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ORIGINAL PAPER

Cadmium and copper induction of oxidative stress and antioxidative response in tomato (*Solanum lycopersicon*) leaves

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Abstract We compare cadmium and copper induced oxidative stress in tomato leaves and the antioxidative enzyme response during a time course of 96 h. Plants were subjected to 25 μM of $CdCl_2$ or $CuSO_4$ and malondialdehyde (MDA) level and activity of guaiacol peroxidase, superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase were determined. The results showed that there was an early increase in the MDA level and in the guaiacol peroxidase activity more pronounced with copper exposure during almost all the time course of the experiment. The activity of superoxide dismutase and catalase was induced very early after cadmium and copper treatment, reached a maximal value after 12 h and then declined but it remained always slightly higher than the control at the end of the experiment. Ascorbate peroxidase activity pathway was similar to superoxide dismutase or catalase with a maximal activity after 48 h of heavy metal exposure. Induction of glutathione reductase activity observed only under copper exposure is maintained during almost all the experimental time. The antioxidative activity developed by tomato leaves is more induced by copper treatment. This can be related to the ability of this metal to induce more than cadmium an accumulation of reactive oxygen species (ROS) at the cellular level. Decline in the antioxidative enzymes activity at the end of the experiment can be a consequence of cadmium- and copper-inducing a further ROS formation that might affect enzymes activity.

Keywords Antioxidative defence · Cadmium · Copper · Oxidative stress · *Solanum lycopersicon*

Abbreviations

APX	Ascorbate peroxidase
ASC	Ascorbate
BSA	Bovine serum albumin
CAT	Catalase
EDTA	Ethylenediaminetetraacetic acid
GPOD	Guaiacol peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
h	Hour
H_2O_2	Hydrogen peroxide
MDA	Malondialdehyde
NBT	Nitro blue tetrazolium
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	Thiobarbituric acid

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TBARS Thiobarbituric acid-reacting substances
TCA Trichloroacetic acid

Introduction

Cadmium (Cd) is a widely spread pollutant with no known biological function. It can reach high levels in agricultural soils and is easily assimilated by plants. Moreover, due to neurotoxic, mutagenic and carcinogenic effects, high water solubility and thereby easy entry into human body via food chain render Cd a dangerous environmental pollutant even at a low concentration (Sanita di Toppi and Gabbrielli 1999). Copper (Cu) is an essential micronutrient for plants that is a component of several electron transport enzymes and is involved in catalyzing the redox reactions in mitochondria and chloroplasts (Marschner 1995). However, Cu also induces toxicity at tissue concentrations slightly above its optimal levels (Fernandes and Henriques 1991). Many study reported cadmium and copper toxicity in plants. At the same high concentration, copper is more toxic than cadmium (Mediouni et al. 2006). However, there are more or less informations in how heavy metals induce plant growth inhibition. Copper and cadmium phytotoxicity could be a consequence of its interference with a number of metabolic processes associated with normal development like nitrogenous and lipid metabolisms and photosynthesis. In fact, Mosulén et al. (2003) reported that the presence of high concentrations of cadmium or copper in the *Chlamydomonas reinhardtii* growth medium induce a NO_3^- uptake inhibition, more pronounced with the supply of Cu^{2+}. Studies carried out by Ouariti et al. (1997), showed that excess copper led to an efficient reduction of lipid content as well as to a strong change in fatty acid composition, compared to excess cadmium. Those changes, particularly in the leaves, would mainly concern the photosynthetic apparatus, reducing, therefore, the redox power and the metabolic energy (Mosulén et al. 2003). In the other hand, photosynthetic apparatus alteration may be the consequent of the disturbance of the chloroplast structure via a degradation of polar lipids (Maksymiec et al. 1995). Indeed, heavy metals are known to involve a breakdown of cells membrane lipid due to the increased accumulation of reactive oxygen species (ROS) mediated-oxidative stress (Rellán-Álvarez et al. 2006).

ROS such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) (Cakmak 2000), may lead to unspecific oxidation of proteins and membrane lipids, resulting in an increased concentration of malondialdehyde (MDA) (Cho and Seo 2005). MDA, a product of lipid peroxidation, has been seen to be greatly accumulated after heavy metal exposure (Zhang et al. 2005; Tripathi et al. 2006; Naser et al. 2008). Heavy metals induced oxidative stress in plants was well established, however, relatively scarce information on the comparison of oxidative damage in plant leaves, in which several physiological and metabolic processes take place, under the same rate of cadmium or copper was available. Furthermore, data in the rapidity of heavy metal induced oxidative stress in plants was also lacked. Measure of oxidative stress biomarkers MDA could foresee when damage would occur and might allow understanding the difference between cadmium and copper toxicity. Due to the fact that it is a redox metal, copper can be more reactive in cells and so can induce an extreme situation of oxidative stress leading to a more strong depressive effect at cellular level. This might explain the ability of this metal to induce more than cadmium alteration in plant growth. Difference in cadmium and copper phytotoxicity can be also related to differences in some defence mechanisms implicated in heavy metal tolerance, like antioxidative system.

Aerobic organisms respond to oxidative stress either by non-enzymatic or enzymatic ROS scavenging systems. Non-enzymatic defence involves glutathione, ascorbic acid, α-tocopherol, β-carotene and other compounds capable of quenching ROS. Enzymes involved in defence include superoxide dismutases (SOD, EC 1.15.1.1), catalase (CAT, EC 1.111.1.6), peroxidases like guaiacol peroxidase (GPOD, EC 1.11.1.7), glutathione reductase (GR, EC 1.6.4.2) and ascorbate peroxidase (APX, EC 1.11.1.11) (Mittler 2002; Razinger et al. 2007). The metalloenzyme SOD is the major O_2^- scavenger and its enzymatic action results in H_2O_2 and O_2 formation. H_2O_2 is eliminated by CAT and by several classes of peroxidases (Parida et al. 2004). On the other hand, reduced glutathione (GSH) and ascorbate (ASC), which are found at high concentrations in chloroplasts and other cellular compartments, are crucial for plant

