Cadmium toxicity causes oxidative stress and induces response of the antioxidant system in cucumber seedlings

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In this study, the effects of cadmium (Cd) on lipid peroxidation, electrolyte leakage, protein oxidation, ascorbate peroxidase (APX; E.C. 1.11.1.11), catalase (CAT; E.C. 1.11.1.6) and superoxide dismutase (SOD; E.C. 1.15.1.1) activities, and ascorbic acid, non-protein thiol groups and total soluble protein contents in cucumber seedlings (*Cucumis sativus* L.) were investigated. Seedlings were grown *in vitro* in an agar-solidified substrate containing four Cd levels as $CdCl_2$ (0, 100, 400, and 1000 µmol L^{-1}) for 10 d. The lowest Cd level decreased the malondialdehyde concentration. Electrolyte leakage increased only at 1000 µmol Cd L^{-1}, whereas protein oxidation and total soluble protein content were enhanced at 400 and 1000 µmol Cd L^{-1}. Activity of APX was inhibited while the activities of CAT and SOD were increased at all Cd concentrations. Ascorbic acid was enhanced at 400 and 1000 µmol Cd L^{-1} whereas non-protein thiol groups were increased at all Cd supplies. The results evidence the importance of the enzymatic and non-enzymatic antioxidant system in response to cadmium toxicity in cucumber seedlings.
Key words: antioxidant enzymes, ascorbic acid, *Cucumis sativus*, heavy metal, lipid peroxidation, protein oxidation

Toxicidade de cádmio causa estresse oxidativo e induz resposta do sistema antioxidante em plântulas de pepino: No presente estudo, os efeitos do cádmio (Cd) sobre a peroxidação lipídica, o extravazamento de eletrólitos, a oxidação protéica, a atividade das enzimas peroxidase do ascorbato (APX; E.C. 1.11.1.11), catalase (CAT; E.C. 1.11.1.6) e dismutase do superóxido (SOD; E.C. 1.15.1.1) e as concentrações de ácido ascórbico, de grupos tióis não-protéicos e de proteínas solúveis totais foram investigados em plântulas de pepino (*Cucumis sativus* L.). As plântulas foram cultivadas *in vitro* em um substrato solidificado com ágar contendo quatro concentrações de Cd na forma de $CdCl_2$ (0, 100, 400 e 1000 µmol L^{-1}), durante 10 d. A menor concentração de Cd diminuiu a concentração de aldeído malônico. O extravazamento de eletrólitos aumentou somente em 1000 µmol Cd L^{-1}, enquanto a oxidação protéica e a concentração de proteínas solúveis totais foram aumentadas somente em 400 e 1000 µmol Cd L^{-1}. A atividade da APX foi inibida, enquanto as atividades da CAT e SOD foram aumentadas em todas as concentrações de Cd. A concentração de ácido ascórbico aumentou sob 400 e 1000 µmol Cd L^{-1}, enquanto a de grupos tióis não-protéicos aumentou em todas as concentrações de Cd. Os resultados evidenciam a importância do sistema antioxidante enzimático e não-enzimático na resposta à toxicidade de cádmio em plântulas de pepino.
Palavras-chave: ácido ascórbico, enzimas antioxidantes, *Cucumis sativus*, metal pesado, oxidação protéica, peroxidação lipídica

INTRODUCTION

Cadmium (Cd) is one of the most important metals in terms of food-chain contamination, because it is readily taken up by the cells of different plant species (Gomes-Junior et al., 2006; Liu et al., 2007). In fact, at least 70% of the Cd intake by humans is originated from plant foods (Wagner, 1993). Consequently, today, environment pollution by Cd is a growing concern in the research community.

Cadmium has been shown to cause many morphological, physiological, biochemical and structural changes in plants, such as growth inhibition, water imbalance and inhibition of seed germination (Benavides

et al., 2005; Mishra et al., 2006a).

Certain heavy metals such as copper and iron can be toxic through their participation in redox cycles like Fenton and/or Haber-Weiss reactions. In contrast, Cd is a non-redox metal unable to perform single electron transfer reactions, and does not produce reactive oxygen species (ROS) such as the superoxide anion ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$), but generates oxidative stress by interfering with the antioxidant defense system (Benavides et al., 2005; Gratão et al., 2005).

In plants, ROS are produced continuously as by-products of various metabolic pathways that are localized in different cellular compartments (Gratão et al., 2005), but under stressful conditions, their formation might be in excess of antioxidant scavenging capacity, thus creating oxidative stress by reaction and damage to all biomolecules, especially proteins, due to the higher rate constants of the reaction of the superoxide anion with amino acid side chains (Davies, 2003). In addition, one of the most damaging effects of oxygen cytotoxic species and their products in cells is the peroxidation of membrane lipids and ion leakage (Gratão et al., 2005; Lee et al., 2007).

