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THE EFFECTS OF EXCESSIVE EXPOSURE TO COPPER IN BEAN PLANTS

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The present study aimed to identify changes in important physiological events related to Cu, malondialdehyde (MDA), nitric oxide (NO), chlorophyll *a* and chlorophyll *b* content in the antioxidative defense system in bean seedlings (*Phaseolus vulgaris* L. cv. Akman) after Cu treatment. The activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were determined. Cu excess was induced in *Phaseolus vulgaris* (cv. Akman) plants by soaking the roots in 100 μM $CuSO_4$ solution for 10 days. Cu content increased in roots, and nitric oxide levels increased remarkably in leaf tissue. Changes in enzyme activity and MDA were observed in root tissue. The highest accumulation of NO was observed in leaf tissue. The study included an assessment of the correlation between heavy metal accumulation in roots, leading to different manifestations of stress, and changes in chlorophyll level. Indications of oxidative stress response were detected by monitoring changes in the activity and content of some components of the antioxidative mechanism. Cu treatment increased the activity of superoxide dismutase, peroxidase and catalase in leaf tissue.

Key words: *Phaseolus vulgaris* L., superoxide dismutase, peroxidase, catalase, nitric oxide, malondialdehyde, copper, chlorophyll, bean.

INTRODUCTION

Heavy metals are very important environmental pollutants. Many of them are toxic even in low concentrations. Pollution of soil and water with heavy metals creates serious problems for the environment and human health. Unlike many other pollutants, heavy metals persist in ecosystems because they cannot be destroyed biologically.

Heavy metals such as Mn, Cu, Fe, Zn and Ni are essential mineral nutrients for higher plants. For example, Cu and Zn in normal concentrations play a crucial role in plant growth and development and act as cofactors in protein and enzyme conformation. It is generally known, however, that heavy metals such as iron, copper, zinc, nickel, manganese, lead and cadmium can cause oxidative stress, eliciting enzymatic and non-enzymatic antioxidative reaction responses and lipid peroxidation in plants.

Copper can induce oxidative stress. Toxicity associated with Cu may be due to oxidative damage to biological macromolecules via redox cycling, glutathione depletion and altered sulfhydryl homeostasis (Stohs and Bagchi, 1995). Free Cu ions are able to bind irreversibly to SH groups involved in the catalytic action of enzymes (van Assche and Clijsters, 1990). Oxidative stress occurs as a result of the emergence of reactive oxygen species such as superoxide radicals, hydroxyl radicals, hydrogen peroxide and singlet oxygen. Reactive oxygen species (ROS) may damage lipids, nucleic acids, proteins, amino acids, carbohydrates and complex molecules produced from all these in cells. Cells can be protected from reactive oxygen species by the combined action of enzymatic antioxidant systems like superoxide dismutase (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6), peroxidase (POD: EC 1.11.1.7) and non-enzymatic antioxidants like ascorbate, glutathione and phenolic compounds. SOD enzyme, one of the protective mechanisms of the enzymatic antioxidant system, exists in different parts of the cell. It catalyzes dismutation of the superoxide radical into hydrogen peroxide and oxygen. Peroxidases use different electron donors (guaiacol, ascorbate) and are named according to the types of donors they use (e.g., guaiacol peroxidase, ascorbate peroxidase). Research shows that the amounts of antioxidant enzymes like SOD and POD increase in plants highly exposed to heavy metals (Acar et al., 2001). Peroxidase in plants plays a crucial role in all phys-

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Abbreviations: SOD – superoxide dismutase; POD – peroxidase; CAT – catalase; NO – nitric oxide; MDA – malondialdehyde.

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iological events related to diminishing growth, such as lignification, cross-connection of cell wall polysaccharides, oxidation of IAA, cell elongation and phenol oxidation (Mocquot et al., 1996). Changes in peroxidase activity can indicate a change in metabolic activity in physiological events such as respiration, photosynthesis, transpiration and gas exchange (MacFarland and Burchett, 2001).

Nitric oxide is a highly reactive molecule, and as a free radical it can scavenge other reactive intermediates and end chain-propagated reactions. The degree of cell damage under heavy metal stress depends on the rate of ROS formation and on the efficiency and capacity of detoxification mechanisms. Nitric oxide seems to exert a protective function also during abiotic stresses. It is reported to increase the drought tolerance of some species by inducing stomatal closure (Mata and Lamattina, 2001). The role of NO as a potent antioxidant has been described (Beligni and Lamattina, 2002). Leshem (2000) suggested that the rapid reaction between O_2^- and NO to form the powerful oxidant peroxynitrite (ONOO–) is a deleterious mechanism. In systems where the toxicity comes predominantly from peroxides, however, these compounds are much more toxic than NO and ONOO–, making NO a protective agent against them (Wink et al., 1993).

