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Copper-Induce Change in Antioxidative System in Maize (*Zea mays* L.)

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Abstract: Maize seedlings treated with various concentrations (25-100 μM) of copper for 15 days. A progressive decrease of root length and biomass with increasing Cu in nutrient solution was observed. The roots accumulated significantly higher amounts of Cu than the above ground parts. Accumulation of copper resulted in more active lipid peroxidation in both roots and shoots, which was attributed to copper-induced additional oxidative stress. Activities of APX (ascorbate peroxidase), GPX (guaiacol peroxidase), GR (glutathione reductase) and CAT(catalase) were higher in both roots and shoots in response to copper accumulation. Changes in lipid peroxidation and antioxidant enzyme activities suggest that oxidative damage may be involved in copper toxicity.

Key words: Antioxidant enzymes, copper (Cu^{2+}), maize, metal toxicity, oxidative stress, APX, GPX, GR, CAT

INTRODUCTION

Excess copper (Cu) in soils result not only from its increasing use industry like mining and smelting but also from its use as presence in sewage sludge amendments (Nicholson *et al*., 2003; Xiong and Wang, 2005). Cu is an essential micronutrients for growth and development of plant. At cellular level, Cu also plays an essential role in signaling of transcription and protein trafficking machinery, oxidative phosphorylation and iron mobilization (Yruela, 2005). Moreover, Cu is required in biological systems as a structural component and catalytic enzyme activity as a cofactor, however it can be a stress factor causing physiological responses that can inhibit plant growth at higher concentrations in soil (Monnet *et al*., 2001). Heavy metal excess may stimulate the formation of free radicals and reactive oxygen species, perhaps resulting in oxidative stress (Dietz *et al*., 1999). Cu-induced generation Reactive Oxygen Species (ROS) has been directly correlated with the damage to proteins and lipids (De Vos *et al*., 1991; Murphy and Taiz, 1997). Evidence from several plant species reveals that Cu caused oxidative stress by mediating the activities of antioxidative enzymes (Luna *et al*., 1994; Gupta *et al*., 1999). The antioxidant protection in plant cells is complex and highly compartmentalized. Superoxide dismutases (SOD) are a family of metalloenzymes catalyzing the dismutation of $O_2^{\cdot -}$-H_2O_2 (Alscher *et al*., 2002). The bulk of H_2O_2 is removed by catalases (CAT), localized in peroxisomes and peroxidases localized in vacuoles, the cell walls and the cytosol (Mittler, 2002). Ascorbate peroxidase (APX) allows the scavenging of small amounts of H_2O_2 in particular parts of the cell like chloroplast and mitochondria (Dat *et al*., 2000). ROS-detoxifiying enzymes are induced during abiotic stress but are also susceptible to oxidative damage (Dietz *et al*., 1999).We were therefore interested in identifying the involvement of antioxidative mechanisms responsible for the Cu-induced oxidative stress in the roots. We also measured the several enzyme activities involved in H_2O_2 metabolism. Plants vary in response to metals, in mechanisms of uptake and of avoiding damage and in the type of damage caused. Maize (*Zea mays*) is one of the most important cereal crops and is relatively sensitive to copper. The aim of the study is to assess the sensitivity of plant growth to copper and an assay to investigate the effects of externally applied substances on the growth of Cu-treated maize roots.

MATERIALS AND METHODS

Seeds of *Zea mays* L. were surface sterilized with 0.1% $HgCl_2$ for 10 min and germinated in a greenhouse on moist filter paper in petri dishes for 3 days. The seedlings were randomly placed in polyethylene pots (10 plants per pot) filled with 300 mL of a modified Hoagland's nutrient solution (Ouzounidou *et al*., 1994).

A randomized block, experimental design with five Cu treatments (0, 25, 50, 75 and 100 μM Cu) and three replicates was used. Copper was supplied as $CuSO_4.5H_2O$. The plants were grown for 15 days in a growth chamber (16 h light/8 h dark) under a light intensity of 15 W m^{-2} at plant level with a day/night temperature regime of 27/22°C and relative humidity of 85%

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(Ouzounidou *et al*., 1993). After 15 days, grown seedlings were taken as the reference material for the experiments done in the presence of added copper.

