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EFFECT OF Cu^{2+} CONCENTRATION ON GROWTH, ANTIOXIDANT ENZYME ACTIVITY AND MALONDIALDEHYDE CONTENT IN GARLIC (*ALLIUM SATIVUM* L.)

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The effects of different concentrations (10^{-5} M, 10^{-4} M, 10^{-3} M) of Cu^{2+} on growth, antioxidant enzyme activity and malondialdehyde (MDA) content were investigated in hydroponically grown *Allium sativum* L. The results indicated that the growth of garlic seedlings was not inhibited under treatment with 10^{-5} M Cu^{2+}. Garlic seedlings exposed to 10^{-4} M and 10^{-3} M Cu^{2+} exhibited significant growth reduction. With increasing Cu^{2+} concentration and treatment time, superoxide dismutase (SOD) activity increased in leaves and roots, and peroxidase (POD) activity increased in leaves. In roots of plants exposed to 10^{-4} M and 10^{-3} M Cu^{2+}, POD activity increased within 9 d and then dropped, but was still higher than in the control at the end of the experiment. Catalase (CAT) activity increased in seedlings grown at 10^{-5} M and 10^{-4} M, whereas a highly toxic level of Cu^{2+} (10^{-3} M) markedly inhibited CAT activity. SOD and POD activity were higher in roots than in leaves, whereas CAT activity was higher in leaves than in roots under both control and Cu^{2+} treatments. There was no obvious effect on MDA content in the seedlings treated with 10^{-5} M Cu^{2+}; at 10^{-4} M and 10^{-3} M Cu^{2+} it increased. The mechanisms of Cu^{2+} toxicity and Cu^{2+} tolerance in garlic are briefly discussed.

Key words: *Allium sativum* L., copper, antioxidant enzymes, malondialdehyde.

INTRODUCTION

Copper is considered an essential microelement for plants. It is required by biological systems as a structural and catalytic component of proteins and enzymes such as Cu and Zn superoxide dismutases (SOD) and ascorbate peroxidases (Ouzounidou, 1994), and it is involved in electron transport in photosynthesis (Lombardi and Sebastiani, 2004; Yang et al., 2004). When absorbed in excess, however, copper can be considered a toxic element, leading to physiological constraints that decrease the vigor of plants and inhibit plant growth (Ouzounidou et al., 1992; Ouzounidou, 1994). It may induce alterations in photosynthetic and respiratory processes, enzyme activity, and membrane integrity (Ouzounidou et al., 1992; Wang et al., 2000; Wisniewski and Dickinson, 2003; Alaoui-Sossé et al., 2004).

It is known that excess heavy metals in cells cause molecular damage to plants either directly or indirectly through the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radical (·OH) and superoxide radicals ($O_2\cdot^-$) (Scandalios et al., 1997; Noctor and Foyer, 1998; Lin and Kao, 2000). An important feature of copper toxicity is the generation of active oxygen species that cause oxidative stress to plants. Evidence that copper causes the production of ROS in plants came from research on *Prunus cerasifera* and *Arabidopsis thaliana* (Lombardi and Sebastiani, 2004). Harmful ROS can damage biological molecules such as lipids, which are altered by peroxidation (Halliwell and Gutteridge, 1984; Aust and Morehouse, 1985; Weckx and Clijsters, 1996). Measurement of malondialdehyde (MDA) levels is routinely used as an index of lipid peroxidation under stress conditions (Shah et al., 2001; Zhang et al., 2005).

In plants there are protective enzymatic mechanisms and non-enzymatic mechanisms to scavenge ROS and alleviate their deleterious effects. The antioxidant enzymes include superoxide dismutase (SOD, E.C. 1.15.1.1), peroxidases (POD, E.C. 1.11.1.7), and catalase (CAT, E.C. 1.11.1.6)

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(Scandalios, 1993; Scandalios et al., 1997). The importance of antioxidant enzymes in preventing oxidative stress by scavenging ROS is generally emphasized (Dawes, 2000). SOD dismutates $O_2^{\cdot-}$ to H_2O_2, and this is decomposed to H_2O by POD and CAT, so that the accumulation of $O_2^{\cdot-}$ and H_2O_2 is effectively prevented (Panla and Thompson, 1984; Scandalios, 1993; Yan et al., 1997; Liu et al., 2002). The excess active oxygen species and peroxide can be cleared and lipid peroxidation prevented by those enzymes working together (van Assche and Clijsters, 1990).

