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Differential physiological responses of two *Salvinia* species to hexavalent chromium at a glance

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ABSTRACT

In plants of *Salvinia rotundifolia* and *Salvinia minima* the effect of two Cr(VI) concentrations (5 and 20 mg L^{-1}) applied for 7 days was assessed by measuring changes in biomass, photosynthetic pigments, Cr accumulation, malondialdehyde (MDA), membrane stability index (MSI), thiols (TT, NPT and PBT), and phenolics (SP and IP). Biomass in *S. minima* was decreased at highest Cr(VI) concentration, but there were no changes in *S. rotundifolia*. Metal accumulation was different in both species. *S. minima* accumulates more metal in fronds, but *S. rotundifolia* accumulates more metal in lacinias. Results also showed that *S. minima* translocates more Cr to fronds than *S. rotundifolia*, but at the whole plant level higher accumulation occurred in this last. Tolerance index (Ti) was higher in *S. rotundifolia*. Chl *b* and carotenoids were decreased only upon exposure to high Cr(VI) concentration in both species. Cr(VI) treatment did not enhance MDA accumulation. Cr exposure had no impact on MSI values when comparing with Cr-untreated values. Thiols in fronds and lacinias showed different distribution patterns between species. IP and NPT were higher in *S. rotundifolia* lacinias that accumulate more Cr than *S. minima* lacinias. Whilst SP and NPT were higher in *S. minima* fronds compared with *S. rotundifolia* ones. This may indicate that these species can cope with Cr(VI) toxicity, either through metal complexation and/or metal reduction or by the scavenging of ROS derived from Cr-induced oxidative stress. Based on Cr accumulation and biomass production, *S. rotundifolia* seems more suitable to remove Cr(VI) from polluted waters.

1. Introduction

Large amounts of toxic Cr(VI) from industries, such as electroplating, leather tanning, cement plants, wood preservation, stainless steel production, dye manufacturing, mining, paints and pigments, metal finishing, metal plating, pulp and paper production and timber processing, and also by leaching from improper sanitary landfills, are released into aquatic systems directly or indirectly (Kumari et al., 2014). In less extent, Cr(VI) can also enter into water bodies through natural leaching from topsoil and rocks (Zayed and Terry, 2003). The most stables valence states of Cr occurring in terrestrial and aquatic ecosystems are trivalent [Cr(III)] and hexavalent [Cr(VI)] (Kotaś and Stasicka, 2000). In aquatic environments, more mobile Cr(VI) exists as inorganic oxyanions (CrO_4^{2-} and $Cr_2O_7^{2-}$), while less mobile Cr(III) occurs under different forms including oxides, hydroxides and sulfates (Chandra and Kulshreshtha, 2004; Dong et al., 2011). However, Cr(VI) and Cr(III) mobilities are heavily influenced by water physicochemical properties such as pH and redox potential (de Jongh et al., 2012).

Cr(VI) is more toxic than Cr(III) due to its high solubility and oxidizing potential, being readily incorporated into cells through sulfate transporters (Singh et al., 2013). Cr(VI) oxyanions are considered as carcinogenic to humans and toxic to plants, and also as priority ecosystem contaminants (Prado et al., 2015a). Cr(VI) accumulation in plants causes severe toxicity symptoms such as reduction of growth and biomass accumulation, changes in photosynthesis and respiration, interference with mineral uptake, and structural alterations (Vernay et al., 2007; Prado et al., 2010a, 2015b). In addition many enzymatic activities related to carbohydrate and nitrogen metabolism are decreased either directly or through the production of reactive oxygen species (ROS) (Tiwari et al., 2009; Singh et al., 2013). Cr(VI) also produces the peroxidation of membrane polyunsaturated fatty acids (PUFAs), and even may cause the death of the plant (Oliveira, 2012).

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Phytoremediation uses plants to remove both organic and inorganic contaminants from polluted soils and waters (Pilon-Smits, 2005). In recent years the phytoremediation has received an increasing attention due to its low cost and also because is environmental friendly. Aquatic species belong to the genera *Salvinia*, *Lemna*, *Spirodela*, *Azolla*, *Eichhornia*, *Wolffia* and *Pistia* have the ability to accumulate large amounts of heavy metals without suffering severe damages (Prasad, 2007). The use of floating macrophytes to remove Cr(VI) from polluted waters, has received much attention due to its fast growth and easy culture in natural and artificial aquatic systems (Rai, 2008). In many floating macrophytes the Cr that is absorbed from the surrounding solution is accumulated and retained mainly on submerged roots (Marbaniang and Chaturvedi, 2014), but its translocation to aerial parts (shoot and leaves) also occurs (Sinha et al., 2002). Several *Salvinia* species have been featured as plants with high capability to remove Cr(VI) from polluted waters (Olguín et al., 2002; Dhir, 2009; Prado et al., 2010b). They are common in tropical and subtropical regions of the world, and grow abundantly in ponds and lagoons, but can also be found floating near the edges of slow moving streams. *Salvinia* plants exhibit high biomass productivity and have the potential to double the population in approximately 3.5 days (Nichols et al., 2000). These characteristics make *Salvinia* species suitable candidates for Cr(VI) phytoremediation. Although responses of *Salvinia* plants to Cr(VI) have already discussed, variation in Cr(VI) tolerance if any among *Salvinia* species has not been worked out in detail. There is not published data on comparative Cr(VI) tolerance, underlying detoxifying mechanisms occurring in different species of *Salvinia*. Thus, our hypothesis assumes that different *Salvinia* species show variations in sensitivity and tolerance strategies to Cr(VI) stress. The aim of this study was investigate (i) biomass production and Cr accumulation, (ii) accumulation of thiols (total, non-protein and protein-bound) and phenolic compounds (soluble and insoluble) in floating and submerged leaves and (iii) membrane stability index and lipid peroxidation (malondialdehyde accumulation), in plants of *Salvinia minima* and *S. rotundifolia* growing in two different Cr(VI) solutions.