To control the level of ROS and to protect the cells, plants possess low molecular weight antioxidants (ascorbic acid, reduced glutathione, carotenoids, toco-pherols) and antioxidant enzymes such as superoxide dismutase (SOD; E.C. 1.15.1.1), ascorbate peroxidase (APX; E.C. 1.11.1.11) and catalase (CAT; E.C. 1.11.1.6) that scavenge ROS (Gratão et al., 2005). Superoxide dismutase is the major superoxide radical scavenger and its enzymatic action results in H_2O_2 and O_2 formation. The product of SOD activity (H_2O_2) is still toxic and must be eliminated by conversion to H_2O in subsequent reactions. CAT and several classes of peroxidases like APX then scavenge the H_2O_2 produced (Benavides et al., 2005; Gratão et al., 2005).

The ascorbate-glutathione cycle seems to be a mechanism of great importance in controlling the cellular redox status, especially after application of heavy metals (Tiryakioglu et al., 2006; Liu et al., 2007). Ascorbic acid (AsA) is a primary as well as a secondary antioxidant. As secondary antioxidant it plays an important role in the regeneration of α-tocopherol (Foyer and Noctor, 2005). In view of the multiple roles of ascorbate, changes in its concentration may have important consequences for cellular and metabolic regulation (Noctor et al., 2000). Also, non-protein thiol groups, especially glutathione, exert several important roles in protection of plants from environmental stress factors, especially in the case of Cd toxicity (Gratão et al., 2005; Tiryakioglu et al., 2006).

Cucumber is an important crop plant and was selected for study because it can be used as an indicator species to assess ecotoxicity of soils polluted by contaminants (Cargnelutti et al., 2006; Pereira et al., 2006) and also due to the insufficient information available on Cd toxicity in this species. In order to obtain more information on the mechanisms involved in the plant response to this metal, in the present study we focused on the effects of Cd on lipid peroxidation, electrolyte leakage, protein oxidation, APX, CAT and SOD activities, and AsA, non-protein thiol groups and total soluble protein concentrations.

MATERIAL AND METHODS

Plant material and growth conditions: Seeds of cucumber (*Cucumis sativus* L. cv. Aodai) obtained from Feltrin Ltd. (Santa Maria, Rio Grande do Sul State, Brazil) were germinated in glass recipients (100 mL) containing 15 mL of medium with four Cd concentrations as $CdCl_2$ (0, 100, 400, and 1000 μmol L^{-1}) diluted in a 0.5% agar solution. The solution containing agar was heated and the cadmium solution was then added. No nutritive solution was added to the agar. The seedlings made use of the seed-stored reserves in the initial stage of development; it should be mentioned that seedlings (up to 10-d-old) did not suffer from any nutrient deficiency, as found in a preliminary experiment [see also Pereira et al. (2006)]. From preliminary analyses on the effect of several Cd concentrations (0-100 μM) on cucumber seedlings it was noted that only the highest concentration led to decreases in growth; thus such a concentration was used in the present experiments. The medium pH was adjusted to 5.5. Each experimental unit consisted of six seeds, totalizing 15 replicates per treatment. The seedlings were maintained in a growth chamber with controlled temperature (25 ± 1°C) and photoperiod (16 h light; light intensity of 35 μmol m^{-2} s^{-1} at the plant level).

Growth analysis: Cucumber growth was determined by measuring the length of the root system (Tennant, 1975) and the total dry weight (plants were left at 65ºC to a constant weight).

Metal determination: Approximately 0.05 g of roots and shoot were digested with 4 mL HNO_3 using the following stages of heating: a) 50ºC for 1 h; b) 80ºC for 1 h; and 120ºC for 1 h in a digester block (Velp, Italy). The samples were then diluted to 50 mL with high-purity water. Concentrations of Cd were determined using an atomic absorption spectrometer (model AAS 5 EA, Analytic, Jena, Germany) equipped with a transversely heated graphite furnace and an autosampler (MPE 5) (Iyengar et al., 1997).

Estimation of lipid peroxidation: The concentration of peroxides was determined as malondialdehyde (MDA) accumulation by the thiobarbituric acid (TBA) reaction as described by El-Moshaty et al. (1993). The plants were homogenized in 0.2 mol L^{-1} citrate-phosphate buffer, pH 6.5, at a proportion of 1:20 (w/v). The homogenate was filtered through two layers of filter paper and then centrifuged at 20,000 *g* at 4°C for 15 min. One milliliter of the supernatant fraction was added to an equal volume of 20% TCA containing 0.5% TBA. Tubes were placed in a 95°C water-bath for 40 min, and then immediately cooled on ice for 15 min. Samples were centrifuged at 10,000 *g* for 15 min. The absorbance of the supernatant at 532 nm was read and corrected for unspecific turbidity by subtracting the value at 600 nm.

