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Antioxidant system activation by mercury in *Pfaffia glomerata* plantlets

N. S. Calgaroto · G. Y. Castro · D. Cargnelutti · L. B. Pereira · J. F. Gonçalves · L. V. Rossato · F. G. Antes · V. L. Dressler · E. M. M. Flores · M. R. C. Schetinger · F. T. Nicoloso

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Abstract Oxidative stress caused by mercury (Hg) was investigated in *Pfaffia glomerata* plantlets grown in nutrient solution using sand as substrate. Thirty-day-old acclimated plants were treated for 9 days with four Hg levels (0, 1, 25 and 50 μM) in the substrate. Parameters such as growth, tissue Hg concentration, toxicity indicators (δ-aminolevulinic acid dehidratase, δ-ALA-D, activity), oxidative damage markers (TBARS, lipid peroxidation, and H_2O_2 concentration) and enzymatic (superoxide dismutase, SOD, catalase, CAT, and ascorbate peroxidase, APX) and non-enzymatic (non-protein thiols, NPSH, ascorbic acid, AsA, and proline concentration) antioxidants were investigated. Tissue Hg concentration increased with Hg levels. Root and shoot fresh weight and δ-ALA-D activity were significantly decreased at 50 μM Hg, and chlorophyll and carotenoid concentration were not affected. Shoot H_2O_2 concentration increased curvilinearly with Hg levels, whereas lipid peroxidation increased at 25 and 50 μM Hg, respectively, in roots and shoots. SOD activity showed a straight correlation with H_2O_2 concentration, whereas CAT activity increased only in shoots at 1 and 50 μM Hg. Shoot APX activity was either decreased at 1 μM Hg or increased at 50 μM Hg. Conversely, root APX activity was only increased at 1 μM Hg. In general, AsA, NPSH and proline concentrations increased upon addition of Hg, with the exception of proline in roots, which decreased. These changes in enzymatic and non-enzymatic antioxidants had a significant protective effect on *P. glomerata* plantlets under mild Hg-stressed conditions.

Keywords Antioxidant system · Brazilian ginseng · Mercury · Oxidative stress · *Pfaffia glomerata* · Plant growth

N. S. Calgaroto · G. Y. Castro · F. T. Nicoloso (✉)
Departamento de Biologia, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil
e-mail: ftnicoloso@yahoo.com; fernandonicoloso@yahoo.com

N. S. Calgaroto · G. Y. Castro · F. T. Nicoloso
Programa de Pós-Graduação em Agronomia, Centro de Ciências Rurais, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil

D. Cargnelutti · L. B. Pereira · J. F. Gonçalves · L. V. Rossato · F. G. Antes · V. L. Dressler · E. M. M. Flores · M. R. C. Schetinger
Departamento de Química, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil

D. Cargnelutti · L. B. Pereira · J. F. Gonçalves · L. V. Rossato · M. R. C. Schetinger
Programa de Pós-Graduação em Bioquímica Toxicológica, Centro de Ciências Naturais e Exatas, Universidade Federal de Santa Maria, Santa Maria, RS 97105-900, Brazil

Introduction

Amongst the metal pollutants, mercury (Hg) is both the best known and the most hazardous metal in the

environment. Considerable amounts of Hg are added to farmland with the application of sludge, fertilizers, pesticides, lime and manures (Patra and Sharma 2000).

Being a transition metal, Hg is able to induce oxidative stress in plants, resulting in lipid peroxidation, and alteration of antioxidant enzyme activities and induction of thiol-containing compounds (Cargnelutti et al. 2006; Rellán-Álvarez et al. 2006; Ortega-Villasante et al. 2007; Zhou et al. 2009). Mercuric ions are able to induce oxidative stress in plants by triggering the generation of reactive oxygen species (ROS), e.g., superoxide anion radical (O_2^-), H_2O_2, and hydroxyl radical (OH·) (Cargnelutti et al. 2006; Ortega-Villasante et al. 2007; Zhou et al. 2008). Moreover, Hg is able to bind with water channel proteins of root cells causing a physical obstruction to the water flow (Zang and Tyerman 1999). Another toxic symptom of Hg accumulation in plants is the decreased levels of chlorophyll and proteins (Cargnelutti et al. 2006), which can affect both photochemical and carbon reduction reactions of photosynthesis (Patra and Sharma 2000).