2. Materials and methods

2.1. Plant material

S. minima and *S. rotundifolia* plants were purchased in a local shop of aquarium plants. Because plants grew in the same aquarium media, is assumed that there are no differences in basal level of mineral nutrients between both species. Plants were thoroughly washed with running tap water to remove plant debris and eventual surface-bound pollutants and/or sediment particles. After washing, plants were transferred to 30-L plastic aquaria containing 1/10Hoagland solution for 3 days under outdoor conditions to generate new and rapidly growing fronds. Uniform plants with fully expanded leaves were transferred to plastic trays containing 150 mL of different Cr(VI) solutions. Cr(VI) solutions were obtained from a stock solution (500 mg L^{-1}) prepared in tap water with analytical grade potassium dichromate ($K_2Cr_2O_7$). Metal solution was prepared using tap water in order to get a similar aquatic media where plants come from. Furthermore, plants were not cultivated in Hoagland's solution to avoid chelation and/or ion competition between Hoagland ions and $Cr_2O_7^{2-}$ oxyanion for cell wall-binding sites (Prado et al., 2010b). For each species and tray 15 plants (~35 g FW) with similar size and shape were exposed to 0 (control), 5 and 20 mg L^{-1} Cr(VI) concentrations during 7 days under controlled conditions: 200 μmol m^{-2} s^{-1} light intensity, 12 h dark/light cycle, 80% relative humidity and 25/20 ± 1 °C day/night temperature. Chromium concentrations were chosen from available data on Cr(VI) concentrations typically occurring in both surface and ground polluted waters, which range from zero to 20 mg L^{-1} chromate (Terry et al., 2014). The pH of recently prepared Cr(VI) solution was 6.7, ranging between 6.6 and 6.8 during the cultivation period for both *Salvinia* species. In control tap water the value of pH ranged between 6.7 and 6.9. According to Kotaś and Stasicka (2000) in oxygenated aqueous solutions Cr(III) species are stable at pH values below 6, whereas at pH ≥ 7Cr(VI) species are predominant. Then, we assumed that Cr(VI) must be the dominant species in treatment solutions. To ascertain that Cr(VI) was the only form presents in treatment solutions, prior cultivation of *S. minima* and *S. rotundifolia* plants and at the ending of the experiment, Cr(VI) concentration was measured by using 1.5-diphenylcarbazide with and without addition of $KMnO_4$. In acid medium the $KMnO_4$ oxidizes completely Cr(III) to Cr(VI) (Memon et al., 2005). The difference between both determinations corresponds to Cr(III) concentration in the treatment solution. Standard deviation obtained for calibration curves with and without $KMnO_4$ was 0.0044, which indicated a good fit of data and within an error limit ≤2%. This ensures high confidence limits of experimental measurements. After Cr exposure, plants were harvested, rinsed in distilled water, and divided in floating (fronds) and submerged (lacinias) leaves. To obtain the fresh weight (FW), samples were weighted immediately after cutting, whereas the dry weight (DW) was determined after drying samples at 80 °C in a hot air oven for 2 days. To chemical determinations, fronds and lacinias were maintained frozen at −20 °C. Biomass of Cr-treated plants was determined by recording the DW of the whole plant and expressed as percentage (%), assuming 100% as the biomass of Cr-untreated plants. Tolerance index (Ti) was calculated as the ratio between mean weight of Cr-treated plants and mean weight of Cr-untreated plants, and expressed as percentage (Reisinger et al., 2008).

2.2. Photosynthetic pigments

Photosynthetic pigments (chlorophyll and carotenoids) were measured at the end of the experiment. Briefly: frond sample (0.1 g FW) was added with 2 mL dimethyl sulfoxide and incubated 12 h at 45 °C in darkness (Chappelle et al., 1992). Chlorophyll and carotenoid concentrations were calculated from absorbance values at 665, 649 and 480 nm by using Wellburn's equations (Wellburn, 1994). Concentrations of photosynthetic pigments were expressed as mg g^{-1} FW.

2.3. Cr accumulation

Oven-dried fronds and lacinias of Cr-untreated and Cr-treated plants were digested in HNO_3 at 115 °C for 15 min following the USEPA 3051 protocol (www.epa.gov/epaoswer/hazwaste/test/pdfs/3051.pdf). Cr was determined by using an atomic absorption spectrophotometer (Perkin–Elmer 373, England), and the concentration expressed as μg g^{-1} DW. A blank of HNO_3 was also measured to ensure the correctness of metal quantification. Accuracy of metal determination was ascertained by addition of a known metal concentration. Overall recovery of Cr associated with digestion process was in the 90–95% range. In Cr-untreated samples, Cr content was below the detection limit. Data were from two independent measurements.

2.4. Lipid peroxidation (MDA accumulation)

Lipid peroxidation was evaluated based on the TBARS determination by the method of Du and Bramlage (1992). Frond and lacinia samples (0.5 g FW) were homogenized with 5 mL 80% ethanol and centrifuged at 3000*g* for 10 min. Resulting supernatants were used to MDA quantification. Briefly: supernatant aliquot (1 mL) was

