RESEARCH ARTICLE

Response of *Cucumis sativus* L. seedlings to Pb exposure

Jamile F. Gonçalves[1,3], Alexssandro G. Becker[2], Luciane B. Pereira[3], João B. T. da Rocha[3], Denise Cargnelutti[3], Luciane A. Tabaldi[4], Vanessa Battisti[3], Júlia G. Farias[3], Amanda M. Fiorenza[3], Érico M. M. Flores[5], Fernando T. Nicoloso[4*] and Maria R. C. Schetinger[3]**

[1] Instituto de Ciências Básicas da Saúde, Departamento de Bioquímica, Programa de Pós-Graduação em Ciências Biológicas - Bioquímica, Universidade Federal do Rio Grande do Sul, CEP 90035-003, Porto Alegre, RS, Brazil.

[2] Centro de Ciências da Saúde, Departamento de Fisiologia e Farmacologia, Programa de Pós-Graduação em Zootecnia - Produção Animal, Universidade Federal de Santa Maria, CEP 97105-900, Santa Maria, RS, Brazil.

[3] Centro de Ciências Naturais e Exatas, Departamento de Química, Programa de Pós-Graduação em Bioquímica Toxicológica, Universidade Federal de Santa Maria, CEP 97105-900, Santa Maria, RS, Brazil.

[4] Centro de Ciências Naturais e Exatas, Departamento de Biologia, Programa de Pós-Graduação em Agronomia - Produção Vegetal, Universidade Federal de Santa Maria, CEP 97105-900, Santa Maria, RS, Brazil.

[5] Centro de Ciências Naturais e Exatas, Departamento de Química, Programa de Pós-Graduação em Química, Universidade Federal de Santa Maria, CEP 97105-900, Santa Maria, RS, Brazil.

[***] Corresponding authors:
[*]Dr. Fernando T. Nicoloso and [**]Dr. Maria Rosa C. Schetinger, respectively
Departamento de Biologia and Departamento de Química, respectively
Centro de Ciências Naturais e Exatas
Universidade Federal de Santa Maria
Santa Maria RS Brazil - 97105-900
Fax: +55 -55 -3220-8978 Phone: +55 -55 -3220-9557
e-mail: ftnicoloso@yahoo.com and mariashetinger@gmail.com, respectively

Received: 17 January 2009; Accepted: 03 November 2009.

ABSTRACT

In this study, the effects of lead (Pb) on growth, photosynthetic pigments concentration, lipid peroxidation, electrolyte leakage percentage (ELP), protein oxidation, aminolevulinate dehydratase (ALA-D; E.C. 4.2.1.24), 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 (AsA), non-protein thiol groups (NPSH) and total soluble protein concentrations in cucumber seedlings (*Cucumis sativus* L.) were investigated. Seedlings were grown *in vitro* in an agar-solidified substrate containing three Pb levels as $(C_2H_3O_2)Pb.3H_2O$ (0, 100, 400, and 1000 μmol L^{-1}) for 10 d. Increasing Pb concentrations in substrate enhanced Pb concentration in both roots and shoot. Pb accumulated at a higher amount in roots. Root length and total fresh weight were decreased at the two highest Pb concentrations. Cucumber showed no reduction in shoot length and total dry weight at any Pb level. The highest Pb concentration decreased water content and ALA-D activity as well as increased malondialdehyde, carbonyls and total soluble protein concentrations. Carotenoids concentration enhanced at 100 and 400 μmol Pb L^{-1}, while chlorophyll concentration and ELP were not affected by Pb stress. Activity of APX was inhibited while the activities of CAT and SOD were increased at all Pb concentrations. AsA was enhanced at 400 and 1000 μmol Pb L^{-1} whereas NPSH were increased only at the highest Pb concentration. Therefore, high Pb-exposure caused oxidative stress, and the antioxidant system of the cucumber seedlings was not sufficient to revert it, contributing for growth reduction.

Key words: antioxidant enzymes, cucumber, lipid peroxidation, Pb, photosynthetic pigments, protein oxidation

RESUMO

Respostas de plântulas de pepino à exposição ao chumbo: No presente estudo, os efeitos do chumbo (Pb) sobre o crescimento, a concentração de pigmentos fotossintéticos, a peroxidação lipídica, a percentagem de extravazamento de eletrólitos (ELP), a oxidação protéica, a atividade das enzimas aminolevulinato desidratase (ALA-D; E.C. 4.2.1.24), 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 (AsA), de grupos tióis não-protéicos (NPSH) 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 três concentrações de Pb na forma de $(C_2H_3O_2)Pb.3H_2O$ (0, 100, 400 e 1000 μmol L^{-1}), durante 10 dias. O aumento da concentração de Pb no substrato ocasionou um aumento da concentração de Pb tanto nas raízes quanto na parte aérea. O Pb foi acumulado em maior quantidade nas raízes. O comprimento radicular e a matéria fresca total foram diminuídos nas duas maiores concentrações de Pb. O pepino não apresentou redução no comprimento da parte aérea e na matéria seca total nos tratamento de Pb. A maior concentração de Pb diminuiu o conteúdo de água e a atividade da ALA-D bem como aumentou as concentrações de aldeído malônico, de grupos carbonil e de proteínas solúveis totais. A concentração de carotenóides aumentou em 100 e 400 μmol Pb L^{-1}, enquanto a concentração de clorofila e a ELP não foram afetadas pelo estresse com Pb. A atividade da APX foi inibida, enquanto as atividades da CAT e SOD foram aumentadas em todas as concentrações de Pb. A concentração de AsA aumentou sob 400 e 1000 μmol Pb L^{-1}, enquanto a de NPSH aumentou somente na maior concentração de Pb. Portanto, a exposição a altas concentrações de Pb causou estresse oxidativo e o sistema antioxidante das plântulas de pepino não foi capaz de reverter esta situação, contribuindo para a redução no crescimento.

Palavras-chave: enzimas antioxidantes, oxidação protéica, Pb, pepino, peroxidação lipídica, pigmentos fotossintéticos

INTRODUCTION

With the rapid development in industry all around the world since the 20th century, the inputs of lead (Pb) to soils have being occurring through the mining and smelting activities, storage battery, gasoline and explosives, the disposal of municipal sewage sludge enriched with Pb as well as fertilizers, herbicides, and pesticides (Sharma and Dubey, 2005). Despite regulatory measures adopted in many countries to limit Pb input in the environment, it continues to be one of the most serious global environmental and human hazards (Sharma and Dubey, 2005). The threat that Pb poses to environment is aggravated by its long-term persistence in soil because Pb was estimated to have a soil retention time of 150-1500 years (Shaw, 1990).

