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Effects of Copper Excess on Growth, H₂O₂ Production and Peroxidase Activities in Maize Seedlings (*Zea mays* L.)

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Abstract: Ten day old mays seedlings (*Zea mays* L., var. Aligreen) cultured in hydroponic medium were treated by toxic amounts of copper (50 and 100 µM of CuSO₄) during seven days. Cupric stress induced changes in growth parameters: The matter productions were more reduced in roots than in shoots. Also, a significant decrease in shoot and root elongation was observed. On the other hand, excess of copper increased significantly endogenous H₂O₂ in the two investigated organs and induced changes in peroxidase activities. Our results showed that in shoots, inducibility of GPX (Guaiacol peroxidase, EC 1.11.1.7), CAPX (Coniferyl alcohol peroxidase, EC 1.11.1.4) and APX (Ascorbate peroxidase, EC.1.11.1.11) was highly significant after application of 100 µM of CuSO₄. While, this effect was not observed in 50 µM Cu-stressed shoots, in roots, data showed that 50 µM of CuSO₄ induced stimulation in GPX and APX activities but ACPX activity remains unchanged. In roots, by contrast, exposure to 100 µM Cu induced significant increase only in ACPX activity.

Key words: Ascorbate peroxidase, coniferyl alcohol peroxidase, copper, guaiacol peroxidase, H₂O₂, *Zea mays* L.

INTRODUCTION

Nowadays, metal pollution constitutes a major problem which threatens the environment by exerting disturbance of the ecosystems leading to dysfunction of the biological system.

Many metals, including Cu, are required as micronutrients for the growth and development of higher plants. Copper plays a key role in many metabolic mechanisms, but it can be strongly toxic at high concentrations. In fact, it has been reported that cupric ions (Cu²⁺) interfere with numerous physiological processes and they may induce several alterations.

The toxicity of heavy metals and detoxification by plants are complicated matters, we have difficulties in fully understanding the relevant biological process (Morrey, 1995; Zenk, 1996; Ma *et al.*, 2001).

The toxicity symptoms of copper excess can be recognized by changes in biochemical and physiological processes (Cristina *et al.*, 2002; Wang *et al.*, 2004) or by organ and intact plant responses, such as growth inhibition, biomass reduction (Jouili and El Ferjani, 2003, 2004) or by changes in plants communities (Folkesson and Anderson-Bringmark, 1988). Significant changes in growth inhibition were accompanied by modification on biochemical and metabolic process. In fact, metabolic

changes in plants can serve as a suitable indicator of copper toxicity (Chen *et al.*, 2002; Wang *et al.*, 2004; Yruela, 2005).

It is well known that transition metals like Cu catalase the formation of hydroxyl radicals (OH[•]) from the non-enzymatic chemical reaction between superoxide (O₂⁻) and (H₂O₂) (Haber-Weiss reaction) (Halliwell and Gutteridge, 1984).

Also, copper can catalase the generation of reactive oxygen species and peroxide compounds, such as O₂⁻ and H₂O₂ by inhibiting photosynthetic electron transport (Kappus, 1985). Cupric ions may also stimulate the production of HO[•] in a Fenton-type reaction (Sandmann and Böger, 1980)

Plants have evolved several mechanisms to prevent or alleviate the damage from these toxic compounds. These mechanisms include scavenging the ROS by non enzymatic systems such as ascorbate and α-tocopherol and the use of an enzymatic antioxidant system that includes Glutathione Reductase (GR), superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidases (GPOD) and ascorbate peroxidases (APX). These enzymes are present in plants and their relative activities change during exposure to stress.

In the case of peroxidases, it was recognized that their activity is a common response to various oxidative

stress factors such as copper toxicity (Teisseire and Guy, 2000; Lin *et al.*, 2004; Devi and Prasad, 2005; Yurela, 2005). In fact, they can catalase the oxidation of numerous organic compounds using hydrogen peroxide as the electron acceptor (Dawson, 1992). These enzymes were reported to be involved in many metabolic pathways, such as auxin catabolism (Gazaryan *et al.*, 1996), pathogen defense (Lagrimini, 1991), phenol oxidation (Quiroga *et al.*, 2000), cross-linking of cell wall proteins (Schnabelrauch *et al.*, 1996) and formation of lignin and suberin (Quiroga *et al.*, 2000).

