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Full Length Research Paper

Oxidative damage and antioxidant response caused by excess copper in leaves of maize

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Adequate copper (Cu^{2+}) concentrations are required for plants, but at higher concentrations it can cause toxic effects. In this study, physiological parameters and antioxidant enzymes were assayed in the leaves of maize variety Zhendan958, which is one of the dominant cultivar of maize (*Zea mays* L.) in China due to its better traits in production, after the seedling was treated with 2 to 8 mM Cu for 96 h. Cu inhibited the bioaccumulation of leaf biomass, but increased the accumulation of Cu in leaves. In addition, Cu stress induced the generation of O_2^- and H_2O_2 as revealed by the histochemical staining and increased malondialdehyde content. It also stimulated the activity of superoxide dismutase, peroxidase, ascorbate peroxidase, glutathione reductase and glutathione S-transferase. Cu stress altered the content of glutathione (GSH) and ascorbic acid (AsA) significantly. These demonstrated that Cu induced the oxidative stress on seedlings of Zhendan958, while the heated antioxidant responses indicated a bioaccumulation potential of this cultivar in the phytoremediation of Cu contaminated soils.

Key words: Maize, superoxide dismutase, peroxidase, ascorbate peroxidase, glutathione reductase, glutathione S-transferase.

INTRODUCTION

Excess copper (Cu) in soils results not only from industrial or mining activities, but also from Cu-based fungicides application (Xiong and Wang, 2005; Michaud et al., 2007). Contaminants are threatening human health by their impact on ecosystems, water and food quality (Lim et al., 2008). Cu is an essential micronutrient for growth and development of plant and plays an important role in many biological processes such as photosynthesis, respiration and the response to oxidative stress (Burkhead et al., 2009; Martínez-Peñalver et al., 2012). However, copper at high concentration is phytotoxic to the plant (Sgherri et al., 2007). As a redox-active metal, Cu enhances reactive oxygen species (ROS) formation by the Fenton-Haber-Weiss reactions (Stohs and Bagchi, 1995). The overproduction of harmful ROS can cause severe damage to membrane lipids, protein synthesis, and DNA (Lequeux et al., 2010). Excessive levels of copper can also cause a range of morphological and physiological disorders. In addition, excess copper can decrease the germination rate, shoot elongation, plant biomass, and water content (Ahsan et al., 2007). Cu toxicity can also lower the mitotic index, inhibit cell division, induce chromosomal aberrations and the structural changes of the cell wall (Liu et al., 2009; Bouazizi et al., 2011).

Plants have evolved certain mechanisms to tolerate copper stress by avoiding the accumulation of free Cu ions in cells (Puig and Peñarrubia, 2009; Burkhead et al., 2009). Moreover, plants can impair oxidative stress by mediating the antioxidant system (Luna et al., 1994; Gupta et al., 1999) which scavenge ROS, thereby preventing the damage caused by the overproduction of ROS, including many antioxidant enzymes such as peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD) and antioxidant compounds such as glutathione (GSH), ascorbic acid (AsA) and carotenoids (Zhang and

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Kirkham, 1996). The generation of proline is also one of the vital responses of plant under Cu toxicity, which is possibly associated with the protection of plant cells against oxidative damage and with signal transduction (Choudhary et al., 2007). This activation of antioxidant enzymes and synthesis of antioxidant and metal-chelating compounds are key factors for tolerance to heavy metals and other abiotic stresses in plant (Foyer and Noctor, 2011).

The accumulation of heavy metals in the seeds and other above-ground parts of maize is becoming a serious problem for agriculture and human health. Maize (*Zea mays* L.) is one of the most important crops in China. Zhendan958 has been the largest acreage maize varieties for five years in china. In the present study, the biomass accumulation and the alterations in oxidative stress and antioxidant responses were investigated in leaves of maize seedlings under Cu stress through a series of biochemical and histochemical assays.

MATERIALS AND METHODS

Seeds of cultivar Zhendan958 of maize were sterilized with 75% ethanol for 15 min, rinsed excessively with distilled water and germinated under dark conditions at 28°C on moist filter paper. Three days later, seedlings selected for uniform growth were transplanted into 3-L hydroponic pots with 10% Hoagland nutrient solution. Healthy seedlings with four full expanded leaves were selected to determine the dry weight of the leaves (dry weight before treatment, DW_{bt}) or cultured in solutions. Cu was supplied as $CuSO_4$ at concentrations of 0, 2, 4 and 8 mM.