Garlic (*Allium sativum* L.) was reported to be capable of tolerating heavy metal stress (Zhao and Bi, 1999). Little is known about the effects of Cu^{2+} on the physiological processes of garlic seedlings such as the antioxidant system or lipid peroxidation. The aim of this investigation was to clarify the effects of different concentrations of Cu^{2+} on the growth of garlic, the activity of antioxidant enzymes (SOD, POD and CAT), and MDA content in garlic plants. The possible mechanisms of garlic seedlings' tolerance of Cu^{2+} stress are discussed in the present study.

MATERIALS AND METHODS

PLANT CULTIVATION, TREATMENT AND GROWTH ANALYSIS

Equal-sized and healthy bulbs of *Allium sativum* L. cloves were chosen. The bulbs had not started to form green leaves or to grow roots. Before starting the experiment, the dry scales of the bulbs were removed. The bulbs were germinated and grown in tap water for five days. Then 20 seedlings were chosen and divided into four groups. Three Cu^{2+} treatment groups were transplanted to three containers (10 cm tall, 20 cm × 20 cm) for 12 days with 2 L Hoagland's nutrient solution (Stephan and Prochazka, 1989) spiked with different Cu^{2+} concentrations: 10^{-5} M, 10^{-4} M and 10^{-3} M. These doses were chosen in view of findings on Cu toxicity in *Allium cepa* (Liu et al., 1995) and *Allium sativum* (Liu and Kottke, 2004) in the range from 10^{-5} M to 10^{-3} M. Cu^{2+} was provided as copper sulfate ($CuSO_4 \cdot 5H_2O$). The solutions (pH 5.5) were prepared in deionized water. Hoagland's nutrient solution was used for the control. The solutions were continuously aerated with an air pump and changed every four days.

The color, number and length of leaves and roots were observed, measured and recorded every three days.

ENZYME ACTIVITY

The second leaf and ~200 mg roots from fresh seedlings were harvested every three days, weighed, washed with distilled water and then homogenized with a mortar and pestle with 5 mL chilled sodium phosphate buffer (50 mM, pH 7.8). The homogenates were centrifuged at 10,000 g for 20 min at 4°C. The supernatant was stored at 4°C and used for SOD, POD and CAT assays.

The activity of SOD was measured according to the modified method of Zhang et al. (2005) in terms of its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. The reaction mixture consisted of 54 mL methionine, 2 mL nitroblue tetrazolium chloride (NBT), 2 mL EDTA-Na_2 and 2 mL riboflavin. An appropriate quantity of enzyme extract was added to the reaction mixture. One unit of SOD was defined as the enzyme amount causing 50% inhibition of NBT reduction. SOD activity is expressed as units per mg fresh weight of seedlings.

The activity of POD was determined as described by Zhang et al. (2005). The reaction mixture in a total volume of 50 mL 100 mM sodium phosphate buffer (pH 6.0) containing 28 μL guaiacol (100%) and 9 μL H_2O_2 (30%) was prepared immediately before use. Then 1 mL enzyme extract was added to 3 mL reaction mixture. When enzyme extract was added to the reaction mixture, the reaction time was recorded immediately. The increase in absorbance was measured at 470 nm at 30 sec intervals up to 2 min using a UV-Vis spectrophotometer (UV-2550, Shimadzu, Japan). The results are presented as ΔOD_{470nm} per min per g fresh weight.

