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[1]Departamento de Química e [2]Departamento de Biologia, [3]Programa de Pós-Graduação em Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brasil.

Corresponding author:

Dr. Maria Rosa Chitolina Schetinger

Departamento de Química

Centro de Ciências Naturais e Exatas

Universidade Federal de Santa Maria

Santa Maria RS Brasil - 97105-900

Fax: 0(14)5532208665

e-mail: mariarosa@smail.ufsm.br

Abstract

In this study, the effects of mercury on the electrolytic leakage percentage (ELP), hydrogen peroxide (H_2O_2) levels, superoxide dismutase (SOD) activity, non-enzymatic antioxidants (ascorbic acid (ASA), carotenoids, non-protein thiol content (SH)), and δ-aminolevulinic acid dehydratase activity (ALAD) were investigated in Cucumis sativus L. seedlings. Cucumber seedlings were exposed to 0 to 500 μM of $HgCl_2$ during 10 and 15 days. Hg-treated seedlings showed elevated ELP only at 500 μM $HgCl_2$ at 15 days. H_2O_2 levels decreased at 10 days at a moderately toxic level of Hg, but H_2O_2 increased at the highest concentration. Increased SOD activity occurred at concentrations lower than 50 μM $HgCl_2$, and decreased at higher concentrations. Increased SH levels at all concentrations were observed at 10 days. ASA content increased at all concentrations with a concomitant decrease at higher concentrations. Carotenoids levels increased at the lowest concentrations, both at 10 and 15 days. ALA-D activity increased at 50 μM $HgCl_2$ at 15 days, and it was inhibited at higher concentrations. Therefore, our results suggest that Hg increased the levels of ROS, provoking an increase in the antioxidant system, which makes up part of the overall expression of Hg tolerance in the seedlings. In addition, the decrease in carotenoids levels and ALA-D activity is a consequence of Hg toxicity.

Keywords: superoxide dismutase, carotenoids, hydrogen peroxide, non-protein thiol groups, cucumber, δ-aminolevulinic acid dehydratase.

1. INTRODUCTION

Heavy metal contamination is one the most serious environmental problems for plant productivity and it is a threat to human health. Due to diverse human activities, such as mining and smelting, metal pollution is becoming a major risk to many ecosystems. Among the pollution-production metals, mercury (Hg) is regarded as a non-essential element, with no known physiological function for plants.

However, anthropogenic inputs associated with agricultural practices, mineral extraction, industrial processes and solid waste management are important contributors to heavy metal contamination of natural ecosystems (Alumaa et al., 2002; Segura-Muñoz et al., 2006). The exposure of several plants species to heavy metals could also arise from the use of some pesticides and fertilizers (Falahi-Ardakani, 1984). One of the characteristic effects of metal poisoning, observable at an early stage, is a reduction in plant cell proliferation and growth (Schützendübel et al., 2001).

Mercury has been demonstrated to stimulate the formation of reactive oxygen species (ROS) (Cho and Park, 2000), which include superoxide radicals ($O_2^{\bullet -}$) hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$), either by direct electron transfer involving metal cations, or as a consequence of metal mediated inhibition of metabolic reactions (Stohs and Bagchi, 1995). Under normal conditions, the ROS are necessary for the correct functioning of plants and can play a role in inter- and intracellular signaling (Foyer and Noctor, 1999).

Hydrogen peroxide is produced during different metabolic processes such as photorespiration in chloroplasts or during the formation of lignin in cell walls (Asada, 1992; Schopfer, 1996). Hydrogen peroxide affects the integrity of cells

because it is a precursor of highly reactive oxygen species such as the hydroxyl radical, which attacks proteins, lipids and nucleic acids (Foyer et al., 1994).

Plants are endowed with a complex antioxidant system to cope with ROS (Smirnoff, 1993). However, when the accumulation of ROS under heavy metal stress conditions exceeds the removing capacity of the antioxidant system, the effects of oxidative damage appears, including oxidation of cellular lipids and proteins, destruction of photosynthetic pigments and inactivation of photosynthetic enzymes (Smirnoff, 1993).

The enzyme δ-aminolevulinic acid dehydratase (ALA-D) is sensitive to heavy metals due to its sulfhydrylic nature (Rocha et al., 1995, Morsch et al., 2002). ALA-D catalyzes the asymmetric condensation of 2 molecules of δ-aminolevulinic acid (ALA) to porphobilinogen (Gibson et al., 1955). The synthesis of porphobilinogen promotes the formation of porphyrins, hemes, and chlorophylls, which are essential for adequate aerobic metabolism and for photosynthesis (Jaffe et al., 2000).

In order to combat metal toxicity, plant cells have antioxidants such as carotenoids, glutathione (GSH) and ascorbate, and also antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR), which participate in scavenging ROS. Glutathione, a sulfur containing tripeptide, plays a prominent role in the defense against free radicals in plants under oxidative stress conditions (De Vos et al., 1992; Ranieri et al., 1993).

Major ROS scavenging mechanisms of plants include SOD and CAT. The balance between SOD and CAT activities in cells is crucial in determining the steady state level of superoxide radicals and hydrogen peroxide (Matés, 2000;

Camp et al., 1997). SOD destroys the free radical superoxide by converting it to peroxide, which can in turn be destroyed by CAT (Matés, 2000). Reactive O_2 species (ROS) are produced in both unstressed and stressed cells. Plants have well-developed defense systems against ROS, which involve both limiting the formation of ROS, as well as improving their scavenger capacity (Foyer et al., 1994, Miquel, 1989).

Heavy metals are toxic to plants if their accumulation levels exceed the detoxification capacity of the plant tissue. Thus, a potentially decisive factor in determining the outcome of oxidative stress is the speed with which plants can activate their antioxidant reserves (Ranieri et al., 1993). Correlation studies have indicated that this response is an important aspect of stress tolerance (Sinha et al., 1996).