defense against oxidative stress. GSH, an intermediary redox metabolite in the ascorbate–glutathione cycle of scavenging H_2O_2, is maintained in the reduced state by GR, using NADPH as a co-factor (Arbona et al. 2003). ASC is a small, water-soluble antioxidant molecule, used as substrate for APX which catalyzes hydrogen peroxide detoxification. ASC oxidation always leads to monodehydroascorbate (MDHA) which is normally converted to ASC by monodehydroascorbate reductase (MDHAR). MDHA, unless quickly reduced by MDHAR, disproportional non-enzymatically into ASC and DHA. DHA is reduced to ASC by the action of DHAR, using GSH as the reducing substrate. Oxidized glutathione is reduced by oxidized GR. Thus, APX, in combination with the effective ascorbate–glutathione cycle, functions to prevent the accumulation of H_2O_2 (Mittova et al. 2000).

The effects of Cd and Cu on the activity of antioxidant enzymes and the involvement of these enzymes in the defense of plant tissues against metal-induced damage remain controversial and varied from plant species, tissues analyzed, concentration and duration of metal exposure. Exposure of pea plants to 20 μM of copper or cadmium had no effect on the SOD, CAT and GR activity, however, treatment with high concentration (100 μM) resulted in a decrease in the antioxidative enzyme activities (Chaoui and El Ferjani 2005). Decrease in SOD (Sandalio et al. 2001), CAT (Shaw 1995), APX and GR (Gallego et al. 1996) activities were also reported under cadmium exposure. Copper resulted in an inhibition in CAT and GR (Chen and Kao 1999) and APX (Drazkiewicz et al. 2003) activities. Conversely, data presented by Tripathi et al. (2006) in *Scenedesmus sp.* showed that both low and high concentrations of copper lead to an increase in CAT and SOD activity after metal exposure. In *Arabidopsis thaliana*, induction in GR activity was observed (Drazkiewicz et al. 2003) under copper exposure. CAT (Sharma et al. 2004; Shah et al. 2001), SOD and POD (Wu et al. 2003; Shaw 1995) and APX (Hegedüs et al. 2001) have been shown to be activated by cadmium treatment. Results in the effect of heavy metals in antioxidative enzymes presented in the literature were carried out for the majority in plants after a long time exposure. Study of antioxidative enzyme activity in plants exposed to a short-term of heavy metal might be for a great importance to understand the preliminary response of plant cells to heavy metal treatment. A relationship between heavy metals-induced-oxidative stress and antioxidative response can be investigated.

This work is a continuation of our attempt to establish a rapid, reliable and accurate assay of heavy metal-toxicities and the mechanism of tolerance especially in the above ground part of plant, in which several physiological and metabolic processes are active. In the present research we compare cadmium and copper induced-oxidative stress in tomato leaves during a time course of metal exposure. In the same conditions, activity of antioxidant enzymes, which can be implicated in the oxidative stress defence, were measured.

Materials and methods

Plant material and growth conditions

The seeds of tomato (*Solanum lycopersicon* Mill. cv. Ibiza F1) were sterilized in 10% hydrogen peroxide for 20 min. The seeds were then thoroughly washed with distilled water and germinated on moistened filter paper at 25°C in the dark. The uniform seedlings were then transferred to continuously aerated nutrient solutions containing KH_2PO_4, 0.5 mM; $Ca(NO_3)_2$, 1.25 mM; KNO_3, 1 mM; $MgSO_4$, 0.5 mM; Fe–K-EDTA, 50 μM; $MnSO_4{\cdot}4H_2O$, 5 μM; $ZnSO_4{\cdot}7H_2O$, 1 μM; $CuSO_4{\cdot}5H_2O$, 1 μM; H_3BO_3, 30 μM; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 1 μM. After an initial growth period of 10 days, 25 μM of $CdCl_2$ or $CuSO_4$ were separately added to the medium.

Plants were grown and treated in a growth chamber (26°C/70% relative humidity during the day, 20°C/90% during the night). A 16 h (daily) photoperiod was used with irradiance light of 150 μmol $m^{-2}s^{-1}$ at the canopy level. Leaves were harvested (3, 6, 12, 24, 48 and 96 h after metal exposure), weighted and stored in liquid nitrogen.

Lipid peroxide determination

Lipid peroxide was determined by measuring the concentration of thiobarbituric acid- reactive substances (TBARS), as described by Alia et al. (1995). The leaves were homogenized in 5% (w/v) trichloroacetic acid (TCA). After centrifugation, the

supernatant was added with 20% TCA containing 0.5% (w/v) thiobarbituric acid (TBA). The mixture was incubated at 95°C for 30 min. The concentration of thiobarbituric acid reacting substances was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Extraction, protein determination

Enzyme extractions were carried out at 4°C. The plant tissue was reduced to powder in liquid nitrogen and extracted at a ratio 1:3 (w/v) fresh weight in 50 mM potassium phosphate buffer (pH 7) containing 1 mM EDTA, 5 mM cysteine and 5% (w/v) insoluble polyvinylpyrrolidone (PVP). For the APX assay, 5 mM ascorbate was added to the extracted buffer. The homogenate was centrifuged at 14,000*g* for 30 min and the supernatant was used for enzyme assays.

Protein content was determined spectrophotometrically at 595 nm as described by Bradford (1976) using bovine serum albumin (BSA) as standard.

Enzyme assay

Total CAT (EC 1.11.1.6) activity was assayed in presence of H_2O_2, according to Chaparro-Giraldo et al. (2000), by monitoring the decrease of absorbance at 240 nm, as H_2O_2 was consumed. Enzyme activity was evaluated using the extinction coefficient of 40 mM^{-1} cm^{-1} for H_2O_2.

Total SOD (EC 1.15.1.1) activity was measured spectrophotometrically at 560 nm according to Beyer and Fridovich (1987), based on the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT). One unit of enzyme activity was defined as the quantity of SOD required for 50% inhibition of NBT reduction.