After harvesting, the root lengths of maize cultivars were measured (cm plant^{-1}) for every copper treatment and control. For determination of Cu content 15 days after exposure to Cu, plants were harvested and separated into shoots and roots. Roots and shoots were washed in distilled water and dried for 48 h at 80°C. Dry plant material was wet digested in cylinders filled with HNO_3-$HClO_4$ (4: 1) at 120-130°C for 5 h. After cooling, metal concentrations (Cu) were determined by a Shimadzu AA-670 atomic absorption spectrophotometer (Ouzounidou, 1994).

Malondialdehyde (MDA) was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer (1968).

For determination of enzyme activities, fresh leaf and root samples (0.5 g) from control and Cu-treated plants were homogenized in 100 mM cold potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and 1% PVP-40. The homogenate was centrifuged at 14000 rpm (18000 g) for 20 min at 4°C and resulting supernatant was used for determination of enzyme activities later.

Catalase (CAT) activity was assayed by measuring the initial rate of disappearance of H_2O_2 (Aebi, 1983). The reaction mixture contained 50 mM potassium phosphate (pH 7) and 10 mM H_2O_2. After enzyme addition, the reaction was monitored by following decomposition of H_2O_2 at 240 nm (extinction coefficient of H_2O_2 = 43.6 mM^{-1} cm^{-1}).

Ascorbate peroxidase (APX) activity was determined by the method of Nakano and Asada (1981). The reaction was developed in 50 mM Tris-HCl (pH 7), 1 mM sodium ASC and 2.5 mM H_2O_2. After the addition of ASC to the mixture, the reaction was followed at 290 nm (extinction coefficient of ASC = 2.8 mM^{-1} cm^{-1}).

Glutathione Reductase (GR) was determined by the method of Foyer and Halliwell (1976). The reaction was developed in 50 mM Tris-HCl (pH 7.5) containing 2.5 mM $MgCl_2$, 0.5 mM GSSG and 0.2 mM NADPH. Oxidation of NADPH was followed at 340 nm (extinction coefficient = 6.2 mM^{-1} cm^{-1}).

GPX activity was measured by the method of Curtis (1971). The Assay medium contained 50 mM phosphate buffer (pH 5.8), 7.2 mM guaiacol, 11.8 mM H_2O_2 and 0.1 mL enzyme extract in a final assay volume of 3 mL the reaction was initiated by the addition of H_2O_2 and change in absorbance at 470 nm was measured (extinction coefficient = 26.6 mM^{-1} cm^{-1}).

For statistical analysis, the experiments were performed in a randomized order. Differences among the copper treatments as well as between the cultivars were tested using SPSS statistical program. For statistical variance analysis of independent data with six replicates was performed using ANOVA and compared with Least Significant Differences (LSD) at 5% level.

All experiments process was conducted in the Urmia university plant physiology Laboratory (September to November 2006).

RESULTS

Figure 1 shows that increasing concentrations of $CuSO_4$ from 25 to 100 μM progressively decreased root length. The differential effect of Cu on root and shoot growth could be accounted for by the fact that Cu accumulated mainly in roots and to a minor extent in shoots. A significant reduction of total dry weight (Fig. 2) was observed for Cu-stressed plants and was explained by a reduction in the root dry weight while the shoot dry weight remained unaffected.

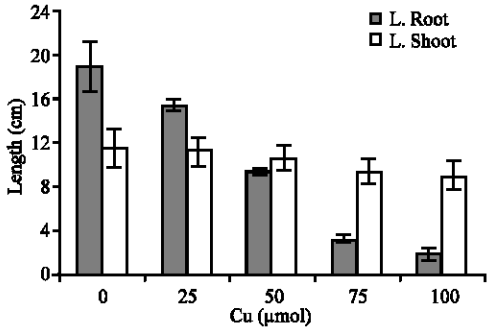

Fig. 1: Effects of Cu-concentrations on root length in maize. Each value is the mean±SD of triplicates

