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RESEARCH ARTICLE

Physiological and biochemical responses of *Eichhornia crassipes* exposed to Cr (III)

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Abstract The effect of exposure of *Eichhornia crassipes* to Cr (III) was assessed by measuring changes in photosynthetic pigments, malondialdehyde, superoxide dismutase, glutathione reductase, catalase, and guaiacol peroxidase activities, as well as Cr concentration in tissues. Cr concentration in roots was significantly higher than in aerial parts and increased with Cr concentration in water. Photosynthetic pigments increased significantly, whereas the activities of antioxidant enzymes varied differently in plant tissues. Low Cr concentrations induced a rapid response of *E. crassipes* during short-term exposure, implying that the antioxidant system conferred redox homeostasis. Results showed that Cr (III) was more toxic at the two highest concentrations and long-term exposure, while it was not harmful but beneficial at the two lowest concentrations and short-term exposure. This work concludes that *E. crassipes* was able to grow under Cr (III) stress by protecting itself with an increase in the activity of its antioxidant system.

Keywords Oxidative stress · Hormesis · Antioxidant enzyme defense system · Macrophyte · Bioaccumulation · Lipid peroxidation

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Introduction

Removal of toxic metals from industrial wastewater is essential from the standpoint of environmental pollution control. The aquatic macrophyte *Eichhornia crassipes* (water hyacinth) has been of particular interest for water remediation, and it has been used in constructed wetlands for heavy metal removal (Tchobanoglous et al. 1989; Vesk and Allaway 1997; Liao and Chang 2004; Jayaweera et al. 2008). This plant has demonstrated a remarkable ability to remove As, Hg, Se, Ag, Pb, Cd, Cr, Ni, and Zn (Wolverton and McDonald 1975; Delgado et al. 1993; Fett et al. 1994; Soltan and Rashed 2003; Maine et al. 2004; Hasan et al. 2007; Alvarado et al. 2008). However, plants used in a constructed wetland should be selected according to both their contaminant uptake capacity and their stress resistance. Hence, when selecting plants, it will be necessary to focus not only on uptake capacity and survival but also on physiological and biochemical responses.

Chromium is not considered an essential element for plant nutrition (Shanker et al. 2004; Mangabeira et al. 2011). However, Cr (III) and Cr (VI) can be taken up by plants using different uptake mechanisms. It was reported that Cr (III) uptake is passive, occurring by simple diffusion, while Cr (VI) uptake is active (Shewry and Peterson 1974; Skeffington et al. 1976). Later, Espinoza-Quiñones et al. (2009) studied Cr (VI) and Cr (III) uptake by *Salvinia auriculata*, *Pistia stratiotes*, and *E. crassipes*, using high-

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resolution XRF technique. These authors concluded that Cr (VI) reduction to Cr (III) occurred during metal sorption by aquatic plants. On the other hand, Cr (VI) is relatively unstable under most environmental conditions, and converts into the less toxic trivalent form in surface waters, especially in the presence of organic matter (Kadlec and Wallace 2009). For these reasons, Cr (III) was chosen for this study.

Above threshold concentrations, Cr (III) can have toxic effects due to its ability to bind various organic compounds resulting in the inhibition of some metalloenzyme systems (Shanker et al. 2004). It has been demonstrated that Cr (III) stimulates the formation of reactive oxygen species (ROS) such as superoxide radicals ($*O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (*OH) either by direct electron transfer involving metal cations or as a consequence of metal-mediated inhibition of metabolic reactions (Stohs and Bagchi 1995). Reactive oxygen species are partially reduced forms of atmospheric oxygen (O_2). They typically result from the excitation of O_2 to form singlet oxygen (O_2^1) or from the transfer of one, two, or three electrons to O_2 to form $*O_2^-$, H_2O_2, or HO^-, respectively. In contrast to atmospheric oxygen, ROS are capable of unrestricted oxidation of various cellular components and can lead to the oxidative destruction of cells (Mittler 2002).