Total APX (EC 1.11.1.11) activity was assayed in presence of ascorbate by following the decline in absorbance of the oxidized ascorbate at 290 nm, according to Chen and Asada (1989). Enzyme activity was calculated using the extinction coefficient of 2.8 mM^{-1} cm^{-1} for ascorbate.

Total GPOD (EC 1.11.1.7) activity was measured spectrophotometrically in presence of guaiacol according to Landberg and Greger (2002). Development of absorbance at 470 nm was measured. Enzyme activity was calculated using the extinction coefficient of 26.6 mM^{-1} cm^{-1} for guaiacol.

Total GR (EC 1.6.4.2) activity was determined by following the rate of NADPH oxidation, as measured by the decrease in the absorbance at 340 nm (Rao et al. 1996).

Statistics

The experiment was repeated at least three times. The data presented here correspond to a representative experiment. The mean values ± SE are reported in the figures. The significance of differences between control and treatment was determined at the 0.05 level of probability.

Results

Effect of Cu and Cd on lipid peroxidation

The level of lipid peroxidation was determined in terms of the thiobarbituric (TBA)-reactive substances, such as MDA. The kinetic responses of the MDA content to 25 μM of $CdCl_2$ or $CuSO_4$ are summarized in Fig. 1. A significant increase in lipid peroxidation was observed in tomato leaves as compared to control seedlings 3 h after the start of cadmium exposure. An important increase in MDA level under copper treatment is observed only after 6 h of exposure. From 24 h, MDA level continued to increase during all the kinetic time with copper treatment to reach a maximum at the end of the experiment. However, during the same period of experiment, the amount of MDA induced by cadmium treatment remained constant and was less than that observed with copper treatment.

Effect of Cu and Cd on the activity of ROS-scavenging enzymes

Guaiacol peroxidase activity

The presence of 25 μM of cadmium or copper induced the capacity of H_2O_2-quenching enzymes such as the guaiacol peroxidase (GPOD). Compared to the untreated control, GPOD activity started to increase in the leaves of tomato plants 3 and 12 h after cadmium and copper exposure, respectively

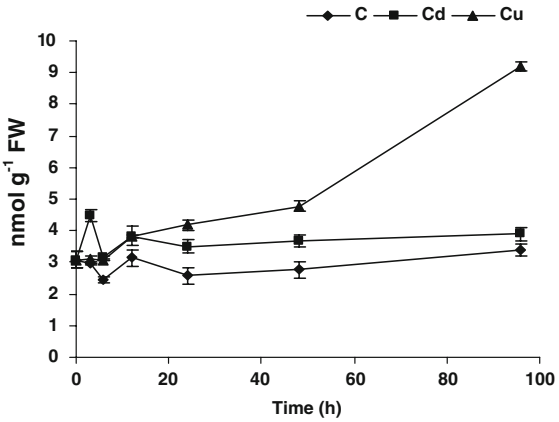

Fig. 1 The level of lipid peroxidation, determined in terms of the TBA-reactive compounds (nmol MDA g^{-1} FW) in the leaves of tomato plants treated with 25 μM $CuSO_4$ (▲) or $CdCl_2$ (■) compared to the control (◆) after different periods of treatment. Each point represents the mean ± SD of three determinations

(Fig. 2) and was more marked with cadmium exposure. From 12 h GPOD activity pursued to increase under cadmium and copper treatment until the end of the experiment and was more pronounced with copper than with cadmium exposure.

Superoxide dismutase activity

In control leaves of tomato seedlings (Fig. 3), the total activity of SOD was stable during the whole experiment. Cd or Cu treatment induced an early increase in the total SOD activity (6 h and 3 h respectively). SOD activity, under Cd and Cu exposure, continued to increase and reached a maximum after 12 h of treatment then decreased and remained slightly higher than the control at the end of the experiment. From 6 h of our experiment, SOD activity under copper exposure exceeded that with cadmium treatment and was maintained until the end of the experiment.

Catalase activity

The activity of catalase remained constant in control seedlings throughout the period investigated (Fig. 4). On the other hand, an early and strong increase in CAT activity was observed after Cd or Cu treatment. CAT activity reached a maximum 6 h after Cd and Cu exposure, and then decreased in the case of copper treatment. In the presence of cadmium catalase activity remained constant till 48 h and decreased. During almost all experimental period, CAT activity was more pronounced with cadmium treatment than with copper.

Ascorbate peroxidase activity

A significant increase of the APX activity was observed (Fig. 5) in the tomato leaves after cadmium or copper-exposed plants. Increase in APX activity was clearly detectable 3 h after heavy metal

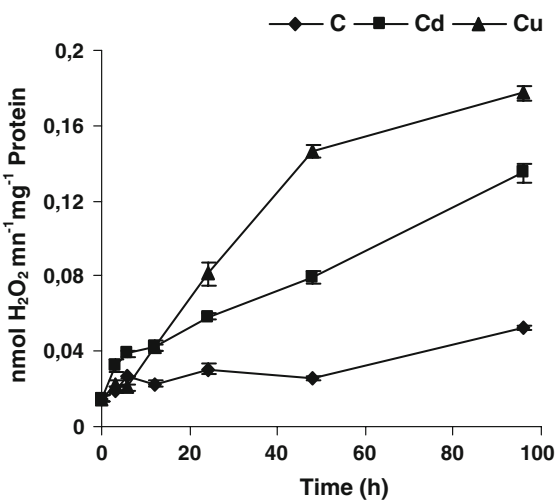

Fig. 2 Time course changes of GPOD activity in leaves from control (◆) or 25 μM of $CdCl_2$ (■) or $CuSO_4$ (▲)-treated tomato seedlings. Each point represents the mean ± SD of three determinations