In the present study the copper toxicity and peroxidases behaviours will be discussed.

Copper accumulation, biomass production and hydrogen peroxide level are determined as an estimate of oxidative stress state in 10 day old maize seedlings treated with 50 μM and 100 μM of CuSO_4 .

Also, we present a description of peroxidases activity modulation under application of cupric stress.

Thus, the purpose of this study is to elucidate the response of peroxidases isoforms (GPX, APX and ACPX) to the copper excess in maize shoots and roots.

MATERIALS AND METHODS

Growth conditions: Seeds of *Zea mays* L., Var. aligreen were germinated on glass plates covered with moist filter paper in darkness at 25°C for 4 days.

The seedlings were grown in plastic pots filled with nutrient solution as previously described by Mazhoudi *et al.* (1997).

Ten days old seedlings were treated for 7 days by addition of 50 and 100 μM^{-1} of CuSO_4 to nutrient medium.

Copper content: Dried shoot and root materials were ground to powder and were wet-digested in 65% nitric acid (1 mL per 0.1 g of dry matter). The digested material was made up in distilled water. The Cu content of shoot and root was determined using atomic absorption spectrophotometer (Perkin Elmer-model 2380, CRGR).

H_2O_2 determination: Hydrogen peroxide levels were determined according to Sergiev *et al.* (1997). Shoot and root tissues (500 mg) were homogenized in ice bath with 5 mL 0.1% (w/v) TCA. The homogenate was centrifuged at 12,000 g for 15 min and 0.5 mL of the supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M KI.

The absorbance of supernatant was measured at 390 nm, the content of H_2O_2 was given on a standard curve.

Peroxidase extraction: Plant material was extracted in 50 mM K-Phosphate buffer (pH 7.0) containing 5 mM sodium ascorbate and 0.2 mM EDTA. The homogenate was centrifuged at 13,000 g for 15 min. The resulting supernatant was considered as soluble enzymatic fraction.

Extraction was performed at 4°C. Protein concentration was determined according to Bradford (1976) using bovin serum albumin as standard protein.

Enzyme assays: Guaiacol peroxidase activity was determined following the increase in absorbance at 470 nm by adding the enzymatic preparation to 2 mL of 9 mM guaiacol and 10 mM hydrogen peroxide in 25 mM K-phosphate buffer (pH 7.0) (Fielding and Hall, 1978).

Coniferyl alcohol peroxidase activity was measured by monitoring the decrease in absorbance at 260 nm (Pedereño *et al.*, 1989). Ascorbate peroxidase activity was determined according to Nakano and Asada (1981).

Statistical analysis: The results presented are the values \pm standard error obtained from at least six replicates. Significant differences between treated and control plants are determined using ANOVA test ($p < 0.05$).

RESULTS

Growth analysis: Exposure to 50 and 100 μM of CuSO_4 reduced significantly seedling growth in both shoots and roots. Copper treatment caused an inhibition in shoot and root elongation (Fig. 1A and B). Also, fresh matter production was reduced by 40 and 75%, in Cu-treated shoots supplemented, respectively, with 50 and 100 μM of CuSO_4 (Fig. 1C and D).

As shown in Fig. 1E and F, Cu exposure induced a significant reduction of both root and shoot dry weights and this effect varied as the concentration of the exogenous Cu.

Copper content: When hydroponically grown maize plants were exposed to 50 and 100 μM of CuSO_4 , copper was highly accumulated in root tissues. In fact, Fig. 2B showed that Cu accumulation is increased from 80.22 $\mu\text{g g}^{-1}$ DW in control to 1085.13 $\mu\text{g g}^{-1}$ DW and 2110.50 $\mu\text{g g}^{-1}$ DW in roots treated with 50 and 100 μM of CuSO_4 , respectively. Cu-treated shoots accumulated 23.17 $\mu\text{g g}^{-1}$ DW of copper and 71.60 $\mu\text{g g}^{-1}$ DW after treatment with 50 and 100 μM of CuSO_4 , respectively (Fig. 2A).