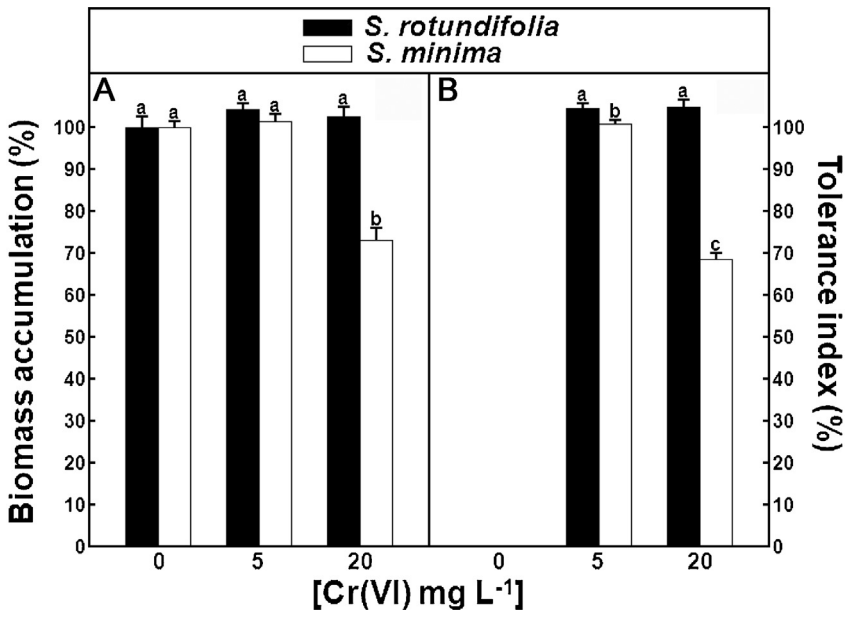

Fig. 1. Effect of Cr(VI) on the biomass production (expressed as%, assuming as 100% the value of Cr-untreated plants) **(A)**, and Tolerance index (expressed as%) **(B)**, in *S. rotundifolia* and *S. minima* plants after a 7-d cultivation period. Data are mean ± SE of three replications (n = 6). Different letters on bars indicate significant differences for each analyzed parameter ($p < 0.05$).

added with 1 mL of 0.5% (w/v) thiobarbituric acid solution in 20% (w/v) trichloroacetic acid. Resulting mixture was heated in boiling water for 25 min, and then quickly cooled on an ice bath. After centrifugation at 3000*g* for 10 min the absorbance was read at 440, 532 and 600 nm. MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol g^{-1} FW.

2.5. Membrane stability index (MSI)

MSI was determined by measuring the electrical conductivity according to Sairam et al. (2002) procedure with minor modifications. Briefly: plant sample (1 g FW) was rinsed with distilled water, placed in a glass tube containing 10 mL of distilled water, and incubated at 40 °C for 30 min in a water bath. After this time the electrical conductivity (E_1) was measured using a conductimeter (Metrohm, Switzerland). Immediately, tubes were boiled in a water bath for 15 min and the electrical conductivity (E_2) was again recorded. MSI was calculated as follow:

$$MSI(\%) = [1 - (E_1/E_2) \times 100]$$

2.6. Total thiols (TT), non-protein thiols (NPT) and protein-bound thiols (PBT)

Frond and lacinia samples (1.0 g FW) were homogenized with 3 mL of 20 mM Tris-HCl buffer (pH 8.6) containing 1 mM dithiotreitol (DDT) to maintain reduced thiols in a cooled mortar and pestle (Linde and Garcia-Vazquez, 2006). Homogenized extracts were centrifuged at 15,000*g* for 10 min at 4 °C and supernatants used for determinations of both TT and NPT. To TT determination, aliquots of supernatants (0.2 mL) were mixed with 0.6 mL of Ellman's reagent (5 mM DTNB, [5,5′-dithiobis-(2-nitrobenzoic acid)], in 100 mM Tris-HCl buffer, pH 8.0), 0.8 mL of 100 mM Tris-HCl buffer (pH 8.0) and 0.5 mL of distilled water. After incubation at 37 °C for 60 min the absorbance was read at 412 nm against a blank without supernatant. NPT were determined as follows: aliquots of supernatants (0.5 mL) were mixed with 0.1 mL of trichloroacetic acid (TCA) 50% (w/v) and 0.4 mL of distilled water and maintained in ice for 20 min. After centrifugation at 15,000*g* for 10 min at 4 °C, supernatants were used to determine NPT as described above. Protein-bound thiols (PBT) were calculated by subtracting the non-protein thiols (NPT) from total thiols (TT). Thiols concentration was calculated from a calibration curve prepared with pure reduced glutathione (GSH), and expressed as mg of GSH equivalent g^{-1} FW.

2.7. Soluble phenolics (SP) and insoluble phenolics (IP)

SP extraction was made with ethanol 96% according to Swain and Hillis (1959) with minor modifications. Briefly: frond and lacinia samples (1.0 g FW) were extracted with 3 mL 96% ethanol, incubated in darkness at room temperature for 48 h, and centrifuged a 3000*g* for 5 min. Supernatants were recovered and used to SP determination. Aliquots of supernatants (0.1 mL) were added with 0.2 mL (1:1, v/v) of diluted Folin–Ciocalteu reagent and 1.8 mL of distilled water. After standing at room temperature for 2 min, 0.8 mL of 7.5% Na_2CO_3 was added and left standing again at room temperature for 5 min. Next the absorbance was read at 760 nm. Precipitates from SP extraction were washed twice with 2 mL ethanol 96% and centrifugation at 3000*g* for 5 min. Washed precipitates were dried a 37 °C for 48 h and used to obtain cell wall-bound phenolics (IP). IP extraction was adapted from Assabgui et al. (1993). Dried samples (0.5 g) were hydrolysed with 2 mL of 2 N NaOH in a water bath at 60 °C for 60 min. After cooling, solutions were slowly acidified to pH with 5 N HCl and extracted with ethyl acetate. Following ethyl acetate fractions were taken near dryness under a stream of N_2 gas and dissolved in 0.5 mL of 96% ethanol. Solubilized phenolics were determined using the Folin–Ciocalteu reagent as described above. Concentrations of SP and IP were determined using a standard curve made with pure phenol and expressed as μmol of phenol equivalent g^{-1} FW.

