

## Research Article

# The Effects of Excess Copper on Antioxidative Enzymes, Lipid Peroxidation, Proline, Chlorophyll, and Concentration of Mn, Fe, and Cu in *Astragalus neo-mobayenii*

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To probe the physiological and biochemical tolerance mechanisms in *Astragalus neo-mobayenii* Maassoumi, an endemic plant around the Cu-rich areas from the North West of Iran, the effect of different copper concentrations at toxic levels on this plant was investigated. Copper was applied in the form of copper sulfate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) in four levels (0, 50, 100, and 150  $\mu\text{M}$ ). We observed no visible symptoms of Cu toxicity in this plant species. During the exposure of plants to excess copper, the antioxidant defense system helped the plant to protect itself from the damage. With increasing copper concentration, superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities increased in leaves and roots ( $P < 0.001$ ) compared with that of the control group. The chlorophyll amount gradually declined with increasing Cu concentrations. However, reduction in the 50  $\mu\text{M}$  level showed insignificant changes. Enhanced accumulation of proline content in the leaves was determined, as well as an increase of MDA content (oxidative damage biomarker) ( $P < 0.001$ ). The results indicated that Cu contents in leaves and roots enhanced with increasing levels of Cu application. The Fe and Mn contents in both shoots and roots significantly decreased with increasing Cu concentration. Finally, the mechanisms of copper toxicity and copper tolerance in this plant were briefly discussed.

## 1. Introduction

Copper, an essential element for normal plant growth and metabolism [1, 2], plays a significant role in a number of physiological processes such as the photosynthetic and respiratory electron transport chains [3], nitrogen fixation, protein metabolism, antioxidant activity, cell wall metabolism, and hormone perception [2, 4, 5]. As a structural and catalytic component of proteins and enzymes, it is also well documented [6] and has been reported to be among the most toxic heavy metals [7]. However, when absorbed in excess quantities, Cu is highly toxic to plant growth potentially leading to physiological disorders that inhibit plant growth [8, 9]. It has been reported that excess Cu, at the cellular level, causes molecular damage to plants via the generation of reactive oxygen species (ROS) and free radicals [10]. Oxidative

stress by formation of ROS and oxidation of biomolecules such as lipids, proteins, nucleic acids, carbohydrates, and almost every other organic constituent of the living cell is an important aspect of Cu toxicity [11–13]. Plant cells can be protected from ROS by enzymatic defense mechanisms like superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) and nonenzymatic defense mechanisms like free amino acids especially proline, ascorbate, and glutathione and phenolic compounds [12, 14]. Free proline is known to accumulate under heavy metal exposure and considered to be involved in stress resistance [15]. In addition, Cu toxicity is related to disturbances in the uptake and transport of other mineral elements [16]. Less is known about the effects of Cu transport and uptake on Fe, Mn, Mg, and other mineral element assimilation. The induced deficiency of mineral content under excess copper from previous investigations is

also available [16–20]. *Astragalus* with nearly 3000 species is generally considered the largest genus of vascular plants. Iran is one of the largest centers of diversity for the genus. It has nearly 750 species and an endemism rate of nearly 50% [21, 22]. It was determined how some physiological and biochemical parameters and Cu, Fe, and Mn concentration in roots and shoots were changed due to excess Cu in *Astragalus* plants grown in heavy metal soils constituting the flora of Iran Northwest.

## 2. Materials and Methods

**2.1. Seeds Germination and Growth Conditions.** *Astragalus* (*A. neo-mobayenii* Maassoumi) seeds were collected from Cu-rich areas (East Azerbaijan Province, Iran) and sterilized in 1% active sodium hypochlorite solution for 5 min, carefully washed by deionized water, and germinated on damp filter paper in the dark. Six-day seedlings were transferred to appropriate light conditions and supplied with 20%, 50%, and the whole Hoagland solution for 10 days. Seedlings were then cultivated in polyethylene pots containing perlite and vermiculite, and treatments were applied after three weeks. Seedlings were grown for 30 d in a growth chamber (greenhouse) at 65% constant relative humidity, 16/8 h day/night regime under  $600 \mu\text{mol m}^{-2} \text{s}^{-1}$  of light intensity, and day/night temperatures 25/20°C. Plants were supplied with the Hoagland nutrient solution (pH 6.2) which contained (macronutrients in mM) 1  $\text{KH}_2\text{PO}_4$ , 2  $\text{MgSO}_4 \cdot 4\text{H}_2\text{O}$ , 5  $\text{KNO}_3$ , and 5  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$  and (micronutrients in  $\mu\text{M}$ ) 9  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 4.6  $\text{H}_3\text{BO}_3$ , 0.8  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.3  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , and 0.1  $\text{H}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$ . Iron was supplied as Fe-EDTA (1.8 mM). Copper in four levels (0, 50, 100, and 150  $\mu\text{M}$ ) as  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  was added to the nutrient solution. The experiment was conducted in four treatments with four replicates. 30 days after treatment, plants were harvested and used for physiological and biochemical analysis.

