM copper, the amount of chlorophyll (a+b) reached a minimum value. In leaves treated with 0.4, 0.5 and 0.6 mM copper were decreased by 19.2%, 26.3% and 31.6%, respectively, compared to the control seedlings (p<0.05). Many studies have demonstrated influences of copper and other heavy metals on chlorophyll content in higher plants. Whereas chlorophyll contents increased in plants cultured in Cu containing growth medium (Balsberg Pahlsson, 1989), other studies found chlorophyll content reduction by copper treatment (Ouzonidou, 1996; Devi and Prasad, 1998; Ralph and Burchett, 1998; Xiong *et al.*, 2006; Tanyolac *et al.*, 2007; Singh *et al.*, 2007). In this study copper treatment also was found to reduce chlorophyll (a+b) content. This may result from chlorophyll degradation and chlorophyll synthesis inhibition to Cu-induced inhibition of ALA-dehydrates (Fernandes and Henriques, 1991). The mechanism of heavy metals on photosynthetic pigments may be owed to three reasons:

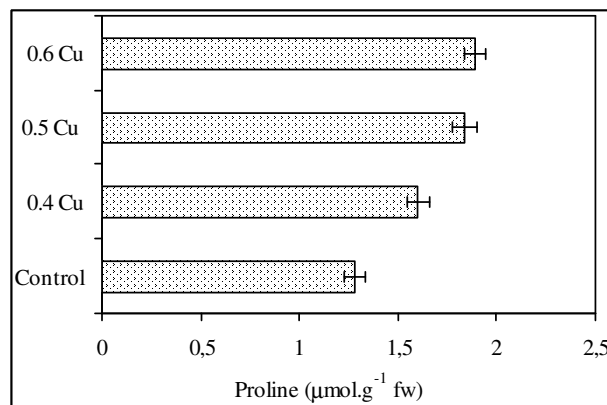
1. Heavy metals enter frond chloroplast and may be over-accumulated causing oxidative stress which may cause damage like peroxidation of chloroplast (Romero-Puertas *et al.*, 2004). Also they can directly destroy the structure and function of



**Fig. 1:** Chlorophyll (a+b) contents ( $\mu\text{mol l}^{-1}$  FW) in the primary leaves of sunflower seedlings grown in different concentrations of copper ( $\text{CuCl}_2$ )



**Fig. 2:** Total protein ( $\text{mg g}^{-1}$  FW) contents in the primary leaves of sunflower seedlings grown in different concentrations of copper ( $\text{CuCl}_2$ )



**Fig. 3:** Proline contents ( $\mu\text{mol g}^{-1}$  FW) in the primary leaves of sunflower seedlings grown in different concentrations of copper ( $\text{CuCl}_2$ )

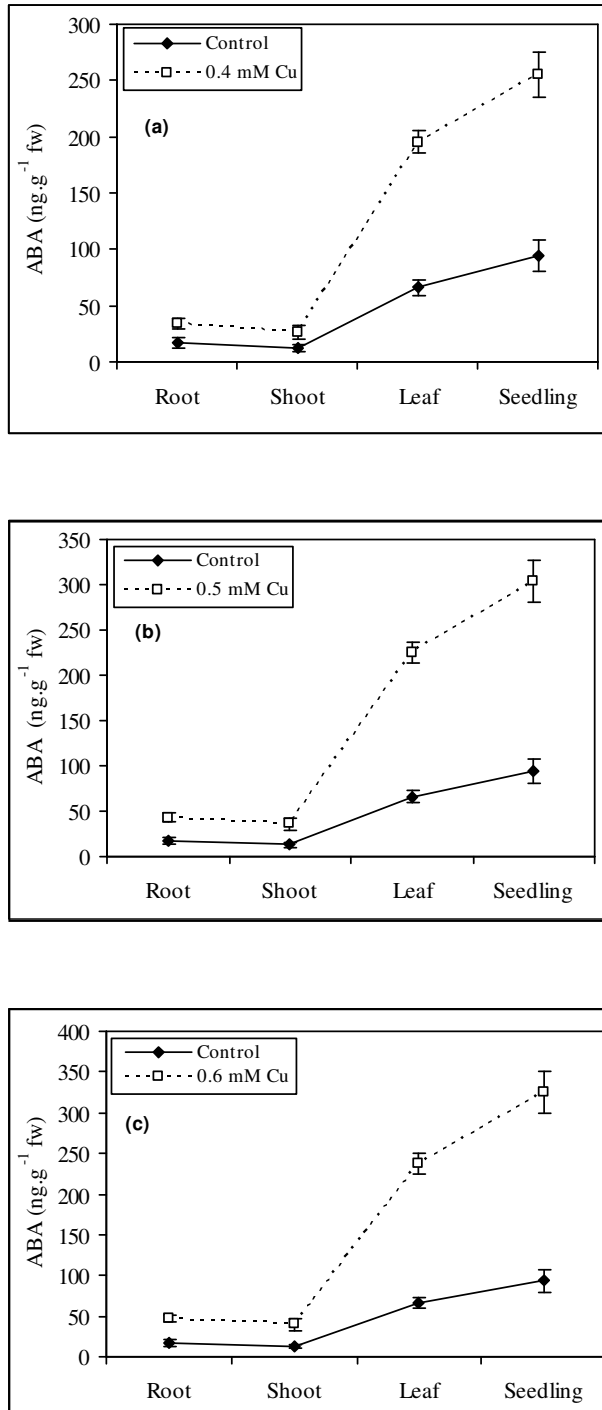
chloroplast by binding with-SH group of enzyme and over all chlorophyll biosynthesis through  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$  or  $\text{Zn}^{2+}$  (Singh, 1995).

