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Seasonal variability of physiological and biochemical aspects of chromium accumulation in outdoor-grown *Salvinia minima*

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ABSTRACT

Seasonal variations in physiological and biochemical parameters of the aquatic fern *Salvinia minima* exposed to different Cr(VI) concentrations were studied. Growth, photosynthetic pigments, soluble carbohydrates, sucrose-related enzymes, lipid peroxidation, phenolics, and Cr accumulation in floating and submerged leaves were analyzed. Cr content was lower in winter than in summer, indicating that active metabolic events occurred in metal uptake. Leaf number and metal concentration factor were higher in summer than in winter. Relative growth rate (R_n) indicated that growth was more affected by Cr in winter than in summer. Biochemical parameters showed great seasonal variations under increasing Cr. Hexose, starch, malondialdehyde and phenolic contents were greatest in winter, but R_n and protein values were lowest. Sucrose content was highest in summer floating leaves. A great seasonal variability was observed in sucrose-related enzymes with the highest activities occurring in winter lipoxygenase was much higher in winter than in summer, indicating a strong lipid peroxidation. Results indicate that in *Salvinia* Cr causes seasonal perturbations in carbohydrate metabolism and oxidative stress by altering both sucrose-related enzymes and lipoxygenase activities. Variability in physiological and biochemical parameters seems to indicate that in outdoor conditions different mechanisms, in terms of Cr accumulation and tolerance, may occur in *S. minima* during summer and winter.

1. Introduction

Aquatic plants possess an immense potential to remove heavy metals from wastewater, but not all have the same effectiveness to metal removal. They differ in both the capacity to accumulate the heavy metal in roots and in the proportion of metal transferred to aerial parts (Suñe et al., 2007). The uptake of heavy metals by aquatic plants is also dependent on many environmental factors (i.e. temperature, light intensity, nutrient availability, salinity, presence of other metals, UV radiation, oxygen level, and herbivore predation) (Duman et al., 2006; Olguín et al., 2007). Hence, the knowledge of effects of environmental factors on physiological and biochemical parameters of the metal-extractive plants has become crucial to design plant-based treatments and to improve the metal removal efficiency of plants in artificial wetlands. Many aquatic ferns have a large potential for metal removal from wastewaters, specially, in tropical and subtropical regions (Olguín et al., 2007; Suñe et al., 2007). *Salvinia* comprises one genus (Salvinia) and about 20 known species found in tropical and temperate regions of the world. They show a high growth rate and great capacity to survive under adverse environmental conditions (Oliver, 1993). All *Salvinia* species can remove Cr from wastewaters and polluted water bodies (Olguín et al., 2007; Dhir et al., 2009). Cr accumulation in *Salvinia* is rapid and involves the passive uptake through adsorption of metal ions onto the plant surface and/or active uptake into plant cells (Suñe et al., 2007). Despite the explosive growth that *Salvinia* exhibits, experiments in controlled-temperature cabinets indicated that it dies when its buds are exposed to temperatures <−3 or >43 °C for 2–3 h (Whiterman and Room, 1991). This fact is very important to heavy metal removal and should be kept in mind to design and construct outdoor artificial wetlands for the treatment of urban and industrial effluents. Temperatures over 40 °C are recorded in many tropical and subtropical industrialized regions, whereas temperature values lowest −3 °C are frequently recorded in many populations of the world. Although many studies have shown the ability of *Salvinia* to remove Cr (Olguín et al., 2007; Prado et al., 2010), there is very limited information related to the effect of the weather on physiological and metabolic parameters involved in the Cr accumulation. The aim of this work was to analyze seasonal variations in growth, carbohydrates, sucrose-related enzymes and oxidative parameters

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that occur in leaves of *Salvinia minima* exposed to different Cr concentrations, in order to ascertain as weather affects the capacity of this species to Cr removal.

2. Materials and methods

2.1. Plant material and growth conditions

S. minima was employed for this study because it is a common and conspicuous representative species of the local macrophyte communities. Plants with approximately same age, size and weight were collected from a heavy metal non-polluted fresh water pond located at 500 m asl (above sea level), Tucumán-Argentina (26°50′S, 65°12′W). Collections were performed in summer (December and February) 2008/2009 and winter (July and August) 2009. After collection, the plants were thoroughly washed under running tap water in order to eliminate sediments, particles and microalgae. Washed plants were put into two 140-L clean plastic tanks containing tap water (130 L) for 2 d under outdoor conditions in the Campus of the School of Natural Sciences (acclimation period). This acclimation period seemed necessary for the *Salvinia* plants to recover from a hypothesized stress due to transplantation and to obtain stable and reliable results. After that, healthy plants with fully expanded leaves and uniform weight were selected, thoroughly rinsed with running tap water in order to eliminate any remainder of sediment and microalgae, and transferred to 1.2-L polystyrene pots. Each pot containing 15 plants (35 ± 2 g wet plant biomass) was added with 1000 mL of a determined $K_2Cr_2O_7$ (thereafter Cr) solution, which was made with tap water (treatment period). No difference in plant growth by using a Cr solution prepared with distilled water was observed (Prado et al., 2010). Pot only added with tap water was used as control treatment. Four replicates for each Cr treatment and control were performed. The experiment was carried out outdoor for 7 d under climatic circumstances prevailing in the city of San Miguel de Tucumán (Tucumán, Province). We chose 7 d as treatment period because preliminary tests showed that *S. minima* plants were able to grow well and stay healthy in tap water without nutrient supply for at least 9 d. We do not use a nutrient solution (i.e. Hoagland mineral solution) to avoid chelation and/or cation competition between Cr and Hoagland's salts. Moreover, the addition of nutrients would result in algae growth that would interfere with Cr uptake by *Salvinia* leaves. Water loss by evaporation and transpiration was compensated daily by adding tap water up to the initial mark in each pot. To avoid a great change in Cr concentration the test solution was renewed over 3 d. The Cr stock solution (1000 mg L^{-1}) was prepared by dissolving potassium dichromate of analytical grade with $\geqslant$99% purity in 1000 mL of tap water. From this stock solution, different concentrations (2, 5, 10 and 20 mg L^{-1}) of Cr solution were prepared. This Cr concentration range was chosen from previous studies that reported Cr concentrations in rivers or industrial effluents from 5 to over 200 mg L^{-1} (Miretzky and Cirelli, 2010). The pH of recently prepared Cr solution was 6.7 that is considered suitable for the growth of aquatic plants. During the experiment, the pH of Cr solution ranged from 6.6 to 6.7, and the control tap water between 6.7 and 6.9. The pH of both tap water and Cr solution was daily measured using a glass combination pH-sensitive electrode coupled to a digital pH meter (Hanna Instrument, Germany). According to Kotaś and Stasicka (2000) in oxygenated aqueous solutions the Cr(III) form, according to thermodynamic calculations, is stable at pH $\leqslant$ 6, while at pH $\geqslant$ 7 the Cr(VI) oxidation form predominates. Then, we assumed that hexavalent chromium would be the dominant species under our conditions. Physico-chemical characteristics of tap water were: pH 7–7.2; EC (μS cm^{-1}) 200; DO (mg L^{-1}) 3; TDS (mg L^{-1}) 100; Turbidity (NTU) < 1; As (μg L^{-1}) 0.2; Cu (μg L^{-1}) 2.2; Fe (μg L^{-1}) 12; Mn (μg L^{-1}) 0.2; NH_4^+ (mg L^{-1}) < 0.02; NO_2^- (mg L^{-1}) < 0.05; NO_3^- (mg L^{-1}) 9; HPO_4^{2-} (mg L^{-1}) < 0.2; HCO_3^- (mg L^{-1}) 50; SO_4^{2-} (mg L^{-1}) 10; Cl^- (mg L^{-1}) 12; Ca^{2+} (mg L^{-1}) 10; Mg^{2+} (mg L^{-1}) 5; Na^+ (mg L^{-1}) 20; K^+ (mg L^{-1}) 5; Cr (μg L^{-1}) < 1.2; Ag^+ (μg L^{-1}) 0.05; Pb (μg L^{-1}) 2.1; Hg (μg L^{-1}) < 0.01; Cd^{2+} (μg L^{-1}) 2.5; CN^- (μg L^{-1}) < 0.001; Zn^{2+} (μg L^{-1}) 3.5; Hardness (mg $CaCO_3$ L^{-1}) 212. DO = dissolved oxygen; EC = electrical conductivity; TDS = total dissolved solutes; NTU = nephelometric turbidity unit), (Servicio Provincial de Agua Potable y Saneamiento, SEPAPYS, Tucumán, Argentina). Water parameters correspond to average of data from the 10 last years.