2.8. Statistics

For all determinations, at least three replicates were analyzed and two independent experiments were performed. Data are presented as the mean of all replicates, and bars represent the standard error. Significant differences were established by using one-way

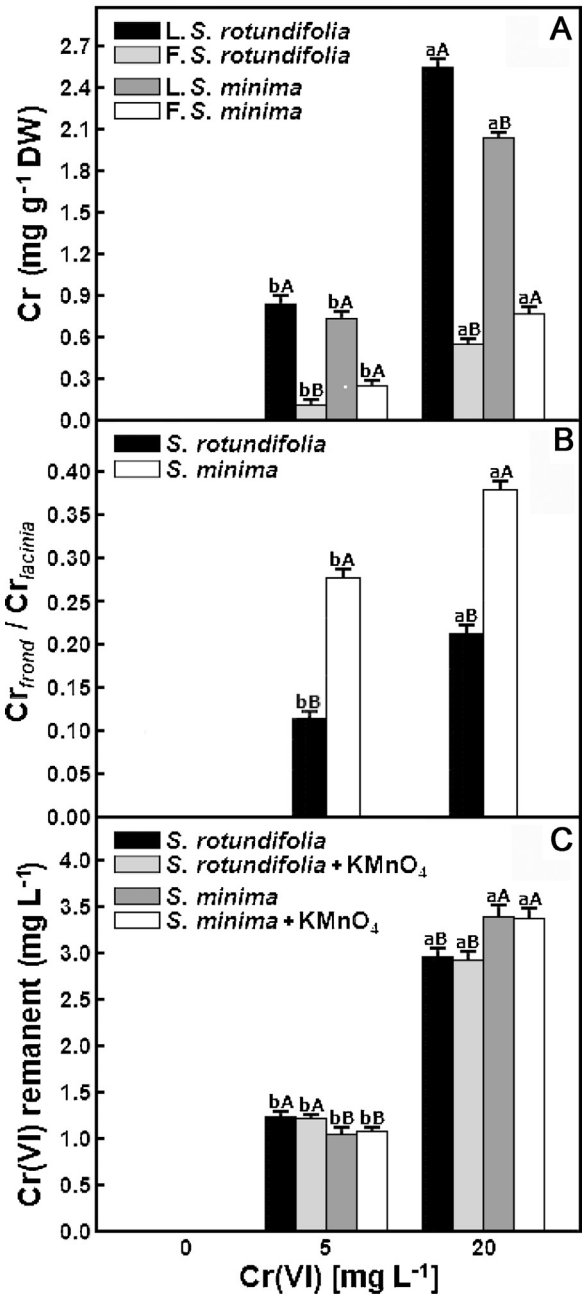

Fig. 2. Metal accumulation in fronds (F) and lacinias (L) of *S. rotundifolia* and *S. minima* plants grown in presence and absence of Cr(VI) during 7 days **(A)**, $Cr_{frond}/Cr_{lacinia}$ ratio **(B)**, and Cr(VI) remanent in the treatment solution at the end of the experiment **(C)**. Data are mean ± SE of three replications (n = 6). For each analyzed parameter different lowercase letters on bars indicate significant differences into each *Salvinia* species. Different uppercase letters denoted significant differences between species for each Cr(VI) concentration ($p < 0.05$).

analysis of variance (ANOVA) and treatment means compared by Tukey's multiple range tests at $p < 0.05$.

3. Results

3.1. Biomass production and Cr accumulation

After a 7-d growth period under highest Cr(VI) concentration the biomass of *S. minima* was reduced significantly ($p < 0.05$), while *S. rotundifolia* biomass was not affected. At low Cr(VI) concentration there were no significant changes in the biomass of both species (Fig. 1A). Frond to lacinia ratio (*frond*/*lacinia*) in Cr-untreated and Cr-treated plants ranged between 3.2 and 4.1 in both species (data not shown). Tolerance index was higher in *S. rotundifolia* (104% and 103%) compared with *S. minima* (101% and 69%), indicating that the former has a greater ability to counteract or cope higher Cr(VI) levels (Fig. 1B). As Cr(VI) concentration increased, metal concentration in both fronds and lacinias of *S. rotundifolia* and *S. minima* plants increased (Fig. 2A). In fronds Cr concentration ranged from 0.10 to 0.55 mg g^{-1} DW in the former, and from 0.20 to 0.78 mg g^{-1} DW in the latter. In lacinias accumulated Cr ranged from 0.85 to 2.57 mg g^{-1} DW in *S. rotundifolia*, and from 0.72 to 2.05 mg g^{-1} DW in *S. minima*, respectively. At the whole plant level, *S. rotundifolia* accumulated 10% more metal than *S. minima*. The $Cr_{frond}/Cr_{lacinia}$ ratio showed significant differences between Cr(VI) concentrations and also between *Salvinia* species. Values ranged from 0.118 to 0.214 in *S. rotundifolia* and from 0.278 to 0.380 in *S. minima*, which indicates a higher metal translocation toward fronds in this last (Fig. 2B). Determination of Cr(VI) remanent in treatment solutions in presence and absence of $KMnO_4$ did not show differences for both *Salvinia* species (Fig. 2C). This indicates that Cr(VI) was the only form of metal accumulated in *Salvinia* plants.

3.2. Photosynthetic pigments

Chlorophyll and carotenoid contents, in general, were more affected by Cr(VI) in *S. rotundifolia* fronds than that in *S. minima* ones. At 20 mg L^{-1} Cr(VI) concentration, Chl *a*, Chl *b* and carotenoids decreased 9%, 38% and 48% in the former whereas in the latter decreased by 8%, 28% and 17%, respectively (Table 1). In Cr-untreated and Cr-treated fronds both Chl *a* and carotenoids were significantly higher ($p < 0.05$) in *S. rotundifolia* compared with *S. minima*, but there was no significant difference in Chl *b* content. The Chl *a*/Chl *b* ratio ranged between 2.63 and 3.87 in *S. rotundifolia*, and from 2.44 to 3.11 in *S. minima*. The Chl (*a* + *b*)/Car ratio ranged between 6.75 and 10.85 in the former and from 8.78 to 9.09 in the latter (Table 1). Of interest, no chlorosis symptoms were observed in fronds of Cr-exposed plants.

3.3. Lipid peroxidation and membrane stability index (MSI)

In both control and Cr-treated fronds and lacinias the accumulation of malondialdehyde (MDA), currently used as an index of lipid peroxidation in plants exposed to adverse environmental conditions, was significantly higher ($p < 0.05$) in Cr-untreated and Cr-treated *S. rotundifolia* plants compared with *S. minima* ones (Fig. 3A). However, Cr treatment did not enhance the accumulation of MDA in both species. The extent of membrane damage was evaluated indirectly by measuring electrolyte leakage from cells. Cr treatment did not have any impact on MSI values of the two species (Fig. 3B).