Electrolyte leakage percentage (ELP) measurement: The ELP was measured using an electrical conductivity meter following Lutts et al. (1996), with some modifications. Seedling samples were washed with distilled water to remove surface contamination, weighed into 5-g portions and placed in individual stoppered vials containing 50 mL of distilled water. These samples were incubated at 25ºC on a shaker (100 rpm) for 24 h. Electrical conductivity of the bathing solution (EC1) was read after incubation. Samples were then placed in thermostatic water-bath at 95ºC for 15 min and the second reading (EC2) was determined after cooling the bathing solutions to 25ºC. The ELP was calculated as EC1/EC2.

Protein oxidation: The reaction of carbonyls with 2,4-dinitrophenylhydrazine (DNPH) was used to determine the amount of protein oxidation, as described by Levine et al. (1990). Cucumber seedlings were homogenized in a 25 mmol L^{-1} K-phosphate buffer containing 10 mL L^{-1} Triton X-100, pH 7.0, at a proportion of 1:5 (w/v). The homogenate was centrifuged at 13,000 *g* for 30 min at 4°C. After the DNPH-reaction, the carbonyl concentration was calculated by absorbance at 370 nm, using the molar extinction coefficient 21 x 10^3 mM cm^{-1}.

Superoxide dismutase (SOD; E.C. 1.15.1.1) assay: The activity of SOD was assayed according to Misra and Fridovich (1972). About 200 mg of cucumber seedlings were homogenized in 5 mL of 100 mmol L^{-1} K-phosphate buffer (pH 7.8) containing 0.1 mmol L^{-1} EDTA, 0.1% (v/v) Triton X-100 and 2% PVP (w/v). The extract was filtered and centrifuged at 22,000 *g* for 10 min at 4ºC, and the supernatant was used for assays. The assay mixture consisted of a total volume of 1 mL, containing glycine buffer (pH 10.5), 1 mmol L^{-1} epinephrine and enzyme material. Epinephrine was the last added component. Adrenochrome formation over the next 4 min was spectrophotometrically recorded at 480 nm. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions used. This method is based on the ability of SOD to inhibit the autoxidation of epinephrine at an alkaline pH. Since the oxidation of epinephrine leads to the production of a pink adrenochrome, the rate of increase of absorbance at 480 nm, which represents the rate of autoxidation of epinephrine, can be conveniently followed. The enzyme has been found to inhibit this radical-mediated process.

Catalase (CAT; 1.11.1.6) assay: Catalase activity were determined from cucumber seedlings homogenized in a solution containing 50 mmol L^{-1} KH_2PO_4/K_2HPO_4 (pH 7.0), 10 g L^{-1} PVP, 0.2 mmol L^{-1} EDTA and 10 mL L^{-1} Triton X-100, at a proportion of 1:5 (w/v). The homogenate was centrifuged at 12,000 *g* for 20 min at 4ºC. The supernatant was used for determination of CAT activity according to the modified method of Aebi (1984). The disappearance of H_2O_2 was monitored by measuring the decrease in absorbance at 240 nm in a reaction mixture with a final volume of 2 mL containing 15 mmol L^{-1} H_2O_2 in 50 mmol L^{-1} KPO_4 buffer (pH 7.0) and 30 µL of the extract.

Ascorbate peroxidase (APX; E.C. 1.11.1.11) assay: To determine the APX activity, cucumber seedlings were homogenized in 50 mmol L^{-1} K-phosphate buffer containing 1 mmol L^{-1} EDTA and 2% PVP (w/v), pH 7.8, at

a proportion of 1:3 (w/v). The homogenate was centrifuged at 13,000 *g* for 20 min at 4°C, and the supernatant used for enzyme activity according to the modified method of Zhu et al. (2004). The reaction mixture in a total volume of 2 mL consisted of 25 mmol L^{-1} sodium phosphate buffer (pH 7.0), 0.1 mmol L^{-1} EDTA, 0.25 mmol L^{-1} ascorbate, 1.0 mmol L^{-1} H_2O_2 and 100 µL extract. The H_2O_2-dependent oxidation of ascorbate was followed by a decrease in absorbance at 290 nm using the molar extinction coefficient 2.8 mM cm^{-1}.