To avoid rain and excessive solar radiation, the pots were put under an iron frame (90-cm height) with a plastic film cover and kept under a tree canopy. In this condition, the temperature of Cr solution was only 1 °C higher than the air temperature during a hot day. Weather conditions occurring at the experimental site, in term of air temperature, wind intensity, rainfall, and sunlight duration, were monitored continuously using an automatic meteorological station (Pegasus EP1000, Argentina). Solution temperature was measured with a portable underwater thermistor (−20 ± 1 to +70 ± 1 °C) connected to a Hobo Temp logger (Onset Computer Corp., Pocasset, USA) and data were recorded every 30 min. Photosynthetic active radiation (PAR) was measured with a Quantum Sensor (Li-190SA, Li-Cor, Lincoln, USA) coupled to a Data-Logger (Li-1000, Li-Cor, Lincoln, USA). PAR measurements were only performed at midday because solar radiation reaching plants was more intense. UV-B measurements were performed with a Silicon photoelectric cell coupled to a photometer/radiometer (PMA2100, Version 1.17, Solar Light Company, Inc., USA). UV-B data were recorded daily between 8:00 AM and 6:00 PM at 1-h interval including sunny and cloudy days to get a better approach of the seasonal radiation. PAR an UV-B measurements were performed under the frame. Time integrated irradiance of the biologically effective UV-B radiation (UV-BBE) was calculated as described by Caldwell et al. (1982).

After Cr exposure, the plants were harvested, rinsed in distilled water, separated in floating and submerged leaves, and used for biochemical studies. In order to minimise any diurnal effect on carbohydrate content and enzyme activities all samples were collected at noon. The wet plant biomass (FW) was immediately determined after harvesting, whereas the dry plant biomass (DW) was determined by drying the weighed wet samples at 80 °C in a hot air oven for 4 d and weighed again. For biochemical analyses, the plants were kept at −20 °C until chemical determinations were carried out.

2.2. Plant growth analysis

The plant development was estimated by determining percentage increase of both green and yellow-brownish (senescent) floating leaves at the end of the experiment. Ten similar plants were marked and the number of leaves recorded for each plant. At beginning of the experiment (0 d), leaf number was assumed as 100%. Seasonal variation of the *S. minima* growth was estimated by determining the relative growth rate (R_n) based on floating leaf number, (Farmer and Spence, 1987).

$$R_n = \frac{\log N_m - \log N_o}{t_m - t_o}$$

where N_m is the mean of floating leaf number at the end of the experimental period; N_o is the mean of floating leaf number at the beginning of the experiment; $t_m - t_o$ is the time interval. R_n was expressed as leaves d^{-1}.

2.3. Photosynthetic pigment and protein contents

Photosynthetic pigments (chlorophyll and carotenoids) were extracted with dimethyl sulfoxide at the end of the experiment, and quantified as described by Wellburn (1994). The protein content was determined according to Lowry et al. (1951) using bovine serum albumin as a standard. Photosynthetic pigment and protein concentrations were expressed as mg g^{-1} FW.

2.4. Cr content

Oven-dried plant tissues (submerged and floating leaves) of Cr-treated and control 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). The total Cr content was determined by atomic absorption spectrophotometry (Perkin–Elmer 373, England), and expressed as μg g^{-1} DW. To ascertain that Cr(VI) was only present in the Cr solution prior to plant growth and at the end of the experiment, the hexavalent chromium was determined at 540 nm as Cr(VI)-diphenylcarbazide complex prior and after addition of $KMnO_4$ in acid medium. Acidic $KMnO_4$ is able to oxidizing completely Cr(III) to Cr(VI).

2.5. Soluble carbohydrate and starch content

Soluble carbohydrate determination in both floating and submerged leaves was conducted as described by Prado et al. (2010). Starch was determined by measuring reducing sugars released after enzymatic hydrolysis (Rosa et al., 2009). Soluble sugar and starch contents were expressed as mg g^{-1} FW and mg maltose equivalent released g^{-1} FW, respectively.

2.6. Sucrose-related enzymes

Sucrose phosphate synthase (SPS), sucrose synthase (SS) and soluble acid invertase (AI) were measured in floating and submerged leaves at the end of the experiment. All enzymes were extracted and determined as described by Rosa et al. (2009). SPS and SS activities were assayed in the synthesis direction.

2.7. Lipoxygenase activity and malondialdehyde content

Lipoxygenase enzyme (LOX) was extracted from *S. minima* leaves as described by Baracat-Pereira et al. (2001). LOX activity was assayed at 234 nm based on the absorption spectrum of the conjugated dienes formed when linoleic acid (used as substrate) was oxidised in the presence of lipoxygenase (Szymanowska et al., 2009). Malondialdehyde (MDA) was determined by thiobarbituric acid reaction (Du and Bramlage, 1992). MDA content was calculated taking the molar extinction coefficient ($\varepsilon = 1.57 \times 10^5$ M^{-1} cm^{-1}), and was expressed as nmol g^{-1} FW.

2.8. Determination of soluble phenolics

Total soluble phenolics were extracted from *Salvinia* leaves with ethanol 96% and determined by using the Folin–Ciocalteau's reagent (Swain and Hillis, 1959). Phenolic content was expressed as μmol phenol equivalent g^{-1} FW.