The generation of ROS, particularly H_2O_2, has been proposed as part of the signaling cascade leading to protection from stresses (Mittler 2002) that can be caused by abiotic factors, such as drought, chilling, heavy metals, or biotic factors, such as pathogen invasion. In order to protect tissues from oxidative stress, plant cells contain both oxygen radical detoxifying (antioxidant) enzymes such as catalase, ascorbate peroxidase and superoxide dismutase, and non-enzymatic antioxidants such as ascorbate and glutathione (Mittler 2002; Gratão et al. 2005; Zhou et al. 2008). Altered antioxidant enzyme activities are frequently used as indicators of stress (Cargnelutti et al. 2006; Ortega-Villasante et al. 2007; Zhou et al. 2008). However, changes in ROS metabolism and antioxidant systems involved in scavenging ROS in plants exposed to Hg have been investigated in few species (Cavallini et al. 1999; Cho and Park 2000; Cargnelutti et al. 2006; Rellán-Álvarez et al. 2006; Ortega-Villasante et al. 2007; Zhou et al. 2008).

The genus of *Pfaffia* belongs to the Amaranthaceae family and has about 90 species distributed throughout Central and South America. In Brazil, 27 species have been described (Taniguchi et al. 1997). Carneiro et al. (2002) showed that an undetermined species of the genus *Pfaffia* exhibited high tolerance to soil contamination, growing quite abundantly in soil mixtures with 90 and 1,450 mg kg^{-1} of Cd and Zn, respectively. In a recent study, Skrebsky et al. (2008) showed that *Pfaffia glomerata* (Spreng.) Pedersen plantlets grown hydroponically seemed to have reasonable degree of Cd tolerance. In line with this and taking into account the high commercial value of *P. glomerata* to the pharmaceutical industries (Nicoloso et al. 2001), it is important to verify whether this species accumulates other metals, shows Hg tolerance and, if so, which mechanisms are involved in Hg tolerance.

Under this context, the present study was designed to analyze the importance of enzymatic and non-enzymatic antioxidants in both roots and shoots of *P. glomerata* plantlets during a 9-day period of exposure to different mercury concentrations, ranging from those observed in moderately contaminated (1 μM) to highly contaminated soils (25 and 50 μM).

Materials and methods

Plant material and growth conditions

Pfaffia glomerata (Spreng.) Pedersen plantlets for tissue culture were obtained from the Brazilian Ginseng Germplasm Program, Universidade Federal de Santa Maria, RS, Brazil. Nodal segments (1.0 cm long) without leaves were micropropagated in MS medium (Murashige and Skoog 1962), supplemented with 30 g l^{-1} of sucrose, 0.1 g l^{-1} of myo-inositol and 6 g l^{-1} of agar according to Nicoloso et al. (2001). Thirty-day-old plantlets grown in vitro were transferred into pots containing washed sand. These plantlets were supplemented daily with nutrient solution containing the following composition: 65.1 mg l^{-1} NH_4Cl, 76.2 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 135.2 mg l^{-1} $MgCl_2 \cdot 6H_2O$, 33.1 mg l^{-1} KH_2PO_4, 181.5 mg l^{-1} KCl, 575.3 mg l^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 0.11 mg l^{-1} $CuSO_4 \cdot 5H_2O$, 0.39 mg l^{-1} $MnCl_2 \cdot 4H_2O$, 0.57 mg l^{-1} $ZnSO_4 \cdot 7H_2O$, 0.04 mg l^{-1} $NiSO_4$, 1.54 mg l^{-1} H_3BO_3, 0.09 mg l^{-1} $H_2MoO_4 \cdot H_2O$ and 13.34 mg l^{-1} $FeSO_4 \cdot 7H_2O$. After 1 month of plantlet acclimation, Hg was added to the nutrient solution as $HgCl_2$ at concentrations of 0 (control), 1, 25 and 50 μM. After 9 days of Hg exposure, 3 plantlets per replicate (each treatment consisted of three replicates) were harvested randomly.

The plantlets were divided into roots and shoot for evaluation of fresh biomass. Three independent and representative tissue samples were used for Hg determination. Fresh samples were used for measurements of H_2O_2, MDA, chlorophyll concentrations, antioxidant enzyme activities and non-enzymatic antioxidant concentrations. Both in vitro and ex vitro cultured plantlets were grown in a growth chamber at 25 ± 1°C on a 16/8 h light/dark cycle with 35 μmol m^{-2} s^{-1} of irradiance by cold fluorescent lamps.

Tissue Hg concentration

For the metal determination, plantlets were oven-dried at 65°C to constant mass. Dried shoot and roots (0.07–0.1 g) were ground and digested with 5 ml HNO_3 and 0.2 ml H_2O in closed Teflon vessels, which were heated at 100°C for 3 h in a digester block (Tecnal TE 007D). The samples were then diluted to 50 ml with high-purity water. Hg concentrations were determined using a Varian Atomic Absorption Spectrophotometer (Spectr AA 600, Australia) equipped with a vapor generative accessory (Varian VGA-76). The content absorbed was expressed as μg g^{-1} dry weight.