In plants, Pb toxicity leads to inhibition of enzyme activities, disturbed mineral nutrition, water imbalance, changes in hormonal status and membrane permeability resulting in growth reduction (Sharma and Dubey, 2005). Pb-treated plants also presented alterations in their photosynthetic apparatus such as distortion of chloroplast ultrastructure, inhibition of Calvin cycle enzymes and synthesis of photosynthetic pigments (Mishra et al., 2006). Reduction in chlorophyll content may be attributed to reduced chlorophyll synthesis due to Pb-inhibition of aminolevulinate dehydratase (ALA-D; E.C. 4.2.1.24) (Prasad and Prasad, 1987; Morsch et al., 2002), which catalyzes the asymmetric condensation of two molecules of aminolevulinic acid (ALA) to porphobilinogen (Gibson et al., 1955).

In addition, one of the most damaging effects of Pb in plants is the induction of oxidative stress due to enhanced production of reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) (Sharma and Dubey, 2005). These ROS are produced during normal metabolism in aerobic organisms, but can cause a severe damage to all biomolecules when produced in larger amounts generating lipid peroxidation and protein oxidation (Gratão et al., 2005).

Plants have several efficient constituents in the enzymatic and non-enzymatic antioxidant defense systems that allow scavenging of ROS and protection of plant cells from oxidative damage (Gratão et al., 2005). The term antioxidant describes any compound capable of quenching ROS without itself undergoing conversion to a destructive radical (Gratão et al., 2005). Antioxidant enzymes like superoxide dismutase (SOD;

E.C. 1.15.1.1), ascorbate peroxidase (APX; E.C. 1.11.1.11), catalase (CAT; E.C. 1.11.1.6), and glutathione peroxidase (GPX; E.C. 1.11.1.9) are considered as those that either catalyses such reactions, or are involved in the direct processing of ROS (Mittler, 2002). SOD is responsible for the dismutation of $O_2^{\cdot -}$ to H_2O_2 and O_2 and is considered to be the first line of defense against ROS, influencing the concentration of $O_2^{\cdot -}$ and H_2O_2, the two Haber-Weiss reaction substrates (Gratão et al., 2005). APX, CAT and GPX subsequently detoxify H_2O_2 in several cell compartments (Gratão et al., 2005). Moreover, non-protein thiol groups (NPSH), glutathione (GSH), ascorbic acid (AsA), tocopherols and carotenoids are examples of non-enzymatic antioxidants (Gratão et al., 2005; Mishra et al., 2006).

Aiming to contribute to a better understanding of the toxicology of this metal, in this paper we present some data showing changes in plant growth, photosynthetic pigments and total soluble protein concentrations, ALA-D activity, enzymatic and non-enzymatic antioxidant capacity, protein oxidation and lipid peroxidation in seedlings of cucumber exposed to lead. Cucumber (*Cucumis sativus*) was selected as the test plant species due to its sensitivity to a wide range of contaminants (An et al., 2004; Cargnelutti et al., 2006; Gonçalves et al., 2007) and also due to its easy germination in laboratory conditions.

MATERIAL AND METHODS

Plant material and growth conditions: Seeds of cucumber (*Cucumis sativus* L. cv. Aodai) obtained from Feltrin Ltd. (Santa Maria, RS) were germinated in glass recipients (100 mL) containing 15 mL of medium containing three Pb concentrations as $(C_2H_3O_2)Pb.3H_2O$ (0, 100, 400, and 1000 μmol L^{-1}) diluted in a 0.5% agar solution. The solution containing agar was heated and the Pb 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 any nutrient deficiency, as found in a previous experiment (Gonçalves et al., 2007). Each experimental unit consisted of 6 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 plant level). The usual range of Pb concentration in the soil goes up to 200 ppm (maximum Pb concentration approximately supplied in the present study), but Pb-strong affected soils contain Pb in the range of 400-800 ppm and in industrialized areas the level may reach up to 1000 ppm (Angelone and Bini, 1992).

Growth analysis: Cucumber growth was determined by measuring the length of the root system (Tennant, 1975) and of the shoot (measured with a ruler). To obtain the fresh weight, excess water was removed with a paper towel after root washing. To obtain dry weight, the plants were left at 65ºC to a constant weight. The water content (WC) of the seedlings was obtained using the formula [(Fresh weight – Dry weight)/ Fresh weight] according to Sridhar et al. (2007).

Metal determination: Approximately 0.05 g of roots and shoot were digested with 4 mL HNO_3 utilizing 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 Pb were determined using a Model AAS 5 EA 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).

Chlorophyll and carotenoids concentrations: Cotyledons were weighed and used for chlorophyll and carotenoids determination. Chlorophyll and carotenoids were extracted following the method of Hiscox and Israeslstam (1979). One hundred milligrams of chopped fresh cotyledons were placed in a vial containing 7 mL dimethylsulphoxide (DMSO). The photosynthetic pigments were extracted from the fluid without grinding at 65ºC by incubating for 2 h. The liquid extract was transferred to a graduated tube and made up to a total volume of 10 mL with DMSO. A 3 mL sample was transferred to a cuvette and the absorbances at 645, 663 and 470 nm were read in order to determine chlorophyll a, chlorophyll b and carotenoids concentrations, respectively. Chlorophyll concentration was calculated following the equation used by Lichtenthaler (1987).

Delta-aminolevulinic acid dehydratase (ALA-D; E.C. 4.2.1.24) assay: Since the cotyledons presented high chlorophyll concentration, they were used in the determination of ALA-D activity. Cucumber cotyledons were homogenized in 10 mmol L^{-1} Tris-HCl buffer, pH 9.0, at a proportion of 1:1 (w/v). The homogenate was centrifuged at 12,000 *g* at 4°C

for 10 min to yield a supernatant (S1) that was used for the enzyme assay. The supernatant was pre-treated with 0.1% Triton X-100 and 0.5 mmol L^{-1} dithiotreithol. ALA-D activity was assayed as described by Morsch et al. (2002) by measuring the rate of porphobilinogen formation. The incubation medium for the assays contained 100 mmol L^{-1} Tris-HCl buffer, pH 9.0. For the enzyme assay, the final concentration of ALA was 3.6 mmol L^{-1}. Incubation was started by adding 100 μL of the tissue preparation to a final volume of 400 μL. The product of the reaction was determined with the Ehrlich reagent at 555 nm using a molar absorption coefficient of 6.1×10^4 $M^{-1}cm^{-1}$ (Sassa, 1982) for the Ehrlich-porphobilinogen salt.