In animal tissues, it has been demonstrated that mercury induces changes of the antioxidant status either by increasing lipid peroxidation and metallothionein (Aschner, et al., 1997), or by decreasing the enzymatic and non-enzymatic antioxidants (Perottoni et al., 2004). However, less information is available for plants. In a previous study with cucumber, we showed the enhancement of lipid peroxidation and alterations in growth and in the activity of some antioxidative enzyme such as catalase and ascorbate peroxidase (Cargnelutti et al., 2006).

Cucumis sativus is known to accumulate toxic metals under laboratory conditions and have been selected as one of the test plant species due to its sensitivity to a wide range of contaminants (Cargnelutti et al., 2006; Pereira et al, 2006).

Thus, the objective of the present study was to contribute to the understanding of the toxicology of mercury. In order to obtain these results, cucumber seedlings were used to evaluate the effect of this metal on the antioxidant system and its relation to ALA-D activity (an enzyme involved in the metabolism of chlorophyll).

2. MATERIAL AND METHODS

2.1. Plant material and growth conditions

Seeds of cucumber (*Cucumis sativus* L.) obtained from Feltrin Ltd. (Santa Maria, RS) were germinated in glass recipients containing 20 mL of 10% of Murashige and Skoog (1962) medium, supplemented with 0.6% agar and various $HgCl_2$ levels. Seedlings were exposed to 0.5, 50, 250 and 500 μM of $HgCl_2$. Seedlings without $HgCl_2$ treatment served as a control group. These concentrations were chosen due the highest mercury concentrations found on contaminated soil ranging from 15 to 300 μg/g dry weight (Cavallini et al., 1999). Moreover, higher mercury content was recorded in plants growing close to highly industrialized areas (Wojciechowska-Mazurek et al., 1995). The medium pH was adjusted to 5.8. Each experimental unit consisted of 6 seeds, totalizing 15 replicates per treatment. After the radicle broke through, 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) for 10 and 15 days. This time was selected to verify if there would be alterations in the biochemical parameters evaluated at a small interval of time.

2.2. Determination of electrolyte leakage percentage

ELP was used to assess membrane permeability and it was measured using an electrical conductivity meter. The procedure used was based on the method of Zhu et al. (2004), with some modifications. Plant samples were separated into 5 g segments and placed in individual stoppered vials containing 50 mL of distilled water after washes with distilled water to remove surface contamination. These samples were incubated at room temperature (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 a thermostatic water bath at 95ºC for 15 min and the second reading (EC2) was determined after cooling the bathing solutions to room temperature. ELP was calculated as EC1/EC2 and expressed in percentage (%).

2.3. Determination of hydrogen peroxide

The H_2O_2 contents of both control and treated seedlings were determined according to Loreto and Velikova (2001). Approximately 100 mg of seedlings were homogenized at 4ºC in 2 mL of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000g for 15 min. 0.5 mL of supernatant was added at 0.5 mL of 10 mM potassium phosphate buffer pH 7.0 and 1 mL of 1M KI. The H_2O_2 content of the supernatant was evaluated by comparing its absorbance at 390 nm with a standard calibration curve. The H_2O_2 content was expressed as µmol/g fresh weight.

2.4. Estimation of antioxidants

2.4.1. Superoxide Dismutase (E.C 1.15.1.1)

The activity of superoxide dismutase was assayed according to Mc Cord and Fridovich (1969). About 200 mg fresh tissues were homogenized in 5 ml of 100 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA, 0.1% (v/v) Triton X-100 and 2% (w/v) polyvinyl pyrrolidone (PVP). The extract was filtered and centrifuged at 22,000 x 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 50 mM glycine buffer (pH 10.5), 60 mM epinephrine and enzyme. Epinephrine was the last component to be added. The adrenochrome formation in the next 4 min was recorded at 480 nm with a UV- Vis spectrophotometer. One unit of SOD activity is expressed as the amount of enzyme required to cause 50% inhibition of epinephrine oxidation under the experimental conditions.

2.4.2. Non-protein thiol content

Non-protein thiol content in seedlings (mg) was measured spectrophotometrically with Ellman's reagent (Ellman, 1959). Reaction was read at 412 nm after the addition of 50 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB) (0.05 ml). Treated seedlings were homogenized in 10 mM Tris/HCl, pH 7.5, centrifuged at 3,000 x g for 10 min, and supernatants were used for total thiol group determination. Non-protein thiol groups were determined in the fraction obtained after mixing 1 volume of supernatant with 1 volume of 10% trichloroacetic acid followed by centrifugation and neutralization (to pH 7.5) as described by Jacques-Silva et al. (2001). A standard curve using cysteine was used to calculate the content of thiol groups in samples, and was expressed as μmol SH g^{-1} fresh weight.

2.4.3. Ascorbic acid content

Ascorbic acid determination was performed as described by Jacques-Silva et al. (2001). Briefly, seedlings were homogenized in 50 mM Tris/HCl, pH 7.5, centrifuged at 3,000 x g for 10 min and protein was removed by dilution with 1 volume of 10% trichloroacetic acid followed by centrifugation. An aliquot of the sample was incubated at 37ºC in a medium containing 4.5 mg/ml dinitrophenylhydrazine, 0.6 mg/ml thiourea, 0.075 mg ml^{-1} $CuSO_4$, and 0.675 mol/l H_2SO_4 (final volume 1 ml). After 3 h, 1 ml of 65% H_2SO_4 was added and samples were read at 520 nm and were expressed as µg ASA g^{-1} fresh weight. A standard curve was constructed using ascorbic acid.