3.4. Total thiols (TT), non-protein thiols (NPT) and protein-bound thiols (PBT)

Contents of TT, NPT and PBPT thiols in fronds and lacinias of the two *Salvinia* species are presented in Fig. 4. In *S. rotundifolia* fronds both TT and PBT contents increased under increasing Cr(VI) concentrations, while NPT showed an inverse pattern being significantly decreased ($p < 0.05$) in Cr-exposed plants. Maximum increases for TT and PBT were 20% and 260% whereas maximum decrease in NPT was 26% under 20 mg L^{-1} Cr(VI). In lacinias, all thiols increased significantly under increasing Cr(VI) concentrations. Maximum increases were 4.8 fold (TT), 133% (NPT) and ~10 fold (PBT) and occurred at 20 mg L^{-1} Cr(VI) concentration (Fig. 4A). In *S. minima* TT and NPT increased significantly under increasing Cr(VI) concentrations with maximum increases of 81% and

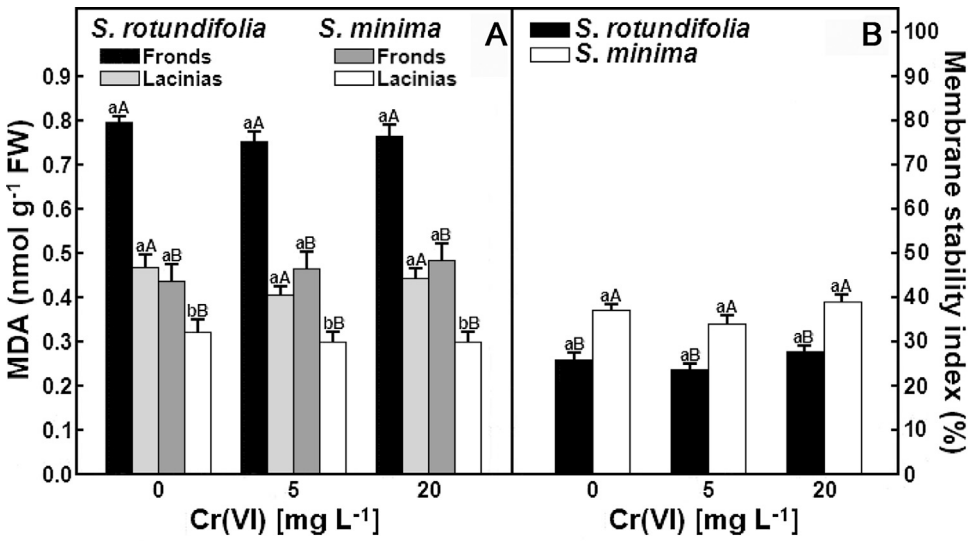

Fig. 3. Effect of Cr(VI) on MDA accumulation **(A)**, and Membrane Stability Index **(B)**, in *S. rotundifolia* and *S. minima* plants after a 7-d cultivation period. Data are mean ± SE of three replications (n = 6). Different lowercase letters on bars indicate significant differences for each organ and each *Salvinia* species. Different uppercase letters denoted significant differences between species, for each organ and each metal concentration ($p < 0.05$).

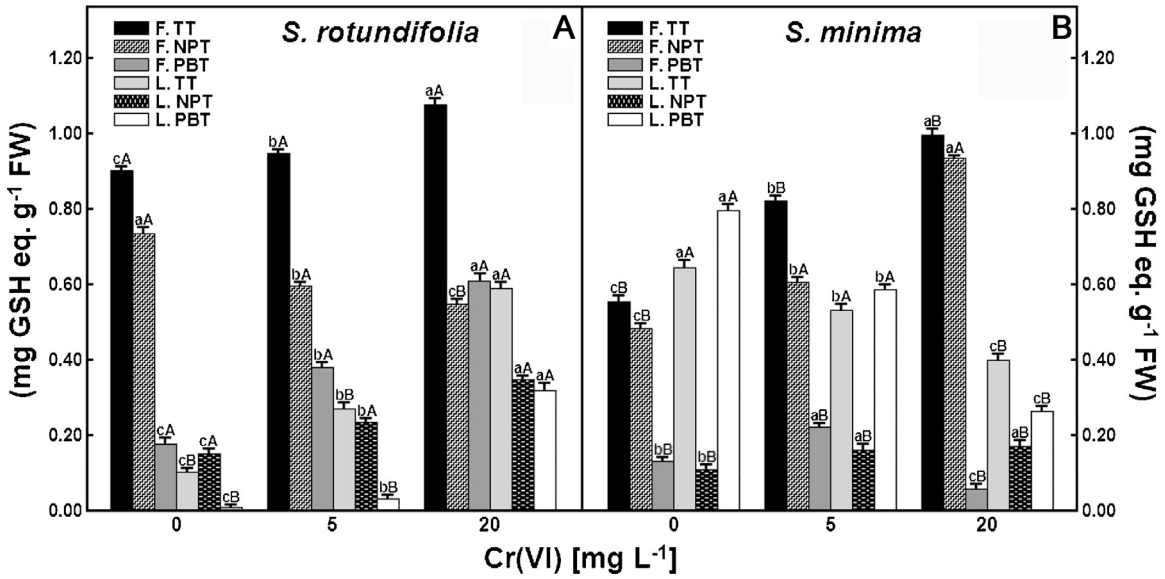

Fig. 4. Accumulation of TT, NPT and PBT in fronds and lacinias of *S. rotundifolia* **(A)** and *S. minima* **(B)** grown in presence and absence of Cr(VI) during 7 days. Data are mean ± SE of three replications (n = 6). Different lowercase letters on bars indicate significant differences for each evaluated parameter and each organ into each analyzed species. Different uppercase letters denote significant differences between species for each evaluated parameter, each organ and each Cr(VI) concentration ($p < 0.05$).