Chlorophyll and carotenoid determination

Fresh biomass (leaves plus stem) was homogenized in 80% ice-cold acetone in the dark and then centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was used for the immediate determination of pigments. Absorbance of the solution was measured at 663, 645, 510 and 480 nm in a spectrophotometer (Celm E-205D) in order to determine the concentration of carotenoids, chlorophyll a and chlorophyll b, respectively, with the help of Arnon's formulae (Arnon 1949). Chlorophyll and carotenoid concentrations were expressed as μg g^{-1} fresh weight.

Delta-aminolevulinic acid dehydratase activity

Shoots 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*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 mM dithiotreithol (DTT). Delta-aminolevulinic acid dehydratase (δ-ALA-D; E.C. 4.2.1.24) activity was assayed as described by Barbosa et al. (1998) by measuring the rate of porphobilinogen (PBG) formation. The incubation medium for the assays contained 100 mM Tris–HCl buffer, pH 9.0 and 3.6 mM ALA. Incubation was started by adding 100 μl of the tissue preparation to a final volume of 400 μl and stopped by adding 350 μl of the mixture containing 10% trichloroacetic acid (TCA) and 10 mM $HgCl_2$. The product of the reaction was determined with the Ehrlich reagent at 555 nm using a molar absorption coefficient of 6.1×10^4 l^{-1} mol^{-1} cm^{-1} (Sassa 1982) for the Ehrlich-porphobilinogen salt. The δ-ALA-D activity was expressed as nmol PBG mg^{-1} protein h^{-1}.

Determination of hydrogen peroxide

The H_2O_2 concentration was determined according to Loreto and Velikova (2001). Approximately 0.1 g of both roots and shoot was homogenized at 4°C in 2 ml of 0.1% trichloroacetic acid (TCA) (w/v). The homogenate was centrifuged at 12,000*g* for 15 min. Then, 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 ml of 1 M KI. The H_2O_2 concentration of the supernatant was evaluated by comparing its absorbance at 390 nm with a standard calibration curve. The hydrogen peroxide concentration was expressed as μmol g^{-1} fresh weight.

Estimation of lipid peroxidation

The level of lipid peroxidation products was estimated following the method of El-Moshaty et al. (1993) by measuring the concentration of malondialdehyde (MDA) as an end product of lipid peroxidation by reaction with thiobarbituric acid (TBA). Fresh tissue samples (0.1 g fresh weight) were ground in 2 ml of 0.2 M citrate–phosphate buffer (pH 6.5) containing 0.5% Triton X-100, using mortar and pestle. The homogenate was filtered through two layers of paper and centrifuged for 15 min at 20,000*g*. One milliliter of the supernatant fraction was added to an equal volume of 20% (w/v) TCA containing 0.5% (w/v) TBA. The mixture was heated at 95°C for 40 min and then quickly cooled in an ice bath for 15 min. After centrifugation at 10,000*g* for 15 min, the absorbance of the supernatant was measured at 532 nm. A correction for non-specific

turbidity was made by subtracting the absorbance value taken at 600 nm. The lipid peroxides were expressed as nmol MDA (mg protein)$^{-1}$, by using an extinction coefficient of 155 l mol^{-1} cm^{-1}.

Catalase assay

Catalase (CAT, E.C. 1.11.1.6) activity was assayed according to the method of Aebi (1984) with some modifications. Fresh samples (1 g) were homogenized in 5 ml of 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 10 g l^{-1} PVP, 0.2 mM EDTA and 10 ml l^{-1} Triton X-100. The homogenate was centrifuged at 12,000*g* at 4°C for 20 min and the supernatant was used for the enzyme assay. CAT activity was determined by monitoring the disappearance of H_2O_2 by measuring the decrease in absorbance at 240 nm from a reaction mixture containing 2 ml of 15 mM H_2O_2 in KPO_4 buffer (pH 7.0) and 30 µl extract. Activity was expressed as ΔE min^{-1} mg^{-1} protein.

Ascorbate peroxidase assay

Ascorbate peroxidase (APX, E.C. 1.11.1.11) activity was measured according to Zhu et al. (2004). The reaction mixture, at a total volume of 2 ml, contained 25 mM (pH 7.0) sodium phosphate buffer, 0.1 mM EDTA, 0.25 mM ascorbate, 1.0 mM H_2O_2 and 100 µl enzyme extract. H_2O_2-dependent oxidation of ascorbate was monitored by a decrease in absorbance at 290 nm ($E = 2.8$ l mol^{-1} cm^{-1}) and activity was expressed as µmol ascorbate oxidated min^{-1} mg^{-1} protein.

Superoxide dismutase assay

Superoxide dismutase (SOD, E.C. 1.15.1.1) activity was assayed according to Misra and Fridovich (1972). About 200 mg of roots and shoots 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 homogenate was centrifuged at 22,000*g* at 4°C for 10 min. 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 component added. Adenochrome 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.