2.9. Data analysis

Salvinia responses from control and exposed groups were compared by using a one-way analysis of variance followed by post hoc Tukey's HSD test ($p < 0.05$) when necessary. Results were the average of three independent experiments and they were represented as mean ± standard deviation (SD). All data analyses were performed using Statistica 6.0 Software (©Statsoft).

3. Results

3.1. Meteorological data

Meteorological data showed that the averages of air and water temperatures did not show significant variation in each season, but they were much higher in summer than in winter. Values of air and water temperatures remained mild (13.5 ± 1.5 and 13.4 ± 1.3 °C) throughout the winter and rose to maximum averages of 31.5 ± 1.5 and 32.3 ± 1.7 °C in summer. Sunlight duration (daylength) was over 13 h in summer and 9 h in winter. Averages of maximum PAR and UV-BBE were 1860 ± 46 μmol m^{-2} s^{-1} and 27.5 ± 3.2 kJ m^{-2} s^{-1} for the summer, and 1050 ± 48 μmol m^{-2} s^{-1} and 13.8 ± 2.0 kJ m^{-2} s^{-1} for the winter, respectively. Furthermore, during the experimental period one and two cloudy days were recorded for summer and winter, respectively (data not shown).

3.2. Growth dynamics and photosynthetic pigments

After 7 d of Cr treatment, the values of R_n in control plants were 0.02 and 0.01 leaves d^{-1} for summer and winter, respectively. In Cr-treated leaves, R_n ranged from 0.02 to 0.01 leaves d^{-1} in summer and from 7×10^{-3} to 4×10^{-3} leaves d^{-1} in winter, indicating that plant growth was more affected by Cr exposure in winter than in summer. Values of the $R_{n(summer)}$ to $R_{n(winter)}$ ratio in Cr-treated plants ranged from 2.5 ± 0.1 to 3.4 ± 0.1 according to metal concentration, whereas in Cr-untreated plants it was 2.6 ± 0.1 (Table 1). At the end of the experiment, the number of green leaves of Cr-untreated plants increased in summer and winter by 34 ± 3 and 13 ± 2% respectively, while under Cr exposure it showed a sustained decrease in both seasons being more pronounced in winter one (data not shown). By contrast the number of yellow-brownish leaves showed different trends in both summer and winter. In the former until 5 mg L^{-1} Cr concentration the percentage of yellow-brownish leaves did not change, but from this point on a pronounced increase was observed reaching at 20 mg L^{-1} Cr concentration an increase of 16 ± 1%. In winter, there was no significant increase in the percentage of yellow-brownish leaves, while in control plants the percentage of yellow-brownish leaves was practically unascertainable. The green to yellow-brownish leaf ratio, an indicator of the plant Cr toxicity, strongly decreased in summer at 10 and 20 mg L^{-1} Cr concentrations whereas in winter a much less pronounced decrease was observed (data not shown).

After 7 d of Cr treatment, the concentrations of photosynthetic pigments were differentially affected by contrasting seasons (Fig. 1). In summer, the chlorophyll *a* content did not show significant changes between control and Cr-treated plants ranging from 0.3 ± 0.03 to 0.2 ± 0.04 mg g^{-1} FW with increasing Cr concentrations. By contrast, the chlorophyll *b* concentration was significantly

Table 1
Relative growth rate (R_n) based on floating leaf number and $R_{n(summer)}/R_{n(winter)}$ ratio for each season with increasing Cr concentrations. Values are means of results from 10 plants ± SD in each Cr treatment. In each column, different superscript letters indicate significant differences ($p < 0.05$).

Cr(VI) (mg L^{-1})	R_n (leaves d^{-1})		$R_{n(summer)}/R_{n(winter)}$ (Ratio)
	Summer	Winter	
0 (control)	0.019 ± 0.001[a]	0.008 ± 0.001[a]	2.57 ± 0.07[a]
2	0.017 ± 0.001[b]	0.007 ± 0.001[b]	2.49 ± 0.07[a]
5	0.017 ± 0.001[b]	0.007 ± 0.001[b]	2.43 ± 0.06[a]
10	0.014 ± 0.001[c]	0.004 ± 0.001[c]	3.28 ± 0.08[b]
20	0.012 ± 0.001[d]	0.004 ± 0.001[c]	3.38 ± 0.07[b]

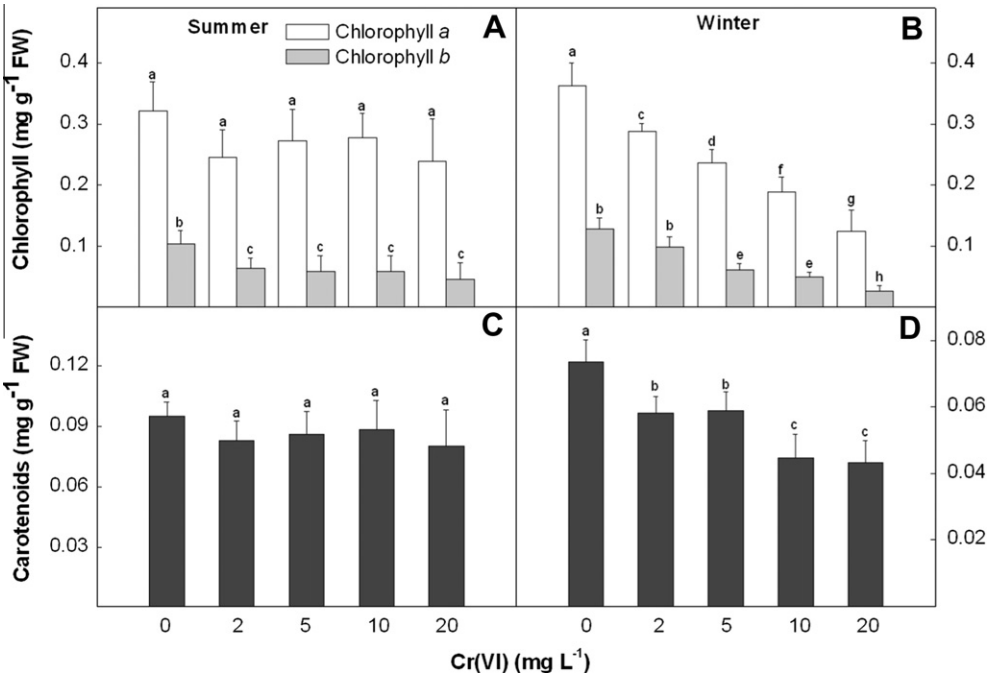

Fig. 1. Seasonal effect of different Cr concentrations on chlorophyll *a* and chlorophyll *b* (A,B), and carotenoid (C,D) contents in floating leaves of *S. minima* growing at outdoor conditions. Means of three independent experiments with four replicates are represented. For each parameter measured into each season, different lowercase letters are significantly different ($p < 0.05$).