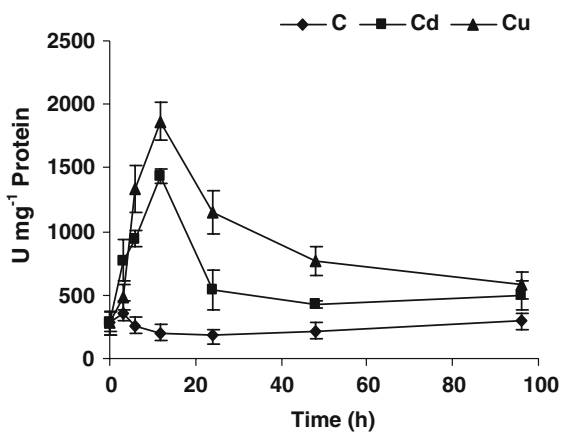

Fig. 3 Time course changes of total SOD activity in leaves from control (◆) or 25 μM of $CdCl_2$ (■) or $CuSO_4$ (▲)-treated tomato seedlings. Each point represents the mean ± SD of three determinations

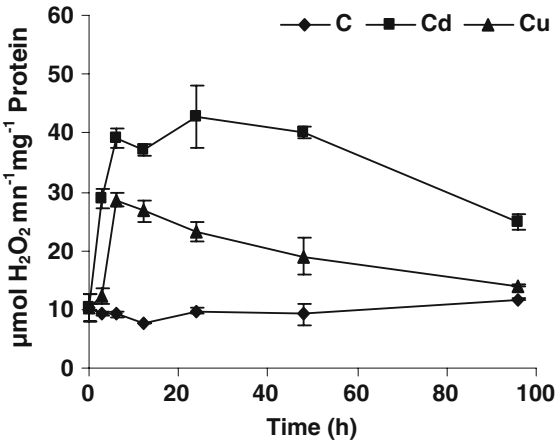

Fig. 4 Time course changes of CAT activity in leaves from control (◆) or 25 µM of $CdCl_2$ (■) or $CuSO_4$ (▲)-treated tomato seedlings. Each point represents the mean ± SD of three determinations

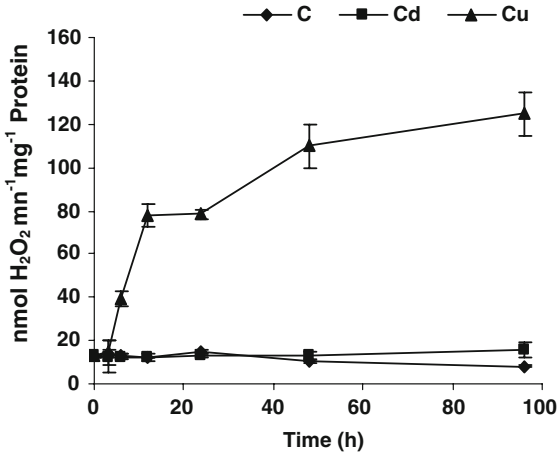

Fig. 6 Time course changes of GR activity in leaves from control (◆) or 25 µM of $CdCl_2$ (■) or $CuSO_4$ (▲)-treated tomato seedlings. Each point represents the mean ± SD of three determinations

application and reached a maximal value after 48 h before slowly decreasing to a level significantly higher than in control plants at the end of the experiment (96 h). The pattern of APX variation was similar after cadmium and copper exposure.

Glutathione reductase activity

In control plants GR activity remained constant during the time course of the experiment (Fig. 6). Cadmium treatment had no effect in GR activity which remained almost equal to control one. Cu exposure led to a significant induction of GR activity from 6 h after treatment. GR activity continued to increase under copper exposure until the end of the experiment.

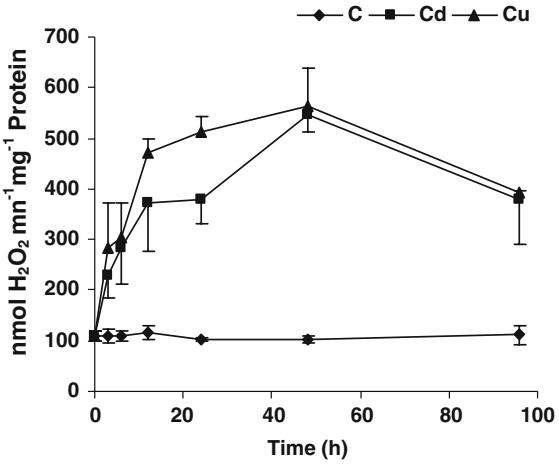

Fig. 5 Time course changes of APX activity in leaves from control (◆) or 25 µM of $CdCl_2$ (■) or $CuSO_4$ (▲)-treated tomato seedlings. Each point represents the mean ± SD of three determinations

Discussion

In our experiments, we used the concentrations of 25 µM of $CdCl_2$ or $CuSO_4$ which were highly toxic for growth of tomato plant. These concentrations were based on earlier works performed in our laboratory (Mediouni et al. 2006). The toxicity of Cd and Cu was postulated as a consequence of heavy metal induces an oxidative stress. There is increasing evidence suggesting that the oxidative stress is a major damaging factor in plants exposed to different environmental stresses (Chaparzadeh et al. 2004; Zancan et al. 2008; Smeets et al. 2008). To estimate oxidative stress, the MDA tissue content had been widely used as an indicator of lipid peroxidation and, thereby, of oxidative damage in heavy-metal-exposed plants (Drazkiewicz et al. 2007). Our results in MDA contents (Fig. 1) showed that, both, Cd and Cu induce an early increase in the amount of lipid peroxidation product. This suggests that, both heavy metals studied, can generate an untimely oxidative stress situation in the leaves of tomato plants. On the other hand, unspecific peroxidases such as guaiacol