2.4.4. Chlorophyll and carotenoids determination

Cotyledons were weighed and used carotenoid determination. Carotenoids were extracted following the method of Hiscox and Israeslstam (1979) and estimated with the help of Arnon's formulae (Arnon, 1949). 0.1 g chopped fresh cotyledon sample was incubated at 65ºC in dimethylsulfoxide (DMSO) until the pigments were completely bleached. Absorbance of the solution was then measured at 470 nm with a spectrophotometer (Celm E-205D). Carotenoid content were expressed as mg g^{-1} fresh weight.

2.5. Estimation of delta-aminolevulinic acid dehydratase (ALA-D; E.C. 4.2.1.24) activity

Cucumber cotyledons were homogenized in 10 mM Tris-HCl buffer, pH 9.0, at a proportion of 1:1 (w/v). The homogenate was centrifuged at 12,000 x g

at 4°C for 10 min to yield a supernatant (S1) that was used for the enzyme assay. At supernatant were added 0.1% Triton X-100 and 0.5 mM dithiothreitol (DTT). ALA-D activity was assayed as described by Morsch et al. (2002) by measuring the rate of porphobilinogen (PBG) formation. The incubation medium for the assays contained 100 mM Tris-HCl buffer, pH 9.0. For the enzyme assay, the final concentration of ALA was 3.6 mM. 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. ALA-D activity was expressed as nmol PBG /mg protein/h.

2.6. Protein determination

In all the enzyme preparations, protein was determined by the method of Bradford (1976) using bovine serum albumin as standard.

2.7. Statistical analysis

The analyses of variance were computed on statistically significant differences determined based on the variance analiys (one-way ANOVA). The results are the means ± S.D. of at least three independent experiments. The mean differences were compared utilizing Duncan's range test.

3. RESULTS

Electrolyte leakage percentage (ELP) represents cell membrane injury. Fig. 1A shows that increased ELP only occurred at a prolonged period of Hg

exposition (15 days), where at 500 μM $HgCl_2$ there was a significant increase of 172.7%, compared to the control.

The effect of $HgCl_2$ on H_2O_2 content, shown in Fig. 1B. The exposure of cucumber seedlings to 50 μM $HgCl_2$ for 10 days decreased the levels of endogenous H_2O_2 by about 60 % in comparison with the control. At the higher concentrations (250 and 500μM $HgCl_2$), a significant increase of H_2O_2 content was observed. On the other hand, 15-day-old seedlings showed increasing H_2O_2 content at the concentrations of 50 and 500 μM $HgCl_2$, while at 250 μM $HgCl_2$ the content of H_2O_2 was decreased by 39%.

Among the various enzymes involved in the abolishment of reactive oxygen species (ROS), superoxide dismutase (SOD) can be considered a key enzyme. SOD activity varied as a function of both exposure time and Hg concentration (Fig. 2A). For 10-day-old seedlings, SOD activity decrease at 0.5 μM $HgCl_2$, increased at 50 μM $HgCl_2$ and decreased again at 250 and 500 μM $HgCl_2$, by about 45% and 37%, respectively. Similarly, 15-day-old seedlings showed the highest level of SOD activity at 50 μM $HgCl_2$ (Fig. 2A) and the lowest level at 250 μM $HgCl_2$ (Fig. 2A).

SH content also varied as a function of both exposure time and Hg concentration (Fig. 2B). For 10- day- old seedlings, SH content increased with all Hg concentrations tested. On the other hand, at the highest exposure time (15 days), SH content increased by about 20% and 232%, at 0.5 and 250 μM $HgCl_2$ respectively, and decreased by about 80% and 75%, at 50 and 500 μM $HgCl_2$ respectively.

The effects of Hg on ascorbic acid content are shown in Fig. 2C. Regardless of the time of Hg exposure, the ASA content increased as a function of Hg

concentration. The maximum accumulation of ASA was 232.6 µg ASA g^{-1} fresh weight in seedlings treated with 500 µM $HgCl_2$ at 10 days of exposure.

The effects of Hg on carotenoid levels of cucumber seedlings are shown in Fig. 2D. Hg-exposure induced a significant increase in carotenoid content up to 50 µM Hg at both 10 and 15 days followed by decrease at higher metal concentrations. At 500 µM HgCl2, the carotenoid content decreased by 67% and 30% at 10- and 15- days, respectively, in comparison with the control.

Hg-exposure induced a significant reduction of ALA-D activity (Fig. 3), and these effects varied with the time of exposure and the concentration of exogenous Hg. At the highest concentration of Hg (500 µM HgCl2), ALA-D activity at 10 and 15- days, was decreased by 99% and 95%, respectively when compared to the control. However, for 50 µM HgCl2 at 15 days, ALA-D activity was increased.

4- DISCUSSION

Heavy metal contamination of soils has markedly increased in the past few decades. Factors such as mining or industrial activities, automotive emission, and use of metal-enriched materials as chemicals fertilizers, farm manures, sewage sludge, and wastewater irrigation can contribute to this contamination (Webber, 1981; Freedman and Hutchinson, 1981).

Our results indicated that in cucumber seedlings, electrolyte leakage percentage (ELP) levels were significantly enhanced, and exposure time and concentration dependent (Fig. 1A). Another study showed that cucumber plants exposed to cadmium the ELP content also increased (Mishra et al., 2006). In a previous study realized by our laboratory, when cucumber seedlings were

exposure by 10 and 15 at mercury, at same concentrations utilized in this experiment, was observed an increase in the mercury levels in both roots and shoot. At 10-and-15-days-old cucumber seedlings, in the concentrations of 0.5, 50, 250 and 500 μM $HgCl_2$, Hg content in the cotyledons was, respectively, 5, 824, 2,686 and 7,066-fold, and 2.4, 542, 1,297 and 2,641-fold higher than the control. Moreover, Hg was more accumulated in root system, which was 10, 2,140, 20,830 and 55,628, and 6, 1,865, 16,018 and 26,006-fold higher than the control, at 10 and 15 days, respectively (Cargnelutti et al., 2006), confirming the Hg intoxication. These observations and others such as an increase in MDA (Cargnelutti et al., 2006) indicate that cucumber plants experienced substantial oxidative damage when exposed to high concentrations of $HgCl_2$ for a prolonged time.