93% at 20 mg L^{-1} Cr(VI). PBT increased significantly (69%) at the lowest Cr(VI) concentration, but strongly decreased at the highest one when comparing with Cr-untreated fronds. Contrarily, in lacinias both TT and PBT strongly decreased in Cr-exposed plants with maximum decreases of 37% and 67% occurring at 20 mg L^{-1} metal concentration. In contrary, NPT increased in Cr-treated lacinias reaching a maximum increase of 43% at the highest concentration of Cr(VI) (Fig. 4B).

3.5. Soluble (SP) and insoluble (IP) phenolics

After 7 days of Cr(VI) treatment, levels of SP and IP in fronds and lacinias of both *Salvinia* species show different distribution patterns (Fig. 5). In Cr-untreated plants, SP were higher in *S. rotundifolia*, while IP were higher in *S. minima*, respectively. SP in fronds of *S. rotundifolia* showed a slight increase (11%) under increasing Cr(VI) concentrations, whereas in lacinias decreased significantly ($p < 0.05$) at the lowest Cr(VI) concentration. Next, a strong increase reaching Cr-untreated value occurred at the high metal concentration. In fronds of *S. minima* the content of SP also decreased at the low Cr(VI) concentration, being increased over Cr-untreated value at the high metal concentration. In lacinias SP were strongly increased in both Cr(VI) concentrations. Maximum increases were 16% (fronds) and 73% (lacinias), respectively (Fig. 5A). IP content in fronds and lacinias of both species was enhanced by Cr(VI) treatment. Maximum increases were 12% (fronds) and 80% (lacinias) in *S. rotundifolia*, and 21% (fronds) and 67% (lacinias) in *S. minima*, respectively (Fig. 5B).

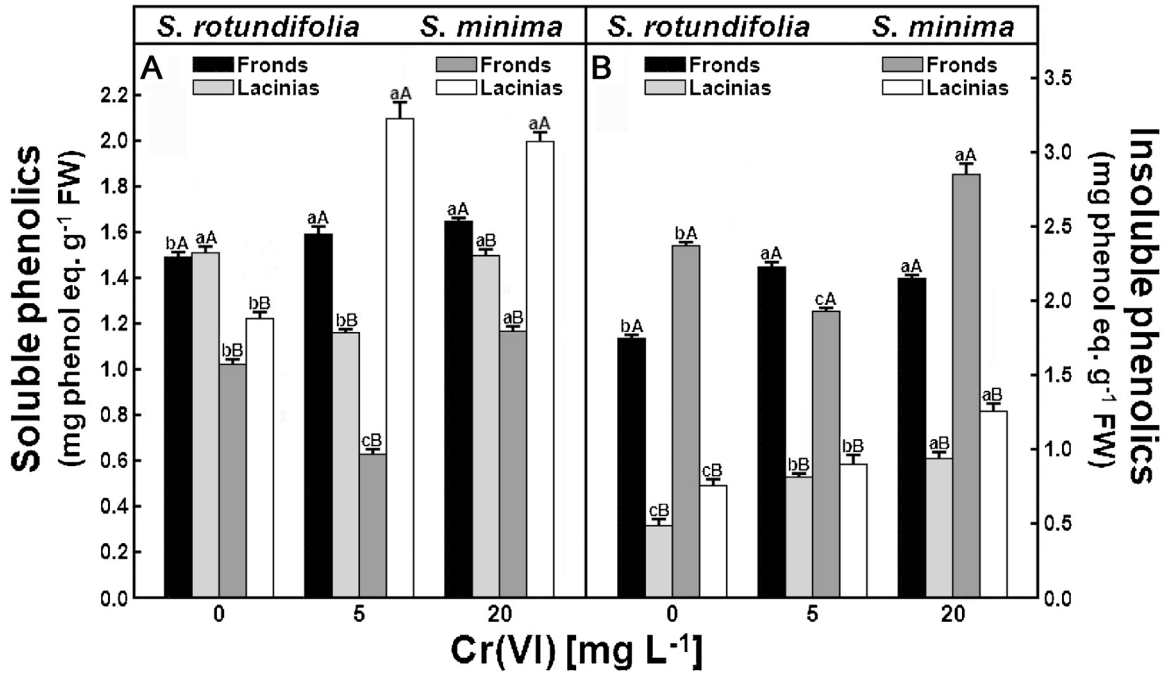

Fig. 5. Accumulation of SP **(A)** and IP **(B)** in fronds and lacinias of *S. rotundifolia* and *S. minima* plants grown in presence and absence of Cr(VI) during 7 days. Data are mean ± SE of three replications (n = 6). Different lowercase letters on bars indicate significant differences for each evaluated parameter and each organ into each analyzed species. Different uppercase letters denote significant differences between species for each evaluated parameter, each organ and each Cr(VI) concentration ($p < 0.05$).

4. Discussion

Even though *Salvinia* species used in this study were obtained from a unique plant nursery (aquarium shop), results of physiological parameters (e.g. photosynthetic pigments, Cr accumulation, and MDA concentration) corresponding to *S. minima* did not show differences with previous results obtained with plants collected from a heavy metal-free pond, where plants grow naturally (Prado et al., 2010a,b; 2012). *Salvinia* species appear as reliable candidates for heavy metal removal from polluted aquatic systems (Dhir, 2009). However, they do not exhibit the same phytoremediation capacity against heavy metals frequently found as contaminants in the environment (Hoffmann et al., 2004; Molisani et al., 2006; Phetsombat et al., 2006; Suñé et al., 2007; Dhir and Srivastava, 2011; Wolff et al., 2012). Then, to get a more efficient phytoremediation of a determined metal, interspecies studies are required to better understand mechanisms involved in metal accumulation and tolerance. The present study, deals with Cr-induced changes in metal accumulation, biomass production and physiological parameters, in two *Salvinia* species used to remove heavy metals (Dhir, 2009). Our results show clearly that *S. rotundifolia* was less affected by Cr(VI) than *S. minima* in terms of biomass production and metal accumulation. According to Sabreen and Sugiyama (2008) the sensitivity of plants to heavy metal stress is linked to growth rate (biomass production), where slower growing plants exhibit higher stress tolerance. However *S. rotundifolia* exhibited slight faster growth and higher metal accumulation than *S. minima*, indicating that differences in Cr(VI) tolerance of *Salvinia* species cannot be simply related to growth rate. Our results clearly also showed different patterns of Cr accumulation in both species. Indeed, dose-response observed in *S. rotundifolia* might be explained by an evolved capacity of this species to retain more metal in lacinias (like root structures) avoiding its excessive translocation to aerial part (fronds). In contrary, *S. minima* exhibits a higher metal translocation capacity to aerial part with less Cr retention capacity in lacinias. In this context, could be expectable a higher Cr-induced toxicity in fronds which, in turn, could limit cellular metabolic activity, giving a reduced biomass production. Hence, *S. rotundifolia* seems to be better adapted than *S. minima* to remove Cr(VI) from polluted waters.