Under normal conditions, the production and destruction of ROS are tightly regulated in cell metabolism. However, under environmental stress, the balance between peroxidative and antioxidative reactions is shifted in favor of the former, which is usually defined as oxidative stress (Foyer et al. 1997). High ROS concentration can damage vital components as proteins, amino acids, and nucleic acids and initiate peroxidation of membrane lipids or proteins. In order to avoid oxidative damage by scavenging ROS, plants possess a complex system of enzymatic and non-enzymatic antioxidants. The main ROS-scavenging pathways in plants include enzymes such as superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), and glutathione reductase (GR), as well as non-enzymatic molecules as ascorbate and glutathione (Noctor and Foyer 1998; Halliwell 2006). The response of these antioxidative enzymes to metal stress varies among plant species and the metal involved. Thus, the knowledge of how plants cope with metal-induced oxidative stress is of considerable importance in understanding the metal tolerance mechanisms developed by plants.

The present study was aimed at evaluating the physiological and biochemical responses of roots and aerial parts of *E. crassipes* to Cr (III) exposure. This work will be helpful to understand how plants are able to adjust enzymatic detoxification strategies in order to prevent oxidative stress induced by metal exposure.

Material and methods

Plant material, growth conditions, and experimental setup

Young plants of *E. crassipes* and water were collected from an unpolluted pond of the Paraná River floodplain. Collected plants were acclimated in the laboratory under controlled conditions, photoperiod of 11–13 h (light–darkness period), temperature of 24±2 °C, 1,400 μmol m^{-2} s^{-1} of photosynthetic photon flux density, and humidity of 50±10 %. After 7 days, only young healthy plants, of a uniform size and a similar weight, were selected for experimental purposes.

Two liters of pond water and one plant (30–40 g of fresh plant biomass) was disposed in plastic experimental pots. Pond water was used to simulate natural aquatic system conditions. The chemical composition of the pond water used in the experiment was (mean±standard deviation) as follows: conductivity=124±1 μS cm^{-1}; dissolved oxygen (DO)=7.6 ±0.10 mg l^{-1}; soluble reactive phosphorus (SRP)=0.035± 0.002 mg l^{-1}; N-$NH4^+$=0.550±0.019 mg l^{-1}; N-NO_3^-= 0.651±0.005 mg l^{-1}; N-NO_2^-=0.008±0.001 mg l^{-1}; Ca^{2+}= 10.3±0.8 mg l^{-1}; Mg^{2+}=3.8±0.5 mg l^{-1}; Na^+=13.7± 1.0 mg l^{-1}; K^+=3.50±0.5 mg l^{-1}; Cl^-=10.6±1.3 mg l^{-1}; SO_4^{2-}=8.0±1.8 mg l^{-1}; HCO_3^-=51.7±0.8 mg l^{-1}; Fe= 5 μg l^{-1}; and Cr = non-detected (detection limit=2 μg l^{-1}).

Cr (III) solution (prepared using $CrCl_3 \cdot 6H_2O$) was added to obtain concentrations of 2.0, 4.0, 6.0, and 8.0 mg l^{-1}. Water pH was maintained between 5.4 and 5.8 to avoid metal precipitation. The study was conducted over 3 days, sampling at periods of 24, 48, and 72 h. Treatments were performed in triplicate with a control in the absence of Cr (III).

After treatments, plants were harvested, rinsed with distilled water, blotted, and separated in aerial parts and roots. Immediately afterwards, samples were frozen using liquid nitrogen for storage at −80 °C, only for enzymatic measurements. The following analytical determinations were performed.

Metal concentration

Dried plant tissues (aerial parts and roots) were ground and digested with a HNO_3/HCl mixture (USEPA 1994). Cr concentrations were determined by atomic absorption spectrophotometry (PerkinElmer AAnalyst 200). Certified standard solutions were used. Detection limits were 0.3 μg g^{-1}. Determinations were carried out in triplicate.

Translocation factor (TF) was calculated as the ratio of Cr concentration in aerial parts to that in roots.

Chlorophyll and carotenoid concentrations

Chlorophyll and carotenoid concentrations were estimated according to the procedure of Arnon (1949). Photosynthetic

pigments were extracted from fresh leaves using 80 % chilled acetone for 48 h in the dark at 3–5 °C without shaking and with the addition of a pinch of magnesium carbonate, to protect and stabilize the pigments. Extracts were filtered and measured by spectrophotometry at 663, 646, and 470 nm. Photosynthetic pigments were reported as chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoid. Pigment concentrations (mg g^{-1} fresh weight (FW)) were calculated by the equations of Wellburn (1994).