Ascorbic acid (AsA) and non-protein thiol group (NPSH) concentrations: Cucumber seedlings were homogenized in a solution containing 50 mmol L^{-1} Tris-HCl and 10 mL L^{-1} Triton X-100 (pH 7.5) and centrifuged at 6,800 *g* for 10 min. To the resulting supernatant 10% TCA was added at a proportion of 1:1 (v/v) followed by centrifugation (6,800 *g* for 10 min) to remove protein. Determination of AsA was performed as described by Jacques-Silva et al. (2001). An aliquot of the sample (300 µL) was incubated at 37ºC in a medium containing 100 µL TCA 13.3%, 100 µL deionized water and 75 µL DNPH. The DNPH solution contained 2% DNPH, 0.23% thiourea, and 0.27% $CuSO_4$ diluted in 49% H_2SO_4. After 3 h, 500 µL of 65% H_2SO_4 was added and samples were read at 520 nm. A standard curve was constructed using *L*(+) ascorbic acid. Non-protein thiols concentration was measured spectrophotometrically with Ellman's reagent (Ellman, 1959). An aliquot of the sample (400 µL) was added to a medium containing 550 µL of 1 mol L^{-1} Tris-HCl (pH 7.4). The developed color was read at 412 nm after the addition of 10 mmol L^{-1} 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 mL). A standard curve using cysteine was used to calculate the concentration of thiol groups in samples.

Protein determination: In all the enzyme preparations, protein was measured by the Coomassie Blue method according to Bradford (1976) using BSA as standard.

Statistical analysis: The experiments were performed using a randomized design. The analyses of variance were computed on statistically significant differences determined based on the appropriate *F*-tests. The results are presented as means ± S.D. of at least three independent replicates. The mean differences were compared using the Tukey test ($P < 0.05$). Three pools of five replicates each ($n = 3$) were taken for all analyses from each set of experiments.

RESULTS

Analysis of Cd concentration and seedling growth: Increasing Cd concentrations significantly enhanced Cd concentration in both roots and shoot. Relative to control plants, root length decreased significantly by 83% in seedlings exposed to 100 µmol Cd L^{-1}, and by 97-98% in seedlings exposed to 400 and 1000 µmol Cd L^{-1} in substrate (Figure 1A). Furthermore, with increasing supply of Cd, the total dry weight showed a clear negative linear response. Total dry weight decreased 32%, 47% and 56%, respectively, at 100, 400 and 1000 µmol Cd L^{-1} in comparison to control seedlings (Figure 1B).

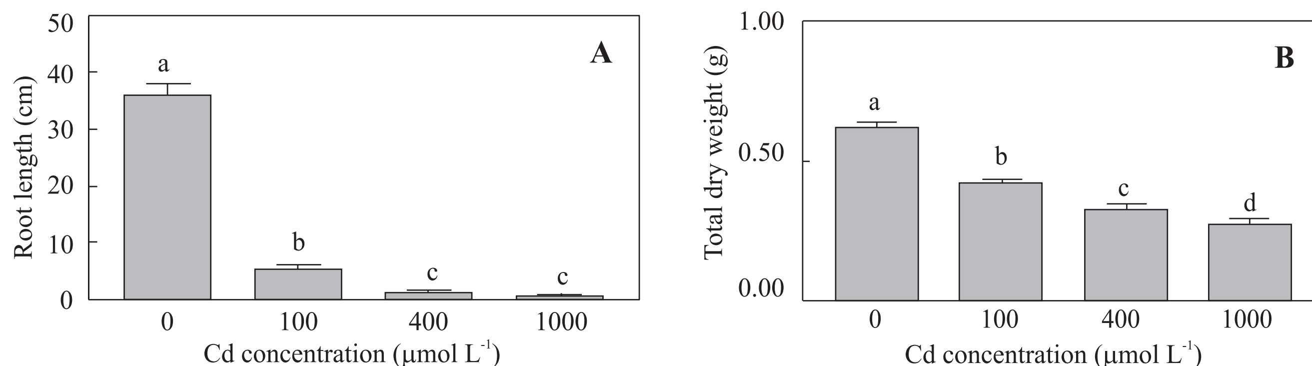


Figure 1. Effect of Cd at different concentrations on root length (**A**) and total dry weight (**B**) of 10-d-old cucumber seedlings. Vertical bars represent SD ($n = 15$). Different letters indicate significant difference among Cd concentrations (one-way ANOVA, Tukey test; $P < 0.05$).

Estimation of lipid peroxidation, ELP, protein oxidation and total soluble protein: The effect of Cd on cell membrane integrity was determined by evaluating MDA levels and ELP of plant tissues. Compared to control seedlings, a significant change (a 20.5% decrease) in MDA concentration was noticed, but only at the lowest Cd concentration (100 µmol L^{-1}) (Figure 2A); conversely, only at the highest Cd supply (1000 µmol L^{-1}) a significant change (a 55% increase) in ELP was found (Figure 2B).

Cucumber seedlings grown with Cd showed an increase in carbonyl formation, which nearly doubled at 1000 µmol Cd L^{-1} as compared to control plants (Figure 2C). Moreover, we observed that total soluble protein content was enhanced 54% and 87%, respectively, at 400 and 1000 µmol Cd L^{-1} in comparison to control seedlings (Figure 2D).