Estimation of lipid peroxidation: The levels of peroxides in the seedling were 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% trichloroacetic acid (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. Seedlings samples were washed with distilled water to remove surface contamination, weighted into 5-g portions and placed in individual stoppered vials containing 50 mL of distilled water. These samples were incubated at room temperature (25ºC) on a shaker (100 rpm) for 24 h. Electrical conductivity of 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 room temperature. 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 superoxide dismutase 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} ethylenediaminetetracetic acid (EDTA), 0.1% (v/v) Triton X-100 and 2% polyvinylpyrrolidone (PVP) (w/v). The extract was filtered and centrifuged at 22,000 *g* for 10 min at 4ºC, and the supernatant was utilized 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 was 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 catalase 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 was 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 groups (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 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, 0.27% $CuSO_4$ diluted in 49% H_2SO_4. After 3 hours, 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) (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 and statistically significant differences determined based on the appropriate *F*-tests. The results are the means ± S.D. of at least three independent replicates. The mean differences were compared utilizing 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 Pb concentration and seedling growth: Increasing Pb concentrations in growth medium significantly enhanced Pb concentration in both roots and shoot. However, Pb accumulated at a higher amount in roots than in the shoot. Pb concentration in roots of 10-day-old seedlings was about 22.5-fold higher than that in shoot at the highest level of Pb in the substrate (1000 μM) (Table 1). Relative to control seedlings, root length was significantly inhibited by 48% and 92% in seedlings exposed to 400 and 1000 μmol Pb L^{-1}, respectively (Figure 1A). However, there was no reduction in shoot length in any Pb treatments (Figure 1B). Total fresh weight decreased 17% and 32%, respectively, at 400 and 1000 μmol Pb L^{-1} in comparison to control seedlings (Figure 1C). Total dry weight was not affected in any Pb treatments (Figure 1D). Relative to control seedlings, the root[1] shoot length ratio showed a strong reduction in seedlings exposed to 400 and 1000 μmol Pb L^{-1} (Figure 1E). On the other hand, the dry fresh^{-1} weight ratio showed an increase only in seedlings exposed to the highest Pb concentration compared to control seedlings (Figure 1F). In addition, at the same concentration, a reduction of 3% in the water content was also observed in Pb-treated seedlings compared to control (Figure 1G).

Table 1. Pb content in root and shoot of 10-d-old cucumber seedlings grown under increasing Pb concentrations.

Pb concentrations (μM)	Pb content (μg g^{-1} DW)	
	Root	Shoot
0	5.17 ± 0.04d	0.933 ± 0.11d
100	6650.00 ± 508.37c	17.15 ± 17.35c
400	27436.33 ± 3022.15b	925.50 ± 0.50b
1000	82087.00 ± 1746.00a	3645.75 ± 65.25a

Data are mean ± S.D. of three pools of 5 replicates each (n=3). Different letters indicate in the columns significant difference among Cd concentrations (one-way ANOVA/Tukey; $p<0.05$).