Enzyme extraction and protein determination

Enzyme extracts were prepared from individual plants (not pooled). The following steps were carried out at 4 °C and performed according to Pflugmacher and Steinberg (1997) with minor modifications. After being ground with liquid nitrogen, 0.3 g of plant material (roots and aerial parts) was homogenized with 0.1 M phosphate buffer (pH 6.5), containing 20 % (*v*/*v*) glycerol, 1.4 mM dithioerythritol (DTE), and 1 mM ethylenediaminetetraacetic acid (EDTA), in a 2:1 *v*/*w* ratio. The homogenate was centrifuged at 12,000×*g* for 15 min at 4 °C, and the supernatant (crude extract) was kept and stored in separate aliquots at −80 °C, prior to enzyme analyses. Protein concentration in the supernatant was determined by the Bradford method using bovine serum albumin as standard (Bradford 1976).

Estimation of lipid peroxidation

The level of lipid peroxidation in the plant samples (roots and aerial parts) was estimated by measuring the concentration of malondialdehyde (MDA), the major thiobarbituric acid (TBA)-reactive material, as described by Heath and Packer (1968). MDA concentrations were calculated following the equation presented by Hodges et al. (1999).

Antioxidant enzyme assays

Catalase activity (*CAT*, *EC 1.11.1.6*) was measured monitoring H_2O_2 decomposition at 240 nm (Maehly and Chance 1954). The final volume reaction mixture contained 50 mM phosphate buffer (pH 7.0), 30 % H_2O_2, and the crude extract. Guaiacol peroxidase activity (*POD*, *EC 1.11.1.7*) was assayed using the procedure of Bergmeyer (1974), in which decomposition of hydrogen peroxide by peroxidase occurs, with guaiacol as hydrogen donor. SOD activity (*EC 1.15.1.1*) was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) according to Beauchamp and Fridovich (1971). GR activity (*EC 1.6.4.2*) was estimated by monitoring the increase in absorbance at 412 nm due to the formation of 2-nitro-5-thiobenzoic acid (TNB) (Smith et al. 1988).

Statistical analysis

Data are the mean values of triplicates and are expressed as means ± standard deviation (SD). After verifying the assumptions of normality and homoscedasticity, analysis of variance (ANOVA) was applied to determine whether significant differences existed in chlorophyll and carotenoid concentrations, lipid peroxidation, enzyme activities, and metal tissue amounts (aerial parts and roots) among the different Cr concentrations and exposure times. Duncan's multiple range test (DMRT) was performed to evaluate significant differences between treatments ($p<0.05$). SPSS Statistics v20.0.0 software was used for all statistical calculations.

Results

Cr accumulation and translocation

There was an increase in Cr accumulation in roots and aerial parts with increasing Cr concentration and exposure times (Figs. 1 and 2). The highest Cr concentrations were determined in roots (Fig. 2). At different exposure times, a positive linear correlation was observed between Cr concentration in plant tissues and initial metal concentration in water. The higher Cr concentration is in water, the higher is the final Cr concentration in plant tissues (aerial parts and roots). The greatest Cr accumulation in aerial parts was observed during the first 48 h for the two highest concentrations, while for the two lowest concentrations, the greatest accumulation occurred in the first 24 h (Fig. 1). However, the greatest Cr accumulation in roots occurred in the first 24 h for all Cr concentrations in water (Fig. 2).

The highest Cr concentrations in aerial parts and roots were 9.59±0.32 and 0.57±0.02 mg g^{-1} DW, respectively. Both concentrations were observed at 8 mg l^{-1} Cr after 72 h of treatment. According to the TFs calculated (between 0.02 and 0.06), Cr was not easily translocated to aerial parts (Table 1).