The activity of CAT was measured following Beers and Sizer (1952). The reaction mixture contained 1.5 mL 200 mM sodium phosphate buffer (pH 7.8), 1.0 mL deionized water and 0.3 mL of 0.1 M H_2O_2 prepared immediately before use, after which 0.2 mL enzyme extract was added. The reduction in absorbance was measured at 240 nm at 30 sec intervals up to 2 min using a UV-Vis spectrophotometer (UV-2550, Shimadzu, Japan). Activity was expressed as ΔOD_{240nm} per min per g fresh weight.

ESTIMATION OF LIPID PEROXIDES

The level of lipid peroxidation products was expressed as malondialdehyde (MDA) according to Buege and Aust (1978). The second leaf and ~200 mg roots from seedlings of each treatment were harvested every three days and weighed, cleaned with distilled water and then extracted in 5 ml 10% trichloroacetic acid (TCA) using a chilled mortar and pestle. The extracts were centrifuged at 4000 g for 10 min. 2 mL of 0.6% 2-thiobarbituric acid (TBA) in 10% TCA was added to the supernatant. The obtained mixture was heated in boiled water for 15 min and then quickly cooled in an ice bath. After centrifugation at 10,000 g (Eppendorf 5210R) for 10 min, the absorbance of supernatant was recorded at

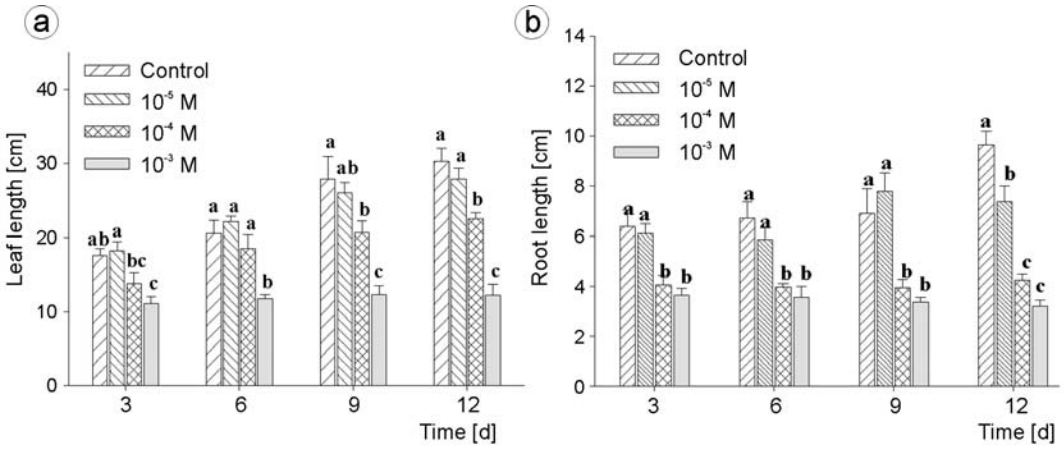

Fig. 1. Effects of different concentrations of Cu^{2+} on growth of *Allium sativum* during 3–12 days. (**a**) Leaves, (**b**) Roots (Vertical bars denote SE). Values with different letters differ significantly from each other ($p < 0.05$, t-test).

532 nm and 450 nm. The blank was 2 mL 0.6% TBA in 10% TCA without the extracts. The MDA concentrations are expressed as n mol g^{-1} fresh weight.

STATISTICAL ANALYSIS

Each treatment was triplicated for statistical validity. Data from this investigation were analyzed with standard statistical software (Sigma Plot 9.0) using means ± standard error (SE). For equality of averages the t-test was applied. Results were considered statistically significant at $p < 0.05$.

RESULTS

EFFECTS OF Cu^{2+} ON SEEDLING GROWTH

The effects of Cu^{2+} on leaves depended on the concentration used. Control seedlings usually had 4 or 5 green leaves when harvested. The seedlings treated with 10^{-5} M and 10^{-4} M Cu^{2+} were more or less the same as the control. Compared to the control, the seedlings grown under 10^{-3} M Cu^{2+} were obviously inhibited in leaf growth and had only 2 or 3 leaves.