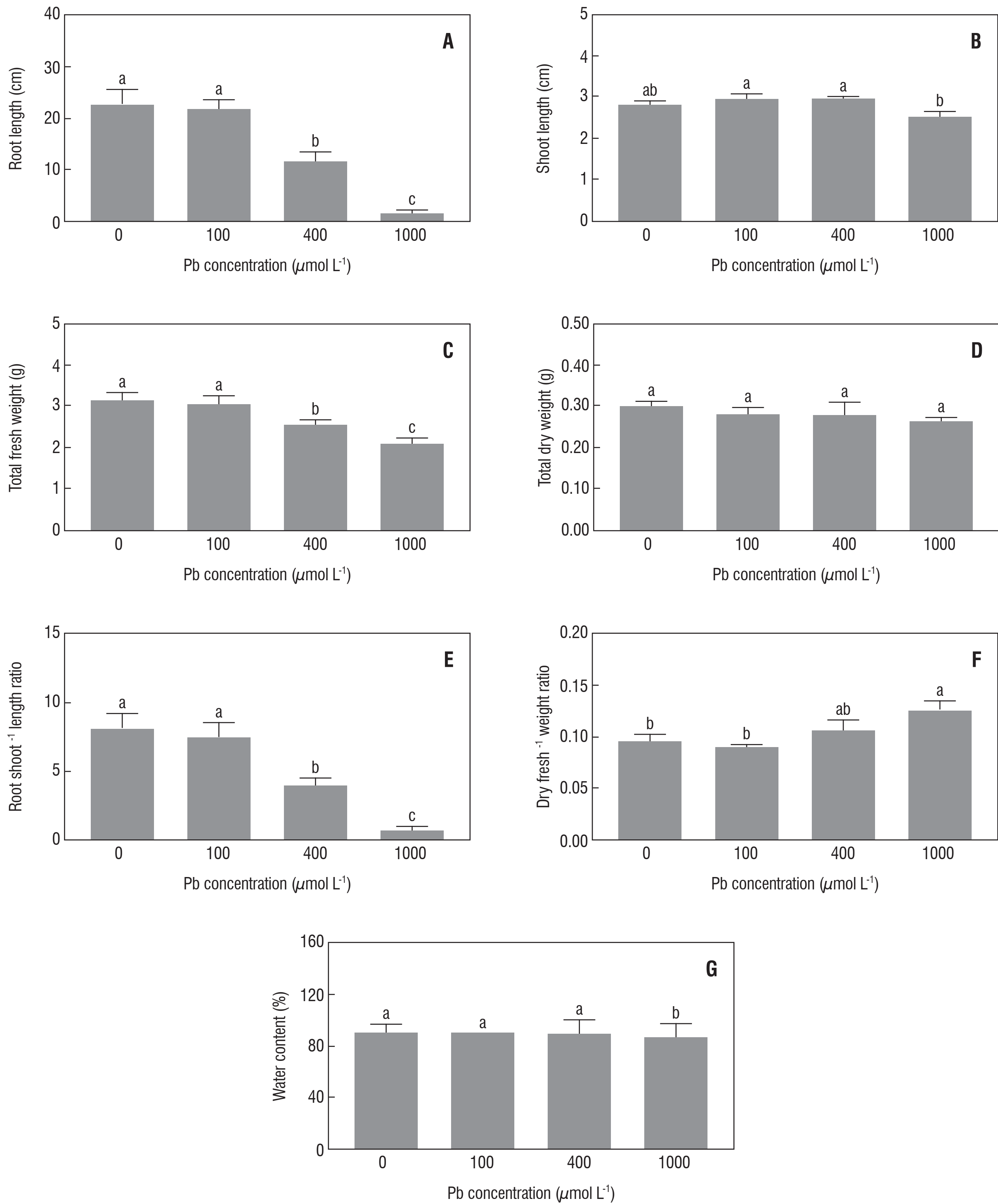


Figure 1. Effect of Pb at different concentrations on shoot (**A**) and root (**B**) lengths, total fresh (**C**) and dry (**D**) weights, root shoot^{-1} length (**E**) and dry fresh^{-1} weight (**F**) ratios and water content (**G**) of 10-d-old cucumber seedlings. Vertical bars represent SD (n = 15). Different letters indicate significant difference among Cd concentrations (one-way ANOVA, Tukey test; $P < 0.05$).

Total carotenoids and chlorophyll concentrations and ALA-D activity: Carotenoids concentration was enhanced 51% and 33%, respectively, at 100 and 400 μmol Pb L^{-1} in comparison to control seedlings (Figure 2A). Total chlorophyll concentration was not affected in any Pb treatments (Figure 2B). Moreover, cotyledon ALA-D activity was reduced only at the highest Pb concentration compared to control seedlings (Figure 2C).

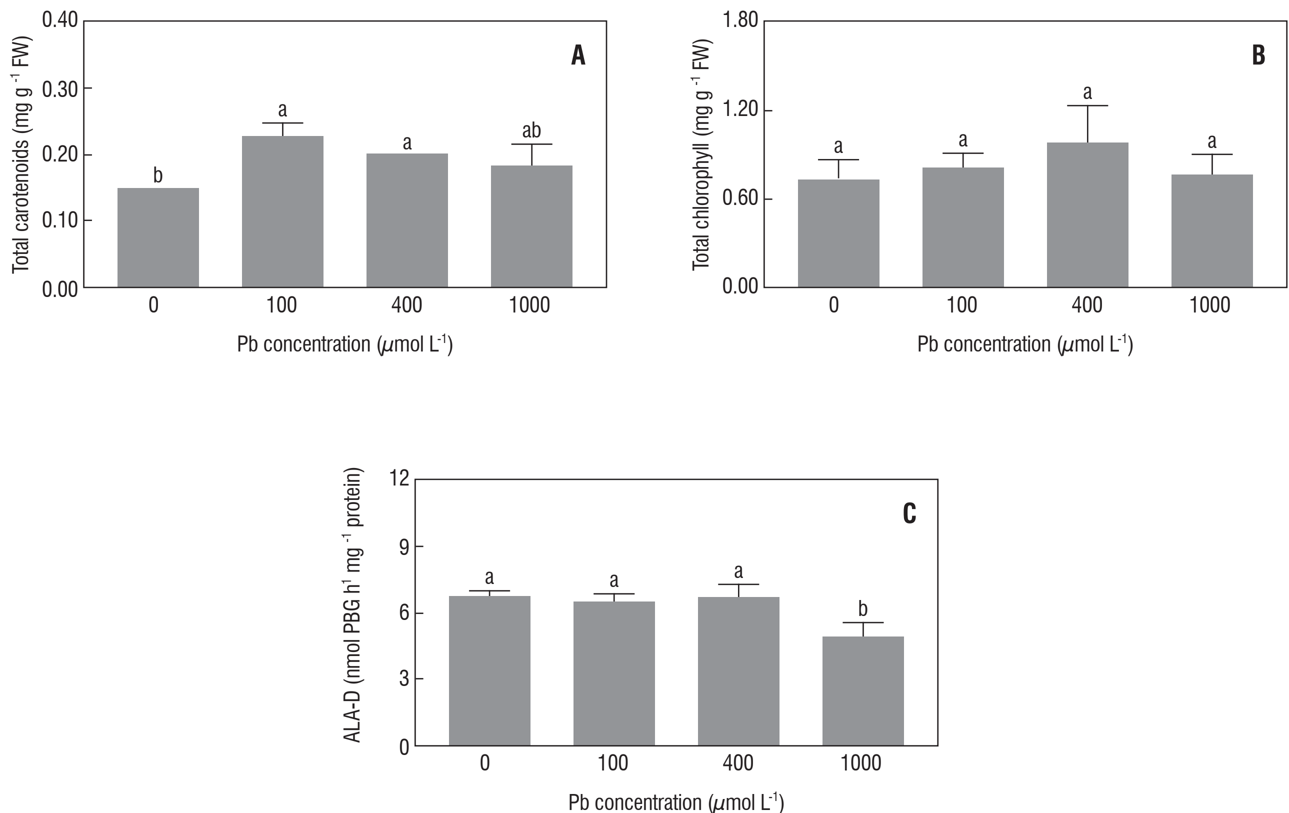


Figure 2. Effect of Pb at different concentrations on total chlorophyll (**A**) and carotenoids (**B**) concentrations and aminolevulinate dehydratase (ALA-D) activity (**C**) of 10-d-old cucumber seedlings. PBG= Porphobilinogen. Statistics as in Figure 1.

Estimation of lipid peroxidation, ELP, protein oxidation and total soluble protein: The effect of Pb on cell membrane integrity was determined by evaluating MDA concentration and ELP of plant tissues. Compared to control seedlings, a significant change (108% increase) in MDA concentration was noticed, but only at the highest Pb concentration (Figure 3A); conversely, ELP measurement was not affected in any Pb treatments (Figure 3B). Cucumber seedlings grown with Pb had an increase in carbonyl formation and total soluble protein concentration only at 1000 μmol Pb L^{-1} when compared with control plants (Figure 3C and 3D).