Antioxidant enzymes activities: A sharp increase in SOD activity following exposure to Cd was noticed, but with no discernible pattern in relation to the Cd doses (Figure 3A). The activity of CAT increased at 100 and 400 µmol Cd L^{-1} (respectively 180% and 173%), and then decreased at the highest Cd supply, although enzyme activity was significantly larger (80%) than in control plants. (Figure 3B). On the other hand, APX activity significantly decreased by 61%, 85% and 79% in seedlings exposed to 100, 400 and 1000 µmol Cd L^{-1} as compared to the control, respectively. No significant difference for APX activity at 400 and 1000 µmol Cd L^{-1} was detected (Figure 3C).

Concentrations of AsA and NPSH: Cadmium treatment led to increased tissue AsA concentration, but only at 400 µmol Cd L^{-1} (49%) and 1000 µmol Cd L^{-1} (63%) (Figure 4A). Furthermore, NPSH concentration increased linearly with increasing Cd supply, as can be deduced from Figure 4B.

DISCUSSION

The environmental degradation, promoted mainly by anthropogenic action, has imposed strong pressure on the quality of ecosystems. The pollution of soil and water by a wide range of contaminants for both plants and animals has become a matter of great concern to researchers. In this sense, the elevated levels of heavy metals such as Cd in the environment are a reality today. Cd occurs naturally at a low concentration in the soil, but its level has been steadily increasing due to mining and

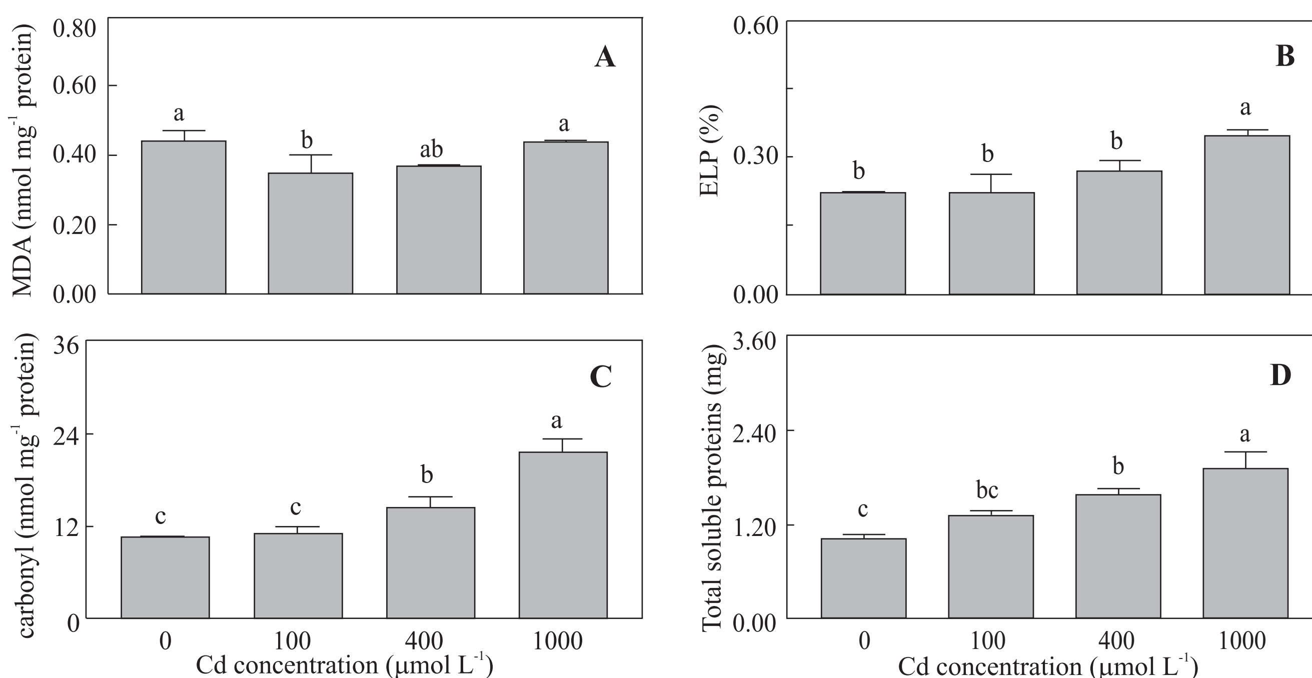


Figure 2. Effect of Cd at different concentrations on malondealdehyde (MDA) concentration (**A**), electrolyte leakage percentage (ELP) (**B**), protein oxidation (**C**), and total soluble proteins content (**D**) of 10-d-old cucumber seedlings. Statistics as in Figure 1.