**2.2. Photosynthetic Pigments' Analysis.** Photosynthetic pigments (chlorophylls and carotenoids) were extracted by 80% acetone and centrifuged at 3000 g for 5 min [23]. Absorbance was determined in supernatant spectrophotometrically at 645 nm (Chlb), 663 nm (Chla), and 470 nm (Car), and according to the Lichtenthaler and Wellburn formulae [24], pigment concentrations were calculated.

**2.3. Enzyme Activity.** The plant material (fresh weight) was homogenized on ice with 5 mL of 50 mmol sodium phosphate buffer (pH 7) including 0.5 mmol EDTA and 0.15 mol NaCl, in a mortar and pestle. The homogenate was centrifuged at 12000 g for 15 min at 4°C. The supernatant was used for enzyme assays. The activity of SOD was determined as described by Chen and Pan [25] in a 3 mL reaction mixture containing 50 mmol sodium phosphate buffer (pH 7), 10 mmol methionine, 1.17 mmol riboflavin, 56 mmol NBT, and 100  $\mu\text{L}$  enzyme extract spectrophotometrically at 560 nm based on the photoreduction of nitroblue tetrazolium (NBT). The blue formazan produced by NBT photoreduction was measured by an increase in absorbance at 560 nm.

An SOD unit was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction.

The activity of CAT was determined as described by Havir and McHale [26] by a decrease in absorbance of the reaction mixture at 240 nm. The activity was assayed for 1 min in a reaction solution composed of 2.9 mL potassium phosphate buffer 50 mmol (2.85 mL, pH 7.0),  $\text{H}_2\text{O}_2$  12.5 mmol (50  $\mu\text{L}$ ), and 100  $\mu\text{L}$  of crude extract. The enzyme activity was calculated using the molar extinction coefficient of  $36 \text{ M}^{-1} \text{ cm}^{-1}$ .

Peroxidase activity was determined based on an increase in absorbance at 470 nm as described by Sakharov and Ardilla [27]. The mixture composed of 2.8 mL guaiacol (3%), 100  $\mu\text{L}$   $\text{H}_2\text{O}_2$  and 100  $\mu\text{L}$  enzyme extract. A POD unit was defined as an increase in absorbance of 1.0 per min.

**2.4. Determination of Lipid Peroxidation.** Lipid peroxidation in roots was determined using thiobarbituric acid test by measurement of malondialdehyde level [28]. Roots were homogenized in 20% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid (TBA). The extracts were centrifuged at 10000 g for 15 min after incubation in 95°C water bath for 30 min and immediately ice bath. The amount of MDA-TBA complex was calculated by its specific absorbency at 532 nm in supernatant. Nonspecific absorbency at 600 nm was also subtracted [29]. The data was obtained as  $\text{nm gr}^{-1} \text{ FW}$  using the extinction coefficient of  $155 \text{ mM}^{-1} \text{ cm}^{-1}$ .

**2.5. Proline Content.** To estimate proline content of shoot, according to the Bates et al. [30] method, samples were homogenized in sulphosalicylic acid. The homogenate was filtered through Whatman's no. 1 filter paper. The filtrate was boiled for 1 hr after adding acetic acid and acid ninhydrin, and absorbance was taken at 520 nm wavelength.

**2.6. Plant Sampling and Digestion.** Roots and shoot were separated and washed by double-distilled water for at least four times. The samples were oven dried at 80°C for 48 hours and then milled by mixer. Homogenate powder was weighted (150 mg) and digested in 10 mL concentrated  $\text{HNO}_3$  at 300°C heating plate. Cooled digests were diluted to 50 mL by double-distilled water and then filtrated by Whatman's no. 1 paper [31].

**2.7. Metal Analysis.** Metal contents of prepared samples were analyzed by ICP-OES spectroscopy (Varian VISTA-MPX) for manganese (Mn), copper (Cu), and (Fe). The metal concentrations were calculated as  $\mu\text{g gr}^{-1} \text{ DW}$ .

**2.8. Statistical Analysis.** Statistical analysis were determined both based on one-way analysis of variance (ANOVA) and least significant difference (LSD) test with SPSS at significance levels of  $P < 0.001$ ,  $P < 0.01$ ,  $P < 0.05$ .