Several reports indicate that exposure to Cr(VI) decreases the chlorophyll content in both aquatic and terrestrial plants (Rai et al., 2004; Panda and Choudhury, 2005; Unnikannan et al., 2011). In the present study only Chl *b* was significantly decreased at the highest Cr(VI) concentration in both species. No change in total chlorophyll content was also reported for Cr-treated *S. natans* plants, and even with a Cr-induced increase of Chl *b* (Dhir, 2009). In agreement no chlorosis symptoms were observed in both *S. rotundifolia* and *S. minima* fronds. Thus, it can be assumed that the decrease of biomass observed in *S. minima* under high Cr(VI) concentration, must be attributed to photosynthesis-unrelated events. However we do not measure CO_2 assimilation, and then this assumption cannot be confirmed. Carotenoids act as light-harvesting accessory pigments, and also can protect the chlorophyll molecule against the oxidation produced by ROS, especially the oxygen singlet ($^1O_2$) generated from triplet excited chlorophyll ($^3Chl^*$). Additionally they can also stabilize lipid bilayers of cell membranes to prevent lipid peroxidation (Ramel et al., 2013). In this study carotenoids of both *Salvinia* species were decreased by Cr(VI) treatment. As a result, the lowest carotenoid content would not produce the detoxification of harmful ROS from Cr-induced oxidative stress. It is well-known that *Salvinia* species (e.g. *S. natans* and *S. auriculata*) have well developed both non-enzymatic and enzymatic antioxidative mechanisms to counteract heavy metal-induced oxidative stress (Dhir et al., 2009; Vestena et al., 2011; Mandal et al., 2013; Thomé Bizzo et al., 2014). In agreement with these findings in a previous study we demonstrated that *S. minima* exposed to Cr(VI) exhibits higher activities of both superoxide dismutase (SOD) and guaiacol peroxidase (G-POD) (Prado et al., 2012), which are key enzymes involved in the detoxification of ROS generated from heavy metal-induced oxidative stress (Mourato et al., 2012). Similar results were observed in *S. rotundifolia* plants exposed to Cr(VI) (Chocobar Ponce, unpublished data). Furthermore, results of the present study showed no changes

Table 1
Effect of Cr(VI) on Chl *a*, Chl *b* and carotenoids contents, and Chl *a*/Chl *b* and Chl (*a* + *b*)/Car ratios in *S. rotundifolia* and *S. minima* fronds after a 7-d cultivation period. Data are mean of three replications ± SE (n = 6). Different lowercase letters in each column for each evaluated parameter and for each species denote significant differences. Different uppercase letters in each row for each evaluated parameter and each Cr(VI) concentration denote significant differences between species ($p < 0.05$).

Cr(VI) (mg L^{-1})	*S. rotundifolia*					*S. minima*				
	Chl *a*	Chl *b*	Chl *a*/Chl*b*	Carotenoids	Chl (*a* + *b*)/Car	Chl *a*	Chl *b*	Chla/Chl*b*	Carotenoids	Chl (*a* + *b*)/Car
	(mg g^{-1} FW)			(mg g^{-1} FW)	(mg g^{-1} FW)				(mg g^{-1} FW)	
0	0.699 ± 0.039^{a,A}	0.266 ± 0.018^{a,A}	2.63 ± 0.12^{b,A}	0.143 ± 0.010^{a,A}	6.75 ± 0.65^{a,B}	0.598 ± 0.042^{a,B}	0.245 ± 0.017^{a,A}	2.44 ± 0.14^{a,A}	0.096 ± 0.008^{a,B}	8.78 ± 0.71^{b,A}
5	0.712 ± 0.045^{a,A}	0.250 ± 0.020^{a,A}	2.85 ± 0.11^{b,A}	0.114 ± 0.009^{b,A}	8.44 ± 0.71^{a,A}	0.567 ± 0.039^{a,B}	0.249 ± 0.026^{a,A}	2.28 ± 0.15^{a,A}	0.093 ± 0.007^{a,A}	8.77 ± 0.90^{a,A}
20	0.638 ± 0.056^{a,A}	0.165 ± 0.018^{b,A}	3.87 ± 0.15^{a,A}	0.074 ± 0.006^{c,A}	10.85 ± 0.80^{a,A}	0.550 ± 0.056^{a,B}	0.177 ± 0.016^{b,A}	3.11 ± 0.21^{b,B}	0.080 ± 0.007^{b,A}	9.09 ± 0.82^{a,A}

in both MDA accumulation and membrane stability index (MSI) in Cr-exposed *S. rotundifolia* and *S. minima* plants, which confirm that *Salvinia* species have effective protective mechanisms against Cr-induced oxidative damage.