When plants are exposed to environmental stressors such as heavy metals, oxidative damage can be caused either directly or indirectly by triggering an increased level of production of reactive oxygen species (ROS) (Shah et al., 2001; Patra et al., 2004). These ROS include superoxide radicals ($O2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2), which are produced during membrane linked electron transport activities as well as by a number of metabolic pathways (Shah et al., 2001) and in turn cause damage to biomolecules such as membranes, proteins and nucleic acids (Sharma and Talukder, 1989). Although the mechanism of Hg-induced H_2O_2 formation is not yet understood known, heavy metals are known to be involved in many ways in the production of ROS (Luna et al., 1994). H_2O_2 is moderately reactive and is a relatively long-lived molecule (half-life of 1 ms), which can be diffused away from its production side. H_2O_2 may inactivate enzymes by oxidizing their thiol

groups. For example, enzymes of the Calvin cycle and superoxide dismutase are inactivated by H_2O_2 (Charles and Halliwell, 1980; Bowler et al., 1994).

Our results clearly indicate that Hg-exposure resulted in increased H_2O_2 content in seedlings (Fig. 1B), which may be due the a decrease in catalase. However, the decreased H_2O_2 accumulation observed at the concentrations of 50 and 250 μM $HgCl_2$, at 10 and 15 days, respectively, could be related to the increased catalase activity. CAT is one of the key enzymes involved in the removal of toxic peroxide, and it decomposes H_2O_2 to water and molecular oxygen (Lin and Kao, 2000). Our results showed that CAT activity may be critical in the scavenging of H_2O_2.

Superoxide dismutase (SOD) scavenges $O_2^{\bullet-}$radicals to protect from cellular oxidative damage. The control of the steady-state $O_2^{\bullet-}$ levels by SOD is an important protective mechanism against oxidative damage, since $O_2^{\bullet-}$ acts as a precursor of more cytotoxic or highly reactive oxygen derivatives, such as peroxynitrite and $HO^{\bullet}$ (Halliwell and Gutteridge, 1999). Therefore, SOD is usually considered the first line of defense against oxidative stress.

In the present study a biphasic effect was observed in the SOD activity at 10 and 15-days. This result also might be attributed to the hormetic dose response. Low concentrations and short exposure times, may produce an effect similar to high concentrations at any period of time. This trend was compatible to SOD activity reported in grape leaves, seedlings of tomato, and in seedlings of Sesbania drummondii cultivated under Hg treatments (Ma, 1998; Cho and Park, 2000; Israr et al., in press). Moreover, with an increase of both exposure time and Hg concentration, there may be an increase in the production of ROS, causing greater damage to tissue cells. Low levels of SOD may be related with

the increase of H_2O_2 levels, because H_2O_2 may inactivate enzymes by oxidizing their thiol groups, as for instance SOD (Charles and Halliwell, 1980; Bowler et al., 1994).

Inorganic mercury in the Hg^{2+} form has a great affinity for SH groups of endogenous biomolecules (Clarkson, 1997). Thus, it is invariably found in cells and tissues attached to thiol-containing proteins and small-molecular-weight thiols such as cysteine and glutathione (GSH). At relatively low toxic or nontoxic doses, mercury increases the renal content of GSH, probably due to the induction of GSH synthesis (Zalups, 2000). However, due to its ability to form stable complexes with Cl^-, OH^-, S^{2-} and S-containing functional groups of organic ligands (Cotton and Wilkinson, 1972), the free Hg^{2+} ion is rarely found under natural field conditions.

Varying responses of Hg induced oxidative stress might be related to the concentration of thiolic groups, since they are consequently able to counteract oxidative stress. Furthermore, the antioxidant property of thiols depends on the oxidation of –SH groups of the tripeptide form transforming it to the disulphide form (Toppi and Gabbrielli, 1999). An increase in the thiol content in Sesbania drummondii was found by Israr et al. (2006) for a short exposure period, which could be due to the inactivation of the reactivity of the metals by a cytoplasmatic detoxification mechanism. Our results showed a higher concentration of the –SH group in Hg-treated seedlings of cucumber at a short exposure time (Fig. 2B). In agreement with Patra et al. (2004), Hg possesses a high affinity for the SH groups, making it a defense mechanism against damage caused by metals. The fast mercury-induced accumulation of GSH and the high stability of Hg $(GSH)_2$ mercaptide complexes suggest that GSH functions as an effective

scavenger of Hg^{2+} ions (Sinha et al., 1996). Our results suggest that thiols also play an important role in Hg detoxification. Moreover, with an increase of exposure time, -SH levels increased at 0.5 and 250 μM $HgCl_2$, and decreased at 50 and 500 μM $HgCl_2$ (Fig. 2B), demonstrating disturbances in the oxidant system.

L-ascorbic acid (ASA) is found in milimolar concentrations in leaves and plays an important role in plant tolerance to stress. ASA is involved in the regulation of photosynthesis, cell expansion, root elongation, and trans-membrane electron transport (Smirnoff, 2000). ASA is an important component of the plant antioxidant defense system and serves as a reductant for the removal of H_2O_2 among other peroxides (Noctor and Foyer, 1998). Vitamin C is the first line defense against oxygen radicals in the water-soluble compartment (Nordberg and Arner, 2001). This vitamin reacts directly with the superoxide, hydroxyl radical and oxygen singlet. ASA was also demonstrated to detoxify mercury in Chlorella vulgaris by donating electrons to free radicals, thus protecting the integrity of –SH groups (Rai, 1979). The increase in antioxidant levels reduces oxidized biomolecules, but these detoxificants are not completely sufficient to protect against visible injury (Ranieri et al., 1993).