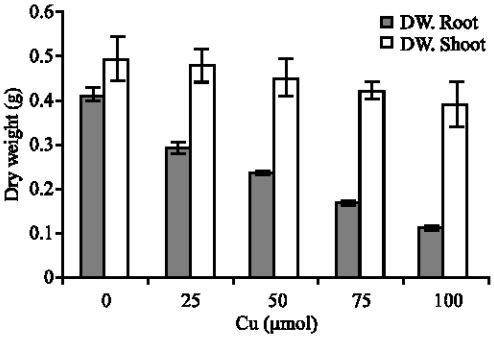

Fig. 2: Effect of Cu-concentrations on root dry weight in maize. Each value is the mean±SD of triplicates

Figure 3 demonstrates that $CuSO_4$ treatment in a significant increase in MDA level, an indicator of lipid peroxidation. The effect of Cu concentration on metal accumulation in leaves and roots were investigated in the present study. Maize plants grown for 15 days in Cu concentrations ranging from 25 to 100 μM showed an increase for all plant organs examined (Fig. 4). Activities of enzymes (APX, GPX, GR and CAT) detoxifying the cells from active oxygen species were measured in $CuSO_4$-treated leaves (Fig. 5). In a significant increase in GPX, APX and GR activities in leaves of maize seedling treated with $CuSO_4$. However, $CuSO_4$ had slightly effect on the activity of CAT in leaves of maize seedlings. Similar to leaves, roots antioxidative enzyme activities increased by Cu treatment (Fig. 5). It is clear that APX, GPX and GR activities in roots had higher than leaves but CAT activity in roots had lower than leaves. In $CuSO_4$ treated plants GPX, APX, CAT and GR activities increase.

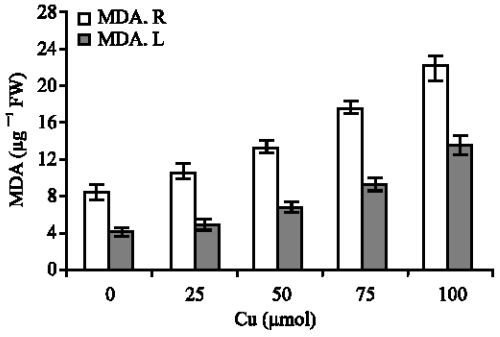

Fig. 3: Effect of Cu-concentrations on MDA content in leaves and roots of maize. Each value is the mean±SD of triplicates

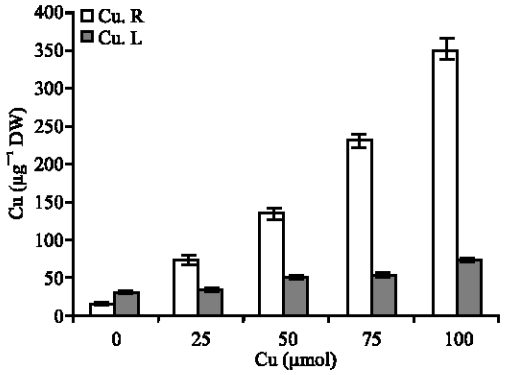

Fig. 4: Effect of Cu-concentrations on Cu content in leaves and roots of maize. Each value is the mean±SD of triplicates