All the experiments were carried out in a growth chamber (GZX-250BS-II, Shanghai Xinmiao instrument Co., Shanghai, China) with the following conditions: 14 h photoperiod, photosynthetic photon flux density 220 ± 20 mol $m^{-2}s^{-1}$, temperature 26 ±1°C/20 ± 1°C day/night and relative humidity of 65 ± 2% / 75 ± 2% day/night. The growth solutions were adjusted to pH 6.2±0.1 and were replaced every two days. After treated with Cu for 96 h, leaves of the seedlings were harvested. Treatments were performed in quartic replicate and there were at least 80 plants in each replicate of each treatment.

Determination of relative increase of leaf biomass and Cu concentration

After harvesting, leaves were extensively washed with distilled water and then samples were oven-dried at 105°C for 20 min and maintained at 80°C for 72 h. The dried material was weighed in order to determine the relative increase of leaf biomass (dry weight after treatment, DW_{at}). Relative increase of leaf biomass = (DW_{at} - DW_{bt})* 100% / DW_{at}. Briefly, 0.2 g of the powdered sample was digested with 10 ml of 10:1 HNO_3-$HClO_4$ solution and heated at 100 to 200°C until near dryness. The cooled residue was dissolved in 5 ml 5% HNO_3 and made up to a final volume of 15 ml with double distilled water (ddH_2O). The Cu concentrations in the extracts were determined by inductively-coupled plasma atomic emission and calculated according to the standard curve.

Histochemical detection of O^{2-} and H_2O_2

The level of O_2^- and H_2O_2 was determined according to the method described by Wang et al. (2011a). O_2^- can react with nitro blue tetrazolium (NBT) to form dark blue precipitate, while H_2O_2 can react with 3,3-dimethoxybenzidine with the help of POD to form a deep brown polymerization. The leaves were exposed to solution with NBT or 1% 3,3-diaminobenzidine (DAB, pH, 3.8; Sigma Chemical CO., USA) for 7 h under light at 25°C after treatments with Cu. Then the leaves were washed with distilled water and then the photographs were obtained immediately after twice boiling in 95% ethanol for 10 min.

Determination of lipid peroxidation

The level of lipid peroxidation in leaf tissues was determined in terms of malondialdehyde (MDA) produced, based on the method described by Wang et al. (2008). Briefly, 0.3 g fresh leaf tissues were ground with 5 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15, 000 g for 10 min and 0.5 ml of the supernatant was mixed with 2 ml of 0.5% 2,4,6-tribromoanisole (TBA) in 20% TCA. The mixture was heated at 90°C for 20 min. After the reaction was stopped, the resultant mixture was centrifuged at 10,000 g for 5 min. The absorbance of the supernatant was measured at 532 nm. The values were corrected for non-specific absorption by subtracting absorbance read at 600 nm. The amount of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Assay of enzyme activity

APX activity was measured in the leaves of the plant by the method of Bradford (1976). Guaiacol POD activity was measured following the method of Upadhyaya et al. (1985). SOD activity was assayed according to the method described by Beauchamp and Fridovich (1971). Catalase (CAT) activity was determined based on the method described by Aebi (1974). The activity of APX was determined according to the method described by Nakano and Asada (1981). GR activity was assayed by following the reduction of NADPH reflected as a change of the absorbance at 340 nm as described by Carlberg and Mannervik (1985) and the glutathione S-transferase (GST) activity was measured according to the method described by Mannervik and Gutenberg (1981).

Determination of non-enzymatic antioxidants

Total GSH and oxidized GSH (GSSG) content were determined using the method described by Anderson (1985). The GSH content was calculated as the difference between the total GSH and GSSG contents. AsA and total ascorbate (AsA + dehydroascorbate (DHA)) were determined using the method of Hodges et al. (1996). The difference between total ascorbate and DHA was considered as AsA.

Statistics and analysis

All data were statistically analyzed using one-way analysis of variance (ANOVA), and differences were considered significant at $p < 0.05$. All statistical analyses used SPSS version 13.0 software.