## 3. Results and Discussion

Total chlorophyll ( $a + b$ ) content varied with Cu levels. With the increasing Cu concentration, the chlorophyll  $a$  and  $b$

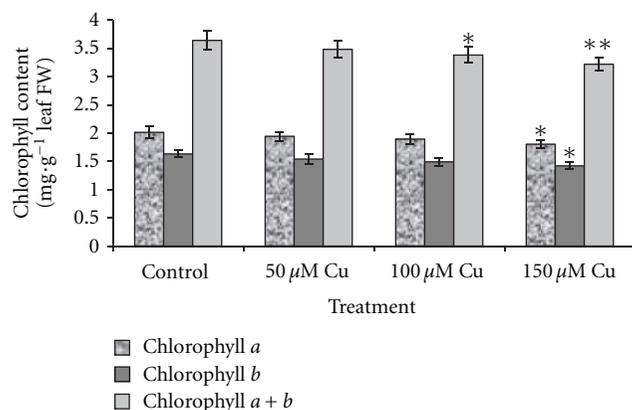


FIGURE 1: Chlorophyll *a* and chlorophyll *b* content and chlorophyll (*a* + *b*) in leaf tissues of *A. neo-mobayenii* grown in different concentrations of copper. Vertical bars represent standard error of the mean ( $n = 4$ ). Asterisks indicate that the mean values are significantly different between treatments and control ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ) according to LSD.

content decreased gradually. However, reduction in the 50 and 100  $\mu\text{M}$  levels showed insignificant changes compared with that of the control group but showed significant change in 150  $\mu\text{M}$  ( $P < 0.05$ ) (Figure 1). Reduction of chlorophyll content in plants due to excess copper was also observed by Quzounidou [32]; Rama Devi and Prasad [33]; Monni et al. [34]; Xiong et al. [35]; Singh et al. [36]. It has been proposed that Cu at toxic concentration interferes with enzymes associated with chlorophyll biosynthesis and protein composition of photosynthetic membranes [37–39]. Also, possibility of Cu-induced Fe deficiency [16] and displacing Mg required for chlorophyll biosynthesis [40] have been proposed as a damage mechanism.

Figure 2 shows the changes of SOD activity in leaves and roots. No significant changes in SOD activity were observed in the leaves under 50  $\mu\text{M}$  Cu concentration, while the activities showed significant increases ( $P < 0.001$ ) under higher level of Cu concentration. Significant increases in root SOD activities under all treatments were observed ( $P < 0.05$  or  $P < 0.001$ ). As Cu concentration increased, the root CAT activity increased significantly ( $P < 0.01$ ). The same result was observed in leaves as shown in Figure 3. Figure 4 shows increased POD activity in both leaves and roots concomitantly with increased Cu level. The increase in POD activity in both was significant ( $P < 0.001$ ). The result of lipid peroxidation in root in the control and treatment groups is shown in Figure 5. MDA level in roots significantly increased with the increase of Cu concentration ( $P < 0.001$ ). Our studied plant was endemic around the Cu-rich area and had adapted to contaminated soils by developing tolerance mechanisms to this metal stress. Many studies reported that internal protective responses to excess copper can vary among plant species and among different tissues [41]. It is well known that when copper is in excess, it catalyzes the formation of ROS and particularly, the highly toxic hydroxyl radicals from Haber-Weiss reaction [42], leading to an increase in MDA as biomarkers of oxidative damages. Hence,

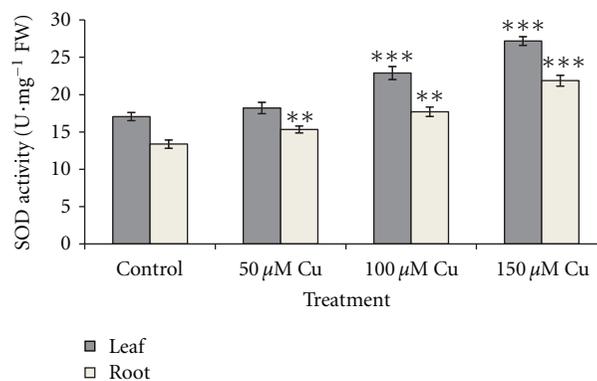


FIGURE 2: Effects of different concentrations of copper on superoxide dismutase (SOD) activity in leaves and roots of *A. neo-mobayenii*. Vertical bars represent standard error of the mean ( $n = 4$ ). Asterisks indicate that the mean values are significantly different between treatments and control ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ) according to LSD.