higher in control than in Cr-treated leaves ranging from 0.11 ± 0.01 mg g^{-1} FW (control) to 0.06 ± 0.01 mg g^{-1} FW (20 mg L^{-1} Cr concentration) (Fig. 1a). Chlorophyll *b* content did not show significant variations from 2 to 20 mg L^{-1} metal concentration. In winter, both chlorophyll *a* and chlorophyll *b* concentrations decreased considerably from 0 mg L^{-1} (control) to 20 mg L^{-1} Cr concentration. Chlorophyll *a* ranged from 0.4 ± 0.04 to 0.1 ± 0.02 mg g^{-1} FW and chlorophyll *b* from 0.13 ± 0.02 to 0.03 ± 0.01 mg g^{-1} FW (Fig. 1b). The chl *a*/chl *b* ratio ranged from 3.1 ± 0.3 to 5.0 ± 0.3 in summer and from 2.8 ± 0.2 to 4.3 ± 0.3 in winter, indicating that chlorophyll *b* was more affected by Cr treatment than chlorophyll *a* in both seasons (data not shown). Carotenoid concentration showed a similar distribution pattern that chlorophyll *a* in both seasons, however, in winter the carotenoid decrease was less pronounced than chlorophyll *a* decrease (Fig. 1c and d). Carotenoid concentration was significantly ($p < 0.05$) higher in summer than in winter ranging from 0.1 ± 0.01 to 0.08 ± 0.01 mg g^{-1} FW in the former and from 0.08 ± 0.01 to 0.05 ± 0.01 mg g^{-1} FW in the latter.

3.3. Cr accumulation

The accumulation of Cr in submerged and floating leaves of *S. minima* in both seasons is shown in Fig. 2. Cr content of both leaves significantly increased when the heavy metal concentration of growth solution was increased. The highest Cr content was observed in summer (3388 ± 355 and 637 ± 56 μg g^{-1} DW for submerged and floating leaves), whereas in winter it strongly decreased (710 ± 65 and 189 ± 18 μg g^{-1} DW for both leaves) (Fig. 2). Maximum Cr concentration in summer floating leaves was nearly 3-fold higher than in winter ones, whereas in submerged leaves it was nearly 5-fold higher. Cr accumulation was not detected in control plants. The metal concentration factor (MCF), defined as the ratio of metal concentration in the plant to initial metal concentration in the feed solution, ranged in summer floating leaves from 45 to 31 with increasing Cr concentrations,

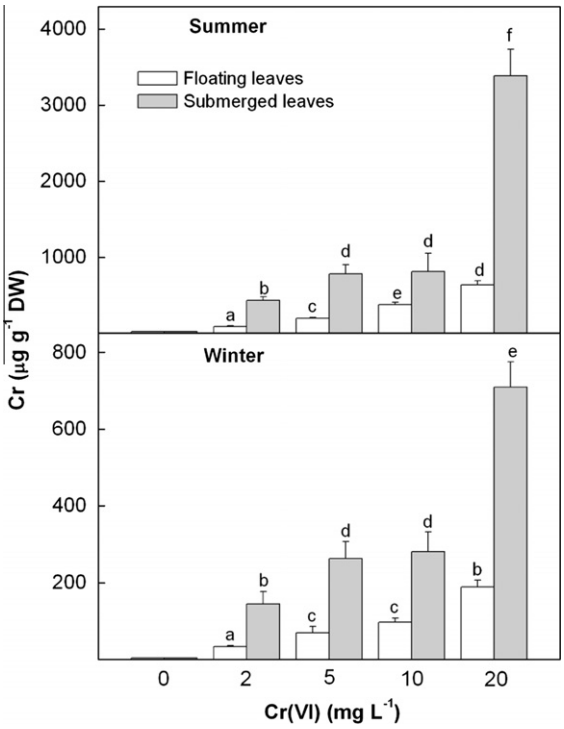

Fig. 2. Cr content in leaves of *S. minima* after a 7-d Cr exposure. Means of three independent experiments with four replicates are represented. For each season, different lowercase letters are significantly different ($p < 0.05$).

while in submerged leaves it ranged between 245 and 171 under similar Cr concentrations. For winter leaves, MCF values ranged from 18 to 9 in floating leaves and from 65 to 36 in submerged ones (data not shown). Concerning to Cr speciation no statistical differences ($p < 0.05$) between $KMnO_4$-treated and $KMnO_4$-un-

treated Cr solutions were observed (data not shown). To calculate the deviation of analytical method of Cr(VI) concentration, a calibration curve was prepared from the ($K_2Cr_2O_7$) stock solution. SD obtained for the calibration curve indicated a good fit of the data and within the error limits. This ensured high confidence limits of the experimental measurements. Hence, Cr(VI) seems to be the unique Cr species occurring in heavy metal solution.

3.4. Carbohydrate and protein content

The exogenous Cr caused seasonal changes in sucrose, reducing sugars and starch content of *Salvinia* leaves. Reducing sugars and starch were higher in winter than in summer, whereas sucrose did not show great seasonal differences (Fig. 3). The distribution pattern of sucrose showed the highest values in summer floating leaves, whereas in winter they were observed in submerged ones (Fig. 3a and b). In summer, the maximum values of sucrose for floating and submerged leaves (0.4 ± 0.03 and 0.04 ± 0.01 mg g^{-1} FW) were observed at 20 mg L^{-1} Cr concentration (Fig. 3a). In winter the maximum sucrose concentrations corresponding to floating and submerged leaves (0.2 ± 0.03 and 0.3 ± 0.04 mg g^{-1} FW) were observed at 2 mg L^{-1} Cr concentration for the former and at 5 mg L^{-1} for the latter (Fig. 3b). Profile analysis of the glucose and fructose concentrations, revealed that in summer the maximum content of glucose (0.1 ± 0.01 mg g^{-1} FW) was observed in floating leaves at 10 mg L^{-1} Cr concentration, whereas in submerged leaves practically there were no significant differences between control and Cr-treated plants (Fig. 3c). In winter, the maximum content of glucose for floating leaves (0.5 ± 0.05 mg g^{-1} FW) was observed at 2 mg L^{-1} Cr concentration, but there was no statistical difference with the control value (0.5 ± 0.05). Submerged leaves showed a similar profile than the summer pattern, but their