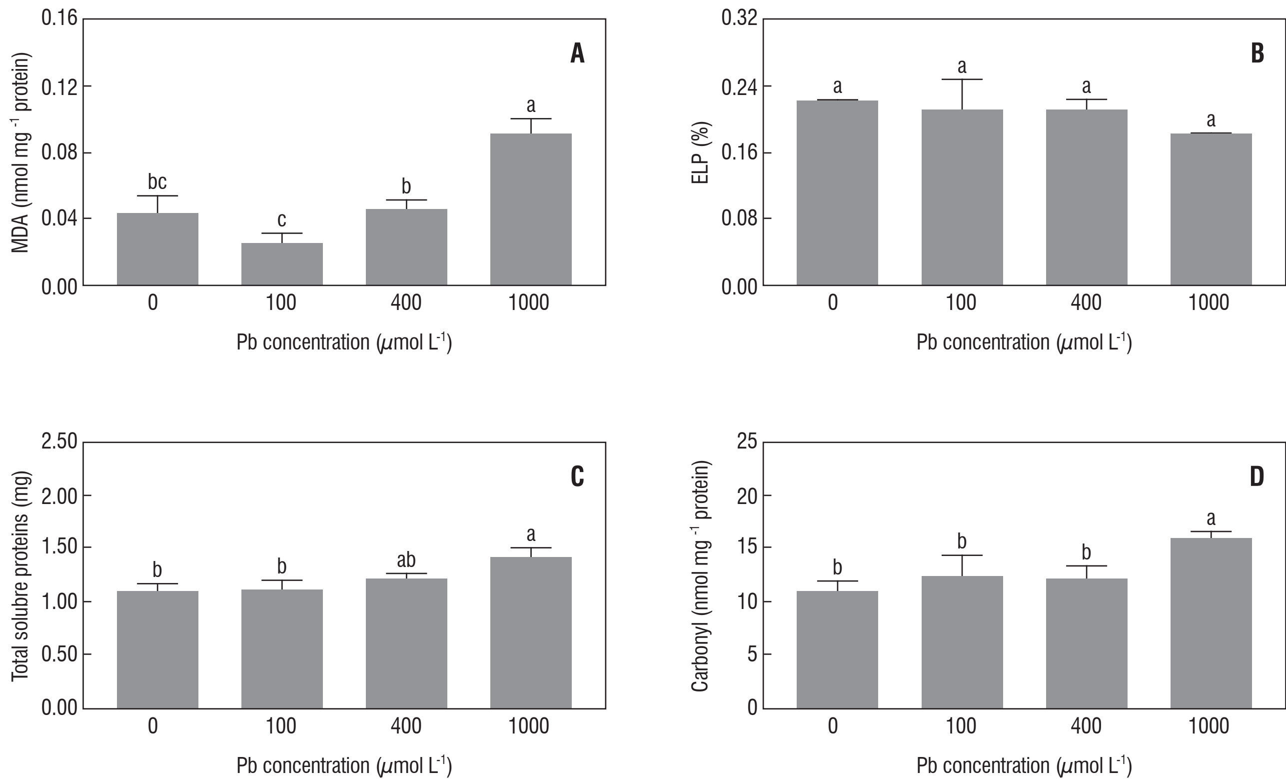
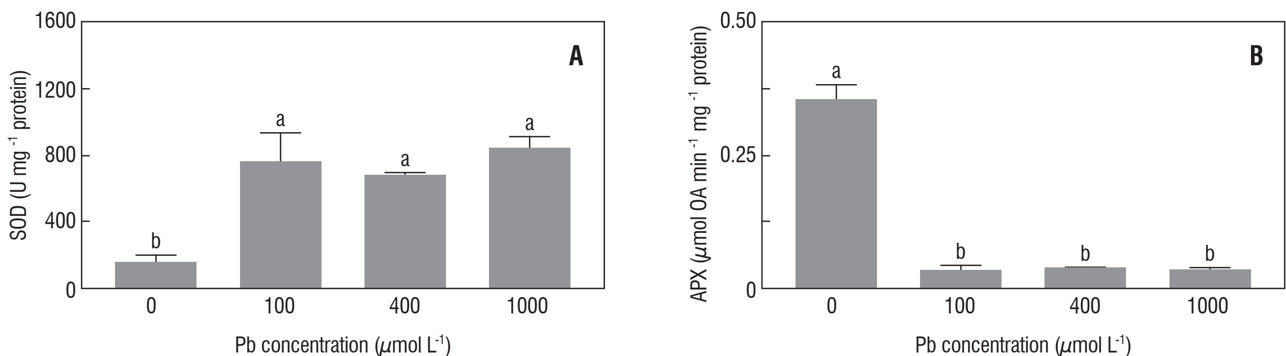


Figure 3. Effect of Pb at different concentrations on malondealdehyde (MDA) concentration (**A**), electrolyte leakage percentage (ELP) (**B**), protein oxidation (**C**), and total soluble proteins content (**D**) of 10-d-old cucumber seedlings. Statistics as in Figure 1.

Antioxidant enzymes activities and AsA and NPSH concentrations: A sharp increase in SOD and CAT activities following exposure to all Pb concentrations was noticed compared to the control seedlings (Figure 4A and 4C). On the other hand, APX activity decreased strongly by 90% in seedlings exposed to any Pb treatments when compared to the control (Figure 4B). Relative to control seedlings, Pb treatment led to increased tissue AsA concentration by 20% and 65%, respectively, at 400 and 1000 μmol Pb L^{-1} (Figure 4D). In contrast, NPSH concentration increased only at the highest Pb concentration (Figure 4E).