RESULTS

Changes of leaf biomass and Cu accumulation

The effect of different Cu concentrations on the leaf biomass of maize plant was presented in Figure 1A. A

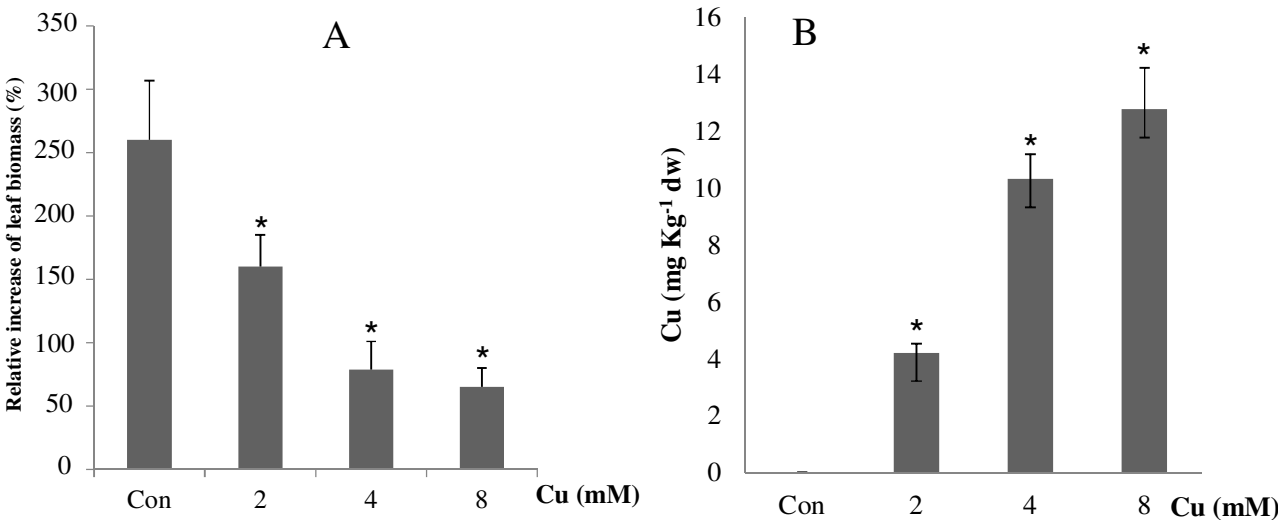

Figure 1. Effects of copper stress on the accumulation of Cu and leaf biomass. Values are the means of three different experiments and errors bars represented SE with n = 4. *Mean values are significantly different between Cu treatments and controls (p<0.05).

significant reduction of leaf biomass was observed between Cu treatments and controls (p<0.05). A gradual decrease in leaf biomass and relative growth rate was also observed with the increase in Cu concentration. Supply of excess-Cu increased the accumulation of Cu in the leaf tissue of plants, the Cu^{2+} content was 4.22, 10.33 and 12.79 mg Kg^{-1} DW in leaves, and was 156, 382 and 473 times of control values under 2, 4 and 8 mM copper, respectively (Figure 1B).

Histochemical detection of O^{2-}, H_2O_2 and lipid peroxidation

Cu-induced oxidative damage of leaves was evidenced by the histochemical staining with NBT (Figures 2A and B) and DAB (Figures 2C and D). Excess copper induced the generation of O_2^- and H_2O_2 in leaves as indicated by the intensified purplish blue formazan and dark brown precipitate in the analysed leaves, respectively. Lipid peroxidation measured in terms of MDA increased in all the stressed plants compared with the control. The MDA content in leaves increased significantly under the Cu^{2+} treatment (Figure 2E), and was positively related to copper concentrations.

Antioxidant enzymes activities

Activities of enzymes (SOD, POD, CAT, GST, APX and GR) detoxifying the cells from reactive oxygen species were measured (Figures 3, 4A and B). In the present study, a gradual increase in the activities of SOD, POD, CAT, GST and GR were observed in excess-Cu plants. The activities of POD and GR increased significantly with the increase of exterior Cu. However, the activities of SOD, CAT and GST in leaves exposed to 4 to 8 mM Cu were significantly higher in comparison with the control. Compared to control, the activity of APX increased about 25.93 and 103.70% in leaves under 2 and 4 mM, respectively, and then declined to control levels (Figure 4A).