peroxidase are considered to be heavy-metal-stress-related enzymes and can be used as stress marker (Castillo 1991). Data presented here (Fig. 2) show that either, cadmium and copper, induced a very early increase in GPOD activity suggesting that both metal lead to a very early induction of oxidative stress in tomato leaves. Because heavy metals were significantly accumulated in plant leaves only from 48 h after starting the supply (Jemal et al. 1998) thereof, it can be concluded that oxidative stress points to the appearance of a signalling response. On the other hand, our results showed that the early increase in the MDA amount and in the GPOD activity was more pronounced with cadmium than with copper. This can be due to the fact that cadmium is a non essential heavy metal consequently the presence of this metal even in a little quantity in the root system can induce a transduction signal in the above ground part of plants. Copper is an essential microelement, so plants may not response to the presence of slight amount of this metal. The increment in MDA level and in GPOD activity continues to increase thereafter with prolonged heavy metal exposure and was more pronounced with Cu than with Cd. This result was similar to that obtained in *Arabidopsis thaliana* plants (Maksymiec et al. 2007) and in *Helianthus annuus* leaf discs (Groppa et al. 2001) treated during 48 h with cadmium and copper. This can be explained by the fact that Cu is the most powerful catalyst of free radical formation and Cu-ions themselves can initiate directly oxidative breakdown of polyunsaturated lipids (De Vos et al. 1993). Conversely, Cd is a non-redox metal, unable to perform single electron transfer reactions, it does not produce directly ROS via Fenton and/or Haber Weiss reactions.

Plant cells respond to elevated levels of oxidative stress by activating their antioxidative defence systems (Mittler et al. 2004). The first group of enzymes involved in this defence assembles the ROS quenching enzymes such as SOD and catalase. In this study, an early increase in CAT and SOD activities was observed in our experimental conditions after cadmium and copper addition (Figs. 3, 4). Both CAT and SOD are important enzymes associated with antioxidative stress in plants and the increase in their activity is well correlated with the augmentation of the MDA rate and the GPOD activity (Figs. 1, 2). Furthermore, the maintenance of physiological activities under heavy metal exposure, as shown by shoot growth, could be related to the increased SOD activity to scavenge active oxygen (Zhang et al. 2005). The continuous increase in SOD and CAT activity during 12 h at 25 μM of $CdCl_2$ or $CuSO_4$ illustrated that ROS accumulated with the incubation time of heavy metal exposure and that this induction of SOD and CAT activity is a response of tomato leaves to heavy metal-induced ROS accumulation. In agreement, induction of SOD and CAT activity in *Arabidopsis thaliana* leaves was concomitant to the rapid increase in H_2O_2 amount after Cd and Cu treatment (Maksymiec and Krupa 2006). In leaves of tomato plants exposed to Cu and Cd a fast activation of CAT and SOD occurred. The time of occurrence is short which might involve a signal factor (Pei et al. 2000).

Later on, CAT and SOD activities reach a maximal value after 12 h of metal-exposure and then decrease significantly remaining slightly higher than the control 4 days after metal treatment. Data in SOD and CAT activity in plants exposed to prolonged heavy metal are very controversial. Results obtained by Baccouch et al. (1998); Wu et al. (2003) and Dong et al. (2006) had defined increases in SOD and CAT activity in response to Cd and Cu but these findings were different from the reports of Schützendübel et al. (2002); Wójcik et al. (2006) which exhibited inhibitory effects on SOD and CAT activity, respectively. The decline in SOD and CAT activity after prolonged Cd or Cu exposure indicated that the scavenging function of both enzymes was impaired with prolonged severe metal stress (Figs. 3, 4). Concurrently to CAT and SOD decrease 12 h after heavy metal exposure, the depressive effect induced by cadmium and copper in tomato leaves, shown by a more increase in MDA amount and the start of visual toxicity symptoms, was more pronounced. Moreover, the more increase in H_2O_2 rate 15 h after Cd and Cu was related to the diminution of CAT and SOD activity (Maksymiec and Krupa 2006). This confirms the implication of these enzymes in plant tolerance.

On the other hand, the early SOD activation was more pronounced with cadmium than with copper. At the same time cadmium seems to be the most inducer of oxidative stress. However, with prolonged metal exposure, induction in SOD activity was higher after treatment with copper. Furthermore, during this period copper induces more than cadmium the accumulation of MDA and the increase in GPOD

activity. SOD activity seems to be dependent in the intensity of oxidative stress in plant cells. This confirms the fact that SOD plays an important role to alleviate oxidative stress by scavenging ROS from cell compartment.

A second group of enzymes that can be activated to control and re-establish the homeostatic equilibrium of the redox status in cells reassembles enzymes of ascorbate–glutathione cycle. In our study, response of two enzymes of the ascorbate–glutathione cycle, APX and GR, was investigated under copper and cadmium exposure. The APX activity dynamics was similar to CAT and SOD. Therefore, we suggest that also this enzyme participate to ROS scavenging. In fact, decline in H_2O_2 level in pumpkin plants is mainly due to the scavenging action of APX (Dipierro et al. 2005). Furthermore enhanced tolerance to oxidative stress in Arabidopsis *gi-3 mutant* is associated with constitutive activation of APX genes (Cao et al. 2006). In contrast to APX, GR activity is induced only with copper treatment. Effect of heavy metals in GR activity was controversial. In barley plants, Finkemeier et al. (2003) observed a 50% reduction of glutathione reductase activity under cadmium exposure depending on external N availability. Decline in GR activity after heavy metals treatment was also reported by Braha et al. (2007); Tripathi et al. (2006). GR did not respond to heavy metal exposure could be explained by the fact that its constitutive activity was sufficient to maintain glutathione in a reduced state (Sharma et al. 2004) and/or through metal binding to SH-groups at the active site of the enzyme (Nagalakshmi and Prasad 2001). Later idea seems to be rejected by some authors who concluded that direct interference by the metals can be excluded, since adding Cd or Cu to the test tube did not affect enzyme activities (Braha et al. 2007). Another reason for decreased glutathione reductase activity could be a lack of redox equivalents such as NADPH. NADPH deficiency can be due to the inhibition of some enzymes producing redox equivalents such as the glucose-6-phosphate dehydrogenase (Braha et al. 2007).