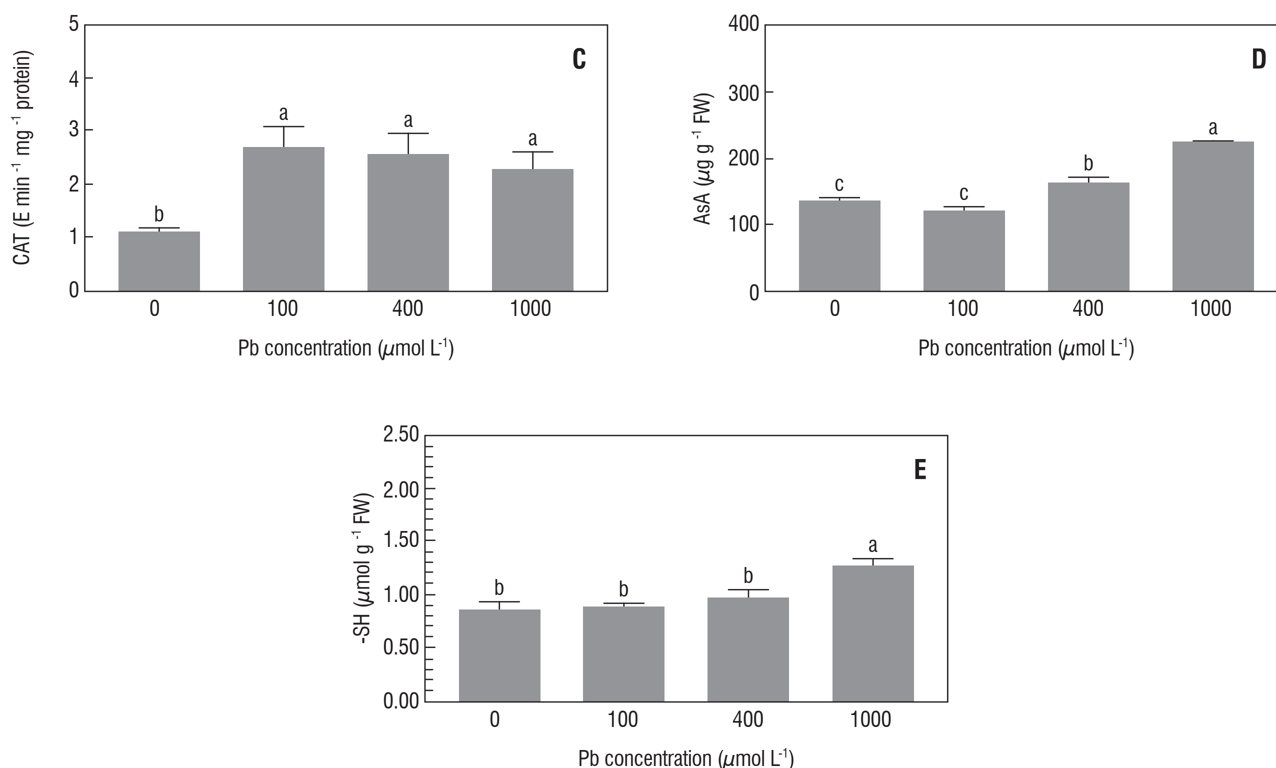


Figure 4. Effect of Pb at different concentrations on superoxide dismutase (SOD) (**A**), catalase (CAT) (**B**), and ascorbate peroxidases (APX) (**C**) activities and ascorbic acid (AsA) (**D**) and non-protein thiol groups (**E**) concentrations of 10-d-old cucumber seedlings. OA= Oxidized ascorbate. Statistics as in Figure 1.

DISCUSSION

In the present study, it was shown that seedling Pb concentration increased with increasing Pb concentration in the growth medium. Moreover, the absorbed Pb is distributed in an organ specific manner with its localization greater in roots than in shoot. According to An et al. (2004) cucumber retain greater amount of metals in the root due to its greater surface area related to numerous thin roots. The Pb accumulation depends upon the species, cultivar, plant organ, the exogenous Pb concentration and the presence of other ions in the environment, but in most cases, it has been reported that the roots accumulate higher Pb amount than the shoot and leaves (Singh et al., 1997; Verma and Dubey, 2003; Sharma and Dubey, 2005; Romeiro et al., 2006). According to Seregin and Ivanov (1997) the limited transport of Pb from roots to other organs is due to the barrier of Casparin strips of the root endodermis that appears to be the major limiting factor restricting Pb transport across endodermis into the central cylinder tissue.

Root length and total fresh weight of cucumber were decreased at the two highest Pb concentrations when compared to the control. On the other hand, cucumber showed no reduction in shoot length and total dry weight at any level of Pb. Thus, Pb toxicity decreased root $shoot^{-1}$ length ratio of cucumber indicating that the decrease in root length was stronger than in shoot length. This result corroborates with that presented by Mishra and Choudhari (1998) in Pb-exposed rice. In fact, Pb toxicity is reported to inhibit growth of several plants (Singh et al., 1997; Mishra et al., 2006; Romeiro et al., 2006; Dey et al., 2007). However, among the different metals tested in cucumber (Cd, Cu, Pb) and in radish (*Raphanus sativus*) (Cd, Hg, Zn, Pb), Pb was the least toxic to growth of seedlings (Morsch et al., 2002; An et al., 2004). The marked root cucumber inhibition observed in this paper might be the result of disturbances either in cell division and/or cell elongation within the root meristem (Sharma and Dubey, 2005 and references herein).

The highest Pb concentration significantly affected cucumber water status causing water deficit as indicated by the decrease in the seedling water content and the increase in dry fresh^{-1} weight ratio. Parys et al. (1998) reported that *Pisum sativum* exposed to Pb showed a reduction in transpiration intensity, osmotic pressure of cell sap, water potential of xylem and relative water content. Decline in transpiration rate and water content in plants by Pb may be due to following reasons: 1) reducing leaf area (the major transpiring organ) (Romeiro et al., 2006) or 2) inducing decreased in stomatal conductance and, consequently, its closure either by reducing the level of compounds that are associated with maintaining cell turgor and cell wall plasticity or by increasing the abscisic acid content (Sharma and Dubey, 2005; Romeiro et al., 2006).

Furthermore, it is reported that Pb adversely affects photosynthesis by causing several disturbances in photosynthetic apparatus as well as pigments such as chlorophyll and carotenoids (Mishra et al., 2006). However, in the present study cucumber carotenoid concentration increased at 100 and 400 μmol Pb L^{-1}. According to Singh et al. (2006) the enhancement in carotenoid level in heavy metals-treated plants is probably a part of strategy adopted by the plant to counteract the toxic effect of free radicals generated under heavy metal toxicity. On the other hand, chlorophyll concentration was not affected in any Pb treatments corroborating with results presented by Olivares (2003) in *Tithonia diversifolia* exposed to Pb through the roadside automotive pollution. One of the most important enzymes involved in the chlorophyll biosynthesis is the aminolevulinate dehydratase (ALA-D) that, in the present study, had its activity reduced only at the highest Pb concentration when compared with the control. Thus, these results allow us to infer that the decreased ALA-D activity was not enough to decrease chlorophyll content in Pb-exposed cucumber. Morsch et al. (2002) observed a different magnitude of ALA-D inhibition in radish leaves after seedling exposure to four metals (Cd, Hg, Zn, Pb) and verified that Pb was the least toxic to ALA-D activity. Moreover, it is interesting to note that the decreased ALA-D activity may result in an accumulation of its substrate, the ALA, which possesses the capacity to enhance the oxidative burst in plant cell (Noriega et al., 2007).