The roots of control seedlings were in good condition, but toxic effects were observed in roots of seedlings exposed to Cu^{2+} stress at 10^{-4} M and 10^{-3} Cu^{2+}. The roots had fewer branches, and the root tips were blue and slightly decomposed. Figure 1 shows that leaf and root growth was progressively inhibited with increasing Cu^{2+} concentration, and that the effect was more significant under treatment with 10^{-4} M and 10^{-3} M Cu^{2+}. The growth of seedlings exposed to 10^{-3} M Cu^{2+} solution stopped; seedlings grown with 10^{-5} M Cu^{2+} were not significantly inhibited.

EFFECT OF Cu^{2+} ON SOD ACTIVITY

Figure 2a shows that the activity of SOD in leaves of control seedlings increased during the first 3 days of growth and then remained stable. It increased slightly in leaves subjected to 10^{-4} M Cu^{2+} versus controls only during the first 3 days of stress. However, seedlings exposed to 10^{-3} M Cu^{2+} showed an obvious increase in SOD activity: it rose 86.7% in leaves after 12 days of the applied stress.

In roots of control seedlings there was no change in SOD activity during 12 days of growth (Fig. 2b). SOD activity increased rapidly under treatment with 10^{-4} M and 10^{-3} M Cu^{2+}; it increased 565.4% in roots exposed to 10^{-3} M Cu^{2+} after 12 days. Roots showed higher SOD activity than leaves.

EFFECT OF Cu^{2+} ON POD ACTIVITY

Increasing levels of Cu^{2+} led to a concomitant increase in POD activity in leaves (Fig. 2c). POD activity in leaves of seedlings exposed to 10^{-4} M Cu^{2+} was higher than in the control only after 3 and 6 days of the applied stress. Plants exposed to 10^{-3} M Cu^{2+} showed an obvious increase in POD activity versus the control and other Cu^{2+} treatments.

POD activity in both the control and stressed seedling roots increased during the early days of growth, reaching maximum at day 9 and then dropping (Fig. 2d). After 12 days, the roots of seedlings growing under exposure to a moderate level of 10^{-4} M Cu^{2+} showed higher POD activity than in the other treatments and the control; such a result was