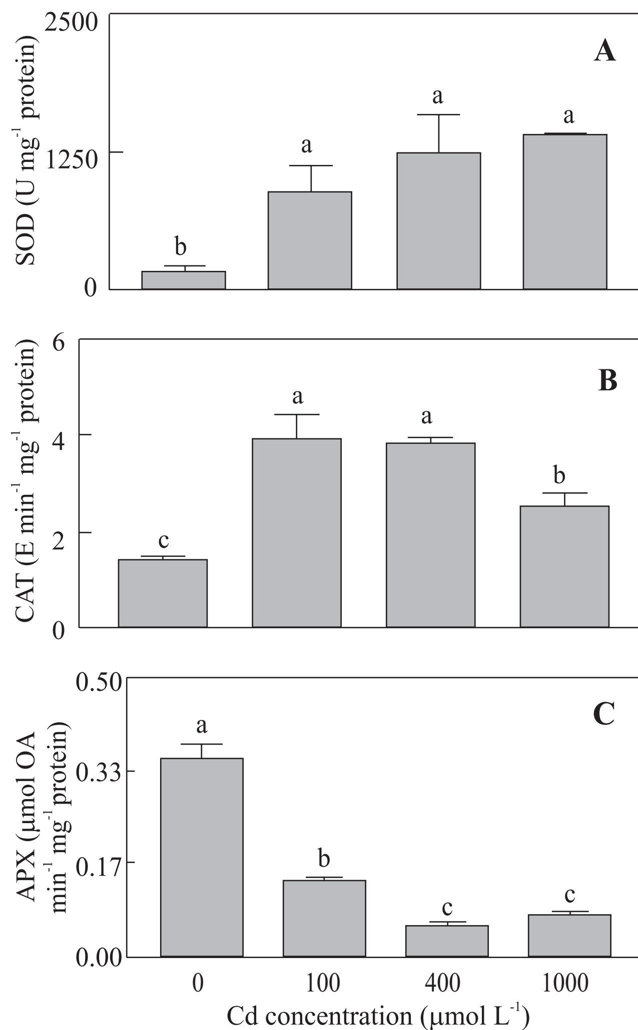
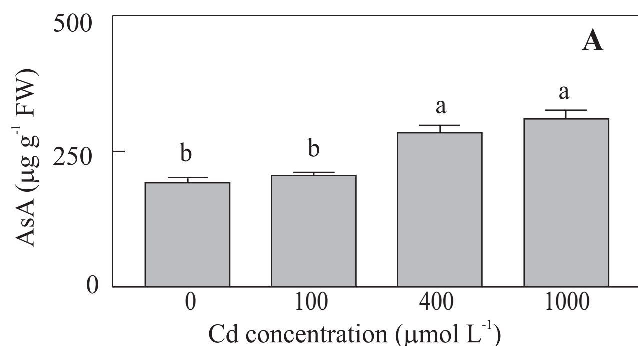


Figure 3. Effect of Cd at different concentrations on superoxide dismutase (SOD) (**A**), catalase (CAT) (**B**), and ascorbate peroxidases (APX) (**C**) activities of 10-d-old cucumber seedlings. OA = oxidized ascorbate. Statistics as in Figure 1.

smelting, dispersal of sewage sludge and the use of Cd-rich phosphate fertilizers (Wagner, 1993; Liu et al., 2007). The Cd concentrations used in our experiments (up to 1000 µmol L^{-1}) are higher than those observed in polluted soils, but these concentrations were chosen to be suitable after preliminary tests in our laboratory and, as expected, increasing Cd concentrations significantly enhanced Cd concentration in roots and in shoot. However, cucumber accumulated significantly higher Cd concentration in roots than in shoot (J.F. Gonçalves et al., unpublished results). Furthermore, even at such high concentrations, potential differences among the plants so far analyzed have been observed in relation to their tolerance of Cd (Tiryakioglu et al., 2006; Ekmekçi et al., 2007).

The present study shows that seedlings of cucumber presented a significant decrease in root length and total dry weight at all Cd treatments. However, it should be noted that although both parameters were affected by Cd supply, the decrease in root length was stronger than in total dry weight. According to Tiryakioglu et al. (2006), the reason for high root sensitivity to Cd might be related to the fact that roots are the first organs to be in contact with Cd, and hence accumulating Cd at much higher amounts than the shoot. Such decrease in growth of Cd-exposed plants has also been reported in other investigations (Vitória et al., 2001; Ferreira et al., 2002; Zhang et al., 2002).