Non-enzymatic antioxidants concentration

In this study, AsA and GSH content increased significantly at 2 mM and then began to decrease at 4 mM Cu (Figures 4C and D).

DISCUSSION

In this study, excess copper caused a significant reduction of leaf biomass (Figure 1A). We also found that excess copper suppressed root growth in a previous study (Wang et al., 2011b). The decrease in growth of maize plant under Cu stress suggested that excess Cu induced toxicity at elevated concentration. This is in accordance with the previous findings of Bouazizi et al. (2010). Cu toxicity is directly correlated with the accumulation of Cu in the plant. The Cu^{2+} content was 4.22, 10.33 and 12.79 mg Kg^{-1} DW in leaves (Figure 1B); however, our early research showed the concentration of Cu was 42.44, 79.68 and 104.22 mg Kg^{-1} DW in roots of maize under 2, 4 and 8 mM copper, respectively (Wang et al., 2011b). Our results suggest that the roots of maize accumulated significantly larger amounts of Cu than the leaves of maize, which reconfirmed an earlier report showing correlation of Cu tolerance and its greater

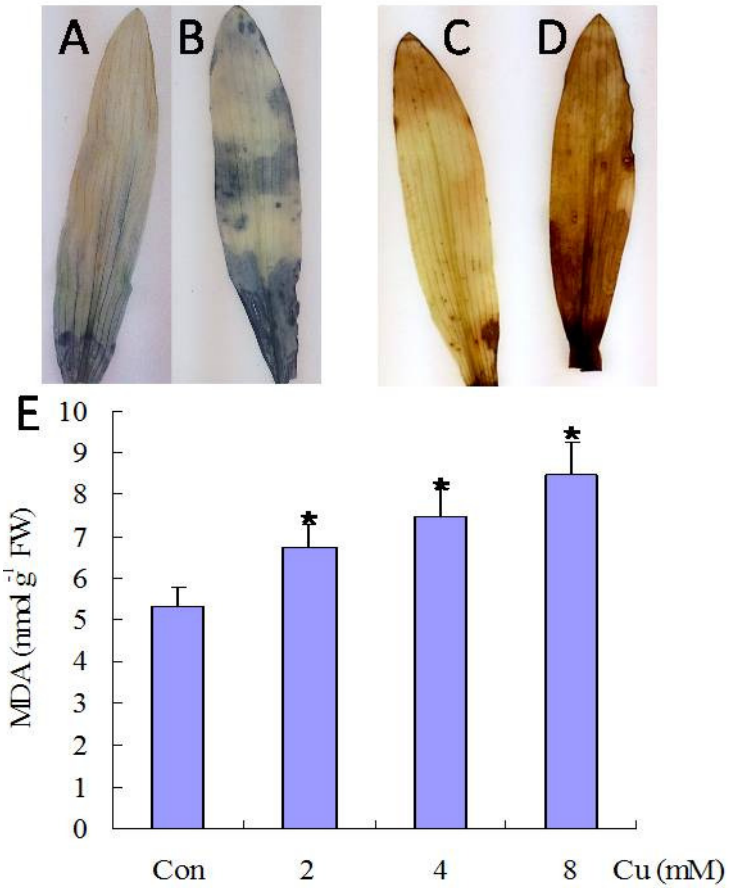

Figure 2. Copper-induced excess O_2^- (A and B), H_2O_2 (C and D) and MDA content (E) in the leaf of Maize. Values are the means of three different experiments and errors bars represented SE with n = 4. *Mean values are significantly different between Cu treatments and controls (p<0.05). MDA, Malondialdehyde.

accumulation in root with poor translocation to shoot in Cu-tolerant plants (Liu and Xiong, 2005).