Oxidative stress occurs in plants exposed to copper at toxic concentrations, activating antioxidant mechanisms. If plants are grown in an environment in which stress is present, excess free radicals accumulate in cells because the mechanism keeping free radicals in balance is compromised. Lipid peroxidation produced by extra free radicals is a sign of the presence of a toxic substance in the environment; the last product of it is malondialdehyde (MDA). Increased MDA is an indicator of physiological stress and aging (Quariti et al., 1997). Many researchers have reported that MDA levels increase in plants under heavy metal treatment (Ozounidou, 1994; DeVos and Schat, 1981; Kennedy and Gonsalves, 1987; Halliwel and Gutteridge, 1984; Asada, 1994; Gille and Singler, 1995). Heavy metals are said to prevent chlorophyll synthesis either by direct inhibition of an enzymatic step or by induction of a major nutrient. Heavy metals such as Cu, Co, Pb and Cd cause a decrease of chlorophyll and protein content in plants. Though copper is a component of both the photosynthetic (plastocyanin) and respiratory electron chains (cytochrome oxidase), and also of various proteins, excess copper in the growth environment causes changes in membrane permeability, chromatin structure, protein synthesis, enzyme activity, photosynthesis and respiratory processes through its phytotoxic effect, and it also causes lipid peroxidation and activates senescence. This study examined how Cu stress specifically affects some important enzymes, and whether damage to lipids resulting from oxidative stress could be the mechanism of these harmful effects. We identified changes in SOD, CAT, POD activity and measured chlorophyll, MDA and NO levels along with the accumulation of Cu in bean roots and leaves.

MATERIALS AND METHODS

PLANT MATERIAL AND TREATMENT

Seeds of bean (*Phaseolus vulgaris* L. cv. Akman) were obtained from the Eskisehir Anatolian Agricultural Research Institute. They were left in half-strength Hoagland culture solution to swell for 24 h (Hoagland and Arnon, 1938), then transferred to germination pots and kept in a growth chamber for 3 days. Bean seedlings were grown as water cultures at 25±2°C under a 15 h photoperiod (200 W m^{-2}; 90% cool white fluorescent and 10% incandescent bulbs) under 65±5% relative humidity for 15 days. The 15-day-old beans were exposed to 100 μM $CuSO_4$, then grown for 10 more days in the same conditions. At the end of this treatment the first expanded leaf and stem and root tissues were taken for analyses. Approximately 1 g each of the root, stem and leaves of bean seedlings were harvested and analyzed for enzyme activity, chlorophyll content, MDA and NO levels. Soluble protein and relative water content (RWC) were also determined. RWC was measured according to Perl-Treves and Galum (1991).

DETERMINATION OF COPPER CONTENT

Cu content in root, stem and leaf tissues was determined by atomic absorption spectrometry as modified by Vinit-Dunand (2002).

ENZYME ASSAY

Total SOD activity was measured spectrophotometrically at 560 nm based on the photoreduction of nitro blue tetrazolium (NBT) as described by Beauchamp and Fridovich (1971), POD enzyme activity was assayed according to Putter (1974), and CAT activity according to Aebi et al., (1984).

ASSAY OF PROTEIN AND NITRIC OXIDE

Total protein amount was measured according to Bradford (1976), and nitric oxide levels were determined with a Cayman Nitrate/Nitrite Colorimetric Assay Kit (LDH method).

DETERMINATION OF LIPID PEROXIDATION

Lipid peroxidation was determined as malondialdehyde (MDA) content after thiobarbituric acid (TBA)

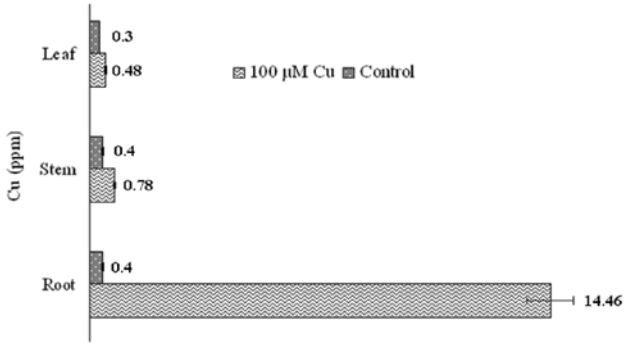

Fig. 1. Amounts of Cu (ppm) in root, stem and leaf tissues of beans after 10 days of incubation with 100 µM Cu.