The difference in GR enzymes responses to cadmium and copper treatment is also observed for the other enzymes studied. In fact, at almost all the time course, at least, SOD, APX and GR are more induced after copper exposure. This can be related to the ability of copper to induce more than cadmium the accumulation of ROS responsible for lipid peroxidation. On the other hand, the more induction of SOD capacity under copper stress at least from 6 h, leads to a supplementary production of H_2O_2 which required a further induction of enzyme activity implicated in H_2O_2-scavenging like CAT and APX. Antioxidative enzyme activity seems to be related to the oxidative stress intensity. Although their role as a defence mechanism against both metal-induced oxidative stress, at least in our experiment condition, antioxidative enzyme activities cannot explain the difference in cadmium and copper induced oxidative stress and consequently the difference in plant toxicity towards these metals. Divergence in the response of some other mechanisms of plant tolerance to heavy metal like phytochelatin might elucidate dissimilarity in plant response to cadmium and copper (Mediouni et al. 2006).

At the end of our experiment, activity of antioxidative enzymes, mainly SOD, CAT and APX seems to be declined under cadmium and copper exposure. SOD is a metalloenzyme containing Fe, Cu/Zn or Mn in its prosthetic groups. Since high concentrations of Cd or Cu have been shown to decrease Fe, Mn and Zn (Gussarsson 1994; Ait Ali et al. 2002) contents in plant tissues it can be speculated that reduction in SOD activity in the leaves of tomato plants subjected to excess Cd or Cu may result from deficiency of metals essential for catalytic action of this enzyme. The lessening in the antioxidative enzymes beyond 12 h of heavy metal exposure can be also a consequence of direct and indirect Cd and Cu induced-ROS accumulation. Study carried out by Escobar et al. (1996) reported that SOD and CAT are inactivated by singlet oxygen and peroxyl radicals. Inactivation of antioxidative enzymes following ROS accumulation has been also observed in rice roots (Demiral and Türkan 2005). Moreover, the formation of ROS during the oxidative burst could cause a substrate overload of SOD leading to its inhibition. SOD induction can be also hindered probably by other factors like the end product (H_2O_2) (Blokhina et al. 2003). On the other hand, an oxidative modification of proteins, which can result in a higher proteolytic degradation, has been reported after heavy metal exposure (Romero-Puertas et al. 2002). Other studies also suggested protein denaturation as important components of metal ions toxicity related responses (Suzuki et al. 2001).

Conclusion

Our findings add new kinetic and comparative information on the metabolism response of plants under heavy metal stress. The results indicate that the early oxidative stress induced was more pronounced with cadmium than with copper, however, after prolonged heavy metal exposure, oxidative stress was mainly enhanced under copper exposure. Tomato leaves seems to be able to respond to heavy metal by the stimulation of the antioxidative enzyme activity that can be implicated in oxidative stress alleviation. In addition, copper induces more than cadmium the activation of the antioxidative response. This can be related to the ability of copper to provoke a more important oxidative stress situation in tomato leaves and therefore suggest that antioxidative response is related to the intensity of oxidative stress induced rather than to the plant sensitivity to cadmium and copper. Difference in cadmium and copper plant response can be explained by a divergence in some other heavy metal defence mechanisms. A long-time exposure to cadmium and copper resulted in a decline in the antioxidative activity due probably to an excess both metals-induced ROS formation that can alter enzyme function.

As cadmium is not a redox-active metal, its ability to induce oxidative stress is most likely an indirect effect. Therefore, further research on the indirect mechanisms of cadmium-induced oxidative stress is required to reveal the underlying molecular and biochemical events. Moreover, that questions the role played by the antioxidant system of roots which are directly exposed to cadmium and copper. This also needs further investigations.

References

Ait Ali N, Bernal MP, Ater M (2002) Tolerance and bioaccumulation of copper in *Phragmiles australis* and *Zea mays*. Plant Soil 239:103–111. doi:10.1023/A:1014995321560

Alia KVSK, Prasad P, Pardha Saradhi P (1995) Effect of zinc on free radicals and proline in *Brassica* and *Cajanus*. Phytochemistry 42(1):45–47. doi:10.1016/0031-9422(94)00919-K

Arbona V, Flors V, Garcia-Agustin P, Gomez-Cadenas A (2003) Enzymatic and non-enzymatic antioxidant responses of *Carrizo citrange*, salt-sensitive citrus rootstock, to different levels of salinity. Plant Cell Physiol 44:388–394. doi:10.1093/pcp/pcg059

Baccouch S, Chaoui A, El Ferjani E (1998) Nickel-induced oxidative damage and antioxidant responses in *Zea mays* shoots. Plant Physiol Biochem 36:689–694. doi:10.1016/S0981-9428(98)80018-1

Beyer WF, Fridovich Y (1987) Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Ann Biochem 161:559–566. doi:10.1016/0003-2697(87)90489-1

Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot (Lond) 91:179–194. doi:10.1093/aob/mcf118

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Ann Biochem 72:248–254. doi:10.1016/0003-2697(76)90527-3

Braha B, Tintemann H, Krauss G, Ehrman J, Bärlocher F, Krauss GJ (2007) Stress response in two strains of the aquatic hyphomycete *Heliscus lugdunensis* after exposure to cadmium and copper ions. Biometals 20:93–105. doi:10.1007/s10534-006-9018-y

Cakmak I (2000) Role of zinc in protecting plant cells from reactive oxygen species. New Phytol 146:185–205. doi:10.1046/j.1469-8137.2000.00630.x

Cao S, Jiang S, Zhang R (2006) The role of GIGANTEA gene in mediating the oxidative stress response and in Arabidopsis. Plant Growth Regul 48:261–270. doi:10.1007/s10725-006-0012-8

Castillo FJ (1986) Extracellular peroxidases as markers of stress? In: Greppin H, Penel C, Gaspar T (eds) Molecular and physiological aspects of plants peroxidases. University of Geneva Press, Geneva, pp 419–426

Chaoui A, El Ferjani E (2005) Effects of cadmium and copper on antioxidant capacities, lignification and auxin degradation in leaves of pea (*Pisum sativum* L) seedlings. C R Biol 328:23–31. doi:10.1016/j.crvi.2004.10.001