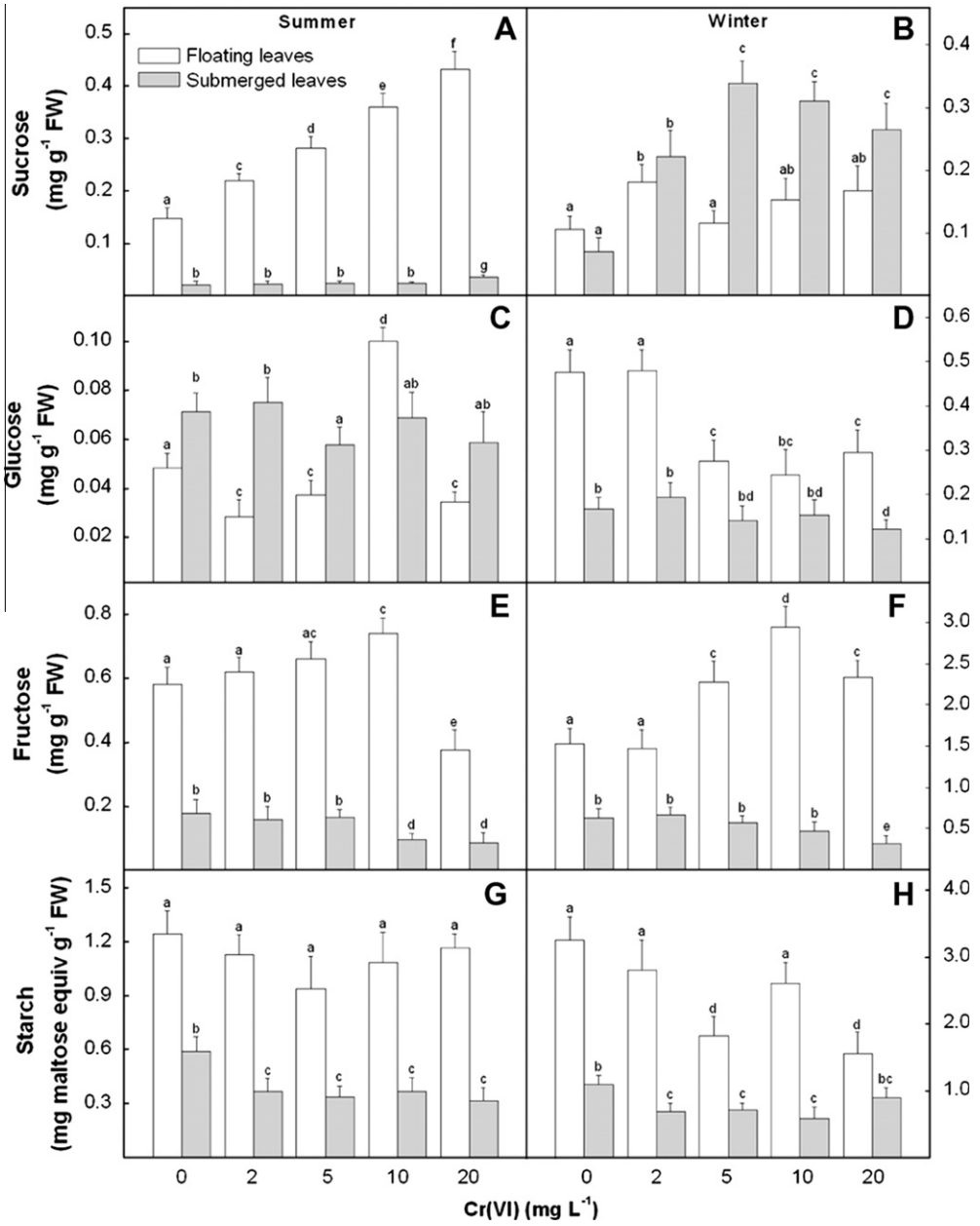

Fig. 3. Seasonal effect of different Cr concentrations on sucrose (A,B), glucose (C,D), fructose (E,F) and starch (G,H) contents in leaves of *S. minima* growing at outdoor conditions. Other details as in Fig. 2.

contents were higher (Fig. 3d). The maximum contents of fructose for floating leaves (0.7 ± 0.05 and 2.9 ± 0.3 mg g^{-1} FW) were observed at 10 mg L^{-1} Cr concentration in both summer and winter. By contrast, in submerged leaves a slight decrease in parallel to Cr increase was observed in both seasons (Fig. 3e and f). Starch content of summer leaves was lower than that winter ones being the highest value observed in floating leaves. Although the distribution pattern in floating leaves was different between summer and winter, in submerged leaves, in general, no seasonal differences were observed (Fig. 3g and h). In summer season, the leaves exposed to Cr solution displayed a similar pattern for protein concentration with the highest values for floating and submerged leaves (0.6 ± 0.04 and 0.5 ± 0.02 mg g^{-1} FW) occurring at 20 mg L^{-1} Cr concentration. In winter submerged leaves there was no statistical difference between control and 2 mg L^{-1} Cr concentration, but from this point on a sustained decrease until the end of the experiment was observed. In contrary, the floating leaves of winter season did not show a significant difference in the protein content under Cr exposure. Maximum protein contents for floating and submerged leaves in winter were 0.17 ± 0.01 mg g^{-1} FW (10 and 20 mg L^{-1}) for the former, and 0.07 ± 0.01 (2 mg L^{-1}) for the later (data not shown).

3.5. Sucrose-related enzymes

Sucrose-related enzyme responses of *S. minima* leaves to Cr exposure are depicted in Fig. 4. In both seasons, all enzymes were differently affected by the Cr treatment even at the lowest tested concentration (i.e. 2 mg L^{-1}). The activity of SPS was much higher in winter than in summer. The pattern of SPS activity in summer floating leaves showed a slow initial decrease up to 2 mg L^{-1} Cr concentration followed by a rapid increase reaching a maximum value of 0.03 ± 0.01 μmol suc-6-P g^{-1} protein min^{-1} at 20 mg L^{-1}. This maximum was 71% higher than that Cr-untreated leaves. In submerged leaves, the enzyme activity was slightly reduced until 2 mg L^{-1} metal concentration and from this point on it was strongly decreased until the end of the experiment. The highest activity (0.08 ± 0.01 μmol suc-6-P g^{-1} protein min^{-1}) corresponded to control leaves (Fig. 4a). In winter, the SPS activity showed relatively similar patterns in both floating and submerged leaves, reaching at 20 mg L^{-1} Cr concentration maximum values of 0.07 ± 0.01 and 0.4 ± 0.01 μmol suc-6-P g^{-1} protein min^{-1} for the former and latter, respectively (Fig. 4b). These values were nearly 4- and 5-fold higher than maximum summer values. Similarly, to SPS the SS activity was much higher in winter than in summer. In summer, the pattern of SS activity of floating leaves showed a progressive increase until 10 mg L^{-1} Cr concentration reaching a maximum activity of 0.07 ± 0.01 μmol suc g^{-1} protein min^{-1} and then it sharply decreased until the end of the experiment. For submerged leaves, an initial strong increase until 2 mg L^{-1} Cr concentration was observed reaching a maximum value of 0.1 ± 0.01 μmol suc g^{-1} protein min^{-1}. From this point on the activity slowly decreased until 10 mg L^{-1} metal concentration and then it strongly decreased until the end of the experiment (Fig. 4c). For winter plants the SS pattern was, in general, similar to SPS profile showing for both floating and submerged leaves