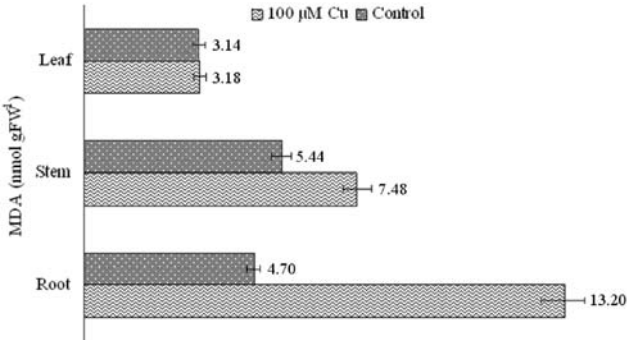

Fig. 2. MDA (nmol gFW^{-1}) levels in root, stem and leaf tissues of beans after 10 days of incubation with 100 µM Cu.

reaction according to Heath and Packer (1968). MDA amount was calculated with a coefficient of 155 mM^{-1} cm^{-1}.

CHLOROPHYLL DETERMINATION

Total chlorophyll was extracted with 80% acetone and quantified according to Arnon (1949).

STATISTICAL ANALYSIS

The data were processed by ANOVA from three replicates of all treatments, and the significance of the results was verified with the Student t-test at p=0.05.

RESULTS

Administration of excess copper in the nutrient solution was followed by an increase of Cu in leaves, and associated symptoms of toxicity. Typical symptoms of Cu toxicity developed 4 days after the beginning of treatment. Necrotic lesions were seen on the leaves of plants treated with 100 µM Cu. These symptoms were associated with a 1.6-fold increase in leaf Cu content. Chlorosis symptoms appeared, reflecting a decrease in chlorophyll *a* and *b*, confirming that excess copper is damaging to the photosynthetic apparatus.

Copper exposure influenced several biochemical and physiological parameters. Cu content and the activity of SOD, CAT and POD increased in roots, and slightly increased in stems and leaves. NO and MDA levels increased in leaves and roots, respectively, and chlorophyll decreased (Figs. 2, 3, 7).

COPPER CONTENT

The Cu concentrations in roots and primary leaves after exposure to copper are shown in Figure 1. Efficient Cu uptake was observed in roots; it increased 36.5-fold versus the control in plants treated with 100 µM Cu. Root growth was significantly reduced and root damage was evident.

The difference in Cu amounts between the control and treatment groups was significant in root tissue ($p < 0.05$) but not in stem or leaf tissue (Fig. 1).

MDA LEVELS

The results of lipid peroxidation in root, stem and leaf of beans in the control and treatment groups are given in Figure 2. In control root tissue the level of MDS was 4.70 nmol MDA/gFW-1, and in root tissue from the treated plants it was 13.02 nmol MDA/g FW. In control stem and leaf tissue the levels were 5.44 nmol MDA/g FW and 3.14 nmol MDA/g FW, respectively, and in stem and leaf tissue from treated plants the levels were 100 µM Cu was 7.48 nmol MDA/g FW and 3.18 nmol MDA/g FW, respectively. The most evident rise in MDA amount versus the control was the 3-fold increase in root tissue; in stem tissue the increase was 1.28-fold, and in leaf tissue 1.08-fold.

The increase in lipid peroxidation after treatment with 100 µM Cu was significant in root tissue but not in stem and leaf tissues.

NITRIC OXIDE LEVELS

Figure 3 presents nitric oxide levels in root, stem and leaf tissues of beans in the control and treated groups. The most evident increase versus the control group was in leaf tissue: a more than 1.82-fold increase of NO amount from the 0.27 mM MDA/g FW control level. In root it increased 1.06-fold and in stem tissue it decreased 1.16-fold.

In plants administered 100 µM Cu the increase in NO in leaf tissue was significant. The corresponding changes in root and stem tissues were not significant.