Chaparro-Giraldo A, Barata RM, Chabregas SM, Azevedo RA, Silva-Filho MC (2000) Soybean leghemoglobilin targeted to potato chloroplasts influences growth and development of transgenic plants. Plant Cell Rep 19:961–965. doi:10.1007/s002990000254

Chaparzadeh N, D'Amico ML, Khavari-Nejad R-A, Izzo R, Navari-Izzo F (2004) Antioxidative responses of *Calendula officinalis* under salinity conditions. Plant Physiol Biochem 42:695–701. doi:10.1016/j.plaphy.2004.07.001

Chen GX, Asada K (1989) Ascorbate peroxidase in pea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol 30:987–998

Chen L-M, Kao C-H (1999) Effect of excess copper on rice leaves: evidence for involvement of lipid peroxidation. Bot Bull Acad Sin 40:283–287

Cho UH, Seo NH (2005) Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. Plant Sci 168:113–120. doi:10.1016/j.plantsci.2004.07.021

De Vos CHR, Bookum VMT, Vooijs R, Schat H, Dekok LJ (1993) Effect of copper on fatty acid composition and peroxidation of lipids in roots of copper tolerant and

sensitive *Silene cucubalus*. Plant Physiol Biochem 31: 151–158

Demiral T, Türkan I (2005) Comparative lipid peroxidation, antioxidant defense systems and proline content in roots of two rice cultivars differing in salt tolerance. Environ Exp Bot 53:247–257. doi:10.1016/j.envexpbot.2004.03.017

Dipierro N, Mondelli D, Paciolla C, Brunetti G, Dipierro S (2005) Changes in the ascorbate system in the response of pumpkin (*Cucurbita pepo L.*) roots to aluminium stress. J Plant Physiol 162(5):529–536. doi:10.1016/j.jplph.2004.06.008

Dong J, Wu F, Zhang G (2006) Influence of cadmium on antioxidant capacity and four microelement concentrations in tomato seedlings (*Lycopersicon esculentum*). Chemosphere 64:1659–1666. doi:10.1016/j.chemosphere.2006.01.030

Drazkiewicz M, Skorzynska-Polit E, Krupa Z (2003) Response of the ascorbate-glutathione cycle to excess copper in *Arabidopsis thaliana* (L). Plant Sci 164:195–202. doi:10.1016/S0168-9452(02)00383-7

Drazkiewicz M, Skorzynska-Polit E, Krupa Z (2007) The redox state and activity of superoxide dismutase classes in *Arabidopsis thaliana* under cadmium or copper stress. Chemosphere 67:188–193. doi:10.1016/j.chemosphere.2006.08.032

Escobar JA, Rubio MA, Lissi EA (1996) SOD and catalase inactivation by singlet oxygen and peroxyl radicals. Free Radic Biol Med 20:285–290. doi:10.1016/0891-5849(95)02037-3

Fernandes JC, Henrique FS (1991) Biochemical, physiological and structural effects of excess copper in plants. Bot Rev 57:246–273. doi:10.1007/BF02858564

Finkemeier I, Kluge C, Metwally A, Georgi M, Grotjohann N, Dietz KJ (2003) Alterations in Cd-induced gene expression under nitrogen deficiency in *Hordeum vulgare*. Plant Cell Environ 26:821–833. doi:10.1046/j.1365-3040.2003.01014.x

Gallego SM, Benavides MP, Tomaro ML (1996) Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. Plant Sci 121:151–159. doi:10.1016/S0168-9452(96)04528-1

Groppa MD, Tomaro ML, Benavides MP (2001) Polyamines as protectors against cadmium or copper-induced oxidative damage in sunflower leaf discs. Plant Sci 161:481–488. doi:10.1016/S0168-9452(01)00432-0

Gussarsson M (1994) Cd- induced alterations in nutrients composition and growth in *Betula pendula* seedlings: the significance of fine roots as primary target for Cd toxicity. J Plant Nutr 17:2151–2163

Hegedüs A, Erdei S, Horvàth G (2001) Comparative studies of H_2O_2 detoxifying enzymes in green and greening barley seedlings under cadmium stress. Plant Sci 160:1085–1093. doi:10.1016/S0168-9452(01)00330-2

Jemal F, Didier-jean L, Ghrir R, Ghorbal MH, Burkard G (1998) Characterization of cadmium binding peptides from pepper (*capsicum annum*). Plant Sci 137:143–154. doi:10.1016/S0168-9452(98)00120-4

Landberg T, Greger M (2002) Differences in oxidative stress in heavy metal resistant and sensitive clones of *Salix viminalis*. J Plant Physiol 159:69–75. doi:10.1078/0176-1617-00504

Maksymiec W, Bednara J, Baszynski T (1995) Responses of runner plants to excess copper as a function of plant growth stages: effects on morphology and structure of primary leaves and their chloroplast ultrastructural. Photosynthetica 31:427–435

Maksymiec W, Krupa Z (2006) The effects of short-term exposure to Cd, excess Cu ions and jasmonate on oxidative stress appearing in *Arabidopsis thaliana*. Environ Exp Bot 57:187–194. doi:10.1016/j.envexpbot.2005.05.006

Maksymiec W, Wójcik M, Krupa Z (2007) Variation in oxidative stress and photochemical activity in *Arabidopsis thaliana* leaves subjected to cadmium and excess copper in the presence or absence of jasmonate and ascorbate. Chemosphere 66:421–427. doi:10.1016/j.chemosphere.2006.06.025

Marschner H (1995) Mineral nutrition of higher plants. Academic Press, London, pp 337–347

Mediouni C, Benzarti O, Tray B, Ghorbel MH, Jemal F (2006) Cadmium and copper toxicity for tomato seedlings. Agron Sustain Dev 26:227–232. doi:10.1051/agro:2006008

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410. doi:10.1016/S1360-1385(02)02312-9

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci 9(10):1360–1385. doi:10.1016/j.tplants.2004.08.009