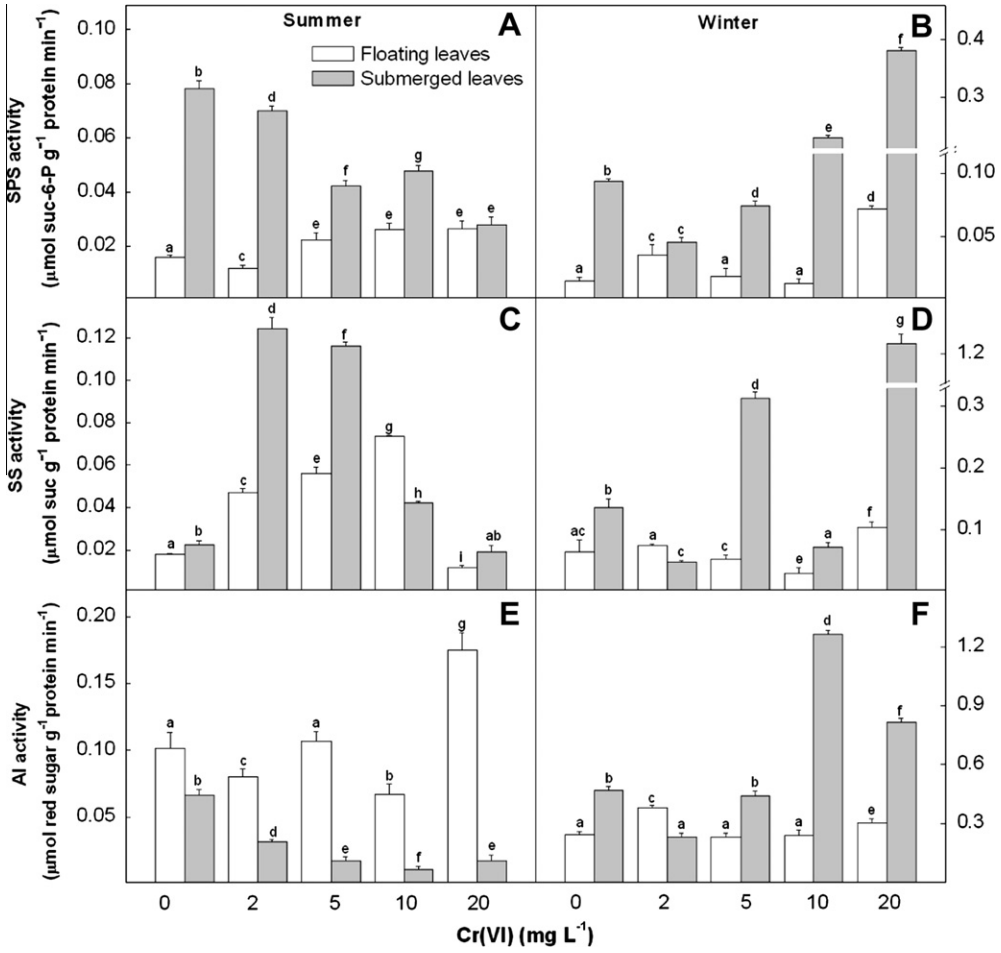

Fig. 4. Seasonal effect of different Cr concentrations on SPS (A,B), SS (C,D) and AI (E,F) activities in leaves of *S. minima* growing at outdoor conditions. Other details as in Fig. 2.

maximum activity values at 20 mg L^{-1} Cr concentration (0.1 ± 0.01 and 1.2 ± 0.03 μmol suc g^{-1} protein min^{-1}) (Fig. 4d). Like SPS the activity of SS was, in general, higher in Cr-treated leaves than in Cr-untreated ones. AI activity was also much higher in winter than in summer. In summer floating leaves the AI activity strongly increased at 20 mg L^{-1} Cr concentration reaching a value of 0.2 ± 0.01 μmol red. sugar g^{-1} protein min^{-1}. However, with the remaining Cr concentrations the AI activity did not show statistical differences compared with the control. In submerged leaves an inverse pattern was observed with the highest activity (0.07 ± 0.01 μmol red. sugar g^{-1} protein min^{-1}) in Cr-untreated leaves (Fig. 4e). In winter floating leaves the AI activity increased until 2 mg L^{-1} Cr concentration, reaching a maximum value of 0.4 ± 0.01 μmol red. sugar g^{-1} protein min^{-1} and then it decreased until 5 mg L^{-1} metal concentration. From this point on the AI activity slowly increased until the end of the experiment. In submerged leaves the AI activity showed a sharply increase until 10 mg L^{-1} Cr concentration with a maximum value of 1.3 ± 0.02 μmol red. sugar g^{-1} protein min^{-1}, and then it decreased until the end of the experiment (Fig. 4f). The AI activity was also higher in winter Cr-treated leaves than in Cr-untreated ones. Interestingly, SS and AI activities of winter submerged leaves were over 10- and 15-fold higher than that summer submerged ones.

3.6. Lipoxygenase activity and MDA accumulation

LOX activity using linoleic acid as substrate was significantly increased in Cr-treated leaves compared with Cr-untreated ones (Fig. 5a and b). In summer floating leaves the LOX activity strongly increased until 10 mg L^{-1} Cr concentration reaching a maximum value of 0.7 ± 0.02 ΔA234 increase g^{-1} protein min^{-1}. From this point on the LOX activity remained almost constant until the end of the experiment. In submerged leaves the LOX activity increased until 5 mg L^{-1} Cr concentration reaching a value of 0.3 ± 0.01 ΔA_{234} increase g^{-1} protein min^{-1} (Fig. 5a). In winter submerged leaves the LOX activity strongly increased until 2 mg L^{-1} Cr concentration, and then remained without significant changes until 10 mg L^{-1}. From this point on the LOX activity again increased until the end of the experiment reaching a maximum value of 23.5 ± 0.8 ΔA_{234} increase g^{-1} protein min^{-1}. In floating leaves a strong initial increase of the LOX activity was also observed at 2 mg L^{-1} Cr concentration. From this point on the LOX activity slowly increased until the end of the experiment reaching a maximum value of 16.5 ± 0.4 ΔA_{234} increase g^{-1} protein min^{-1} (Fig. 5b). LOX activity was also much higher in winter leaves than in summer ones. MDA content in summer season did not vary significantly between Cr-treated and Cr-untreated leaves (Fig. 5c). By contrast, in winter it significantly increased with maximum values of 0.6 ± 0.02 and 0.4 ± 0.01 nmol g^{-1} FW for floating and submerged leaves, respectively (Fig. 5d).

3.7. Soluble phenolics

Total soluble phenolics in floating leaves strongly increased by Cr exposure in both summer and winter, leading to increase over 3- and 4-fold compared with Cr-untreated leaves at 20 mg L^{-1} Cr concentration. Maximum phenolic concentrations for both summer and winter were 1.9 ± 0.1 and 4.6 ± 0.1 μmol phenol equivalent g^{-1} FW, respectively. In submerged leaves an opposite trend was observed being the lowest content recorded at 20 mg L^{-1} Cr concentration in both seasons (Fig. 6).