Ascorbic acid concentration

Ascorbic acid (AsA) determination was performed as described by Jacques-Silva et al. (2001). Both roots and shoots were homogenized in a solution containing 50 mmol l^{-1} Tris–HCl and 10 ml l^{-1} Triton X-100 (pH 7.5), centrifuged at 6,800*g* for 10 min. To the 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. An aliquot of the sample (300 µl) was incubated at 37°C in a medium containing 100 µl 13.3% TCA, 100 µl deionized water and 75 µl 2,4-Dinitrophenylhydrazine (DNPH). The DNPH solution contained 2% DNPH, 0.23% thiourea, 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 group concentration

Non-protein thiol (NPSH) concentration was measured spectrophotometrically with Ellman's reagent (Ellman 1959). Root and shoot samples were homogenized in a solution containing 50 mmol l^{-1} Tris–HCl and 10 ml l^{-1} Triton X-100 (pH 7.5), centrifuged at 6,800*g* for 10 min, and NPSH was determined in a fraction obtained after mixing 1 volume of supernatant with 1 volume of 10% TCA followed by centrifugation (6,800*g* for 10 min) and neutralization (to pH 7.4) with 1 M Tris–HCl as described by Jacques-Silva et al. (2001). The reaction was read at 412 nm after the addition of 0.05 ml of 10 mM 5-5-dithio-bis (2-nitrobenzoic acid) (DTNB). A standard curve using cysteine was used to calculate the concentration of NPSH in the samples and was expressed as µmol SH g^{-1} fresh weight.

Proline concentration

Proline determination was performed as described by Bates et al. (1973). Approximately 0.25 g of fresh tissues were homogenized in 5 ml of 10% aqueous sulfosalicylic acid. The homogenate was centrifuged at 5,000*g* at 4°C for 20 min. Two milliliters of supernatant were used to react with 2 ml acid-ninhydrin and 2 ml of glacial acetic acid in a test tube for 1 h at 100°C, and the reaction terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene, mixed vigorously with a test tube stirrer for 20 s. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance was read at 520 nm using toluene as a blank. The proline concentration was determined from a standard curve and calculated on a fresh weight basis as follows: [(μg proline/ml × ml toluene)/115.5 μg/μmole]/[(g sample)/5] = μmol proline/g of fresh weight material.

Protein determination

In all the enzyme preparations, protein was determined by the method of Bradford (1976) using bovine serum albumin as standard and was expressed in mg l^{-1}.

Statistical analysis

The analyses of variance were computed for statistically significant differences determined based on the appropriate *F*-tests. The results are the means ± SD of at least three independent replicates. The mean differences were compared utilizing Tukey test at $P < 0.05$.

Results and discussion

Plants may survive in heavy metal-contaminated environments by preventing metals from entering into the cytoplasm and/or detoxifying them inside the cytoplasm (Hall 2002). When metals are initially absorbed by roots, some of them are trapped in the cell wall, which reduces the amount of metal that enters the cytoplasm (Gratão et al. 2005). In the present study, the root Hg concentration was about 11-fold higher than that found in shoots, with 713 μg g^{-1} dry weight of Hg in roots treated with 50 μM Hg (Table 1). This result is in accordance with that obtained in *Zea mays* seedlings grown in 30 μM Hg for 7 days (Rellán-Álvarez et al. 2006). We also verified the effect of Hg on the growth of nodal segments of *P. glomerata* grown in vitro, and observed (data not shown) that as the plant organ used initially did not contain roots, Hg treatment as low as 1 μM had a significant suppressive effect on tissue growth. This demonstrates that roots are very important to avoid Hg uptake into the protoplasm. In addition, it indicates that roots serve as a partial barrier to the transport of Hg to shoots (Cavallini et al. 1999; Patra and Sharma 2000).

Although shoots accumulated less Hg than roots, the shoot Hg concentration increased about 24-fold at the highest external concentration of Hg when compared to the control, reaching about 68 μg g^{-1} dry weight (Table 1). Mercury accumulated in the shoot of control plants to a similar extent than plants treated with 1 μM Hg, whereas the concentration in roots of plants non-exposed to Hg was just over the detection limit (Table 1). This might be caused by the uptake of volatile Hg by the leaves via stomata. Patra et al. (2004) reported that Hg salts in soil may be

Table 1 Tissue Hg concentration and fresh weight of shoot and roots of *Pfaffia glomerata* plantlets exposed to treatment for 9 days

Treatments	Hg concentration (μg g^{-1} dry weight)		Fresh weight (g $plant^{-1}$)	
	Shoot	Root	Shoot	Root
Control	2.8 ± 0.4 cA*	0.6 ± 0.01 cB	2.4 ± 0.13 a*	0.7 ± 0.10 a
1 μM Hg	3.1 ± 0.2 cB	4.4 ± 0.09 cA	2.3 ± 0.18 a	0.7 ± 0.07 a
25 μM Hg	25.9 ± 3.9 bB	531 ± 15.4 bA	2.2 ± 0.16 a	0.6 ± 0.04 ab
50 μM Hg	67.7 ± 7.6 aB	713 ± 120 aA	1.5 ± 0.04 b	0.5 ± 0.01 b

Data represent mean values ± SD based on independent determination

* Mean values followed by the same lower-case letters in the column, and capital letters in the line did not differ significantly by Tukey test of $P < 0.05$

reduced by biological and chemical reactions to metallic or methylated compounds, which may volatilize and be taken up through the leaves in plants grown in enclosed spaces. Uptake of gaseous Hg via stomata was also observed in laboratory studies (Cavallini et al. 1999; Iglesia-Turiño et al. 2006).