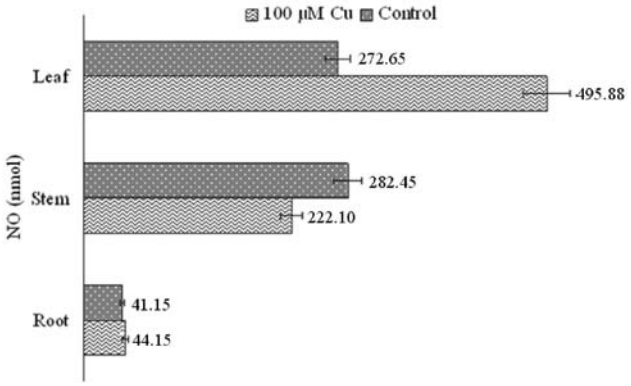

Fig. 3. NO (nmol) levels in root, stem and leaf tissues of beans after 10 days of incubation with 100 µM Cu.

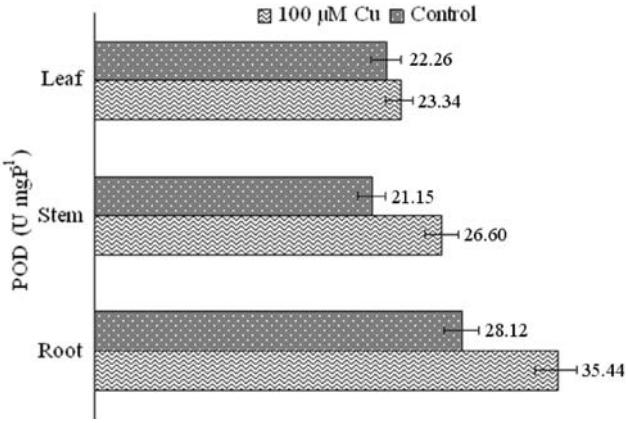

Fig. 5. POD activity (U mgP^{-1}) levels in root, stem and leaf tissues of beans after 10 days of incubation with 100 µM Cu.

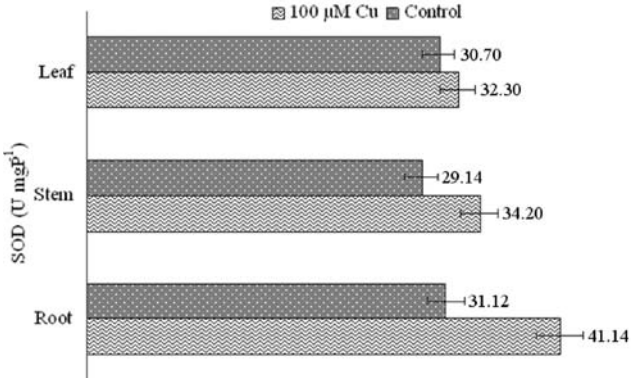

Fig. 4. SOD activity (U mgP^{-1}) levels in root, stem and leaf tissues of beans after 10 days of incubation with 100 µM Cu.

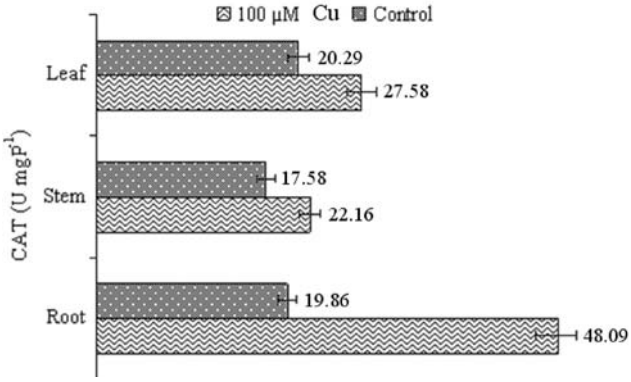

Fig. 6. CAT activity (U mgP^{-1}) levels in root, stem and leaf tissues of beans after 10 days of incubation with 100 µM Cu.

SOD ENZYME ACTIVITY

Data on total SOD enzyme activity in root, stem and leaf tissues of the control and treatment groups are given in Figure 4. The increase in total SOD enzyme activity in treated plants was highest, and significant, in root tissue (1.32-fold); the increases in stem and leaf tissue were not significant.

CAT ENZYME ACTIVITY

Figure 5 presents CAT enzyme activity in root, stem and leaf tissues of beans from the control group and treatment groups. The highest CAT enzyme activity after administration of 100 µM Cu was in root tissue (48.09±3.27 U/mg protein), followed by 27.58±2.64 U/mg protein in leaf tissue and 22.16±1.12 U/mg protein in stem tissue. The increase in CAT enzyme activity was highest in root tissue (2.42-fold).