The young leaves of Cu-stressed plants are suggestive of oxidative damage as a consequence of increased O_2^- and H_2O_2 production in these plants. High accumulation of O_2^- and H_2O_2 in the leaves of Cu-stressed plants was visualized by histochemical staining (Figures 2B and D). Lipid peroxidation is also a well-characterized consequence of oxidative stress and leads further to cell and tissue damage. MDA is the decomposition product of lipid peroxide, a reliable indicator of lipid peroxidation. In this case, membrane destabilization and fusion are directly correlated with MDA production. Its increase shows that plants are under high-level oxidative stress (Wang et al., 2009). In this study, the MDA content of treated plants under Cu stress were significantly higher than control (Figure 2E). Our results further proved that excess Cu increased lipid peroxidation and induced oxidative stress in leaves of Cu-stressed plants. Similar observation was reported in radish leaves (Sun et al., 2010).

The copper toxicity results in the production of ROS, which in turn can cause membrane damage in plant. To overcome this, cells are equipped with enzymatic and non-enzymatic mechanisms to eliminate or reduce its damaging effects. The importance of antioxidant enzymes is their ability to scavenge ROS and thereby prevent oxidative damage. As the first line of defense against ROS in plant, SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide. SOD activities in stressed plants increased with the increase in Cu concentration (Figure 3A). Moreover, increase in SOD activity is related to oxidative stress tolerance (Scandalios, 2002) and may be attributable to an increase in apoplastic and symplastic copper-zinc superoxide dismutase (CuZn-SOD) activity (Apel and Hirt, 2004; Zhang et al., 2010).

POD and CAT are important enzymes that scavenge H_2O_2 (the major product of SOD) by degrading H_2O_2 to water and oxygen. The POD activities in leaves exposed to 2 to 8 mM Cu were significantly higher when compared with control (Figure 3B). Similar results were reported in many plants under Cu stress, including sweet potato (Kim