Shoot and root fresh weight decreased only upon addition of 50 μM Hg (Table 1). At this level of Hg, the shoot and root fresh weights were about 15.5 and 20% lower, respectively, than that of the control. This result is in agreement with the findings of other authors (Cargnelutti et al. 2006; Zhou et al. 2008) demonstrating that only high levels of Hg became strongly phytotoxic to cells. On the other hand, maize and tomato seedlings exposed to lower Hg levels suffered clear symptoms of phytotoxicity (Cho and Park 2000; Rellán-Álvarez et al. 2006). Mercury is known to inhibit water uptake via aquaporins on plasma membranes in higher plants (Zang and Tyerman 1999), which might explain the detrimental effect of high concentrations of Hg on the fresh weight of *P. glomerata* plantlets.

In the present study, there was no effect of Hg on the chlorophyll and carotenoid concentrations (Fig. 1a). This result might be related to the reduction in fresh weight (Table 1), which would lead to an increase in the concentration of cellular components. In addition, young expanding leaves are smaller in Hg-treated plants than in control plants, probably increasing the concentration of cellular components. It has been suggested that the reduction in chlorophyll content in the presence of heavy metals is caused by an inhibition of chlorophyll biosynthesis (Pereira et al. 2006) which may have been caused, in part, by the reduction of δ-ALA-D activity. δ-ALA-D is sensitive to metals due to its sulfhydrylic nature (Rocha et al. 1995). This enzyme catalyzes the condensation of two 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). Altered δ-ALA-D activity concomitant with reduced chlorophyll contents has been reported in many terrestrial plants exposed to various metals (Cargnelutti et al. 2006; Skrebsky et al. 2008; Gonçalves et al. 2009). In the present study, plantlets of *P. glomerata* exposed to 1 μM of Hg showed increased δ-ALA-D activity (Fig. 1b), but did not show any change in the chlorophyll concentration (Fig. 1a). The stimulating effects of low heavy metal concentrations on several plant growth parameters is normally related to the hormetic effect, which probably represents an over-compensation response to a disruption in the homeostasis of the organism (Aina et al. 2007). Skrebsky et al. (2008) reported that there was an increase in both root and shoot biomass of *P. glomerata* plantlets at low Cd levels (20 and 40 μM), which was ascribed to the so-called hormetic effect. Khan et al. (2008) observed similar phenomenon, where 10 μM Cd enhanced the activities of leaf superoxide dismutase, ascorbate peroxidase, glutathione reductase and carbonic anhydrase, net photosynthetic rate and plant dry mass of *Tritucum aestivum.* These data suggest that the synergy between the activities of an antioxidant enzyme and an enzyme involved in chlorophyll biosynthesis (δ-ALA-D) helped to maintain chlorophyll and plant biomass at low Hg levels.

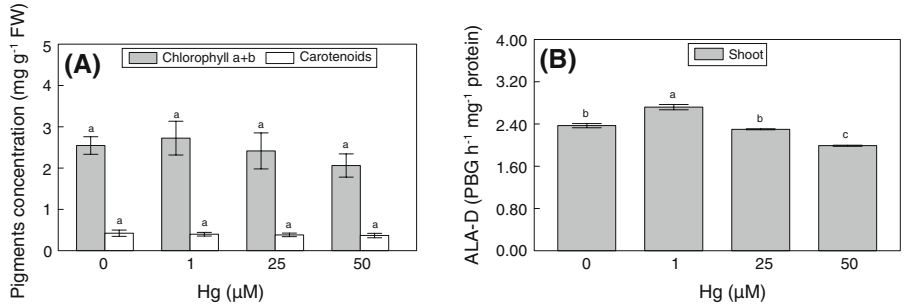

Fig. 1 Effect of increasing Hg concentration on the pigment concentration (**a**) and δ-aminolevulinic acid dehydratase (δ-ALA-D) activity (**b**) in *P. glomerata* plantlets. Data represent the mean ± SD of three replicates. *Identical letters* indicate no significant differences among the Hg concentrations ($P < 0.05$) according to Tukey's multiple range test