POD ENZYME ACTIVITY

POD enzyme activity data for root, stem and leaf tissue from the control and treatment groups are given in Figure 6. The highest POD enzyme activity, 34.44 U/mg protein, was in root tissue of bean plants after copper treatment. POD enzyme activity after copper treatment was 24.60 U/mg protein in stem tissue and 23.34 U/mg protein in leaf tissue.

The highest increase in POD enzyme activity versus the control was in root tissue (1.26-fold). The increase in POD enzyme activity in root tissue was significant; in stem and leaf tissue the differences were not significant.

CHLOROPHYLL AMOUNT AND CHLOROPHYLL *a*/*b* RATIO

As seen in Figure 7, the amount of chlorophyll *a* was 2.57 mg/mL and of chlorophyll *b* was 2.45 mg/ml in

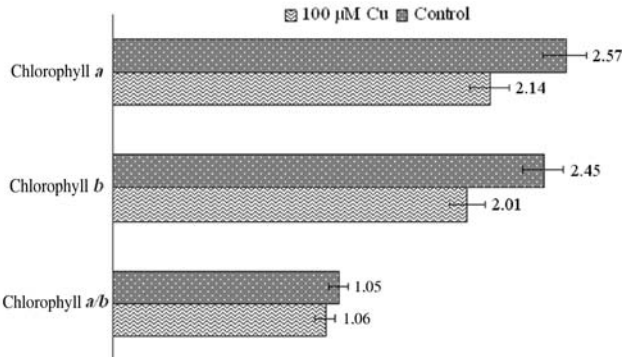

Fig. 7. Chlorophyll *a* and chlorophyll *b* content (mg/ml) and chlorophyll a/b ratio in leaf tissues of beans after 10 days of incubation with 100 μM Cu.

control leaf tissues of beans; after treatment with 100 μm Cu, the level was 2.14 mg/ml for chlorophyll *a* and 2.01 mg/mL for chlorophyll *b*. The chlorophyll *a*/*b* ratio remained about the same.

There was no statistical difference in chlorophyll *a* and chlorophyll *b* amount in leaf tissue between the control and treated plants.

DISCUSSION

The chemistry of copper should alter antioxidant production, cause lipid peroxidation, and increase NO in bean. The main aim of the present study was to identify some biochemical changes after administration of excess Cu. On day 4 of treatment with 100 μM Cu, copper was accumulated in bean root, stem and leaf tissue. Accumulation was higher in root tissues, perhaps the result of a tolerance mechanism developed by the plant in order to reduce heavy metal stress. Some copper-tolerant plants prevent copper from reaching stems and leaves by keeping it in their roots; other plants not tolerant to copper have no such mechanism, and more heavy metal can move from roots to stems (Fernandez and Henriquez, 1991). Limitation of transfer of copper to stems and leaves is suggested to explain copper tolerance in plants (Ozounidou, 1994). High concentrations of heavy metals are reported in metal-tolerant plants (Wu et al., 1975; Baker, 1978; Thomas et al., 1998).

In our study, copper treatment caused a decrease in chlorophyll *a* and *b* in leaf, but the decrease was not statistically significant.

Thomas et al. (1998) reported that chlorophyll in plants dosed with 12.5 μmol/l Cu decreased by 80%. Monni et al. (2000) reported a decrease in chlorophyll content by 15–30% in areas polluted with Cu-Ni. Chettri et al. (1998) reported a decrease in chlorophyll levels *Cladonia rangiformis* after dosing with Cu, Zn and Pb. The decrease of chlorophyll after Cu dosing may be due to blocking of enzymes acting in chlorophyll synthesis or to degradation of chlorophyll. Van Assche and Clijsters (1990) and Luna et al. (1994) reported that copper stopped the formation of chlorophyll and caused destruction of chlorophyll. In our study the decrease of chlorophyll was not statistically significant, perhaps because the roots obstructed the passage of Cu to leaf tissues.

In our study, lipid peroxidation after copper treatment was highest in root tissue. The increases in lipid peroxidation in stem and leaf tissues were not statically significant. In studies related to heavy metal stress, copper was found to be quite effective in forming toxic oxygen types and starting the process of lipid peroxidation (Halliwel and Gutteridge, 1984; Luna et al., 1994; Girotti, 1985; Weckx and Clijsters, 1996; Aust et al., 1985).