Mittova V, Volokita M, Guy M, Tai M (2000) Activities of SOD and the ascorbate-glutathione cycle enzymes in subcellular compartments in leaves and roots of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennelli*. Physiol Plant 110:42–51. doi:10.1034/j.1399-3054.2000.110106.x

Mosulén S, Domínguez MJ, Vigara J, Vílchez C, Guiraum A, Vega JM (2003) Metal toxicity in *Chlamydomonas reinhardtii*. Effect on sulfate and nitrate assimilation. Biomol Eng 20:199–203. doi:10.1016/S1389-0344(03)00053-4

Nagalakshmi N, Prasad MNV (2001) Responses of glutathione cycle enzymes and glutathione metabolism to copper stress in *Scenedesmus bijugatus*. Plant Sci 160:291–299. doi:10.1016/S0168-9452(00)00392-7

Naser A, Anjum NA, Umar S, Ahmad A, Iqbal M, Khan NA (2008) Sulphur protects mustard (*Brassica campestris L.*)from cadmium toxicity by improving leaf ascorbate and glutathione Sulphur protects mustard from cadmium toxicity. Plant Growth Regul 54:271–279. doi:10.1007/s10725-007-9251-6

Ouariti O, Boussama N, Zarrouk M, Cherif A, Ghorbal MH (1997) Cadmium- and copper-induced changes in tomato membrane lipids. Phytochem 45:1343–1350. doi:10.1016/S0031-9422(97)00159-3

Parida AK, Das AB, Mohanty P (2004) Defense potentials to NaCl in a mangrove, *Bruguiera parviflora*: differential changes of isoforms of some antioxidative enzymes. J Plant Physiol 161:531–542. doi:10.1078/0176-1617-01084

Pei Z-M, Murata Y, Benning G, Thomine S, Klüsener B, Allen GJ et al (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nat 406:731–734. doi:10.1038/35021067

Rao MV, Paliyath G, Ormrod DP (1996) Ultraviolet-B- and ozone-induced biochemical changes in antioxidant

enzymes of *Arabidopsis thaliana*. Plant Physiol 110:125–136. doi:10.1104/pp.110.1.125

Razinger J, Dermastia M, Drinovec L, Drobne D, Zrimec A, Koce JD (2007) Antioxidative responses of duckweed (*Lemna minor L.*) to short-term copper exposure. Environ Sci Pollut Res 14(3):194–201. doi:10.1065/espr2006.11.364

Rellán-Álvarez R, Ortega-Villasante C, Álvarez-Fernández A, Del Campo FF, Hernández LE (2006) Stress Responses of *Zea mays* to Cadmium and Mercury. Plant Soil 279:41–50. doi:10.1007/s11104-005-3900-1

Romero-Puertas M, Palma JM, Gomez M, Dong-Hee L, Sandalio LM (2002) Cadmium causes the oxidative modification of proteins in pea plants. Plant Cell Environ 25:677–686. doi:10.1046/j.1365-3040.2002.00850.x

Sandalio LM, Dalurza HC, Gomez M, Romero-Puertas MC, Del Rio LA (2001) Cadmium-induced changes in the growth and oxidative metabolism of Pea plants. J Exp Bot 52:2115–2126

Sanita di Toppi L, Gabbrielli R (1999) Response to cadmium in higher plants. Environ Exp Bot 41:105–130. doi:10.1016/S0098-8472(98)00058-6

Schützendübel A, Nikolova P, Rudolf C, Polle A (2002) Cadmium and H_2O_2-induced oxidative stress in *Populus X canescens* roots. Plant Physiol Biochem 40:577–584. doi:10.1016/S0981-9428(02)01411-0

Shah K, Kumar RG, Verma S, Dubey RS (2001) Effect of cadmium on lipid peroxidation, superoxide anion generation and activities of antioxidant enzymes in growing rice seedlings. Plant Sci 161:1135–1144. doi:10.1016/S0168-9452(01)00517-9

Sharma SS, Kaul S, Metwally A, Goyal KC, Finkemeier I, Dietz K-J (2004) Cadmium toxicity to barley (*Hordeum vulgare*) as affected by varying Fe nutritional status. Plant Sci 166:1287–1295. doi:10.1016/j.plantsci.2004.01.006

Shaw BP (1995) Effects of mercury and cadmium on the activities of antioxidative enzymes in the seedlings of *Phaseolus aureus*. Biol Plant 37(4):587–596. doi:10.1007/BF02908843

Smeets K, Ruytinx J, Semane B, Van Belleghem F, Remans T, Van Sanden S et al (2008) Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. Environ Exp Bot 63:1–8. doi:10.1016/j.envexpbot.2007.10.028

Suzuki N, Koizumi N, Sano H (2001) Screening of cadmium-responsive genes in *Arabidopsis thaliana*. Plant Cell Environ 24:1177–1188. doi:10.1046/j.1365-3040.2001.00773.x

Tripathi BN, Mehta SK, Amar A, Gaur JP (2006) Oxidative stress in *Scenedesmus sp* during short- and long-term exposure to Cu^{2+} and Zn^{2+}. Chemosphere 62:538–544. doi:10.1016/j.chemosphere.2005.06.031

Wójcik M, Skórzyńska-Polit E, Tukiendorf A (2006) Organic acids accumulation and antioxidant enzyme activities in *Thlaspi caerulescens* under Zn and Cd stress. Plant Growth Regul 48:145–155. doi:10.1007/s10725-005-5816-4

Wu F, Zhang G, Dominy P (2003) Four barley genotypes respond differently to cadmium: lipid peroxidation and activities of antioxidant capacity. Environ Exp Bot 50:67–78. doi:10.1016/S0098-8472(02)00113-2

Zancan S, Suglia I, La Rocca N, Ghisi R (2008) Effects of UV-B radiation on antioxidant parameters of iron-deficient barley plants. Environ Exp Bot 63:71–79

Zhang H, Jiang Y, He Z, Ma M (2005) Cadmium accumulation and oxidative burst in garlic (*Allium sativum*). J Plant Physiol 162:977–984. doi:10.1016/j.jplph.2004.12.010