The effects of Hg on H_2O_2 formation and lipid peroxidation are shown in Fig. 2. Plants exposed to 1 μM Hg showed an increase in the H_2O_2 concentration in both shoot and roots (Fig. 2a), whereas lipid peroxidation (Fig. 2b) did not change, when compared to the control. At the same level of Hg, a significant increase in the activity of SOD (Fig. 3a) and CAT (Fig. 3b) was observed in shoots. Therefore, it seems that a low concentration of Hg in the substrate was enough to activate the antioxidant system which aims to protect important metabolic enzymes, such as δ-ALA-D (Fig. 1b). However, δ-ALA-D activity decreased at the highest concentration of Hg used, where it was 20% lower than that of the control. Interestingly, a significant increase in lipid peroxidation in shoots was only observed at 50 μM Hg, which was about threefold higher when compared to the control (Fig. 2b). This demonstrates that the antioxidant system was unable to avoid Hg stress at high Hg levels.

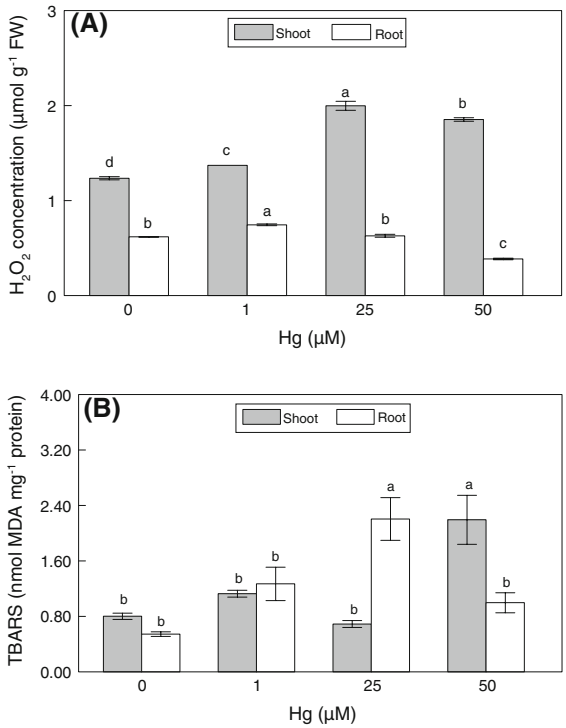

Fig. 2 Effect of Hg on H_2O_2 (**a**) and lipid peroxidation (**b**) of *P. glomerata* plantlets. Data represent the mean ± SD of three replicates. *Identical letters* indicate no significant differences among the Hg concentrations ($P < 0.05$) according to Tukey's multiple range test

At 25 μM Hg, shoot lipid peroxidation was significantly increased, when compared with the control (Fig. 2b), indicating that *P. glomerata* could not tolerate high Hg concentrations. Lipid peroxidation was also higher in roots at 25 μM Hg, even though the H_2O_2 concentration did not differ from that of the control (Fig. 2a). In contrast, SOD activity (Fig. 3a) was inhibited while CAT (Fig. 3b) and APX (Fig. 3c) activities were not affected. Therefore, the significant increase in ascorbic acid (AsA) and non-protein thiol

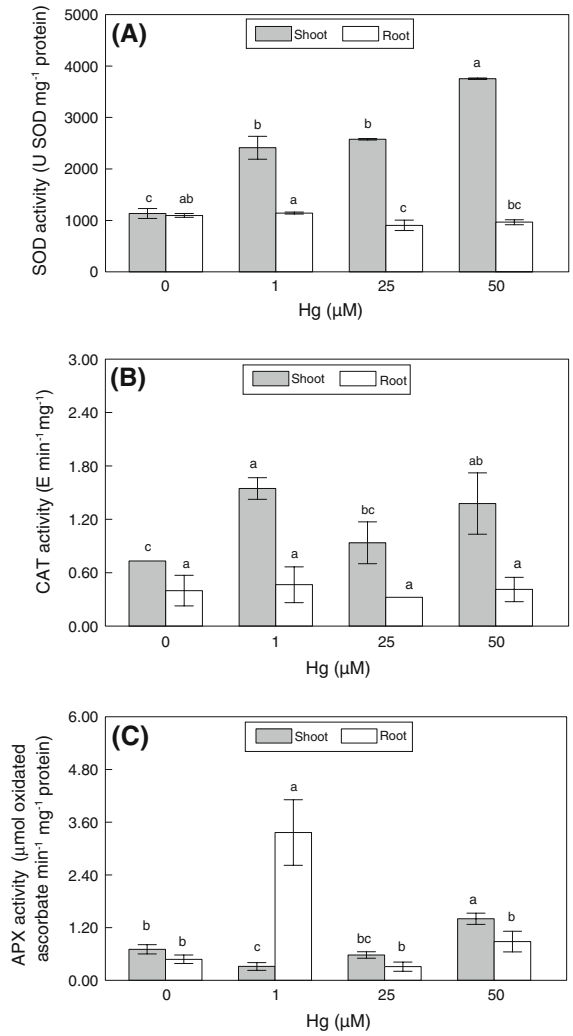

Fig. 3 Effect of Hg on superoxide dismutase (SOD) (**a**), catalase (CAT) (**b**) and ascorbate peroxidase (APX) (**c**) activities of *P. glomerata* plantlets. Data represent the mean ± SD of three replicates. *Identical letters* indicate no significant differences among the Hg concentrations ($P < 0.05$) according to Tukey's multiple range test