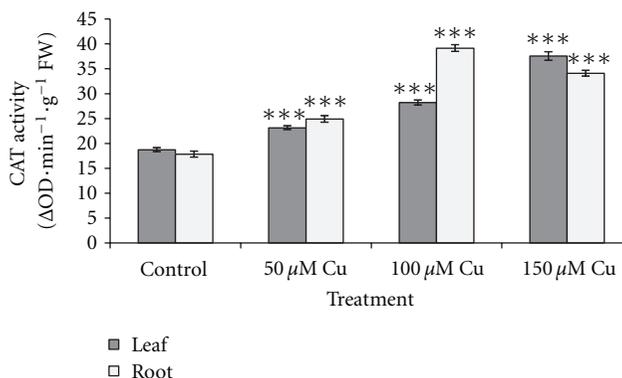


FIGURE 3: Effects of different concentrations of copper on catalase (CAT) activity in leaves and roots of *A. neo-mobayenii*. Vertical bars represent standard error of the mean ( $n = 4$ ). Asterisks indicate that the mean values are significantly different between treatments and control ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ) according to LSD.

in response to the presence of excess Cu, plants increased the antioxidant responses due to increased generation of ROS. Accordingly, it was observed an excess Cu in plants inducing defense genes responsible for antioxidant enzymes, including SOD, POD, and CAT, which contribute to the removal of ROS [43–46]. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. The enhanced activity of catalase demonstrated that any hydrogen peroxide formed as a result of SOD activity was consumed by catalase and/or peroxidase. This indicated that these enzymes were known as a mediator of oxidative damage and might be sufficient to protect biomolecules of some parts of plants against ROS attack [13].

Figure 6 shows Cu-induced proline accumulation in shoots. The proline content increased substantially with increasing Cu concentrations ( $P < 0.001$ ). This may be because synthesis of proline is considered to be one of the first metabolic responses to stress and acts osmoregulator,

TABLE 1: Effects of excess copper on Cu, Mn, and Mg contents of the shoots and roots of *A. neo-mobayenii*.

Cu ( $\mu\text{M}$ )	Shoot			Root		
	Cu ( $\mu\text{g/g DW}$ )	Mn ( $\mu\text{g/g DW}$ )	Fe (mg/g DW)	Cu ( $\mu\text{g/g DW}$ )	Mn ( $\mu\text{g/g DW}$ )	Fe (mg/g DW)
Control	12.32	42.9	147	8.14	24.67	93
50	23.69	42.72 <sup>NS</sup>	146	14.73	26.12	85
100	31.92	39.14	119	18.12	26.89	67
150	44.58	36.12	106	24.29	25.09	53

Each value is the mean of the four replications.

All the values are significant at  $P < 0.01$ .

NS: nonsignificant.

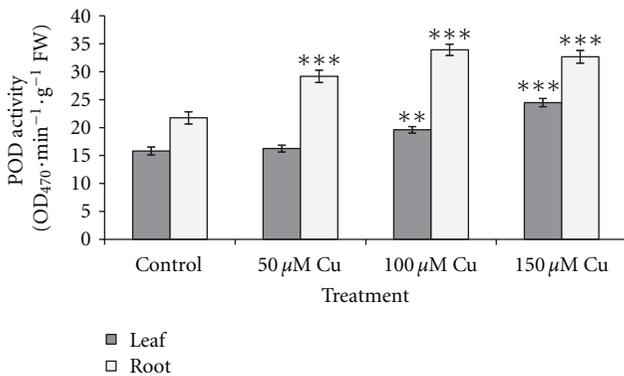


FIGURE 4: Effects of different concentrations of copper on peroxidase (POD) activity in leaves and roots of *A. neo-mobayenii*. Vertical bars represent standard error of the mean ( $n = 4$ ). Asterisks indicate that the mean values are significantly different between treatments and control ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ) according to LSD.

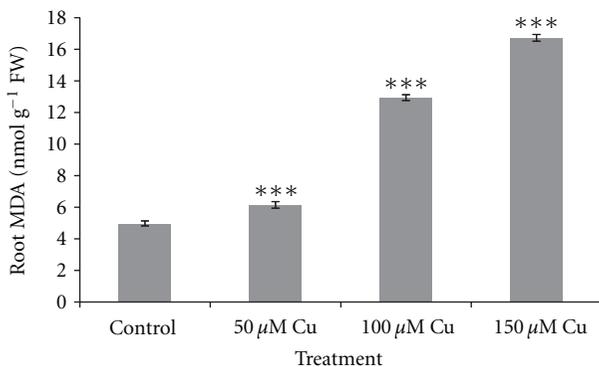


FIGURE 5: MDA levels in roots of *A. neo-mobayenii* grown in different concentrations of copper. Vertical bars represent standard error of the mean ( $n = 4$ ). Asterisks indicate that the mean values are significantly different between treatments and control ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ) according to LSD.

stabilizer of protein synthesis, a metal chelator, and a hydroxyl radical scavenger [47–49].