In the present investigation, ASA levels increased at all the concentrations of Hg at 10 and 15 days. Similarly, Sinha et al. (1996) reported an increase in ascorbic acid content in Baccopa monieri plants treated with Hg, showing a significant increase in ASA content during the initial period of metal exposure.

Carotenoids are a part of the photosynthetic pigment, playing an important role in the protection of chlorophyll under stress conditions. Moreover, they are known to quench the photodynamic reactions leading to loss of chlorophylls,

replace peroxidation and collapse of membrane in chloroplasts (Knox and Dodge, 1985). In the present study, an increase in the carotenoid content was found at low concentrations (up to 50 μM $HgCl_2$), corroborating with a study in chromium treated Pistia stratiotes (Sinha et al., 2005). These findings demonstrate that the antioxidant power of carotenoids protects chlorophyll against the attack of free radicals. Contrarily, above 250 μM Hg, a significant decline in carotenoid content was observed at both 10- and 15- days (Fig. 2D). Several studies have shown that Hg in the substrate decreased the levels of photosynthetic pigments, chlorophylls and carotenoids, at a prolonged duration of exposure. Hg also strongly inhibits the photosynthetic electron transport chain, being that photosystem II (PS II) is the most sensitive target (Bernier et al., 1993; Bernier and Carpentier, 1995).

The reduced synthesis of porphobilinogen, the committed precursor of chlorophyll, in chill- and heat-stressed wheat seedlings, demonstrated that the inhibition of chlorophyll biosynthesis is partly due to the impairment of ALA biosynthesis (Tewari and Tripathy, 1998). In this study a decrease in ALA-D activity was observed at concentrations higher than 50 and 250 μM $HgCl_2$, at 10 and 15 days of exposure, respectively. Similarly, Morsch et al. (2002) showed that Hg was a potent inhibitor of the ALA-D activity in radishes. ALA-D reduced activity, that is sensitive to the mercury due to his nature sulfidrílica (Rocha et al., 1995), may lead the inhibition of chlorophyll biosynthesis (Sinha et al, 1996). Therefore, the ALA-D activity could be used as a sensitive marker for the presence of heavy metals in soils.

At a low concentration (50 μM $HgCl_2$) and at a longer exposure time (15 days), an increase in ALA-D activity was observed (Fig. 3). This effect may be

due to an increase of enzyme synthesis by the plant homeostasis system. At low metal concentrations, the pool of enzymatic activity was not inhibited.

In conclusion, the increase of ELP and H_2O_2 production in cucumber seedlings might be related to decreased superoxide dismutase content with a consequent increase in membrane and protein damage (such as ALA-D). Mercury stress increased the levels of ascorbic acid, total SH, and carotenoids in seedlings of C. sativus at the initial period of exposure. These antioxidant systems in seedlings seem to bind the metal in a form that renders its harmless, making the seedlings tolerant at low concentrations and short exposure times. However, the antioxidant systems are not able to protect from the toxicity caused by higher levels of Hg and increased exposure times, resulting in the negative effects observed in the growth of cucumber seedlings. Moreover, at 50 μM $HgCl_2$ a compensatory effect was observed in relation to the antioxidant defense system, causing the possible induction of enzyme synthesis, as for instance, SOD. Considering the results as a whole, we hope to contribute to a better understanding of the oxidative stress conditions generated by mercury in plants.

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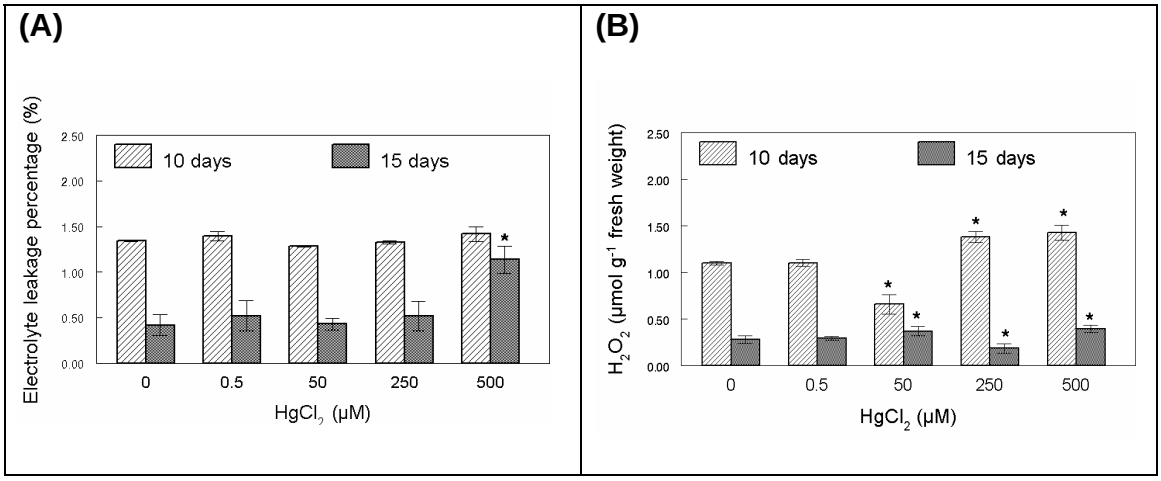
LEGEND OF THE FIGURES

Figure 1. Effect of increasing concentration of $HgCl_2$ on the electrolyte leakage percentage (A) and hydrogen peroxide content (B) at 10- and 15-days- old cucumber seedlings. Data represent the mean ± S.D. of three independent experiments. *Different from control to $p<0.05$.

Figure 2. Effect of increasing concentration of $HgCl_2$ on the superoxide dismutase activity (A), and –SH groups (B), ascorbic acid (C) carotenoid content (D) of 10- and 15-days- old cucumber seedlings. Data represent the mean ± S.D. of three different experiments. *Different from control to $p<0.05$.