2. Heavy metal ions inhibit uptake and transportation of other metal elements such as Mn, Zn and Fe by antagonistic effects and therefore the fronds lose the capacity of synthesis of pigments (Gardea-Torresdey *et al.*, 2004; Lin and Wu, 1994).
3. Heavy metals are known to the direct inhibition of an enzymatic step (Van Assche and Clijsters, 1990; Sharma *et al.*, 2005). In addition, excessive Cu induces leaf chlorosis which is due to peroxidative breakdown of pigments and membrane lipids and in reduction of pigment content (Maksymiec, 1997; Shainberg *et al.*, 2001; Liu *et al.*, 2004).

These plants had significantly lower total protein contents than control plants (Fig. 2). Total protein content in seedlings decreased, by 15%, 27.5% and 37.5% with increasing of Cu concentrations compared to the control seedlings, respectively. Protein content in organisms, an important indicator of reversible and irreversible changes in metabolism, is known to respond to a wide variety of stressors such as natural and xenobiotic (Singh and Tewari, 2003). It was reported that Cd resulted in a significant inhibition of protein level in *Brassica juncea* L. and root tips of barley seedlings (Singh and Tewari, 2003; Liu *et al.*, 2005). It showed that excessive Cu reduced protein amount of many plant species (Chen *et al.*, 2001; Singh *et al.*, 2007).

Proline content in detected sunflower leaves increased with the increase of copper concentration (Fig. 3). Proline content increased, change between 25% - 48% in detected leaves treated with  $\text{CuCl}_2$  ( $p < 0.05$ ). This result is in agreement with those of other investigators using different plants as experimental materials (Chen *et al.*, 2001; Bassi and Sharma, 1993; Schat and Sharma, 1997). Proline is known to accumulate under heavy metal exposure and considered to involve in stress resistance. Proline accumulation in plants under heavy metal stress is induced by a Cd-imposed decrease of the plant water potential and that the functional significance of this accumulation would lie in its contribution to water balance maintenance (Costa and Morel, 1994). They also suggested that proline-mediated alleviation of water deficit stress could substantially contribute to Cd tolerance of the plant. Proline increases the stress tolerance of the plants through such functions as osmoregulation, the protection of enzymes against denaturation, and the stabilization of protein synthesis (Kuznetsov and Shevyakova, 1997). In addition, proline could be involved in metal chelation in the cytoplasm (Farago and Mullen, 1979), especially in case of metals

with a preference for nitrogen or oxygen co-ordination over sulphur co-ordination.



**Fig. 4:** ABA [ng g<sup>-1</sup> (F.W.)] contents in the sunflower seedlings grown in different concentrations of copper (CuCl<sub>2</sub>) (a = 0.4 mM Cu, b = 0.5 mM Cu, c = 0.6 mM Cu)

In copper-treated seedlings the ABA was significantly more effective than control seedlings ( $p < 0.01$ ). ABA contents of the root increased with increasing concentration of this metal (Fig. 4a, 4b, 4c). In root treated with 0.4, 0.5 and 0.6 mM copper, ABA content increased by 100%, 152% and 176%, respectively, compared to the control plants ( $p < 0.01$ ). Copper concentrations (0.4, 0.5 and 0.6 mM) required for a significant level ( $p < 0.01$ ) of the ABA contents increase were 100%, 176% and 207% the shoot of the seedlings as compared to the control seedlings, respectively. The increase in copper concentration in primary leaves caused significant ABA accumulation. In primary leaf treated with 0.4, 0.5 and 0.6 mM copper, ABA content increased by 171%, 223% and 245%, respectively, compared to the control plants ( $p < 0.01$ ). The dynamic balance ABA between biosynthesis and degradation determines the amount of ABA present. These two processes are influenced by development, environmental factors such as light and water stress, and other growth regulators (Cutler and Krochko, 1999). Many kinds of environmental stresses are known to stimulate ABA synthesis, and ABA has been suggested to have a central function in cross-adaptation (Addicott and Van Steninc, 1983). For example, as a response to water deficit, there is an increase in the endogenous ABA levels that rapidly limits water loss through transpiration by reducing stomatal aperture. ABA is also involved in other aspects of stress adaptation such as cold acclimation and root morphogenesis in response to stress (Campalans *et al.*, 1999). In general, plants possess physiological mechanisms that enable them to resist elevated heavy metal concentrations in their substrate. Heavy metal resistance can be based on either avoidance or tolerance mechanism. Plants can be protected externally against metals they can tolerate high tissue concentrations through specific some physiological mechanism (Baker, 1987). One of these mechanisms is that; to hinder the transmission of the heavy metals from roots to shoot. There are a lot of reports which deal with hindering the transmission of the salts from roots to shoot by increasing the ABA content in the roots of the plants, which are under the salt stress (Gomez-Cadenas *et al.*, 1998). Under water stress conditions induced by mannitol solutions (0-0.66M) applied to the apical 12 mm of intact roots of maize (cv. LG11), growth inhibition, a decreased in the osmotic potential of cell sap and a significant accumulation of ABA were observed (Rivier and Pilet, 1983). Heavy metals stress increases the content of the ABA, reason of this probably application of heavy metals because this makes absence of water; or hindering the transmission of the heavy metals from roots to shoot.

The toxic effects of copper on sunflower were discussed in this paper. Our results demonstrated copper adverse effects on total chlorophyll content. Copper adverse effect also was shown protein content. It has been determined that, as a response to heavy metals generated stress, plants increase their proline and abscisic acid.

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