The Cu content in shoots and roots increased significantly with an increase in the level of applied Cu. The accumulations in shoots were higher than that of roots in all treatments. Fe content in both shoots and roots reduced

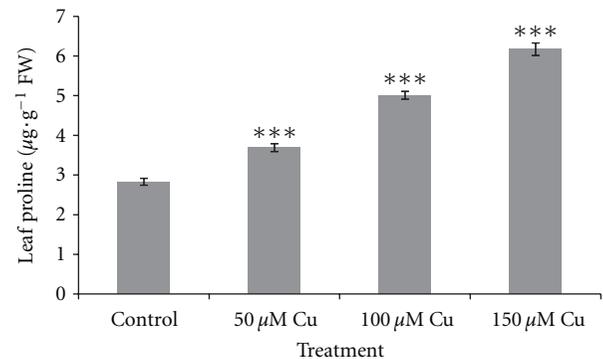


FIGURE 6: Proline contents in leaves of *A. neo-mobayenii* grown in different concentrations of copper. Vertical bars represent standard error of the mean ( $n = 4$ ). Asterisks indicate that the mean values are significantly different between treatments and control ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ ) according to LSD.

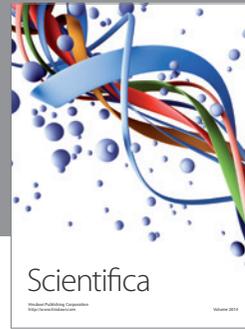
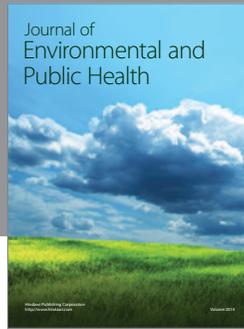
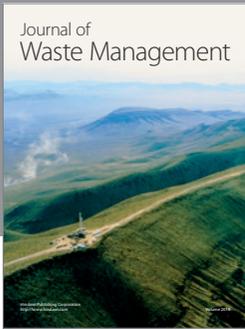
with increasing Cu concentration in the medium. However, a slight increase was observed in the lower level of applied Cu. The Mn content decreased insignificantly at higher levels of applied Cu. In roots increased levels of Mn were observed (Table 1). The results are in close conformity with the findings that an elevated copper application resulted in an increase in plant Cu content [8, 50–52]. In high concentration of copper application, the copper levels in leaves were above the threshold for copper toxicity [4]. On the other hand, normal growth of studied plants without any visible symptoms of Cu toxicity implied that this plant was tolerant to toxic levels of Cu. In addition, translocation of copper to the shoots was suggested as a strategy to explain the copper tolerance mechanism developed by plant in order to reduce copper stress. Thus according to the present study, this plant could be suitable for phytoextraction [53, 54].

Interference of Cu and Cd with the root uptake of mineral nutrients has been observed [55, 56]. Moreover, antagonistic effects of Cu and Fe have been suggested by many workers and often occur in plants grown under Cu toxicity [17, 57–59]. Also, competition of copper with Mn for transport sites in plasmalemma has been reported [60, 61]. In this study reduction of Mn with increasing levels of copper was observed. However, Mn contents in leaves did not drop below the critical deficiency range [4].