Protein content and H_2O_2 production: The amount of total proteins was increased by 53% in roots treated with 100 μM of CuSO_4 (Fig. 2D). This stimulation was not

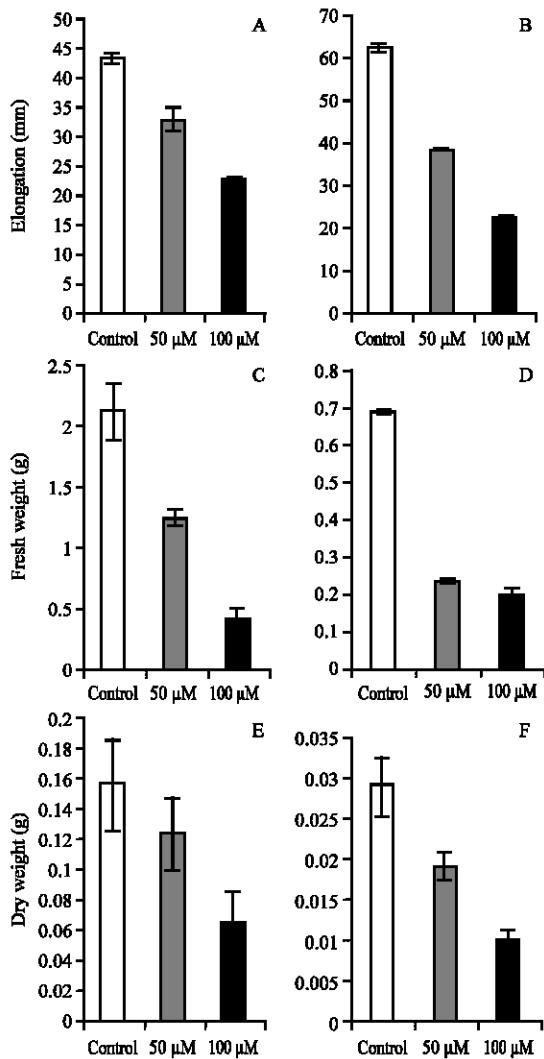


Fig. 1: Elongation, fresh and dry weight production of shoots (A, C and E) and roots (B, D and F) of maize seedlings exposed to 50 and 100 µM of CuSO₄ for up to 7 days. Data are mean values of three independent experiments. SE are indicated by vertical bars

observed in root after exposure to 50 µM Cu. In shoots, the protein content was not affected by cupric stress (Fig. 2C). The effect of copper excess on H₂O₂ formation is shown in Fig. 2E and F. The H₂O₂ generation increased significantly in treatment with supplemented Cu (especially at 100 µM). In shoots, Fig. 2E showed a significant enhancement of H₂O₂ production with 50 and 100 µM Cu-treatment. In roots, the H₂O₂ production was especially increased in plants treated with 100 µM of CuSO₄.

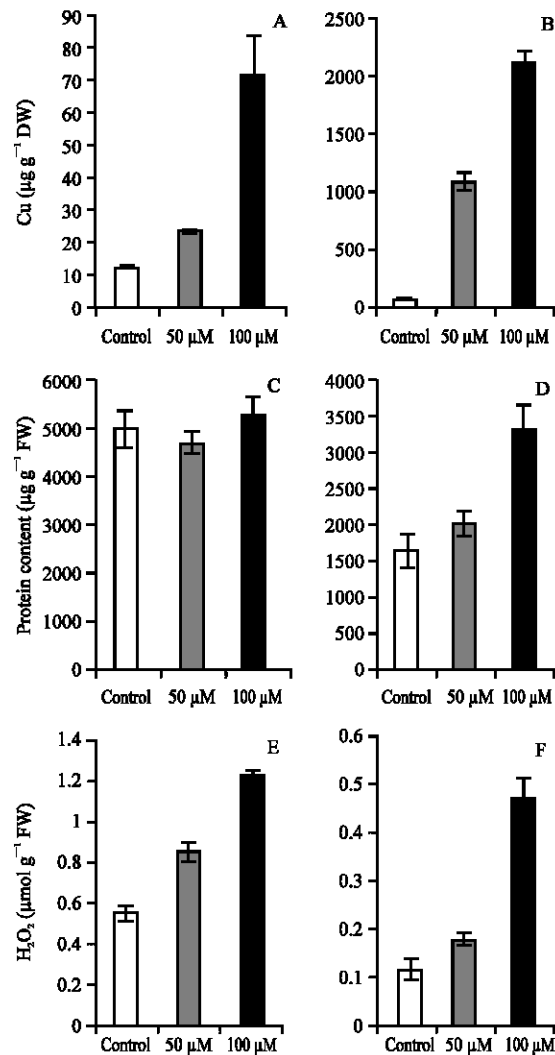


Fig. 2: Copper accumulation, protein content and H₂O₂ level in shoots (A, C and E) and roots (B, D and F) of maize seedlings exposed to 50 and 100 µM of CuSO₄ for up to 7 days. Data are mean values of three independent experiments. SE are indicated by vertical bars