4. Discussion

S. minima exposed to different Cr concentrations under outdoor conditions showed seasonal changes in growth and biochemical parameters. The aerial biomass (floating leaves), indicated by the green leaf number, was significantly higher in summer than in winter. Agreeing with this fact R_n was also higher in summer than in winter (Table 1). The $R_{n(summer)}/R_{n(winter)}$ ratio increased at high-

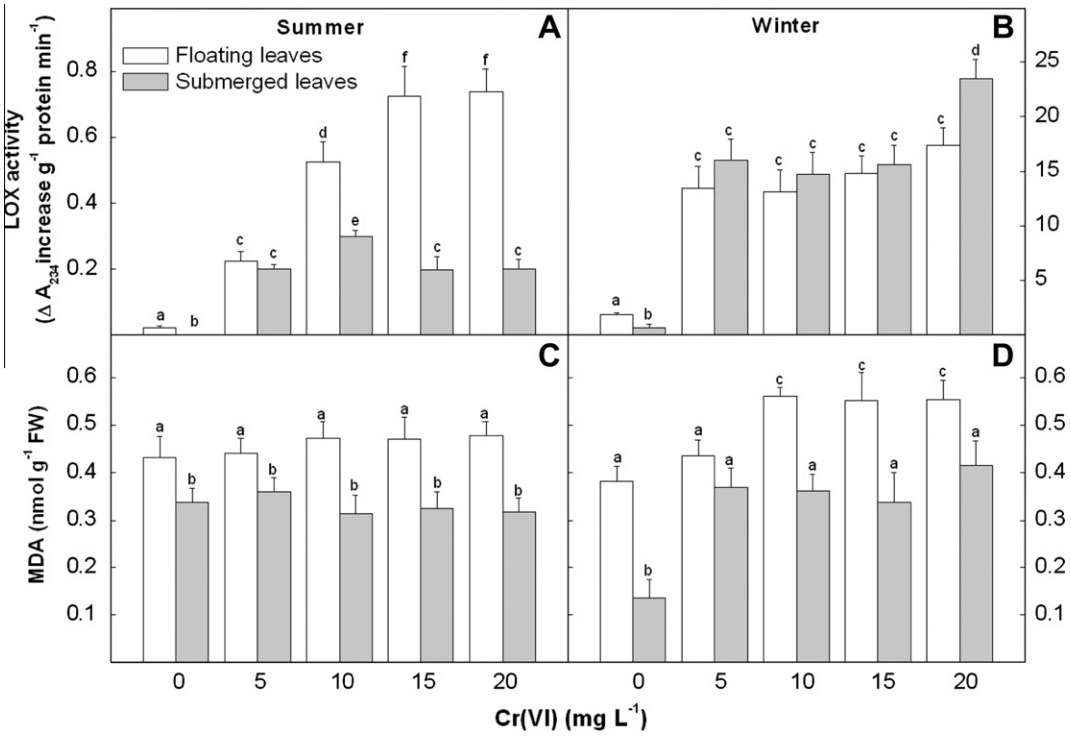

Fig. 5. LOX activity (A,B) and MDA content (C,D) of Cr-treated leaves of *S. minima* growing for 7-d under outdoor conditions. Other details as in Fig. 2.

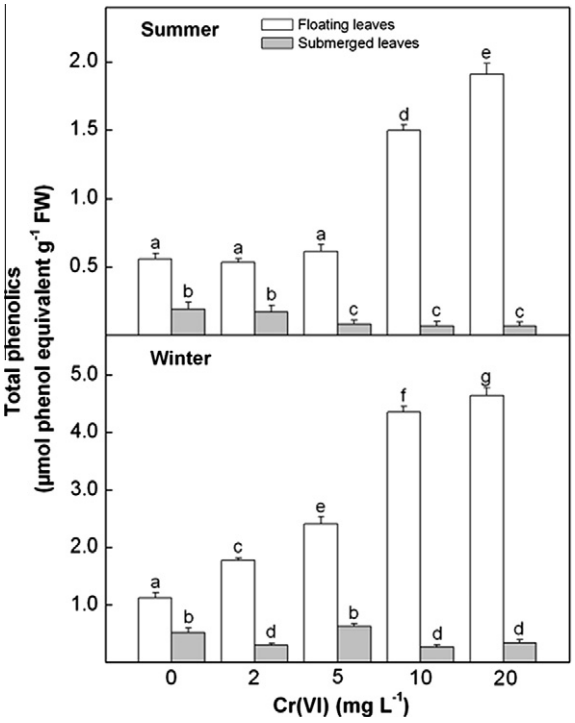

Fig. 6. Soluble phenolic content in leaves of *S. minima* after a 7-d Cr exposure. Other details as in Fig. 2.

est Cr concentrations indicating that *S. minima* growth is more affected by Cr exposure in winter than in summer. This agrees with previous studies that reported that *Salvinia* tends to reduce growth itself to cope-up with low temperatures (Oliver, 1993). Furthermore, it was also noted that in winter *S. molesta* is able to maintain high population densities in water bodies whose larger thermal capacity dampens temperature fluctuations (Whiterman and Room, 1991). Agreeing with the latter assumption in this study a relative growth rate near to 39% of the summer value was observed for winter-grown *S. minima*. This relatively high growth rate may be due to mild winter at the study site where frost temperatures are absent. The presence of yellow-brownish leaves in Cr-treated plants was greater in summer than in winter indicating a more severe Cr-induced senescence in summer-grown plants. Because significant leaf senescence was observed in macrophytes growing in shallow ponds under high water temperatures, we believe that the highest percentage of yellow-brownish leaves observed in summer is a symptom of both high temperature- and Cr-induced senescence.