Figure 3. Effect of increasing concentration of $HgCl_2$ on delta-aminolevulinic acid dehydratase activity of 10- and 15-days- old cucumber seedlings. Data represent the mean ± S.D. of three different experiments. *Different from control to $p<0.05$.

Figure 1


(A)
Electrolyte leakage percentage (%)
2.50
2.00
1.50
1.00
0.50
0.00
10 days
15 days
*
0
0.5
50
250
500
HgCl2 (μM)
(B)
H2O2 (μmol g-1 fresh weight)
2.50
2.00
1.50
1.00
0.50
0.00
10 days
15 days
*
*
*
*
*
0
0.5
50
250
500
HgCl2 (μM)

Figures 2

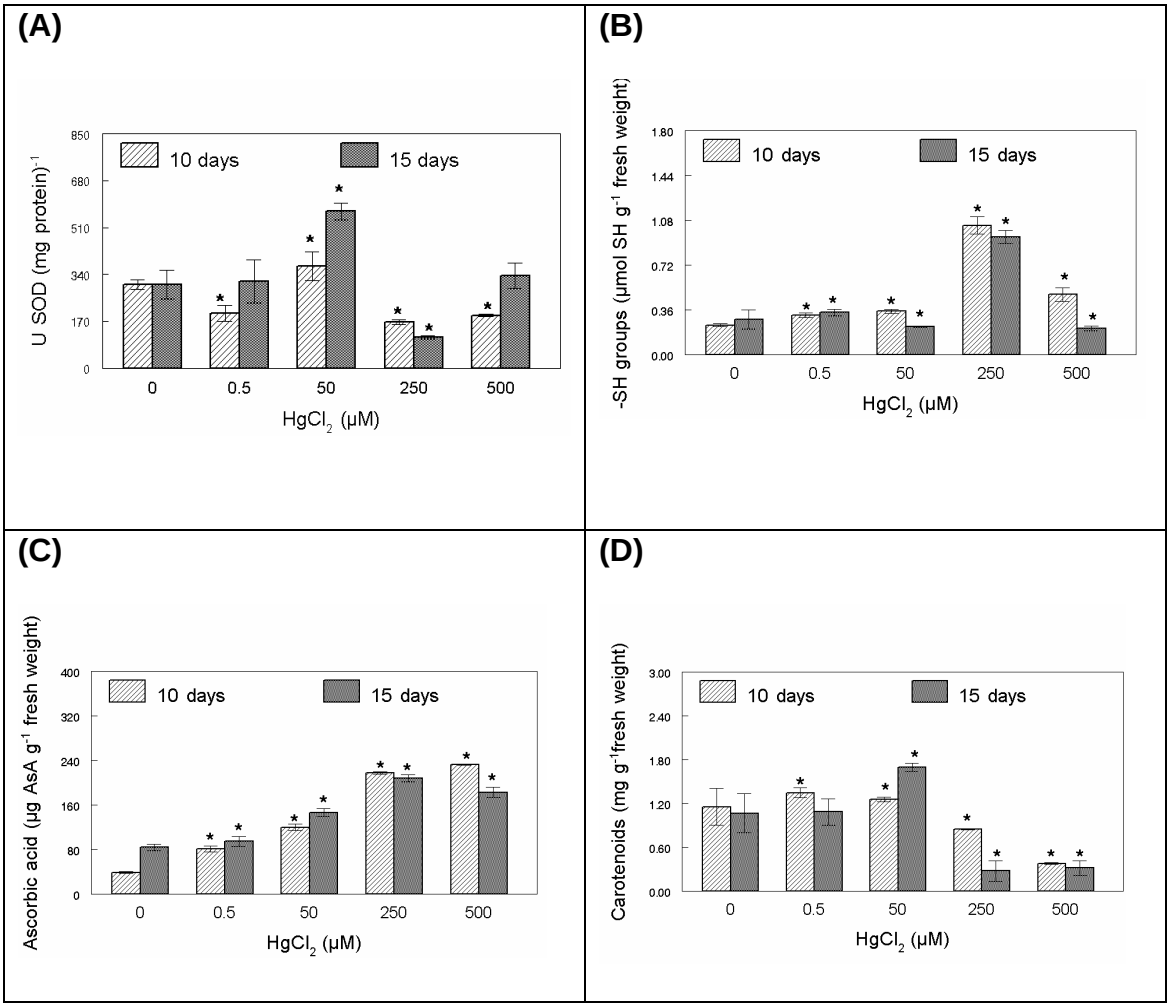
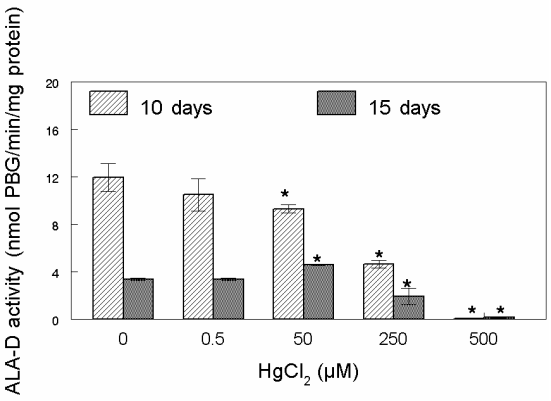

(A)
U SOD (mg protein)$^{-1}$
850
680
510
340
170
0
10 days
15 days
0
0.5
50
250
500
$HgCl_2$ (μM)
(B)
-SH groups (μmol SH g^{-1} fresh weight)
1.80
1.44
1.08
0.72
0.36
0.00
10 days
15 days
0
0.5
50
250
500
$HgCl_2$ (μM)
(C)
Ascorbic acid (μg AsA g^{-1} fresh weight)
400
320
240
160
80
0
10 days
15 days
0
0.5
50
250
500
$HgCl_2$ (μM)
(D)
Carotenoids (mg g^{-1}fresh weight)
3.00
2.40
1.80
1.20
0.60
0.00
10 days
15 days
0
0.5
50
250
500
$HgCl_2$ (μM)