Copper has been found to decrease neutral lipid and total lipid content in tomato seedling, and this decrease was seen in the lipid levels of membranes of organelles such as the chloroplast (Quariti et al., 1997; Ozounidou, 1994). Copper and cadmium were reported to increase lipoxygenase activity, catalyzing lipid peroxidation, especially of unsaturated fatty acids; as a result of these reactions, various radicals were formed, and these led to degradation of membrane structure (DeVos and Schat, 1981). Increasing concentrations of MDA, which is a product of lipid peroxidation, are an indicator of oxidative stress after heavy metal dosing; the increase correlates with the increase of metal concentrations (Wu et al., 2003; Mazhoudi et al., 1997). In our study, both lipid peroxidation and Cu accumulation occurred mostly in root tissue, indicating oxidative stress and lipid peroxidation due to the presence of free radicals in the roots. Peroxidase enzyme activity in root tissue increased more than in the control group; the increase in stem and leaf tissue was not statistically significant. Previous studies have found a positive relationship between increased POD enzyme activity and amounts of heavy metals such as Cu, Pb and Zn in plant tissues (Girotti, 1985; Mazhoudi et al., 1997; Mocquot et al., 1996). In studies using heavy metals such as Cu, Pb and Zn, peroxidase activity was reported to be the result of the presence of free metal ions, which cannot bind to cell walls and cannot undergo phytotoxic metal accumulation or be collected in vacuoles (Woźny and Krzesłowska, 1993). Peroxidase activity and photosynthetic pigments are sensitive indicators of heavy metal stress and can be used to anticipate events on the organism level (Wu et al., 2003; MacFarlane and Burchett, 2001).

In our experiment the most evident increase in SOD, POD and CAT activity versus the control was seen in root tissue; the increases in stem and leaf tissues were not significant. These enzymes remove

superoxide radicals, which are harmful to cell membranes and occur as a result of aerobic metabolism. Hydrogen peroxide radicals, the end product, are formed through catalase and peroxidase activity.

Research on NO in plants has gained considerable attention due to its function in plant growth and development and as a key signaling molecule in different intracellular processes in plants (Gouvea et al., 1997; Leshem, 1996). In plants, enzymatic production of the signal molecule NO either is constitutive or is induced by different biotic/abiotic stresses. Nitric oxide seems to exert a protective function during abiotic stresses. Studies on the effects of heavy metal accumulation in plants have shown it to be highly phytotoxic, inhibiting growth and causing death (Thomas et al., 1998; Girotti, 1985).

In our study, the inhibitory effect of heavy metal treatment was accompanied by increased activity of superoxide dismutase, peroxidase and catalase, higher in seedlings incubated with copper. The most evident increase in NO versus the control group was in leaf tissue. The increases in SOD, POD and CAT enzyme activity in leaf and stem tissues were not statistically significant.

Our data on Cu accumulation in leaves are consistent with what is known about differences between plant tissues in the mobility and binding properties of this micronutrient. A large proportion of Cu absorbed by plants is retained in the roots (Foy et al., 1978). The chemistry of Cu should result in differences in antioxidant protection, which could be linked to specific damage to some chloroplast proteins. There was some stimulation of SOD activity, perhaps reflecting an efficient membrane protection mechanism, as Cu is known to be particularly damaging to membranes (Maksymiec, 1997). Peroxidase activity has been associated with the formation of necrotic brown spots, which in other work were found to be localized accumulations of oxidized and precipitated Mn compounds (El-Jaoual and Cox, 1998). Peroxidase induction has been observed as a general response of higher plants to toxic amounts of heavy metals (Van Assche and Clijsters, 1990).

The main aim of the present work was to identify changes in the levels of key enzymes, lipid peroxidation, NO and chlorophyll related to CO_2 fixation and nitrogen metabolism in leaves under the influence of Cu. Leaf chlorophyll decreased when copper was present in the nutrient solution. This study demonstrated the development of oxidative stress and oxidative damage to proteins after Cu dosing and accumulation of the heavy metal in leaf tissue. The changes in the investigated parameters were more drastic only after incubation with 100 μM Cu for 10 days; SOD, CAT and POD activity and MDA level increased in roots, and NO increased considerably in leaf tissues while chlorophyll was reduced. Our results show that the levels of three major enzymes involved in the antioxidative system (SOD, CAT, POD) are sensitive to heavy metal toxicity.

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