Effect of copper excess on peroxidase activities: Results presented in Fig. 3 showed that exposure to excess of copper induced changes in activity of peroxidases: Guaiacol, Coniferyl alcohol and Ascorbate peroxidases. In fact, in shoots, these changes are significant only after treatment with 100 µM of CuSO₄. At this concentration, the stimulation of GPX, APX and ACP is estimated to 46, 49 and 77%, respectively.

In the case of roots, data showed that 50 µM of CuSO₄ induced stimulation in GPX and APX activities but ACPX activity remains unchanged (Fig. 3A, C and E).

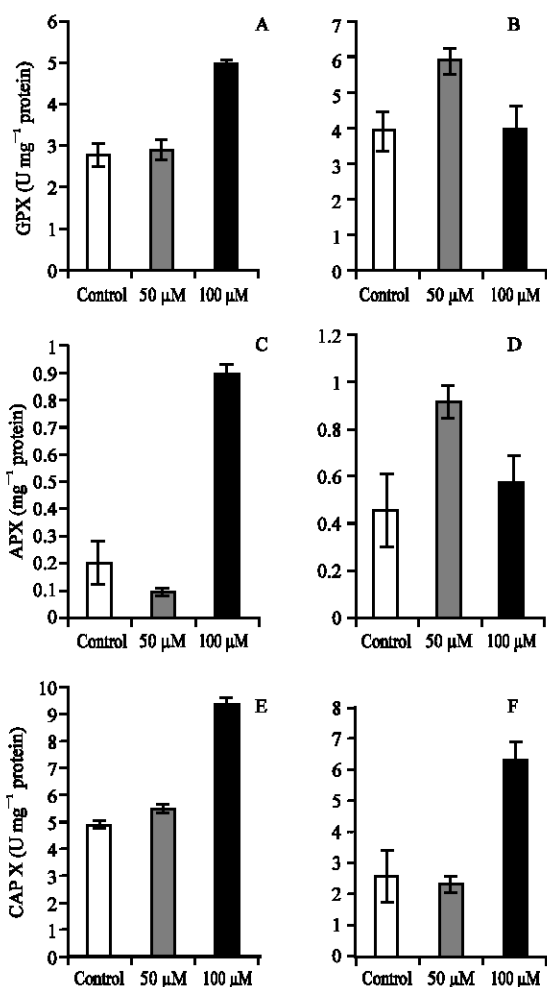


Fig. 3: Activities of guaiacol, coniferyl alcohol and ascorbate peroxidases in shoots (A, C and E) and roots (B, D and F) of maize seedlings exposed to 50 and 100 µM of CuSO₄ for up to 7 days. Data are mean values of three independent experiments. SE are indicated by vertical bars

In roots, by contrast, exposure to 100 µM Cu induced significant increase only in the activity of coniferyl alcohol peroxidase (Fig. 3B, D and F).

DISCUSSION

In the present study, we have examined the effect of copper excess (50 and 100 µM) on growth, H₂O₂ content and some peroxidase activities in roots and shoots of maize seedlings.

We are focused to investigate biochemical and physiological responses to cupric stress in both shoots and roots. Roots are in direct contact with the metal ions supplied to nutrient solution. Root architecture plays an

important role in the transport of elements to aerial plant parts. After uptake by this organ, metal is transported into the shoots and interfere with important cellular processes such as photosynthesis and respiration (Marshner, 1995; Prasad and Strzalka, 1999; Yrueala, 2005).

In this study, growth parameters were determined to estimate stress state.

Marked differences in copper toxicity symptoms (length reduction, dry and fresh weight reduction, H₂O₂ production) were observed in maize treated with excess of copper. This is in agreement with previous studies showing that heavy metals like copper caused depression of growth (Jouili and El Ferjani, 2003). The presence of 50 and 100 µM CuSO₄ in the nutrient medium inhibited considerably the fresh and dry matter production and reduced length in both shoots and roots, though the inhibition is more pronounced in roots than in shoots.