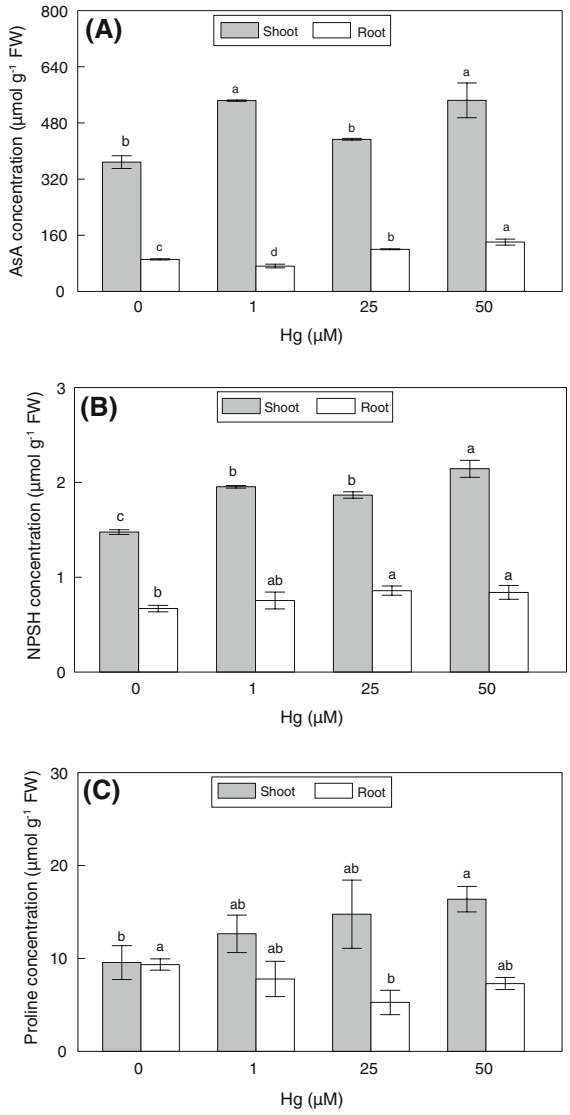

Fig. 4 Effect of Hg on **a** ascorbic acid (AsA), **b** non-protein thiol compounds and **c** proline concentration of *Pfaffia glomerata* plantlets. Data represent the mean ± SD of three replicates. *Identical letters* indicate no significant differences among the Hg concentrations ($P < 0.05$) according to Tukey's multiple range test

(NPSH) concentrations (Fig. 4a, b) was not enough to protect roots from lipid peroxidation, causing a depressive effect on fresh biomass (Table 1).

When compared to the control, SOD activity markedly increased in shoots (Fig. 3a) exposed to increasing Hg levels, and paralleled the levels of H_2O_2 formed in these tissues. However, at 50 μM of Hg the shoot H_2O_2 concentration was lower than that found at 25 μM Hg (Fig. 2a). This reduction in H_2O_2 levels at 50 μM Hg, surprising in light of the increased SOD activity, might have been due to increased CAT (Fig. 3b) and APX (Fig. 3c) activities in shoots. Another possibility is that under acute stress conditions (50 μM Hg), H_2O_2 accumulation probably decreased because there was increased cell death in the plants suffering from Hg toxicity. A similar result was found by Ortega-Villasante et al. (2007). In roots, the highest H_2O_2 concentration occurred at 1 μM Hg (Fig. 2a). This result may be related to the hormetic effect. It is noteworthy that the highest level of root APX activity was observed at this Hg concentration, whereas CAT activity did not differ among Hg treatments. In general, the concentration of 1 μM Hg was sufficient to induce alterations in enzyme activities, and APX showed the highest sensitivity, reaching maximal activity at this concentration. This result suggests that the Hg-triggered antioxidant capacity might be responsible for the removal of excessive H_2O_2. Similar results were reported by Ortega-Villasante et al. (2007) and Zhou et al. (2008).