Many previous studies have reported the relationship between Pb and oxidative stress in several plants (Dey et al., 2007); however, information focused on the response of cucumber seedlings is rather scarce. In this study, increased ROS generation was found in cucumber seedlings under the highest Pb treatment as indicated by the MDA and carbonyl production related to lipid peroxidation and protein oxidation, respectively. Furthermore, we also observed that Pb increased total soluble protein concentration only at the highest concentration and had no influence in ELP measurement from cucumber. Lipid peroxidation involves oxidative degradation of polyunsaturated fatty acyl residues of membranes and has been found in different Pb-stressed plants (Reddy et al., 2005; Choudhury and Panda, 2005; Mishra et al., 2006) and levels of carbonylated proteins and total soluble protein concentration increase in plants undergoing oxidative stress associated with heavy metals (Cargnelutti et al., 2006; Gonçalves et al., 2007). This increase in protein content may be due to *de novo* synthesis of stress proteins (Verma and Dubey, 2003; Mishra et al., 2006).

In order to repair the damage initiated by ROS, plants have evolved complex antioxidant defense system that include both enzymatic and non-enzymatic constituents (Choudhury and Panda, 2005). In the present study, we verified that all Pb concentrations increased SOD activity in cucumber seedlings. The higher SOD activity could possibly be the result of both a direct effect of heavy metal ions and an indirect effect mediated via an increase in levels of O_2^- or also due to *de novo* synthesis of enzymatic protein (Chongpraditnum et al., 1992; Dey et al., 2007). Thus, a decreased O_2^- concentration is to be expected, but in parallel with an increased production of H_2O_2 (Dey et al., 2007). The enzymes CAT and APX are the major responsible for the reduction of H_2O_2 to water. Then, it is worth noting that in this paper both SOD and CAT activities were increased by Pb stress which corroborates with the idea that in order to obtain a powerful scavenging of toxic oxygen forms, the overproduction of the H_2O_2-gererating SOD must always be combined with increased levels of H_2O_2–metabolizing catalase and/or peroxidades (Mittler, 2002; Gonçalves et al., 2007). We also observed that while CAT activity was increased, APX activity was decrease by Pb treatments. This result indicates that the activity of these enzymes are compensated because they have similar roles in the plant cell (Verma and Dubey, 2003; Gonçalves et al., 2007).

In the literature contradictory results have been reported concerning the response of plant antioxidant enzymes to Pb

stress. Wheat (*Triticum aestivum*) exposed to Pb treatments ranging from 200 to 800 ppm during 12 days showed a concentration-depend increase in CAT and SOD activities (Reddy et al., 2005). However, Dey et al. (2007) reported that wheat exposed to 200 to 2000 μM Pb for 7 days presented an induction in SOD and a decline in CAT activities. Moss (*Taxithelium nepalense*) grown in the presence of 100 and 1000 μM Pb for 12 hours showed an increase in SOD and CAT activities, but increased SOD and decreased CAT activities were observed at 24 hours of metal exposure (Choudhury and Panda, 2005). Pb concentrations (500 and 1000 μM) stimulated SOD and APX activities as well as diminished CAT activity in rice plants growing for 20 days (Verma and Dubey, 2003). Coontail (*Ceratophyllum demersum*) exposed to Pb treatments (1 - 100 μM) for 1 - 7 days showed, in general, increased activities of SOD, APX and CAT at lower Pb concentrations and a decline with increase in duration and treatment (Mishra et al., 2006).

Among the non-enzymatic antioxidants, we measured AsA and NPSH concentrations because they are indispensable for plants tolerate the cellular metal load (Mishra et al., 2006; Gonçalves et al., 2007). AsA is quantitatively the predominant antioxidant in plant cells and plays important roles as an antioxidant and as a modulator of plant development through hormone signaling (Pastori et al., 2003). Cucumber AsA concentration was enhanced at the two highest Pb concentrations when compared to the control, indicating that AsA is involved in antioxidant response of this plant to Pb toxicity (Choudhury and Panda, 2005). In relation to NPSH concentration, it was observed an increase only at the highest Pb concentration. This enhancement may be due to the increased phytochelatin biosynthesis (Misrha et al., 2006; Gonçalves et al., 2007). Phytochelatins are very important in metal detoxification, and are also considered as biomarkers of metal toxicity and their synthesis is induced by many metals including Pb (Mishra et al., 2006). Interestingly, similar results concerning antioxidant system have been found in a recent study on cadmium-exposed cucumber, suggesting that the response of this plant to both heavy metals likely occurred in a same way (Gonçalves et al., 2007).

Taken together, the results of the present study showed that Pb toxicity affected more root than shoot of cucumber seedlings as indicated by growth analysis and it might be related to great Pb retention in root tissue. Our results suggest that the enzymes SOD and CAT, rather than APX, appear to play a pivotal role in scavenging oxidative stress in Pb-exposed cucumber. We also demonstrated an increase in the carotenoids, AsA and NPSH concentrations, suggesting its participation as antioxidants in response to Pb stress. Nonetheless, the decrease in water content and ALA-D activity as well as the increase in MDA, carbonyls, and total soluble protein concentrations in the cucumber exposed only at 1000 μmol Pb L^{-1} indicate that the quantity of ROS exceeded the capacity of the antioxidant defensive system. Therefore, Pb-treatment caused oxidative stress, and the antioxidant system of the seedlings was not sufficient to overcome it.

Acknowledgements: The authors wish to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação e Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

REFERENCES

Aebi H (1984) Catalase *in vitro*. Methods Enzymol. 105:121-126.

An Y-J, Kim Y-M, Kwon T-I, Jeong, S-W (2004) Combined effect of copper, cadmium, and lead upon *Cucumis sativus* growth and bioaccumulation. Sci. Total Environ. 326:85-93.

Angelone M, Bini C (1992) Trace elements concentrations in soil and plants of western Europe. In: Adriano DC (ed), Biogeochemistry of Trace Metals, pp.19-60, Lewis Publishers, Boca Raton, London.

Bradford M (1976) A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

Cargnelutti D, Tabaldi LA, Spanevello RM, Jucoski GO, Battisti V, Redin M, Linares CEB, Dressler VL, Flores EMM, Nicoloso FT, Morsch VM, Schetinger MRC (2006) Mercury toxicity induces oxidative stress in growing cucumber seedlings. Chemosphere 65:999-1006.

Chongpraditnum P, Mori S, Chino M (1992) Excess copper induces a cytosolic Cu, Zn-superoxide dismutase in soybean root. Plant Cell Physiol. 33:239–244.

Choudhury S, Panda SM (2005) Toxic effects, oxidative stress and ultrastrustural changes in moss *Taxithelium nepalense* (Schwaegr.) Broth. under chromium and lead phytotoxicity. Water Air Soil Pollut. 167:73-90.