## References

- [1] R. K. Sharma and M. Agrawal, "Biological effects of heavy metals: an overview," *Journal of Environmental Biology*, vol. 26, no. 2, pp. 301–313, 2005.
- [2] I. Yruea, "Copper in plants," *Brazilian Journal of Plant Physiology*, vol. 17, no. 1, pp. 145–156, 2005.
- [3] F. Van Assche and H. Clijsters, "Effect of metals on enzyme activity in plants," *Plant Cell Environment*, vol. 13, pp. 195–206, 1990.
- [4] H. Marschner, *Mineral Nutrition of Higher Plants*, Academic Press, London, UK, 1995.
- [5] J. A. Raven, M. C. W. Evans, and R. E. Korb, "The role of trace metals in photosynthetic electron transport in O<sub>2</sub>-evolving organisms," *Photosynthesis Research*, vol. 60, no. 2-3, pp. 111–149, 1999.
- [6] M. Pilon, S. E. Abdel-Ghany, C. M. Cohu, K. A. Gogolin, and H. Ye, "Copper cofactor delivery in plant cells," *Current Opinion in Plant Biology*, vol. 9, no. 3, pp. 256–263, 2006.
- [7] T. Li and Z. -T. Xiong, "A novel response of wild-type duckweed (*Lemna paucicostata* Hegelm.) to heavy metals," *Environmental Toxicology*, vol. 19, pp. 95–102, 2004.
- [8] G. Ouzounidou, "Root growth and pigment composition in relationship to element uptake in *Silene compacta* plants treated with copper," *Journal of Plant Nutrition*, vol. 17, no. 6, pp. 933–943, 1994.
- [9] V. Caspi, M. Droppa, G. Horváth, S. Malkin, J. B. Marder, and V. I. Raskin, "The effect of copper on chlorophyll organization during greening of barley leaves," *Photosynthesis Research*, vol. 62, no. 2-3, pp. 165–174, 1999.
- [10] J. Liu, Z. Xiong, T. Li, and H. Huang, "Bioaccumulation and ecophysiological responses to copper stress in two populations of *Rumex dentatus* L. from Cu contaminated and non-contaminated sites," *Environmental and Experimental Botany*, vol. 52, no. 1, pp. 43–51, 2004.
- [11] A. Murphy and L. Taiz, "Correlation between potassium efflux and copper sensitivity in 10 *Arabidopsis* ecotypes," *New Phytologist*, vol. 136, no. 2, pp. 211–222, 1997.
- [12] O. Acar, I. Türkan, and F. Özdemir, "Superoxide dismutase and peroxidase activities in drought sensitive and resistant barley (*Hordeum vulgare* L.) varieties," *Acta Physiologiae Plantarum*, vol. 23, no. 3, pp. 351–356, 2001.
- [13] R. Mittler, "Oxidative stress, antioxidants and stress tolerance," *Trends in Plant Science*, vol. 7, no. 9, pp. 405–410, 2002.
- [14] S. Gao, R. Yan, M. Cao, W. Yang, S. Wang, and F. Chen, "Effects of copper on growth, antioxidant enzymes and phenylalanine ammonia-lyase activities in *Jatropha curcas* L. seedling," *Plant, Soil and Environment*, vol. 54, no. 3, pp. 117–122, 2008.
- [15] F. B. Wu, F. Chen, K. Wei, and G. P. Zhang, "Effect of cadmium on free amino acid, glutathione and ascorbic acid concentrations in two barley genotypes (*Hordeum vulgare* L.) differing in cadmium tolerance," *Chemosphere*, vol. 57, no. 6, pp. 447–454, 2004.
- [16] E. Pätsikkä, M. Kairavuo, F. Šeršen, E. M. Aro, and E. Tyystjärvi, "Excess copper predisposes photosystem II to photoinhibition in vivo by outcompeting iron and causing decrease in leaf chlorophyll," *Plant Physiology*, vol. 129, no. 3, pp. 1359–1367, 2002.
- [17] W. Schmidt, "Mechanisms and regulation of reduction-based iron uptake in plants," *New Phytologist*, vol. 141, no. 1, pp. 1–26, 1999.
- [18] Y. Chen, J. Shi, G. Tian, S. Zheng, and Q. Lin, "Fe deficiency induces Cu uptake and accumulation in *Commelina communis*," *Plant Science*, vol. 166, no. 5, pp. 1371–1377, 2004.