Effects of Cr on photosynthetic pigments

Cr accumulation in macrophytes produced significant physiological responses. Results indicated a significant increase in photosynthetic pigments of *E. crassipes* exposed to Cr (III) (Fig. 3). Chl *a* and *b* exhibited a significant increase for all the concentrations and exposure times analyzed. However, Chl *a* for 2 and 4 mg l^{-1} Cr at 72 h and Chl *b* for 2 mg l^{-1} Cr at 48 h and 2, 4, and 6 mg l^{-1} Cr at 72 h showed no significant differences compared to the control. The highest photosynthetic pigment

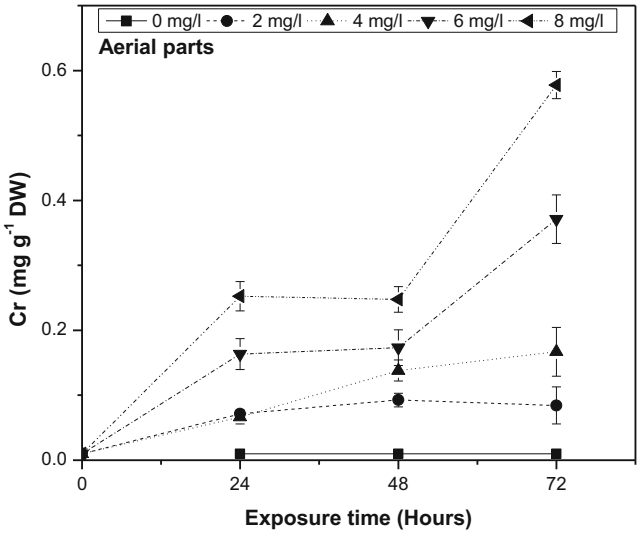

Fig. 1 Cr accumulation in aerial parts of *E. crassipes* at different concentrations and exposure times. All the values are mean of triplicates±SD

concentrations were observed at 24 h for the two lowest Cr concentrations and at 48 h for the two highest Cr concentrations used. The highest Chl *a* concentrations were 0.68± 0.05 and 0.64±0.03 mg g^{-1} FW for 2 and 4 mg l^{-1} Cr at 24 h, while they were 0.72±0.05 and 0.63±0.06 mg g^{-1} FW for 6 and 8 mg l^{-1} Cr at 48 h. At 24 h, the highest Chl *b* concentrations were 0.31±0.02 and 0.30±0.01 mg g^{-1} FW for 2 and 4 mg l^{-1} Cr, whereas at 48 h, they were 0.35± 0.01 and 0.31±0.01 mg g^{-1} FW for 6 and 8 mg l^{-1} Cr, respectively. At 72 h, Chl *a* and *b* concentrations decreased compared with those obtained at 24 and 48 h but without significant differences regarding the control.

A significant increase in carotenoid concentrations was observed for all Cr concentrations at 24 and 48 h, followed by a decrease at 72 h, when no significant differences were

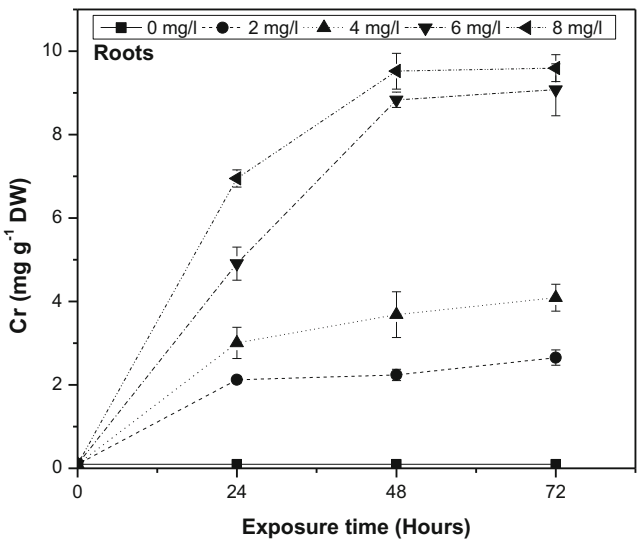

Fig. 2 Cr accumulation in roots of *E. crassipes* at different concentrations and exposure times. All the values are mean of triplicates±SD

Table 1 Translocation factor for Cr accumulation in plants

Cr concentrations (mg l^{-1})	Exposure time (h)		
	24	48	72
2	0.0335	0.0369	0.0317
4	0.019	0.0375	0.0408
6	0.0333	0.0192	0.042
8	0.032	0.026	0.0602

found with respect to the control. This response also agreed with what was observed for Chl *a* and *b*.