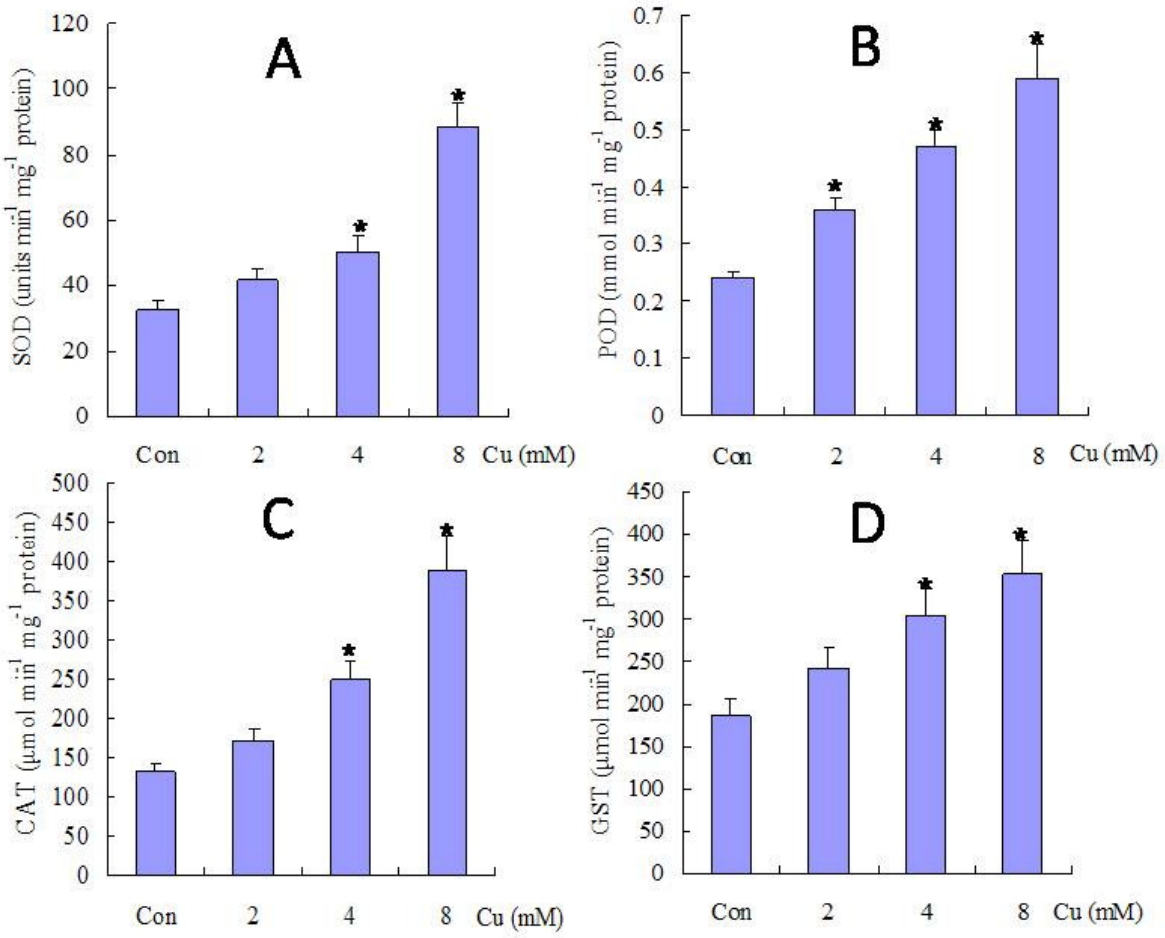

Figure 3. Effects of copper on SOD, POD, CAT and GST activities in the leaves of maize. Values are the means of three different experiments and errors bars represent SE with n = 3. *Mean values are significantly different between Cu treatments and controls (p<0.05). POD, Peroxidase; SOD, superoxide dismutase; GST, glutathione S-transferase; CAT, catalase.

et al., 2010) and *Plagiomnium cuspidatum* (Wu et al., 2009). The increased POD activity may contribute to the enhancement of cell wall under high Cu (Kováčik et al., 2010). Catalase is the most universal oxidoreductase that metabolizes the peroxide liberated in the peroxisome following the conversion of glycolate during photo-respiration (Qureshi et al., 2007). In this study, the activities of POD and CAT of stressed plants were significantly higher than those of control (Figures 3B and C), suggesting the Cu tolerance of Zhendan958 maize variety. GST also plays important roles in combating different biotic and abiotic stresses, including heavy metal stress, through the amelioration of oxidative damage (Dixit et al., 2011). Glutathione transferases can eliminate membrane lipid peroxides such as 4-hydroxyalkenals, as well as products of oxidative DNA degradation such as base propanol by conjugating them with GSH (Bartling et al., 1993; Berhane et al., 1994). GST activities increased significantly under Cu treatments (Figure 3D). The increased GST levels may be propitious to protect organisms from oxidative stress.

APX and GR are important components of the ascorbate-glutathione cycle response for the removal of H_2O_2. APX utilizes ascorbate as its specific electron donor to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate (MDHA). MDHA can be reduced to AsA by the action of NADPH-dependent MDHA reductase. MDHA is a radical with a short lifetime that if not rapidly reduced, disproportionates to AsA and DHA, which is further reduced to AsA by the action of DHA reductase, using GSH (Chao et al., 2010). GR catalyzes the reduction of GSSG to GSH with the accompanying oxidation of NADPH. In our study, the activity of GR increased significantly with the increase in Cu concentration when compared to control. However, the activity of APX reached the highest point at 4 mM Cu stress and then decreased (Figures 4A and B). These results suggest that the modulation of APX in response to