- [19] A. D. Rombolà, Y. Gogorcena, A. Larbi et al., "Iron deficiency-induced changes in carbon fixation and leaf elemental composition of sugar beet (*Beta vulgaris*) plants," *Plant and Soil*, vol. 271, no. 1-2, pp. 39–45, 2005.
- [20] H. Lequeux, C. Hermans, S. Lutts, and N. Verbruggen, "Response to copper excess in *Arabidopsis thaliana*: impact on the root system architecture, hormone distribution, lignin accumulation and mineral profile," *Plant Physiology and Biochemistry*, vol. 48, no. 8, pp. 673–682, 2010.
- [21] J. M. Lock and K. Simpson, *Legumes of West Asia*, Kew, Royal Botanic Gardens, Canada, 1991.
- [22] A. A. Maassoumi, *Astragalus in the Old World*, Tehran, Iran, 1998.
- [23] D. Arnon, "Copper enzymes in isolated chloroplast: polyphenoloxidase in *Beta vulgaris*," *Plant Physiology*, vol. 24, pp. 1–15, 1949.
- [24] H. Lichtenthaler and A. Wellburn, "Determination of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents," *Biochemical Society Transactions*, vol. 603, pp. 591–592, 1983.
- [25] C. N. Chen and S. M. Pan, "Assay of superoxide dismutase activity by combining electrophoresis and densitometry," *Botanical Bulletin of Academia Sinica*, vol. 37, no. 2, pp. 107–111, 1996.
- [26] E. A. Havir and N. A. McHale, "Biochemical and developmental characterization of multiple forms of catalase in tobacco leaves," *Plant Physiology*, vol. 84, pp. 450–455, 1987.
- [27] I. Y. Sakharov and G. B. Ardila, "Variations of peroxidase activity in cocoa beans during their ripening, fermentation and drying," *Food Chemistry*, vol. 65, no. 1, pp. 51–54, 1999.
- [28] R. L. Heath and L. Packer, "Photoperoxidation in isolated chloroplasts—I. Kinetics and stoichiometry of fatty acid peroxidation," *Archives of Biochemistry and Biophysics*, vol. 125, no. 1, pp. 189–198, 1968.
- [29] C. H. De Vos, R. Vooijs, H. Schat, and W. Ernst, "Cooper-induced damage to the permeability barrier in roots of *Silene cucubalus*," *Journal of Plant Physiology*, vol. 135, pp. 165–169, 1989.
- [30] L. S. Bates, R. P. Waldren, and I. D. Teare, "Rapid determination of free proline for water-stress studies," *Plant and Soil*, vol. 39, no. 1, pp. 205–207, 1973.
- [31] N. Lavid, Z. Barkay, and E. Tel-Or, "Accumulation of heavy metals in epidermal glands of the waterlily (*Nymphaeaceae*)," *Planta*, vol. 212, no. 3, pp. 313–322, 2001.
- [32] G. Ouzounidou, "The use of photoacoustic spectroscopy in assessing leaf photosynthesis under copper stress: correlation of energy storage to photosystem II fluorescence parameters and redox change of P700," *Plant Science*, vol. 113, no. 2, pp. 229–237, 1996.
- [33] S. Rama Devi and M. N. V. Prasad, "Copper toxicity in *Ceratophyllum demersum* L. (Coontail), a floating macrophyte: response of antioxidant enzymes and antioxidants," *Plant Science*, vol. 138, no. 2, pp. 157–165, 1998.
- [34] S. Monni, M. Salemaa, and N. Millar, "The tolerance of *Empetrum nigrum* to copper and nickel," *Environmental Pollution*, vol. 109, no. 2, pp. 221–229, 2000.
- [35] Z. T. Xiong, C. Liu, and B. Geng, "Phytotoxic effects of copper on nitrogen metabolism and plant growth in *Brassica pekinensis* Rupr.," *Ecotoxicology and Environmental Safety*, vol. 64, no. 3, pp. 273–280, 2006.
- [36] D. Singh, K. Nath, and Y. K. Sharma, "Response of wheat seed germination and seedling growth under copper stress," *Journal of Environmental Biology*, vol. 28, no. 2, pp. 409–414, 2007.