Figure 3


ALA-D activity (nmol PBG/min/mg protein)
20
16
12
8
4
0
10 days
15 days
*
*
*
*
*
*
0
0.5
50
250
500
HgCl2 (µM)

4. DISCUSSÃO

A presença de mercúrio alterou o crescimento das plântulas de pepino levando ao dano das raízes, o que segundo Godbold & Huttermann (1986), pode ter conseqüências sérias na absorção de nutrientes e no fornecimento de água para a parte aérea das plântulas em crescimento. Também, concentrações altas de mercúrio aumentaram o peso seco das raízes (Artigo, Fig. 2E), o que de acordo com Arduini et al. (2004) indica a formação de agregados nos tecidos vegetais, através da proliferação anormal das células da raiz induzida pelo mercúrio.

Por outro lado, as concentrações altas de mercúrio reduziram a biomassa da parte aérea, confirmando o poder tóxico desse elemento em plantas (Artigo, Fig. 2F). O aumento do peso fresco das raízes em baixas concentrações de mercúrio (50 μM de $HgCl_2$) está relacionado ao efeito hormético, que representa uma alta compensação devido ao rompimento da homeostase. O efeito hormético é observado em relação a diferentes fatores: compostos químicos orgânicos e inorgânicos, Al, deficiência de um elemento essencial ou estimulação de reações de defesa que levam a ativação geral do metabolismo (CALABRESE, 1999; BARCELÓ & POSCHENRIEDER, 2002; CALABRESE & BLAIN, 2005). Stebbing (1998) relatou que o efeito homético geralmente pode ser definido como um efeito estimulatório que acontece quando concentrações altas de uma substância têm efeitos negativos (por exemplo, inibição de crescimento), e concentrações baixas produzem efeitos positivos (por exemplo, estimulação do crescimento) (CALABRESE & BALDWIN, 2000). Zhang & Tyerman (1999) relataram que o mercúrio inibe a absorção de água via aquaporinas na membrana plasmática em plantas superiores, o que poderia

explicar a redução do peso fresco das plântulas sob o efeito de concentrações altas de mercúrio, observado neste estudo.

O conteúdo de mercúrio nas raízes e cotilédones das plântulas de pepino aumentou com o aumento da concentração deste elemento adicionada ao substrato. As plântulas de pepino acumularam uma maior concentração de mercúrio nas raízes, quando comparada aos cotilédones, indicando que as raízes servem como uma barreira parcial para o transporte de mercúrio até a parte aérea das plântulas (CAVALLINI et al., 1999).

O $HgCl_2$ (0,5 – 500 µM) causou um declínio dependente do tempo e da concentração no conteúdo de clorofila (Artigo, Fig. 2A) em cotilédones. Sabe-se que os íons mercúrio podem substituir íons metálicos nos pigmentos fotossintéticos, causando uma redução nas taxas fotossintéticas (XYLANDER et al., 1996), e, também podem atuar reduzindo os níveis de pigmentos fotossintéticos (clorofila e carotenóides) em um tempo prolongado de exposição. Também, neste estudo, uma diminuição na atividade da enzima ALA-D foi observada em concentrações maiores do que 50 e 250 µM de $HgCl_2$, a 10 e 15 dias de exposição ao metal, respectivamente. Este resultado confirma o poder inibitório do mercúrio sobre a atividade da ALA-D (MORSCH et al., 2002). Entretanto, baixas concentrações (50 µM $HgCl_2$) e exposição por 15 dias, levou a um aumento na atividade da ALA-D (Manuscrito, Fig. 3). Esse efeito pode ser devido a um aumento da síntese de moléculas de enzimas pelo sistema homeostático das plantas, e baixas concentrações do metal não foram suficientes para inibir a enzima. Além disso, nossos resultados demonstram uma relação entre o conteúdo de clorofila e a atividade da ALA-D, indicando

que além da destruição de pigmentos, ocorre síntese diminuída de clorofila através da redução da atividade da ALA-D.

Foi demonstrado neste trabalho, que o mercúrio aumentou os níveis de MDA, de proteínas carboniladas (Artigo, Fig. 2B e 2C) e a porcentagem de vazamento de eletrólitos (ELP) (Manuscrito, Fig. 1A), sendo que esse aumento foi dependente do tempo de exposição e da concentração do metal. Estes aumentos indicam que as plântulas de pepino experimentam dano oxidativo substancial às proteínas e aos lipídios das membranas, o que levou ao vazamento de íons e à redução da permeabilidade da membrana plasmática. Também, a acumulação de grupos carbonil em plântulas de pepino, indica que a quantidade de ROS excedeu a capacidade do sistema de defesa antioxidante.

No presente estudo, um efeito bifásico foi observado para a atividade da catalase aos 10 dias de exposição ao metal, o que também pode ser atribuído à resposta hormética. Este efeito está relacionado a um mecanismo estimulatório em baixas concentrações e uma inibição em altas concentrações de um composto (CALABRESE & BALDWIN, 2000). Além disso, aos 10 dias, o efeito inibitório do mercúrio na atividade da catalase coincidiu com uma diminuição no conteúdo de proteína solúvel. Sabe-se que as altas concentrações de mercúrio podem levar à precipitação de proteínas (PATRA & SHARMA, 2000), reduzindo assim a função de algumas enzimas. A perda da função das proteínas sugere que as defesas antioxidantes estão reduzidas e os níveis de ROS aumentados, levando ao dano das células teciduais. Também, nossos resultados indicam que a exposição ao mercúrio resulta em um aumento no conteúdo de H_2O_2 nas plântulas (Manuscrito, Fig. 1B), que

coincidiu com uma diminuição na atividade da catalase. No entanto, a diminuição da acumulação de H_2O_2 observada a 50 e 250 μM de $HgCl_2$, aos 10 e 15 dias, respectivamente, poderia ser correlacionada com o aumento da atividade da catalase.