Despite being a non-redox metal, and thus not directly producing ROS (Benavides et al., 2005), Cd can interfere with antioxidant defense systems. Under stressful conditions the protective system can be overridden by a rapid production of large amounts of ROS, leading to



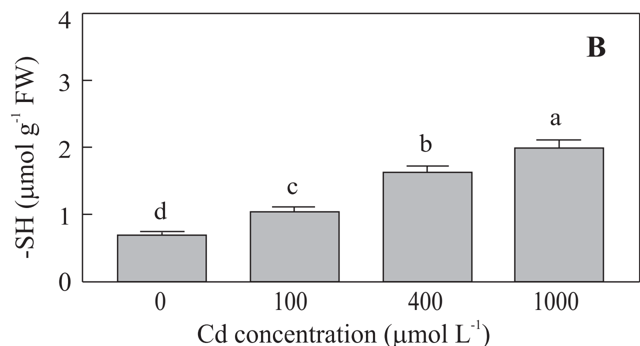


Figure 4. Effect of Cd at different concentrations on ascorbic acid (AsA) (**A**) and non-protein thiol groups concentrations (**B**) of 10-d-old cucumber seedlings. Statistics as in Figure 1.

various structural modifications in proteins (Cargnelutti et al., 2006). These oxidative modifications are characterized by the formation of carbonyl derivatives on side chains of histidine, arginine, lysine, and proline residues (Shacter et al., 1994). Our data demonstrated that the seedling exposure to Cd exceeding 100 µmol L^{-1} caused a remarkable increase in carbonyl formation, indicating that Cd promoted a high protein oxidation. This result is in agreement with that reported by Arvind and Prasad (2005) and Rellán-Álvarez et al. (2006) who noticed carbonyl accumulation in *Ceratophyllum demersum* and *Zea mays* plants exposed to Cd. In addition to oxidation of proteins, an increased total soluble protein content in cucumber seedlings exposed to Cd concentrations was found. In another study, Cargnelutti et al. (2006) showed that Hg-treated cucumbers presented increased total soluble protein content and protein oxidation. Increase in protein content is possible due to *de novo* synthesis of stress proteins provoked by metal exposure (Verma and Dubey, 2003). These stress proteins may constitute enzymes involved in GSH and phytochelatin biosynthesis and those required for Krebs cycle, as well as antioxidants and some heat shock proteins (Mishra et al., 2006b).

Measurement of MDA, a common product of lipid peroxidation, is routinely used as an index of lipid peroxidation under stressful conditions (Mishra et al., 2006a; Lee et al., 2007). In the present study the MDA level decreased upon addition of 100 µmol Cd L^{-1}, indicating lower lipid peroxidation. We suggest that the decrease in MDA concentration in the whole seedling may be due to a decrease in polyunsaturated fatty acid concentration relative to saturated fatty acids, which has also been reported in cucumber under stressful conditions (Kramer et al., 1991). Furthermore, Sinha et al. (1996) reported that a low Hg concentration lowered both the MDA and polyunsaturated fatty acid concentrations in root and leaf tissues of *Bacopa monnieri*. Moreover, membrane damage was evaluated indirectly with conductivity measurements of solute leakage from cells (Ekmekçi et al., 2007). Interestingly, in the present study the ELP was only increased upon addition of Cd at the highest level. Increased ELP has been reported by other investigations working with other plant species exposed to a range of Cd concentrations (e.g., Mishra et al., 2006a; Ekmekçi et al., 2007 and Lee et al., 2007). Cadmium, like other class B metals, has strong affinity towards nitrogen- and sulfur-containing ligands and proteins. So, it forms bridges within proteins leading to distorted membrane ion channels and leakage of ions (Mishra et al., 2006a).

In order to scavenge ROS and to avoid oxidative damage, plants possess several antioxidant enzymes. Superoxide dismutase, the first enzyme in the detoxifying process, converts superoxide radicals to H_2O_2 at a very fast rate (Gratão et al., 2005). The enhanced SOD activity we observed is consistent with previous reports in which other plant species were treated with Cd (Vitória et al., 2001; Gomes-Junior et al., 2006). Increase in SOD activity may be linked to an increase in superoxide radical formation as well as to *de novo* synthesis of enzyme protein (Verma and Dubey, 2003), which in turn may be associated with an induction of genes of SOD by superoxide-mediated signal transduction (Fatima and Ahamad, 2005). Due to the action of SOD, or by direct formation in biochemical pathways like photorespiration, H_2O_2 concentration is expected to increase inside the cell. Although H_2O_2 takes part in several important functions in plant cells (Foyer and Noctor, 2005), control of its build-up is essential to prevent oxidative damage to membranes and proteins. In fact, the increased CAT activity as found herein, which can be associated with H_2O_2 scavenging, was also observed by Vitória et al. (2001) in *Raphanus sativus* and by Gomes-Júnior et al. (2006) in *Coffea arabica* under Cd-stress conditions. This increase suggests a compensatory mechanism of defense against oxidative stress caused by toxic metal concentrations and can be explained by increase in its substrate to maintain the level of H_2O_2 as an adaptive mechanism of the plants (Cargnelutti et al., 2006). Furthermore, the combined action of CAT and SOD is critical in mitigating the effects of oxidative stress, since their roles in the cell metabolism are complementary (Benavides et al., 2005). In this sense, it is interesting to note that both SOD and CAT activities increased in Cd-treated cucumbers and that it is widely agreed that plants resist oxidative stress by increasing components of their intrinsic defensive system (Benavides et al., 2005).