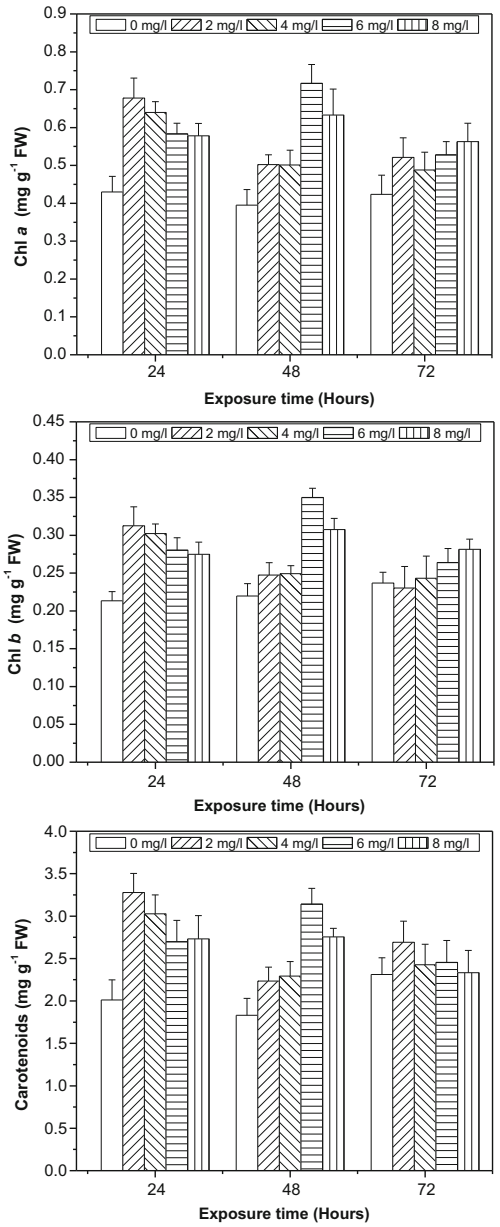

Fig. 3 Effects of Cr on photosynthetic pigments in the leaves of *E. crassipes*. All the values are mean of triplicates±SD

Effects of Cr on CAT, POD, SOD, and GR activity

Enzyme activities were affected by Cr (III) exposure both in roots and aerial parts (Fig. 4).

CAT activity increased significantly only at 24 h for 8 mg l^{-1} Cr (45.39±2.42 μmol min^{-1} mg^{-1} prot) in aerial parts, decreasing below control values after 72 h of exposure, which was probably due to the harmful effect of ROS by this time. POD activity showed a significant increase regarding the control for all treatments and exposure times analyzed. However, it is important to note that POD activity showed a bell-shaped concentration–response trend for Cr concentrations of 6 and 8 mg l^{-1}: the activity level increased accompanying the rise in Cr concentrations, up to a maximum value and then decreased. This decline was observed at 72 h of exposure (Fig. 4). Similarly to POD, SOD activity increased significantly for all treatments: at 72 h, it showed a decrease compared to the other times, which indicated a bell-shaped concentration–response trend, except for Cr concentration of 8 mg l^{-1}. A significant increase in GR activity was observed only at 48 and 72 h.

In roots, CAT activity rose significantly for all treatments. At the two lowest Cr concentrations, the CAT enzyme showed a bell-shaped response with a maximum activity at 48 h and a decrease at 72 h. A significant increase compared to the control was observed for POD activity for all Cr treatments. At different exposure times, a positive linear correlation was observed between POD activity and initial metal concentration in water. SOD activity increased significantly in all treatments and presented a bell-shaped response, except for the concentration of 2 mg l^{-1} Cr. Maximum concentrations for SOD activity were observed at 48 h for 4, 6, and 8 mg l^{-1} Cr concentrations. GR activity increased significantly for all Cr concentration and times. The pattern of GR enzyme response was similar to that of POD response, showing a positive linear correlation with the initial metal concentration.

Effects of Cr on lipid peroxidation

The effects of Cr on MDA concentration are presented in Fig. 5. Compared to controls, MDA concentrations decreased significantly in aerial parts at 24 and 48 h for all treatments, except at 2 mg l^{-1} Cr at 48 h, which showed no significant differences regarding the control. The magnitude of MDA reduction ranged from 10 to 22 % with regard to the control. However, a significant increase was observed at 72 h for all Cr concentrations, indicating an enhanced lipid peroxidation by this time. The maximum increase (20 % above the control) was observed for 2 mg l^{-1} Cr.