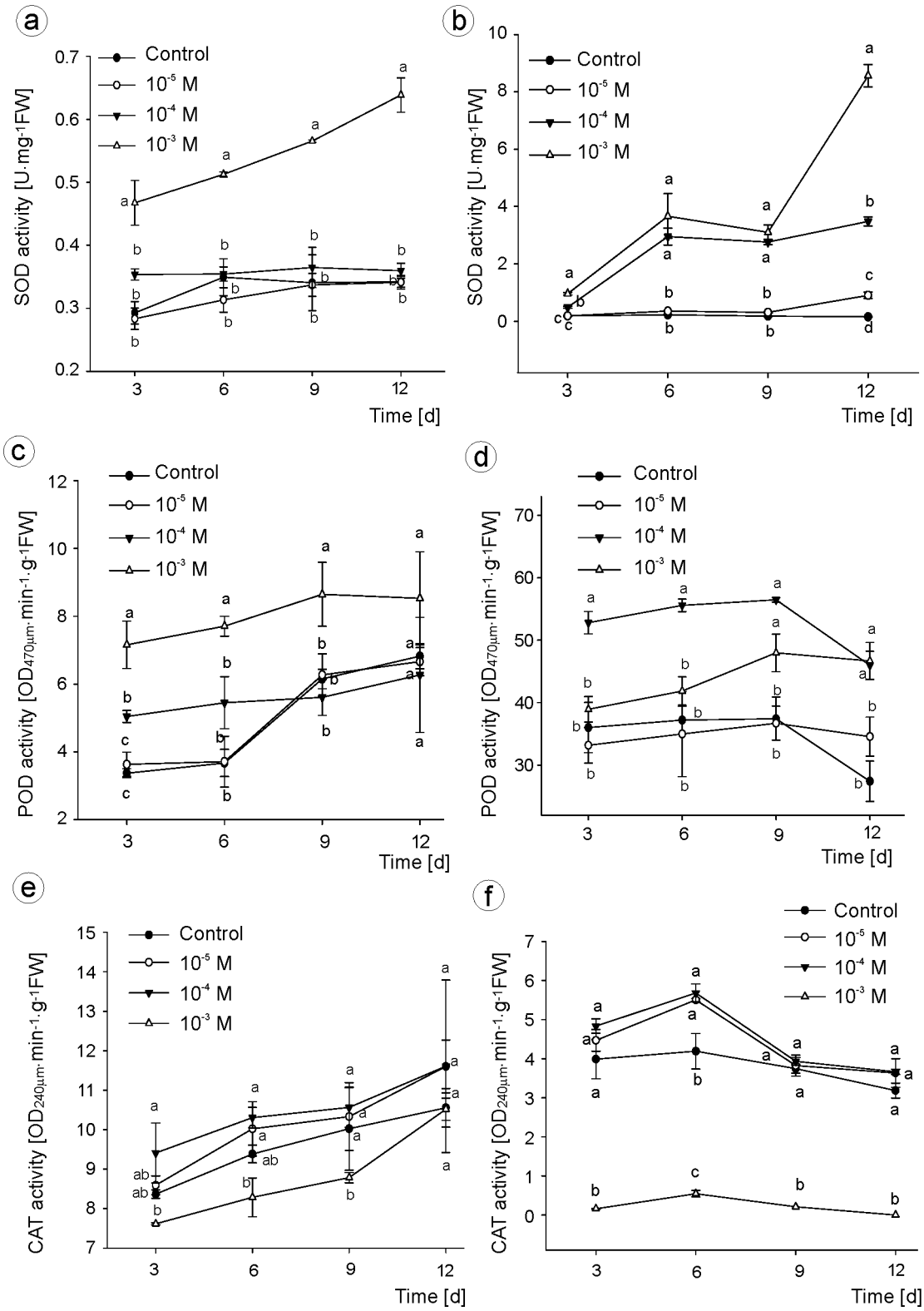

Fig. 2. Effects of different concentrations of Cu^{2+} on the activities of three antioxidant enzymes in *Allium sativum* exposed to Cu^{2+} stress over 12 days. (**a**) SOD in leaves, (**b**) SOD in roots, (**c**) POD in leaves, (**d**) POD in roots, (e) CAT in leaves, (**f**) CAT in roots. Vertical bars denote SE. Values with different letters differ significantly from each other ($p < 0.05$, t-test).