In this work, APX activity, a H_2O_2-scavenger that belongs to the ascorbate-glutathione cycle, was inhibited at all Cd concentrations tested. Such a decrease has also been reported in some Cd-treated plants (Zhang et al., 2002; Gomes-Júnior et al., 2006). The reduction in APX activity may be due to GSH depletion and a subsequent

reduction in the ascorbate–glutathione cycle (Gomes-Júnior et al., 2006). The reduction in GSH could be caused by an increased rate of phytochelatin synthesis induced by Cd ions (Gomes-Júnior et al., 2006). Ascorbate peroxidase could be responsible for the fine modulation of ROS for signaling, and its reduced activity would lead to a deleterious imbalance in ROS production and scavenging (Gomes-Júnior et al., 2006), leading to signaling alterations in other physiological processes in cucumber seedlings. Furthermore, the decreased activity of APX was apparently compensated for by the increased activity of other H_2O_2-degrading enzymes like CAT (Mishra et al., 2006a).

The response of antioxidant enzymes to Cd can vary among species and among different tissues (Vitória et al., 2001; Ekmekçi et al., 2007). Either activation or inhibition of APX, CAT and SOD activities have been related to effects of Cd toxicity in plants. The reason for such inconsistent results with the effects of Cd seems to be due to differences in the plant organs studied (root, stem, leaf), duration and concentration of Cd utilized and plant species (or genotypes) under investigation (Tiryakioglu et al., 2006).

In fact, in this work only changes in enzymatic activities, such as SOD, CAT or APX were detected, but not alterations of enzyme expression. The different levels of enzymatic activity are not exclusively dependent on enzyme synthesis, but on various other factors as well. Thus, in futures studies it will be necessary to analyze the effects of Cd on antioxidant enzymes activities by native polyacrylamide gel electrophoresis to verify possible differences in activity among specific isoenzymes as shown by some authors (Vitória et al., 2001; Gomes-Júnior et al., 2006; Ferreira et al., 2007; Lee et al., 2007). Furthermore, it would be interesting to evaluate the activity of other enzymes involved in the antioxidant system, such as glutathione reductase, in order to further understand the oxidative stress caused by Cd in cucumber.

To understand the contribution of the non-enzymatic antioxidants in the response of cucumber seedlings to Cd toxicity, we examined their AsA and NPSH concentrations. Cucumber AsA concentration was enhanced at the two highest Cd concentrations compared to the control, indicating that AsA is involved in antioxidant response of this plant to Cd toxicity. Similarly to our results, other investigations have shown increased AsA for other plant species exposed to Cd (Tiryakioglu et al., 2006; Liu et al., 2007). Of the antioxidants found in plants, AsA is the most abundant and has diverse physiological roles (Noctor, 2006), being a substrate for APX in addition to directly scavenging superoxide, hydroxyl radicals, and singlet oxygen (Chen and Gallie, 2004). With regard to NPSH, its increased concentration at all Cd concentrations is also consistent with other investigations which have shown increased NPSH in plants exposed to Cd (Mishra et al., 2006a,b; Tiryakioglu et al., 2006). Such enhancement may be due to an increase of phytochelatins and is correlated with the observed reduction in APX activity. The synthesis of phytochelatins could mitigate Cd toxicity by complexing the metal and is an important mechanism of plant adaptation to long periods of metal exposure (Romero-Puertas et al., 2007). This increase in the level of thiols may be due to stimulation of enzymes of the sulfate reduction pathway such as adenosine 5'-phosphosulfate reductase and serine acetyltransferase (Noctor et al., 1998). The enhancement of AsA levels together with the marked increase in NPSH concentration possibly reflects a defense reaction to enhanced production of ROS (Tiryakioglu et al., 2006) and indicates the ability of cucumber to tolerate the cellular metal load.

We showed that MDA concentration decreased upon addition of the lowest Cd concentration whereas ELP increased in plants exposed to highest Cd supply, demonstrating that plasma membrane structure was affected by Cd in the substrate. Also, Cd strongly interfered with the oxidative metabolism of cucumber seedlings by increasing protein oxidation and AsA, non-protein thiol groups and total soluble protein content. Besides, the enzymes SOD and CAT, rather than APX, could act as important components of the antioxidant defense system mechanisms to cope with metal-induced oxidative injury. Nonetheless, despite these Cd-induced changes, cucumber growth was extremely decreased in the presence of this metal demonstrating that other enzymes or metabolites not evaluated in this work could have been affected by Cd stress.

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