O efeito bifásico observado para a atividade da enzima superóxido dismutase (SOD) aos 10 e 15 dias, esteve de acordo com os resultados obtidos em outros estudos (MA, 1998; CHO & PARK, 2000; ISRAR et al., 2006). Concentrações baixas do mercúrio induzem a ativação da SOD devido ao efeito compensatório produzido pelas plântulas em resposta ao metal. Entretanto, altas concentrações do mercúrio inibem a atividade da SOD. Esta inibição está relacionada aos níveis aumentados de H_2O_2, pois, de acordo com Charles & Halliwell (1980) e Bowler et al. (1994), o H_2O_2 pode inativar as enzimas através da oxidação dos seus grupos tióis.

Um declínio nas atividades das enzimas superóxido dismutase, catalase e ascorbato peroxidase em plântulas tratadas com mercúrio sugerem uma possível demora na remoção do $O_2^{\bullet -}$, H_2O_2 e dos peróxidos tóxicos nas células, levando a um aumento na peroxidação lipídica e oxidação protéica.

Em relação aos antioxidantes não enzimáticos, nossos resultados mostraram um aumento no conteúdo de grupos –SH por um curto período de exposição ao mercúrio (Manuscrito, Fig. 2B). O aumento dos grupos –SH leva à inativação da reatividade do mercúrio por mecanismos de detoxificação citoplasmáticos. De acordo com Patra et al. (2004) e Clarkson (1997), o mercúrio possui alta afinidade por grupos -SH, o qual representa um mecanismo de defesa contra o dano celular causado pelo metal. Além disso, com o aumento do tempo de exposição ao mercúrio, foram observados níveis

aumentados de grupos –SH nas concentrações de 0,5 e 250 μM de $HgCl_2$. Por outro lado, nas concentrações de 50 e 500 μM de $HgCl_2$, os níveis de grupos –SH foram reduzidos em plântulas de pepino (Manuscrito, Fig. 2B), demonstrando distúrbios no sistema de defesa antioxidante.

Na presente investigação, os níveis de ASA aumentaram nas amostras de plântulas crescidas em todas as concentrações de mercúrio tanto aos 10 quanto aos 15 dias, evidenciando um mecanismo removedor de ROS ativo. Além disso, um aumento no conteúdo de carotenóides foi encontrado em baixas concentrações de mercúrio (acima de 50 μM de $HgCl_2$), demonstrando também o poder antioxidante dos carotenóides que protege a clorofila contra o ataque dos radicais livres. Contrariamente, acima de 250 μM de $HgCl_2$, foi observado um declínio no conteúdo de carotenóides aos 10 e 15 dias (Manuscrito, Fig. 2D), evidenciando a destruição de pigmentos pelo mercúrio e a redução do sistema de defesa antioxidante nas plântulas.

Portanto, nossos resultados mostraram que altas concentrações de mercúrio induzem estresse oxidativo, evidenciado pela inibição de enzimas antioxidantes, pela redução dos níveis de antioxidantes não enzimáticos (exceto o ácido ascórbico), pela inibição da enzima delta-ALA-D e pela redução no conteúdo de clorofila. Além disso, os níveis elevados de H_2O_2, a peroxidação lipídica, as proteínas oxidadas e a porcentagem de vazamento de eletrólitos indicam o dano às membranas e as proteínas, o que contribui para o vazamento de íons através das membranas celulares. No entanto, em baixas concentrações e durante 10 dias de exposição ao metal, foi observado uma ativação do sistema de defesa antioxidante. Esse fator indica mecanismos de

detoxificação ativos o que poderia estar relacionado com um processo de tolerância das plântulas de *C. sativus* ao mercúrio.

5. CONCLUSÕES

- Concentrações altas de mercúrio inibiram a atividade das enzimas catalase, ascorbato peroxidase e superóxido dismutase, e baixas concentrações do metal (50 μM de $HgCl_2$) exibiram um efeito compensatório em relação as enzimas catalase e superóxido dismutase. O estresse causado pelo mercúrio elevou os níveis de ácido ascórbico, -SH total e carotenóides em plântulas de *C. sativus* durante o período inicial de exposição e em baixas concentrações de $HgCl_2$, sugerindo que o sistema de defesa antioxidante estava ativo em baixos níveis de mercúrio e atuando na remoção das ROS. Níveis altos de mercúrio e um prolongado período de exposição causaram estresse oxidativo em *C. sativus*, indicando que as defesas antioxidantes das plântulas não foi capaz de proteger a toxicidade causada pelo metal.

- O mercúrio aumentou a peroxidação lipídica, o conteúdo de H_2O_2, a oxidação de proteínas e a porcentagem de vazamento de eletrólitos. Isto sugere que o mercúrio possa induzir dano às membranas e às proteínas, levando a perda de íons através das membranas celulares, bem como à diminuição da atividade das enzimas de plântulas de pepino.

- O mercúrio inibiu a atividade da delta-ALA-D, assim como reduziu os níveis de clorofila em plântulas de *C. sativus* durante os 10 e 15 dias de exposição ao metal, indicando que o mercúrio influenciou no processo fotossintético, alterando, consequentemente, o crescimento das plântulas de pepino.

- Níveis altos de mercúrio e um período prolongado de exposição resultaram em efeitos negativos observados no crescimento das plântulas de pepino, pelo qual pode-se concluir que o mercúrio absorvido pelos tecidos produziu efeitos tóxicos no metabolismo das plântulas, levando à perda de matéria seca.

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