In roots, MDA concentration increased significantly at 24 h at the highest Cr concentrations (4, 6, and 8 mg l^{-1} Cr), indicating a rise in lipid peroxidation with the increasing Cr concentrations. The greatest increase (38 % above the control) was observed for 8 mg l^{-1} Cr. A bell-shaped response was observed at 48 and 72 h, being the highest increases 31.43± 1.49 and 41.58±1.14 nmol g^{-1} FW at 48 h (4 mg l^{-1} Cr) and at 72 h (6 mg l^{-1} Cr), respectively.

Discussion

Although Cr (III) is a non-essential element for plants, *E. crassipes* accumulated it efficiently, especially at low concentrations, in agreement with other authors (Delgado et al. 1993; Soltan and Rashed 2003).

E. crassipes accumulated higher Cr concentrations in roots than in the aerial parts of the plant (Figs. 1 and 2), in agreement with many authors (Ingole and Bhole 2003; Maine, et al. 2004; Paiva et al. 2009; Hadad, et al. 2011; Mangabeira et al. 2011). Low concentration of the metal in the aerial parts of *E. crassipes* may be due to the slow mobility of metal after being taken up by roots. Zhang et al. (2008) employed Fourier transform infrared (FTIR) spectrometry to study the root cell walls of Cr-treated plants, suggesting that –OH and COO– groups were associated with Cr in aqueous solutions, which might constitute a mechanism for Cr accumulation. Mangabeira et al. (2004) employed ion microscopy to detect large amounts of Cr in the vascular cylinder of *E. crassipes* roots and stems. In addition, these authors observed Cr in the transport parenchyma, indicating that such cells were responsible for carrying metal from roots to leaves.

Cr accumulation can reduce growth, induce chlorosis in young plants, reduce pigment concentration, and cause ultrastructural modifications of the chloroplast and cell membranes (Barton et al. 2000; Chatterjee and Chatterjee 2000; Cervantes et al. 2001; Panda and Choudhury 2005). However, in this study, the concentration of photosynthetic pigments increased at low Cr concentrations after short-term exposure (Fig. 3). This could be associated with an increase rate of chlorophyll synthesis or decline in pigment catabolism. In agreement with our results, Paiva et al. (2009) found that Cr (III) might eventually increase photosynthesis and chlorophyll concentration. In another study, El-Bassam (1978) reported that low Cr (III) concentrations promote plant growth and also stimulate chlorophyll synthesis and photosynthetic activity. Similar observations were made by Bonet et al. (1991), who found that low Cr (III) concentrations could enhance chlorophyll concentration by improving availability of biologically active Fe in plant tissue. It is known that low or moderate heavy metal concentrations do not affect or even enhance some metabolic functions such as mineral nutrition, photosynthesis, and growth, whereas high metal concentrations reduce metabolic functions (Mysliwa-Kurdziel et al. 2004; Gratão et al. 2005). This phenomenon, which describes that substances can be beneficial at low doses and lethal at higher doses, has been