Dey SK, Dey J, Patra S, Pothal D (2007) Changes in the antioxidative enzyme activities and lipid peroxidation in wheat seedlings exposed to cadmium and lead stress. Braz. J. Plant Physiol. 19:53-60.

Ellman GL (1959) Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82:70–77.

El-Moshaty FIB, Pike SM, Novacky AJ, Sehgal OP (1993) Lipid peroxidation and superoxide production in cowpea (*Vigna unguiculata*) leaves infected with tobacco ringspot virus or southern bean mosaic virus. Physiol. Mol. Plant Pathol. 43:109-119.

Gibson KD, Neuberger A, Scott JJ (1955) The purification and properties of delta-aminolevulinic acid dehydratase. Biochem. J. 61:618-676.

Gonçalves JF, Becker AG, Cargnelutti D, Tabaldi LA, Pereira LB, Battisti V, Spanevello RM, Morsch VM, Nicoloso FT, Schetinger MRC (2007) Cadmium toxicity causes oxidative stress and induces response of the antioxidant system in cucumber seedlings. Braz. J. Plant Physiol. 19(3):223-232.

Gratão PL, Polle A, Lea PJ, Azevedo RA (2005) Making the life of heavy-metal stressed plants a little easier. Funct. Plant Biol. 32:481-494.

Hiscox JD, Israelstam GE (1979) A method for the extraction of chlorophyll from leaf tissue without maceration. Can. J. Bot. 57:1132-1134.

Iyengar GV, Subramanian KS, Woittiez JRW (1997) Elemental Analysis of Biological Samples. Principles and Practice. CRC Press, Boca Raton.

Jacques-Silva MC, Nogueira CW, Broch LC, Flores EMM, Rocha JBT (2001) Diphenyl diselenide and ascorbic acid changes deposition of selenium and ascorbic acid in liver and brain of mice. Pharmacol. Toxicol. 88:119–125.

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER (1990) Determination of carbonyl concentration in oxidatively modified proteins. Methods Enzymol. 186:464-478.

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. 148:350-382.

Lutts S, Kinet JM, Bouharmont J (1996) NaCl-induced senescence in leaves of rice (*Oriza sativa* L.) cultivar differing in salinity resistance. Ann. Bot. 78:389–398.

Mishra A, Choudhari MA (1998) Amelioration of lead and mercury effects on germination and rice seedling growth by antioxidants. Biol. Plant. 41:469-473.

Mishra S, Srivastava S, Tripathi RD, Kumar R, Seth CS, Gupta DK (2006) Lead detoxification by coontail (*Ceratophyllum demersum* L.) involves induction of phytochelatins and antioxidant system in response to its accumulation. Chemosphere 65:1027-1039.

Misra HP, Fridovich I (1972) The role of superoxide anion in the autoxidation of epinephrine and simple assay for superoxide dismutase. J. Biol. Chem. 244:6049-6055.

Mittler, R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7:405-410.

Morsch VM, Schetinger MRC, Martins AF, Rocha JBT (2002) Effects of cadmium, lead, mercury and zinc on δ-aminolevulinic acid dehydratase activity from radish leaves. Biol. Plant. 45:85-89.

Noriega GO, Balestrasse KB, Batlle A, Tomaro MA (2007) Cadmium induced oxidative stress in soybean plants also by the accumulation of δ-aminolevulinic acid. Biometals 20:841-851.

Olivares E (2003) The effect of lead on the phytochemistry of *Tithonia diversifolia* exposed to roadside automotive pollution or grown in pots of Pb-supplemented soil. Braz. J. Plant Physiol. 15:149-158.

Parys E, Romanowaska E, Siedlecka M, Poskuta J (1998) The effect of lead on photosynthesis and respiration in detached leaves and in mesophyll protoplasts of *Pisum sativum*. Acta Physiol. Plant. 20:313-322.

Pastori GM, Kiddle G, Antoniw J, Bernard S, Veljovic-Jovanovic S, Verrier PJ, Noctor G, Foyer CH (2003) Leaf vitamin C contents modulate plant defense transcripts and regulate genes that control development through hormone signaling. Plant Cell 15:939–951.

Prasad DDK, Prasad ARK (1987) Altered δ-aminolaevulinic acid metabolism by lead and mercury in germinating seedlings of Bajra (*Pennisetum typhoideum*). J. Plant Physiol. 127:241-249.

Reddy AM, Kumar SG, Jyothsnakumari J, Thimmanaik S, Sudhakar C (2005) Lead induced changes in antioxidant metabolism of horsegram (*Macrotyloma uniflorum* (Lam.) Verdc.) and bengalgram (*Cicer arietinum* L.). Chemosphere 60:97-104.

Romeiro S, Lagôa AMMA, Furlani PR, Abreu CA, Abreu MF, Erismann NM (2006) Lead uptake and tolerance of *Ricinus communis*. Braz. J. Plant Physiol. 18:483-489.

Sassa S (1982) δ-Aminolevulinic acid dehydratase assay. Enzyme 28:133-145.

Seregin IV, Ivaniov VB (1997) Histochemical investigation of cadmium and lead distribution in plants. Fiziol. Rast. 44:915-921.

Sharma P, Dubey RS (2005) Lead toxicity in plants. Braz. J. Plant Physiol. 17:33-52.

Shaw AJ (1990) Heavy metal tolerance in plants: evolutionary aspects. CRC Press, Florida.

Singh RP, Tripathi RD, Sinha SK, Maheshwari R, Srivastava HS (1997) Response of higher plants to lead contaminated environment. Chemosphere 34:2467-2493.

Singh S, Eapen S, D'Souza SF (2006) Cadmium accumulation and its influence on lipid peroxidation and antioxidative system in an aquatic plant, Bacopa monnieri L. Chemosphere 62:233-246.

Sridhar BBM, Han FX, Diehl SV, Monts DL, Su Y (2007) Effects of Zn and Cd accumulation on structural and physiological characteristics of Barley plants. Braz. J. Plant Physiol. 19:15-22.

Tennant D (1975) A test of a modified line intersect method of estimating root length. J. Ecol. 63:995-1001.

Verma S, Dubey RS (2003) Lead toxicity induces lipid peroxidation and alters the activities of antioxidant enzymes in growing rice plants. Plant Sci. 164:645-655.

Zhu Z, Wei G, Li J, Qian Q, Yu J (2004) Silicon alleviates salt stress and increases antioxidant enzymes activity in leaves of salt-stressed cucumber (*Cucumis sativus* L.). Plant Sci. 167:527-533.