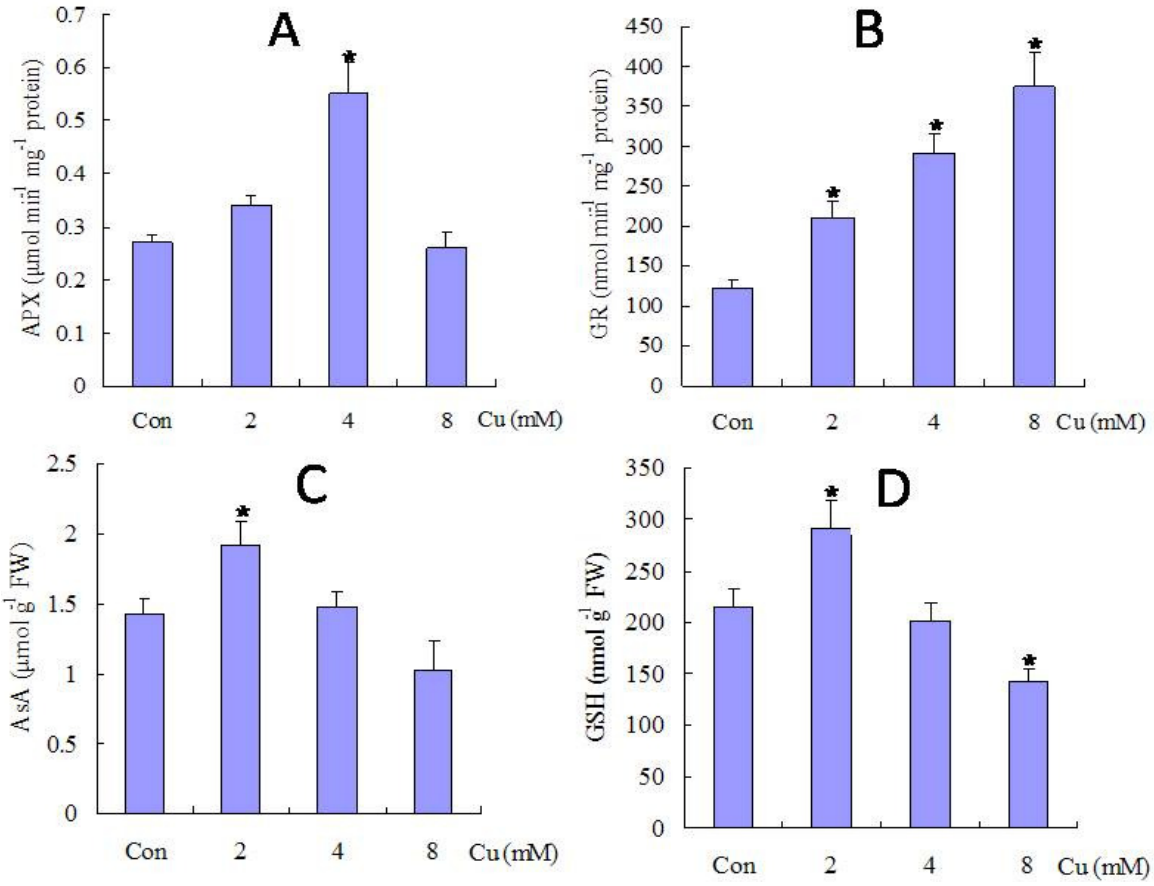

Figure 4. Effects of copper on GR and APX activity and AsA and GSH content in leaves of Maize. Values are the means of three different experiments and errors bars represent SE with n = 4. *Mean values are significantly different between Cu treatments and controls (p<0.05). APX, Ascorbate peroxidase; GR, glutathione reductase; GSH, glutathione; AsA, ascorbic acid.

stress had a limit beyond which they would be damaged by stress, and at the same time, it implies that maize leaves had been damaged under 8 mM Cu concentration. On the other hand, the high activation of GR observed in maize leaves after exposure to 8 mM Cu (Figure 4B) suggested that GR also played an important role in the adjustment of metabolism to excess Cu.

AsA and GSH are the most important antioxidant metabolites reacting directly with different ROS and participating in the ascorbate-glutathione cycle (Foyer and Noctor, 2011). AsA is an effective scavenger for phenoxy radicals by reducing these radicals and thus inhibiting phenol oxidation. GSH, the most abundant intracellular free thiol and an important antioxidant in thylakoid membranes of chloroplasts, quenches oxygen radicals directly or in conjunction with a-tocopherol (Alscher, 1989). In this study, AsA and GSH content increased significantly at 2 mM and began to decrease (Figures 4C and D). Increased AsA and GSH may enhance the ability of plants to cope with oxidative stress at low concentration. In addition, the reduction on GSH at 4 to 8 mM in maize leaves in spite of higher GR activities observed indicated that the mechanism of antioxidant defense was by enhanced oxidation of GSH. These results further imply that maize leaves have been damaged under 8 mM Cu concentration.

Regarding the present findings, excess Cu increased the Cu accumulation in leaves, inhibited leaf growth and induced oxidative stress, thus enhancing ROS production and increasing lipid peroxidation. The antioxidant responses were stimulated as indicated by the increased antioxidant enzyme activities and antioxidant contents. Our results demonstrate the ability of Zhendan958 to tolerate the excess Cu induced oxidative stress.

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