The response of enzymes involved in attenuation of ROS (SOD, APX or CAT) to heavy metals greatly depends on the species, plant age and growth conditions (Gratão et al. 2005; Gonçalves et al. 2009; Tabaldi et al. 2009). Plants have multiple genes coding SOD and different isoenzymes of SOD are specifically targeted to chloroplasts, mitochondria, peroxisomes, cytosol and apoplasts. Zhou et al. (2008) found five SOD enzymes in alfalfa leaves treated with Hg concentrations from 1 to 40 μM, where isoforms III and IV showed a progressive increase with the Hg concentration, and isoforms I, II and V showed a "low–high–low" pattern. Interestingly, in the present study the pattern of total SOD activity was significantly different between roots and shoots, where it linearly increased in shoots and decreased in roots. Therefore, this may be due to diverse responses from different SOD isoforms in these tissues.

ROS levels can be also controlled by non-enzymatic antioxidants (Noctor and Foyer 1998). Therefore, we measured the accumulation of AsA and NPSH, the two major components of plant cells that mediate H_2O_2 in some cellular compartments (Mittler 2002). As shown in Fig. 4a, the shoot AsA concentration increased only at 1 and 50 μM Hg. On the other hand, the AsA concentration in roots

decreased at 1 μM Hg but increased at 25 and 50 μM Hg, when compared to the control. According to Horemans et al. (2000), AsA is involved in the regulation of photosynthesis, cell expansion, and cell proliferation. Moreover, exposure to oxidative stress increases tissue ascorbate accumulation and results in an enhanced of ascorbate-dependent detoxification processes (Noctor and Foyer 1998; Horemans et al. 2000; Mittler 2002).

In the present study, the NPSH concentration in both shoot and root increased concomitantly with increasing Hg concentrations (Fig. 4b). Treatment with 50 μM Hg resulted in the maximum accumulation of NPSH, which was 50% higher than that of the shoot in control plants. Thiol-based complexing substances are comprised of several acid-soluble sulphydryl-components, such as cysteine, glutathione (GSH and hGSH) and phytochelatins (PCs). GSH is a key component of the antioxidant network that scavenges ROS either directly or indirectly by participating in the ascorbate–glutathione cycle (Horemans et al. 2000). Recently Ortega-Villasante et al. (2007), observed the transient activation of genes related to GSH/hGSH metabolism in Hg-treated alfalfa seedlings. In the present study, it seems that both NPSH and AsA were important to counteract the toxic effect of Hg on *P. glomerata* plants. In contrast Zhou et al. (2008), found that GSH might be more sensitive to Hg^{2+} than ascorbate in *Medicago sativa* plants. However, immobilization and deactivation of heavy metals by natural compounds in plants, such as PCs, is the major mechanism in counteracting heavy metal toxicity (Hall 2002). Recently Chen et al. (2009), elucidated the important roles of PCs in Hg tolerance of *Brassica chinensis*, not only because of their sequestration of free Hg^{2+} but because they reduce oxidative stress in cells.

Proline has several functions during stress: osmotic adjustment (Voetberg and Sharp 1991), osmoprotection (Kishor et al. 2005), free radical scavenging and as an antioxidant (Sharma and Dietz 2006). Backor et al. (2004) found that proline treatment produced a significant increase in chlorophyll *a*, chlorophyll *b*, chlorophyll $a + b$ and total carotenoids of the tolerant strain of *Trebouxia erici* exposed to Cu, which significantly increased the yield of photosystem II. Also, proline improved salt tolerance by up-regulating stress-protective proteins (Khedr et al. 2003) and reduced oxidation of lipid membranes (Demiral and Turkan 2004). In the present study, the shoot proline concentration was slightly, but not significantly, increased by Hg levels up to 25 μM, and at 50 μM Hg level it increased by 80% when compared to the control (Fig. 4c). In addition, the root proline concentration was significantly decreased at 25 μM Hg, when compared to the control. Interestingly, increasing Hg levels did not alter the chlorophyll and carotenoid concentrations. Therefore, our results suggest that the increase in proline content might account for the stability of photosynthetic pigments in *P. glomerata.*

These results present circumstantial evidence of the occurrence of oxidative stress in *P. glomerata* plantlets exposed to Hg and of the importance of the antioxidant system in regulating Hg-induced oxidative damage in *P. glomerata*. However, at 50 μM of Hg, the antioxidant system of the plantlets was not able to reverse Hg-mediated damage. Moreover, from our present investigation we can conclude that the translocation of Hg from root to shoot is very low, and as roots of this species are commonly used in Brazilian folk medicine, the harvest of plants in Hg-polluted areas might be hazardous since this species has been shown to be quite Hg tolerant.

Under Hg stress conditions, the analysis of different antioxidant enzymes (SOD, CAT, APX, etc.) by non-denaturating polyacrylamide gel electrophoresis has shown that they present several isoforms in different organs of various plant species (Rellán-Álvarez et al. 2006; Ortega-Villasante et al. 2007; Zhou et al. 2008). Our results show that *P. glomerata* plants under acute toxicity (50 μM) and prolonged treatment (9 days) partially followed the biochemical pattern of other species. Therefore, it would be interesting to investigate in depth the presence and activity of different antioxidant isoforms in this species.

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