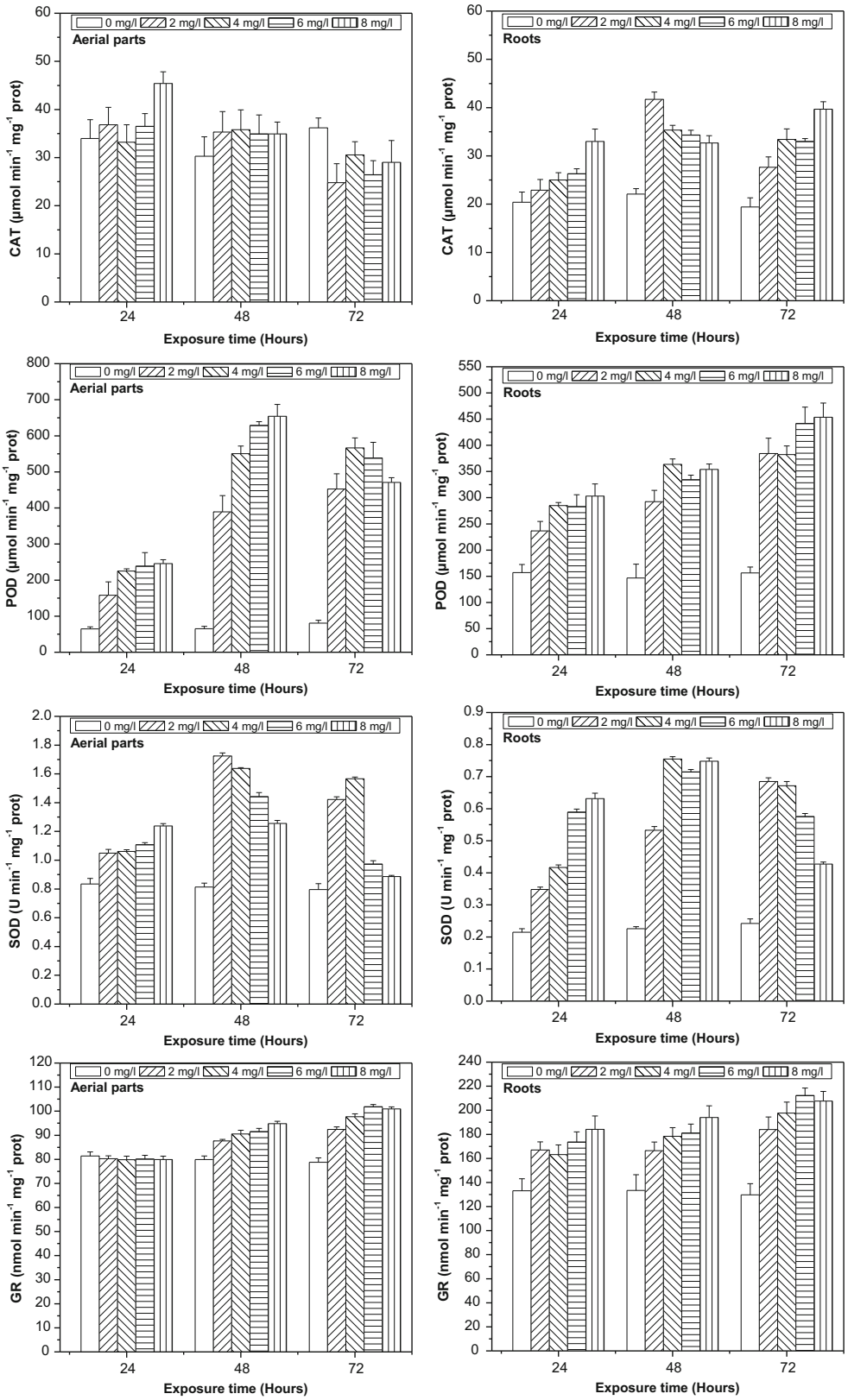

Fig. 4 Effects of Cr on antioxidant enzyme activities of *E. crassipes*, in aerial parts and roots. All the values are mean of triplicates±SD

explained over a century ago as the Arndt-Schulz law (Calabrese 2005), introducing the term "hormesis" in organisms against a disruption of homeostasis due to induced environmental stress. Overcompensation occurs as a result of

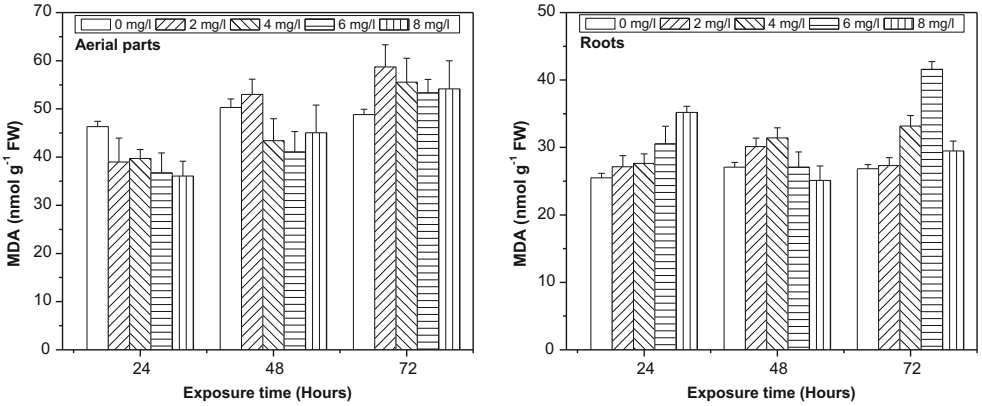

Fig. 5 Effects of Cr on lipid peroxidation of *E. crassipes*, in aerial parts and roots. All the values are mean of triplicates±SD

a process of transfer of resources to recover its homeostasis slowly, resulting in a hormetic response. Some authors have proposed that hormesis would be triggered by certain types of stress, such as the production of ROS (Cedergreen et al 2007). For this reason, the activity of enzymes associated with oxidative stress as biomarkers to detect metal-induced toxicity was analyzed.