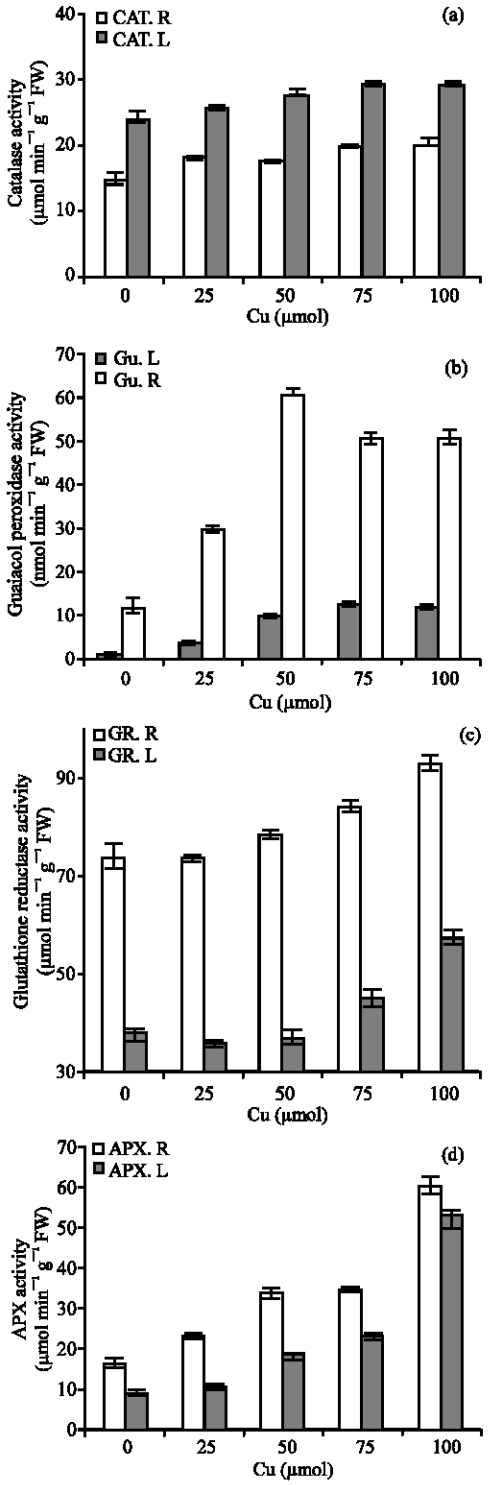

Fig. 5: Effect of Cu-concentrations on the activities of catalase (a), guaiacol peroxidase (b), glutathion reductase (c) and ascorbate peroxidase (d) in leaves and roots of maize. Each value is the mean±SD of triplicates

DISCUSSION

Cu^{2+} is required by biological systems as a structural and catalytic enzyme component and in the soil Cu^{2+} can be a stress factor by causing physiological responses that can decrease the vigour of the plants and inhibit plant growth (Ouzounidou, 1994). The Cu^{2+} content in roots increased with increasing solution concentrations of Cu^{2+}. Cu on *Z. mays* in which a large amount of heavy metal was accumulated in the roots (Liu *et al*., 2001). Ouzounidou *et al*. (1994) observed that at high external copper concentrations, the copper sensitive plants contained more copper in their shoots than the copper tolerant plants, suggesting that sensitive plants transport more Cu^{2+} from the root to the shoot than tolerant ones. Early researches showed that Cu reduced cell increment of many plant species and root biomass of maize and the tolerance of plant to Cu differed significantly among plant species (Yang and Kuboi, 1991; Ouzounidou *et al*., 1994). This research indicated that, Cu is highly toxic to maize growth and development. Present research also showed that root was more sensitive to Cu than other parts of maize plant. Root dry weight was largely reduced at high levels of Cu concentration (75 and 100 μM). While the weight of shoot dry weight was not or only slightly affected.

The optimum biomass production of maize occurred in the control nutrient solution. Increasing Cu supply resulted in a striking decrease of root biomass indicating alterations of physiology and metabolism. Biomass loss (dry weight) under metal treatment has also been reported (Lolkema *et al*., 1984; Stiborova *et al*., 1987; Verkleij *et al*., 1989). Growth performance, either in terms of biomass yield or growth rate, provides a ready means of assessing intraspecific differences in the effects of metal treatment on plants (Baker *et al*., 1989). Copper at increasing concentrations progressively reduced extension growth rates of maize roots with the largest Cu concentration (100 μM) causing major disturbances to root growth. The inhibitory effect of Cu on root growth is due to the reduction in cell divisions (Agarwal *et al*., 1987; Kahle, 1993) while reduction in cell elongation may also occur, since root biomass production increases under 100 μM Cu-treatment.