Investigations of the effects of heavy metals on aquatic macrophytes suggested that the metal accumulation was depending upon seasonal variations, but results were controversial (Duman et al., 2006). Some studies reported high metal concentrations for autumn and relatively low levels during spring, whereas others indicated high contents during spring or summer and low ones in winter (Brekken and Steinnes, 2004). In agreement with the latter finding, our data showed the highest content of Cr in summer-grown *Salvinia* plants (Fig. 2). The amount of metal that is taken up by plants is dependent on its availability, which it is controlled by physicochemical factors such as metal solubility, water temperature and pH (Duman et al., 2006). According to Zumdahl (1992), seasonal variations in water temperature have no direct effect on metal solubility whereas the increase of pH is generally accompanied by a decrease of the metal solubility. In this study, no seasonal differences in the pH value of Cr solution was observed and then the lowest Cr content found in winter may be related to a decrease in metal uptake induced by temperature-dependent metabolic changes. In agreement with this hypothesis our data showed that the distribution pattern of soluble sugars of Cr-treated leaves was differently affected in both summer and winter. In general the sucrose concentration was higher in summer floating leaves than in winter ones (Fig. 3a and b), probably, as result of the photosynthesis increased by both higher temperature and longer daylength. It is well known that in unstressed growing plants the synthesized sucrose is translocated to growing tissues where is either hydrolyzed by invertase or sucrose synthase and the produced hexoses channelled toward glycolytic cycle and pentose phosphate pathway (Devi et al., 2007). However, under stress conditions (i.e. cold, heavy metals) the synthesis and accumulation of sucrose might act as an effective sink for the excess of ATP through the alternative respiratory pathway (Prado et al., 2010). Agreeing with this assumption, our data show an increase in sucrose concentration under Cr exposure in both summer and winter seasons (Fig. 3a and b). Similar results were observed in *S. minima* under Al exposure (Gardner and Al-Hamdani, 1997). According to Farmer and Spence (1987) soluble carbohydrates, mainly hexoses, are higher in winter-grown plants due to a low leaf materials production. In agreement with this fact, our data showed both a less growth rate and a significantly higher concentration of soluble hexoses in winter-grown *Salvinia* plants (Fig. 3c–f). Furthermore, many aquatic macrophytes show an accumulation of storage carbohydrates (i.e. starch) at the end of summer. This carbohydrate cycle is accompanied by starch depletion mainly in winter, but also during the spring when the plant growth takes place. Although this cycle could contribute to explain the much higher hexose concentrations observed in *Salvinia* winter leaves, the leaf starch content was higher in winter than summer (Fig. 3g and h). Hence, the observed accumulation of glucose and fructose in Cr-exposed winter leaves may reflect a distinct carbohydrate cycle depending upon both low temperature and heavy metal concentration. Despite that heavy metals toxicity affects the carbohydrate metabolism producing an accumulation of soluble sugars; they also perturb the water movement into leaves (Perfus-Barbeoch et al., 2002). In that context, the accumulation of soluble sugars in Cr-exposed leaves could also provide an adaptive mechanism to maintain a favourable intracellular osmotic potential. However, further studies are needed to clarify this topic.

To elucidate the influence of two contrasting seasons on soluble carbohydrate metabolism, we studied the activity of sucrose-related enzymes SPS, SS and AI in both Cr-treated and Cr-untreated leaves. Although enzyme activities were predominantly higher in Cr-treated leaves there were significant seasonal variations (Fig. 4). All activities were much higher in winter than in summer. The significant increase of SPS activity observed in winter floating leaves at 20 mg L^{-1} Cr concentration may indicate that sucrose-synthesizing capacity is not affected by both low temperature and heavy metal. AI and SS activities were also significantly higher at the highest Cr concentrations (Fig. 4c–f). Although this fact could lead to apparent wasteful hydrolysis of sucrose, the released hexoses could be metabolized to sustain a pool of NADPH, which could be utilized either to sustain biosynthetic reactions or NADPH-dependent ROS-detoxifying enzymes (Møller, 2001). Then *Salvinia* could switch carbohydrate pathway from the primary to secondary metabolism to enhance the synthesis of protective metabolites (i.e. phenolics) and/or contribute to defense against the Cr-induced oxidative damage. In agreement with this hypothesis, our results showed the highest levels of leaf soluble phenolics and both MDA content and LOX activity in winter season. In summer submerged leaves exposed to Cr the SPS and AI activities decreased when compared with the control (Fig. 4a and e). Although this fact is not clear to understand, we believe that it could be related to a

higher growth metabolic demand in Cr-untreated than in Cr-treated plants.

In many aquatic macrophytes, the decrease of photosynthesis, promoted by increased Cr concentration in the nutrient solution, was associated with ROS-induced inhibition of important enzymes of chlorophyll biosynthesis i.e. α-aminolevulinic acid dehydrogenase and photochlorophllide reductase (Sankar-Ganesh et al., 2008). Moreover, the loss of chlorophyll under Cr stress was also related to lipid peroxidation (Sinha et al., 2005). In the present study Cr exposure did not affect the content of chlorophyll *a* of summer leaves, while the chlorophyll *b* concentration was significantly decreased (Fig. 1a). In winter, the content of both chlorophyll *a* and chlorophyll *b* decreased with increasing Cr concentrations (Fig. 1b). The carotenoid content was also decreased with increasing Cr concentrations in *Salvinia* winter leaves (Fig. 1d). In Cr-exposed plants, the decrease of carotenoid content has been related to interference on their synthesis by the heavy metal through oxidative damage (Panda and Choudhury, 2005). Then, in Cr-exposed *S. minima* leaves the ROS and lipoxygenase-catalyzed lipid peroxidation seem to be a common feature involved in the seasonal regulation of both chlorophyll and carotenoid synthesis. Since the accumulation of Cr was much higher in submerged than in floating leaves it is expected that a more severe oxidative damage occurs in the first ones. In agreement with this assumption, LOX and MDA increases were higher in submerged leaves than in floating ones. Phenolics have a great antioxidative capacity based on the ability of their phenolic hydrogen to scavenge ROS (Rice-Evans et al., 1996). Estimation of phenolic compounds can be utilized to indicate the resistance of aquatic macrophytes to oxidative damage induced by heavy metals (Dai et al., 2006). In our study, a considerable accumulation of soluble phenolics in Cr-exposed floating leaves was observed in both seasons. By contrast, in submerged leaves, in general, Cr exposure decreased the content of soluble phenolics (Fig. 6). This result, apparently anomalous, may be produced by the highest levels of polymerized phenolics (i.e. lignin) localized in the cell wall of Cr-treated leaves (Podazza, personal communication). Agreeing with this fact, the polymerized phenolics are not extracted with ethanol 96% that is used for soluble phenolics extraction, and then a less phenolic content could be estimated. In agreement with this assumption it has been demonstrated that the main mechanism for Cd accumulation in aquatic roots occurs by metal binding to polymerized phenols (Kováčik and Klejdus, 2008). Proteins are especially prone to ROS attack and can be considered reliable indicators of the oxidative stress. In Cr-treated *S. minima* plants the content of soluble proteins decreased in winter submerged leaves with increasing Cr concentrations whereas it remained without significant changes in floating leaves. However, in summer the protein content was higher in Cr-treated than in Cr-untreated leaves. Although we cannot completely explain this opposite trend, we suggest that in winter the lowest metabolic activity determines a low Cr uptake, which is not able to reach the threshold to induce a de novo protein synthesis. Additional experiments are being planned to clarify these subjects and will be reported elsewhere.

5. Conclusions

According to our results, the metal accumulation in *S. minima* depends upon seasonal variation being higher in summer than in winter. This fact suggests that plant's metabolic activity is responsible for metal uptake. Data show a great variability in physiological and biochemical parameters. We have also emitted some considerations to explain the observed seasonal variability in terms of carbohydrate metabolism and oxidative stress. Based on the obtained results we conclude that in *S. minima* growing in outdoor conditions different seasonal mechanisms might come into play in terms of Cr accumulation and detoxification.

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