ROS are produced in plants as the result of mainstream enzymatic reactions, but they also appear to be an unavoidable accident, e.g., $O_2^{\bullet-}$ formed by the chloroplast electron transport chain. Although physiological concentrations of ROS have important functions in stress signaling, high concentrations can cause oxidative stress leading to cell death, if they are not detoxified. This fact turns plants into the organisms which are most exposed to oxidative stress. Membrane lipids are the main cellular targets that are susceptible to damage, and lipid peroxidation is believed to be a free radical-mediated process (Repetto et al. 2012; Sharma et al. 2012). Cr (III) toxicity can disturb the balance between ROS generation and removal, thus causing MDA accumulation in plants, which results in oxidative damage. As a defensive mechanism, antioxidative enzymes play an important role in protecting cells by scavenging ROS. In this study, the highest Cr concentrations induced a significant increase in aerial parts of MDA at 72 h. This result indicated that excess Cr accumulation caused oxidative stress over time. On the other hand, a significant decrease in MDA concentration was observed at 24 and 48 h regarding the control, which indicated that a better protective mechanism might be operating in the plants at an earlier exposure. In roots, an increase in MDA concentrations associated with increasing Cr (III) concentration in water was observed at 24 h, revealing oxidative damage to lipids. In general, bell-shaped concentration–enzyme activities response could be observed. A significant increase in enzyme activity was observed at the two lowest Cr concentrations, and a decrease was noted for the two highest Cr concentrations and exposure times. The decline observed could be interpreted as a sign of cytotoxicity due to a ROS overproduction which led to a drop in the antioxidant system activity that could not avoid metal-induced damage. The induction of the activities of a particular group of enzymes is considered to play an important role in the cellular defense strategy against oxidative stress (Van Assche et al. 1990; Møller et al. 2007). The increase in enzyme activity observed in *E. crassipes* showed an increased ability to adapt to moderate Cr concentrations by developing the enzymatic antioxidative defense system.

The presence of a hormetic response could be established by the relationship between Cr accumulation and antioxidant defense activation. This hormetic effect could be due to plant adaptation through the activation of defenses by cross signaling because a defense mechanism implies the existence of metal-sensing systems that regulate gene expression via cellular signaling transduction cascades (Poschenrieder et al. 2013). It is important to note that this study was not conducted to investigate a possible hormetic response but to elucidate the effects of Cr on the physiology and biochemistry of *E. crassipes*. It is also important to highlight that the present study was performed under controlled laboratory conditions using pond water as medium. Poschenrieder et al. (2013) established that test plants—even under controlled conditions—have suboptimal growth rates due to latent stresses and their performance is supposed to be the best. However, these circumstances may make low concentrations of toxic ions contribute to stress alleviation and growth stimulation. Therefore, contaminants could induce beneficial homeostatic responses at suitable spacing of doses, being responsible for enhanced defense ability to repair damaged cells in organisms (Calabrese and Baldwin 2003a, b).

Conclusions

Cr accumulation was observed to be higher in roots than in aerial parts. Cr (III) sorption occurred mainly during the first 24 h in roots, whereas in aerial parts, it occurred at 24 and 48 h, for the two lowest and for the two highest Cr (III) concentrations, respectively, affecting physiological and biochemical parameters of this plant:

- Low MDA concentrations measured in *E. crassipes* indicate a reduced oxidative stress. The rapid increase in plant defense response was induced by low Cr concentrations, implying that the antioxidant defense system enzymes attempted to guarantee the redox homeostasis.
- The presence of hormesis induced by low Cr concentrations in *E. crassipes* could be related to the defense antioxidant mechanism or to the continuity of photosynthetic activity. However, the hormetic response may occur as a result of numerous mechanisms. Further work is needed to research the underlying mechanisms of hormesis produced by Cr.
- *E. crassipes* has the ability to adapt to moderate Cr concentrations by developing an antioxidative defense system.

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