Beyond of enzymatic antioxidative systems both thiol and phenolic compounds have not been extensively studied regarding to Cr-induced oxidative stress in *Salvinia* species. Thiols are molecules biologically relevant due to intrinsic reactivity of the nucleophilic −SH moiety. They participate in cellular redox homeostasis, as well as in chelation and/or complexation of heavy metals (Pivato et al., 2014). Moreover, non-protein thiols (NPT) such as reduced glutathione (GSH) and cysteine (Cys), and thiol-rich metal binding polypeptides (PBT) such as metallothioneins (MTs) and phytochelatins (PCs) are also able to act as ROS-scavenger molecules (Yadav, 2010; Hassinen et al., 2011). In fronds of both *Salvinia* species, total soluble thiols (TT) increased significantly under increasing Cr(VI) concentrations, but was more pronounced in *S. minima*. By contrast, NPT increased in this last only. In contrary, in *S. rotundifolia* both TT y NPT increased strongly in Cr-exposed lacinias. Since TT and NPT have been used to indirect estimation of MTs and PCs in plants exposed to heavy metals (Patra et al., 1994), could be assumed that in fronds and lacinias of *Salvinia* species, thiol-rich metal binding polypeptides could be involved in tolerance mechanisms against higher amount of Cr translocated from lacinias to fronds in *S. minima* and higher Cr amount retained in roots in *S. rotundifolia*. In agreement with our assumption there are some reports on the synthesis of both MTs and PCs induced by Cr(VI) in plants (Diwan et al., 2010; Teixeira et al., 2013). Additionally, the accumulation of other thiol-containing proteins such as cysteine proteins (Giles et al., 2003) in Cr-exposed fronds and lacinias of *S. rotundifolia* could also contribute to chelate Cr(III) derived from the reduction of Cr(VI), like occurs in algal cells (Rocchetta et al., 2003). Reduction of Cr(VI) to Cr(III) has been demonstrated in both *S. auriculata* and *S. minima* species (Espinoza-Quiñones et al., 2009; Chocobar Ponce et al., 2014). Contrarily, decreases occurring in PBT of Cr-treated *S. minima* plants could reflect a disturbance of sulphur metabolism, like that observed in both Cd- and Cr(VI)-stressed plants (Herbette et al., 2006; Schiavon et al., 2012). Since higher concentrations of thiols occurred in fronds of both *Salvinia* species, is might be expected that thiol compounds also play another protective role against Cr(VI) toxicity in fronds of *Salvinia* plants.

Phenolics such as hydroxycinnamic acids, flavonoids, proanthocyanidins and their relatives constitute the most abundant secondary metabolites found in mono- and dicotyledonous plants. Polymeric phenolics, such as lignin, suberin, and melanin, can also be commonly found in plants (Caretto et al., 2015). Phenolics, like other secondary metabolites, are essential for plant defence mechanisms against both biotic and abiotic stresses (Pereira et al., 2009). There is growing evidence on the association between exposure to heavy metals and accumulation of phenolic compounds (Sgherri et al., 2003; Posmyk et al., 2009; Tolrà et al., 2009). Like to thiols, phenolic compounds can act as antioxidant molecules either through their ability to chelate metal ions or their capacity to scavenging ROS derived from metal-induced oxidative stress (Michalak, 2006). However, effectiveness of phenolics in protecting against oxidative stress depends on their reactivity towards ROS and the reactivity of the antioxidant phenoxyl radicals towards critical biomolecules such as proteins and lipids (Sakihama et al., 2002). Indeed the reduction of phenoxyl radicals by intracellular reductants such as non-protein thiols (GSH and cysteine) and ascorbate, as well as by enzymes or intermediates of mitochondria electron transport recycles phenolic antioxidants, and thus enhancing antioxidant protection (Kagan and Tyurina, 1998). Therefore, reactivity of phenoxyl radicals should be considered as a critical factor in heavy metal tolerance. However, phenolics can also act

as pro-oxidants by chelating metal in a manner that maintains or increase their catalytic activity or by reducing metals, thus increasing their ability of form free radicals (Pereira et al., 2009). Since no lipid peroxidation was observed in our study, these phenolic traits do not seem to be present in both *Salvinia* species. Thus, a close interconnectivity between phenolics, thiols and heavy metal tolerance, is expectable that occurs in *Salvinia* species. In this regards, both NPT and cell wall-bound phenolics (IP) in lacinias of both *Salvinia* species increased under increasing Cr(VI) concentrations, but increase was more pronounced in lacinias of *S. rotundifolia* that accumulate higher levels of metal than lacinias of *S. minima* plants. In fact, these results agree with previous reports indicating that increases of both cell wall structural polymerized phenolics i.e. lignin and suberin, and cell wall-bound polyphenols, are related to heavy metal accumulation in roots of aquatic and terrestrial plants (Ederli et al., 2004; Kováčik and Klejdus, 2008). Contrarily, soluble phenolics (SP) and NPT were higher in *S. minima* fronds that accumulate more Cr than *S. rotundifolia* fronds. In this regard, SP have been related to vacuolar detoxification of H_2O_2 in leaves of stressed plants (Yamasaki et al., 1997; Michalak, 2006). Since, changes in phenolics and thiols in heavy metal-stressed plants may depend on the plant age, organ, heavy metal, stress time, and species/variety (Skórzyńska-Polit et al., 2004; Ernst et al., 2008), more studies must be addressed.

Together, results of this study provide new insights on the role of both thiols and phenolics into the mechanism of Cr(VI) tolerance, which can be meaningful to better selection of *Salvinia* species to remove heavy metals from polluted waters.

5. Conclusions

It is evident from our results that *S. rotundifolia* seems to be better adapted to cope with Cr-induced stress than *S. minima* in terms of biomass production and metal accumulation. Although fast growth rate and abundant biomass production are desirable characteristics of plants for phytoremediation purposes (Rai, 2008); caution should be taken when choosing *Salvinia* species because of their explosive growth in different aquatic environments. In fact, *Salvinia* species can become in invasive plants in both natural and artificial wetlands, if are not handled by qualified workers and/or management practices are inadequate. Hence, it is always recommended to make a screening of the local aquatic macrophytes to find potentially useful species that can be used to remove Cr(VI), and without representing any risk to the natural environment.

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