In fact, Ait Ali *et al.* (2002) suggested that maize roots are more sensitive to copper excess than shoots. It has been reported that this sensibility could response to cupric ions are mainly accumulated in roots. Accordingly, our results showed that growth reduction observed on Cu-treated roots coincided with a considerable accumulation of Cu ions especially at 100 µM of exogenous CuSO₄.

The reduction of growth can be justified as the result of the inhibition of mitotic activities of meristematic cells (Gabbrielli *et al.*, 1990).

Wainwright and Woolhouse (1977) suggested that growth inhibition can be a consequence of lost of cellular turgor due to a flight of K⁺ and another solute.

On the other hand, the application of cupric stress (at 100 µM) induced in roots the increase of protein content which was probably attributable to the neo-synthesis of stress-protein implicated in defense mechanism of plants.

Our results also shown that cupric stress induced a marked enhancement in the level of H₂O₂ (Fig. 2E and F). The increase of H₂O₂ content after CuSO₄ exposure may be caused by several biochemical perturbations on plant cells. It has been reported that decrease of enzymatic and non enzymatic free radicals scavengers caused by heavy metals may contribute to the shift in the balance of free radical metabolism toward H₂O₂ accumulation (Doke *et al.*, 1994; Lin *et al.*, 2005). Also, Sandmann and Böger (1980) reported that Cu²⁺ ions at toxic concentration inhibit photosynthetic electron transport, promoting the formation of O₂⁻, H₂O₂ and HO[•].

Although, H₂O₂ accumulation was considerate as potent compound damaging cell structure, it has been reported that generation of active oxygen species, particularly H₂O₂, during abiotic stress could be considerate as part of the signalling cascade leading to

protection from this stress (Prasad *et al.*, 1994; Alvarez *et al.*, 1998; Yruela, 2005). In fact, H₂O₂ can act as a local signal and also as a diffusible signal for the induction of defensive genes in adjacent cells (Gaspar *et al.*, 1982).

Thus, the overproduction of free oxygen could be quenched by the induction of specific enzymes such as peroxidases, since these enzymes were reported to reduce H₂O₂ using phenolic compounds or flavonoids as donors of electrons such as guaiacol, coniferyl alcohol and ascorbate. Guaiacol may be considered as a substrate for peroxidases involved in the protection against peroxidative process (Gaspar *et al.*, 1982). Ascorbate is a specific substrate for APX, which participate in ascorbate-glutathione pathway (Foyer, 1993), whereas coniferyl alcohol is a substrate specific for peroxidases involved in the lignification process.

In the present study, we noted an increase in peroxidase activity in shoots and roots exposed to 100 µM of CuSO₄ (Fig. 3A). This stimulator effect of cupric stress on peroxidase activity was also shown by other studies (Van Assche and Clijsters, 1990; Jouili and El Ferjani, 2004) which suggested that this phenomenon represent an early mechanism of defense against excess of copper and an appropriate protection against overproduction of hydrogen peroxide.

On the other hand, we noted that stimulator effect of copper on peroxidase activities is not observed in shoots treated with 50 µM of CuSO₄. The differential behavior of peroxidases could be related to the level of H₂O₂ produced under stress conditions. In fact, enhancement of peroxidase activity especially APX (at 100 µM of CuSO₄) coincided with a high stimulation of H₂O₂ production. This stimulation is more pronounced in shoots treated with 100 µM of CuSO₄ in comparison of those stressed with 50 µM of CuSO₄.

In roots, only coniferyl alcohol peroxidase activity was increased in treatment by 100 µM CuSO₄. Accordingly, it was shown that lignifying peroxidases were generally stimulated in plants exposed to cupric stress (Chen *et al.*, 2002; Jouili and El Ferjani, 2003, 2004). This enhancement of lignifying peroxidases could be related to the activation of lignification process which occur an important role in defense mechanism of plants under stress conditions (Chen *et al.*, 2002). So, it seems that maize seedlings mobilize several biochemical processes to mitigate Cu-damage.

In conclusion, the response of peroxidase activities to the cupric stress is more pronounced in shoots than in roots and probably related to the metal concentration and to the H₂O₂ levels. So, further research is required to investigate the molecular process of peroxidases response to a various amount of copper.

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