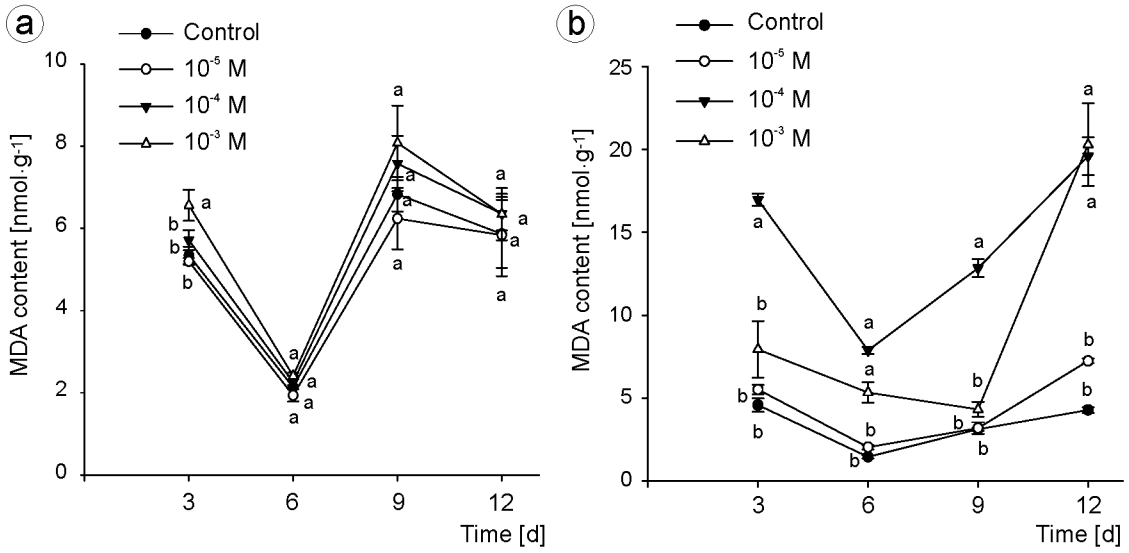

Fig. 3. Effects of different concentrations of Cu^{2+} on MDA content in *Allium sativum* exposed to Cu^{2+} stress over 12 days. (**a**) Leaves, (**b**) Roots. Vertical bars denote SE. Values with different letters differ significantly from each other ($p < 0.05$, t-test).

not found for the 10^{-3} M treatment. In 12-day seedlings, POD activity under 10^{-3} M, 10^{-4} M and 10^{-5} M Cu^{2+} stress were obviously higher than in the control plants. Both the controls and Cu^{2+} treatments showed higher POD activity in shoots than in roots.

EFFECT OF Cu^{2+} ON CAT ACTIVITY

Figure 2e,f shows increased CAT activity in both leaves and roots concomitantly with increased Cu^{2+} level, except at the 10^{-3} M dose. Roots growing in the 10^{-5} M and 10^{-4} M Cu^{2+} treatments showed CAT activity higher than in the control only after 6 days, but CAT activity was markedly inhibited under treatment with 10^{-3} M Cu^{2+}. At that level, inhibition was more evident in roots than in leaves. CAT activity in both control and tested seedlings increased in leaves over time but decreased in roots.

EFFECT OF Cu^{2+} ON MDA CONCENTRATION

MDA content in the leaves of garlic seedlings grown under different concentrations of Cu^{2+} was not significantly enhanced versus the control (Fig. 3a). Figure 3b shows that the MDA concentration in roots of plants exposed to 10^{-3} M and 10^{-5} M Cu^{2+} was not significantly higher than in the control, but in the 10^{-4} M Cu^{2+} treatment the increase was significant. In 12-day seedlings, MDA content was increased in roots of seedlings growing in the presence of 10^{-4} M Cu^{2+}. Under all Cu^{2+} treatments the MDA concentration was higher in roots than in leaves.

DISCUSSION

The results from this investigation indicate that the growth of garlic seedlings was not significantly inhibited under exposure to 10^{-5} M Cu^{2+}, while seedlings exposed to 10^{-4} M and 10^{-3} M Cu^{2+} exhibited significant growth reduction. This inhibition was more evident in roots than in leaves. Cu^{2+} accumulates mainly in roots; in garlic, Wang et al. (2001) found that small amounts of Cu^{2+} translocated to leaves at concentrations of 10^{-4} M and 10^{-5} M Cu^{2+}. Data from the present investigation showed that garlic tolerates moderate Cu^{2+} levels; 10^{-3} M Cu^{2+} exceeded its Cu toxicity thresholds, and the seedlings exposed to that dose did not grow any further during the experiment.

SOD is a crucial component of plants' antioxidative defense systems (Cakmak and Horst, 1991; Scandalios, 1993). In metal stress studies the activity of SOD is greater, as it is engaged in maintenance of the defense system of plants subjected to oxidative stress (Slooten et al., 1995). Three distinct types of SOD have been detected in plants, which can be classified according to their metal cofactors Mn, Fe and Cu/Zn. Mn-SOD is located in the mitochondria and peroxisomes; Fe-SOD, which is not found in all plants, is associated with chloroplasts; and the abundant Cu/Zn SODs are located in the cytosol, chloroplasts and peroxisomes (Bowler et al., 1992; Del Rio et al., 1998). Our results showed that Cu^{2+} dosing (10^{-3} M in leaves, and 10^{-3} M and 10^{-4} M in roots) resulted in a considerable rise of SOD activity. The increase in SOD activity in response to stresses

has been attributed to synthesis of the enzyme (Cakmak and Horst, 1991; Slooten et al., 1995).