Roots of *Zea mays* accumulated larger amounts of Cu than the above-ground parts, particular the leaves. Present results are in agreement with a number of recent reports which indicate that metals accumulate more in the roots than in the shoot leaves (Harmens *et al*., 1993; Ouzounidou, 1994; Ouzounidou *et al*., 1992; Wu and Lin, 1990). However, according to Van Assche and Clijsters (1990) only part of the heavy metal taken up by the plant is phytotoxic, namely the fraction which interferes with cellular metabolism. Consequently, the metal bound to cell walls or accumulated in vacuoles has no physiological effect.

Hydrogen peroxide (H_2O_2) is a constituent of oxidative plant metabolism. It is a product of peroxisomal and chloroplastic oxidative reactions (Del Rio *et al*., 1992). H_2O_2 itself is an active oxygen species. H_2O_2 can also react with superoxide radicals to form more reactive hydroxyl radicals in the presence of trace amounts of Fe or Cu (Thompson *et al*., 1987). The hydroxyl radicals initiate self-propagating reactions leading to peroxidation of membrane lipids and destruction of proteins (Asada and Takahashi, 1987; Bowler *et al*., 1992; Halliwell, 1987).

The formation of TBARS (thiobarbituric acid reagent for sorbic acid) in plants exposed to adverse environmental conditions is an indicator of free-radical formation in the tissues and it may be used as an index of lipid peroxidation in biological systems (Heath and Packer, 1968). Plant cell membranes are generally considered to be primary sites of metal injury. Membrane destabilization is frequently attributed to lipid peroxidation due to an enhanced production of toxic oxygen free radicals after exposure to metal. There is considerable evidence that inorganic exposure results in the generation of ROS in plants (Hartley-Whitaker *et al*., 2001).

The copper toxicity resulted in the production of ROS, which in turn can cause membrane damage in copper-sensitive plants. Antioxidant enzymes are considered to be an important defense system of plants against oxidative stress caused by metals (Weckx and Clijsters, 1996). The results of this study show differential responses of the anti-oxidative enzymes to copper in the different parts of plant. The production of anti-oxidant enzymes as a function of copper concentration applied was evident in all tissues of the plants assayed during the present study.

CAT is an important enzyme against oxidative stress, being able to scavenge H_2O_2, which is the major product produced by Super Oxid Dismutase (SOD) (Asada, 1992). CAT is a universally present oxidoreductase that decomposes H_2O_2 to water and molecular oxygen (Lin and Kao, 2000). CAT activity was found to be significantly higher in leaves than in roots Cu treatment plants, suggesting that, possibly, CAT mediated the removal of H_2O_2 and toxic peroxides in this hyper-accumulator of copper. In turn, there may be a decrease in the free radical-mediated lipid peroxidation under copper toxicity.

Ascorbate peroxidase is required to scavenge H_2O_2, produced mainly in the chloroplast and other cell organelles and to maintain the redox state of the cell

(Asada, 1992). Effect of increasing concentrations of copper induced in the growth medium on GR activity in leaf and root tissues of maize. The present results indicate an enhancement in the activity of APX in response to a copper stress in maize.

Present results indicate an enhancement of GPX activity upon exposure to copper, suggesting that this enzyme serves as an intrinsic defense tool to resist Cu-induced oxidative damage in maize. Induction of GPX activity in plants has also been reported under toxic levels of other metals like Al, Cd and Zn (Cakmak and Horst, 1991; Chaoui *et al*., 1997; Shah *et al*., 2001). Under sub-lethal metal toxicity conditions, the level of GPX activity has been used as a potential biomarker to evaluate the intensity of systemic stress (Mittal and Dubey, 1991; Shah *et al*., 2001). GR, which catalyses the NADPH-dependent reduction of oxidized glutathione, showed significantly increased activity with copper treatment in maize.

The results of the present study suggest that Cu-induced increases in the levels of anti-oxidant enzymes may represent a secondary defensive mechanism against oxidative stress, which is not as direct as phytochelatins and vacuolar compartmentalization. The generation of oxidative stress could be characteristic of a mechanism of metal toxicity in plants. However, more information is needed at the sub-cellular and molecular levels in order to gain deeper insight into the mechanisms of copper toxicity, as well as copper accumulation in copper tolerant and sensitive species.

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