- [37] F. C. Lidon and F. S. Henriques, "Limiting step in photosynthesis of rice plants treated with varying copper levels," *Journal of Plant Physiology*, vol. 138, pp. 115–118, 1991.
- [38] W. Maksymiec, R. Russa, T. Urbanik-Sypniewska, and T. Baszynski, "Effect of excess Cu on the photosynthetic apparatus of runner bean leaves treated at two different growth stages," *Physiologia Plantarum*, vol. 91, no. 4, pp. 715–721, 1994.
- [39] M. F. Quartacci, C. Pinzino, C. L. M. Sgherri, F. Dalla Vecchia, and F. Navari-Izzo, "Growth in excess copper induces changes in the lipid composition and fluidity of PSII-enriched membranes in wheat," *Physiologia Plantarum*, vol. 108, no. 1, pp. 87–93, 2000.
- [40] H. Küpper, I. Šetlík, E. Šetliková, N. Ferimazova, M. Spiller, and F. C. Küpper, "Copper-induced inhibition of photosynthesis: limiting steps of in vivo copper chlorophyll formation in *Scenedesmus quadricauda*," *Functional Plant Biology*, vol. 30, no. 12, pp. 1187–1196, 2003.
- [41] F. Passardi, C. Cosio, C. Penel, and C. Dunand, "Peroxidases have more functions than a Swiss army knife," *Plant Cell Reports*, vol. 24, no. 5, pp. 255–265, 2005.
- [42] B. Halliwell and J. M. C. Gutteridge, "Oxygen toxicity, oxygen radicals, transition metals and disease," *Biochemical Journal*, vol. 219, no. 1, pp. 1–14, 1984.
- [43] M. E. Alvarez and C. Lamb, "Oxidative burst mediated defense responses in plant disease resistance," in *Oxidative Stress and the Molecular Biology of Antioxidant Defenses*, J. G. Scandalios, Ed., pp. 815–839, Cold Spring Harbor Laboratory Press, New York, NY, USA, 1997.
- [44] F. Navari-Izzo, M. F. Quartacci, C. Pinzino, F. DallaVecchia, and C. L. M. Sgherri, "Thylakoid-bound and stromal antioxidative enzymes in wheat treated with excess copper," *Physiologia Plantarum*, vol. 104, no. 4, pp. 630–638, 1998.
- [45] M. Drazkiewicz, E. Skorzynska-Polit, and Z. Krupa, "Response of the ascorbate-glutathione cycle to excess copper in *Arabidopsis thaliana* (L.)," *Plant Science*, vol. 164, no. 2, pp. 195–202, 2003.
- [46] H. Wang, X. Q. Shan, B. Wen, S. Zhang, and Z. J. Wang, "Responses of antioxidative enzymes to accumulation of copper in a copper hyperaccumulator of *Commoelina communis*," *Archives of Environmental Contamination and Toxicology*, vol. 47, no. 2, pp. 185–192, 2004.
- [47] M. E. Farago and W. A. Mullen, "Plants which accumulate metals—part 4. A possible copper-proline complex from the roots of *armeria maritima*," *Inorganica Chimica Acta*, vol. 32, no. C, pp. L93–L94, 1979.
- [48] S. Siripornadulsil, S. Traina, D. P. S. Verma, and R. T. Sayre, "Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae," *Plant Cell*, vol. 14, no. 11, pp. 2837–2847, 2002.
- [49] V. V. Kuznetsov and N. I. Shevyakova, "Stress responses of tobacco cells to high temperature and salinity. Proline accumulation and phosphorylation of polypeptides," *Physiologia Plantarum*, vol. 100, no. 2, pp. 320–326, 1997.
- [50] V. Kumar, D. V. Yadav, and D. S. Yadav, "Effects of nitrogen sources and copper levels on yield, nitrogen and copper contents of wheat (*Triticum aestivum* L.)," *Plant and Soil*, vol. 126, no. 1, pp. 79–83, 1990.
- [51] H. Panou-Filotheou, A. M. Bosabalidis, and S. Karataglis, "Effects of copper toxicity on leaves of oregano (*Origanum vulgare subsp. hirtum*)," *Annals of Botany*, vol. 88, no. 2, pp. 207–214, 2001.
- [52] J. Cambrollé, E. Mateos-Naranjo, S. Redondo-Gómez, T. Luque, and M. E. Figueroa, "Growth, reproductive and photosynthetic responses to copper in the yellow-horned poppy, *Glaucium flavum* Crantz," *Environmental and Experimental Botany*, vol. 71, no. 1, pp. 57–64, 2011.
- [53] D. C. McCain and J. L. Markley, "More manganese accumulates in maple sun leaves than in shade leaves," *Plant Physiology*, vol. 90, pp. 1414–1421, 1989.
- [54] J. Yoon, X. Cao, Q. Zhou, and L. Q. Ma, "Accumulation of Pb, Cu, and Zn in native plants growing on a contaminated Florida site," *Science of the Total Environment*, vol. 368, no. 2–3, pp. 456–464, 2006.
- [55] D. T. Clarkson and U. Luttge, "Mineral nutrition: divalent cations, transport and compartmentation," *Progress in Botany*, vol. 51, pp. 93–100, 1989.
- [56] R. B. Harrison, C. L. Henry, and D. Xue, "Magnesium deficiency in Douglas-fir and Grand fir growing on a sandy outwash soil amended with sewage sludge," *Water, Air, and Soil Pollution*, vol. 75, no. 1–2, pp. 37–50, 1994.
- [57] C. D. Foy, R. L. Chaney, and M. C. White, "The physiology of metal toxicity in plants," *Annual Review of Plant Physiology*, vol. 29, pp. 511–566, 1978.
- [58] G. Ouzounidou, I. Ilias, H. Tranopoulou, and S. Karataglis, "Amelioration of copper toxicity by iron on spinach physiology," *Journal of Plant Nutrition*, vol. 21, no. 10, pp. 2089–2101, 1998.
- [59] L. Lombardi and L. Sebastiani, "Copper toxicity in *Prunus cerasifera*: growth and antioxidant enzymes responses of in vitro grown plants," *Plant Science*, vol. 168, no. 3, pp. 797–802, 2005.
- [60] B. F. Hulagur and R. T. Dangarwala, "Effect of zinc, copper and phosphorus fertilization on the uptake of iron, manganese and molybdenum by hybrid maize," *Madras Agricultural Journal*, vol. 69, pp. 11–16, 1982.
- [61] F. C. Lidon and F. S. Henriques, "Copper toxicity in rice; a diagnostic criteria and its effect on Mn and Fe contents," *Soil Science*, vol. 154, no. 2, pp. 130–135, 1992.



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