POD is also an important enzyme, able to scavenge H_2O_2, which is a major substance degraded by SOD. The role of POD as a stress enzyme in plants has been widely accepted. POD activity has been suggested as a potential biomarker for sublethal metal toxicity in plant species (Agawal and Pandey, 2004). Induction of POD activity has been documented under many stress conditions such as NaCl stress (Agawal and Pandey, 2004), high temperature (El-shintinawy et al., 2004) and toxic concentrations of Cu, Pb, Zn, Cd and Al (Cakmak and Horst, 1991; Radotic et al., 2000; Guo T R et al., 2004). In our experiments we observed enhancement in POD activity due to increasing concentrations of Cu^{2+}, and also depending on the duration of stress and the organ. When Cu^{2+} was assimilated, pernicious peroxide was produced, accumulating with time and level of exposure. Consequently, POD activity was stimulated by the accumulation of peroxide in the plants. Enhancement of POD activity in garlic reflects its great capacity to acclimate to lower Cu^{2+} stress by rapidly engaging an antioxidative defense system. However, the decline in POD activity in roots after prolonged Cu^{2+} exposure, especially at 10^{-3} M, indicates that the scavenging function of POD was impaired with prolonged, severe Cu^{2+} stress. We ascribe the decrease of POD activity to disturbed metabolism caused by excessive Cu^{2+} in seedlings. That POD activity increased much more in roots than in leaves can be explained by the probability that the glutathione/ascorbate cycle was operating at a high rate in order to detoxify the ROS formed in the roots.

CAT is the most universal oxidoreductase, which scavenges H_2O_2 to O_2 and H_2O. Our results indicated that the CAT activity response differed between roots and leaves, and that the reaction was dependent on the level and duration of stress. CAT activity declined markedly in roots exposed to 10^{-3} M Cu^{2+}. Reduced CAT activity has been regarded as a general response to many stresses, suggested to be due to inhibition of enzyme synthesis or to a change in assembly of enzyme submits (MacRae and Ferguson, 1985). Possibly CAT is a less efficient H_2O_2 scavenger than POD because of its low substrate affinity, and is more sensitive to high Cu^{2+} levels than SOD and POD, as seen by the decline in CAT activity.

The level of the antioxidant enzymes SOD, POD and CAT may determine the sensitivity of plants to lipid peroxidation (Kanazawa et al., 2000). A deficiency of this activity may result in the enhancement of free radical-mediated lipid peroxidation. In this study, under moderate dosing with 10^{-4} M and 10^{-5} M Cu^{2+}, the activity of SOD, POD and CAT in the seedling changed with the extent of stress, presumably due to the activation of zymogen or to triggering of the defense system by reactive oxygen species. CAT activity decreased at the high level of 10^{-3} M Cu^{2+}, indicating that the ability these antioxidant enzymes to eliminate ROS is limited. Higher SOD and POD activity than CAT activity at 10^{-3} M Cu^{2+} suggests that SOD and POD provide a better defense mechanism against Cu-induced oxidative damage in garlic seedlings.

MDA is an oxidized product of membrane lipids, and accumulates when plants are exposed to oxidative stresses. MDA concentration is commonly considered a general indicator of lipid peroxidation as well as stress level (Chaoui et al., 1997; Ding et al., 2004). In our study, MDA concentrations in roots exposed to 10^{-4} M and 10^{-3} M Cu^{2+} increased versus the control, indicating that Cu^{2+} indirectly produced superoxide radicals, resulting in increased lipid peroxidative products and oxidative stress in garlic plants. MDA concentrations did not greatly increase during most of the experimental period under 10^{-5} M Cu^{2+} stress.

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