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Uptake of chromium by *Salvinia minima*: Effect on plant growth, leaf respiration and carbohydrate metabolism

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ABSTRACT

Metabolic responses to chromium (Cr) exposure and metal uptake were investigated using *Salvinia minima* plants. Cr treatment reduced the dry weight of floating and submerged leaves, while photosynthetic pigments were not affected. Measurements of respiratory oxygen uptake with and without inhibitors (KCN and SHAM) demonstrated that total respiration, alternative oxidase capacity and residual respiration were higher in Cr-treated than in Cr-untreated leaves, but the highest values were observed in floating leaves. Cr affected the soluble sugar content. Sucrose concentration was, in general, higher in Cr-treated than in Cr-untreated leaves, while the glucose concentration showed an inverse pattern. Cr also affected soluble acid invertase activity, but affectation trend was different between both leaves. Highest values of invertase activity were observed in Cr-treated floating leaves. According to our data soluble acid invertase and sucrose seem to be related to alternative oxidase capacity and residual respiration in floating and submerged leaves exposed to Cr. Thereby, this study constitutes an important contribution to understand metabolic relationships between mitochondrial respiration, alternative respiratory pathway and soluble carbohydrates in plants exposed to heavy metals.

1. Introduction

Heavy metals such as Cr, Cu, Pb, Sb, Hg, Ni and Cd are nowadays among the most important pollutants in surface and ground water [1]. They are often discharged by many industries, such as metal plating facilities, mining operations and tanneries, which can lead to contamination of freshwater and marine environments [2]. At present, a great body of information accumulated concerning plants' capability of accumulation and responses of functional systems to various heavy metals [1,3]. 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 [4,5]. The exposure of plants to heavy metals also stimulates the formation of reactive oxygen species (ROS) which can damage different macromolecules (lipids, proteins, nucleic acids), thereby to affect both mitochondrial respiration and carbohydrate metabolism [6–8]. Plant mitochondrial respiration comprises the cytochrome pathway (cyanide-sensitive) and the alternative pathway (cyanide-resistant) that includes an alternative oxidase activity (AOX) [9]. Beyond the branch point (ubiquinone), the alternative pathway does not contribute to generation of a protonmotive force, in contrast to the cytochrome pathway [10]. The biological function of AOX in thermogenic tissues is well known, but in non-thermogenic tissues it is not still completely understood [9]. It seems to be that its role is to allow the turnover of the Krebs cycle under high cytosolic energy charge or to protect cells against oxidative stress [10]. Furthermore, recent findings also suggest that function of the alternative pathway is to allow a flexible control of ATP synthesis and sucrose level to maintain a growth rate and homeostasis relatively stable [11]. Despite that heavy metals increase alternative respiratory pathway [7,12,13], the information about relationships between heavy metals, carbohydrate metabolism, and AOX is scarce [6].

Like other trace metals, chromium (Cr) in their most commonly occurring forms: trivalent chromium [Cr(III)] and hexavalent chromium [Cr(VI)] can produce beneficial or detrimental effects on plant metabolism being the latter most toxic and more readily assimilated by biotic systems [14]. Hexavalent chromium is highly soluble in water and forms strong divalent anionic oxi-

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dants [14]. Traditional technologies to remove and recover the Cr from polluted environments include different methods such as redox processes [15], ion-exchange [16], reverse osmosis [17], membrane separation [18], electrolysis [19], chemical precipitation [20] and absorption [21]. However, most of them are expensive and do not exhibit high treatment efficiency, especially at metal concentrations ranging between 10 and 100 mg L^{-1} [22]. Thereby, cheaper and effective extraction techniques are needed to enhance the Cr removal. Plant-based methods (phytoextraction) seem to be highly competitive among emerging techniques and they have been accepted worldwide [23]. Phytoextraction of Cr depends on metal-accumulating plants to absorb, transport and concentrate polluting Cr from the soil and/or water inside the plant [22]. Furthermore plants can reduce the toxic Cr(VI) to nontoxic Cr(III) giving an useful tool to get the detoxification and environmental cleanup in situ [15,24]. Several plant species such as *Eichhornia crassipes, Typha latifolia*, *Carex lurida*, *Prosopis* spp., *Allium sativum*, *Leptospermum scoparium*, and *Polypogon monspeliensis* have been identified that are excellent hyperaccumulators of Cr from contaminated soils [15,25,26]. Moreover, these plants are able to reduce a high percentage of Cr(VI) to give Cr(III) [14]. Hydroponically grown plants can also directly absorb, precipitate, and concentrate toxic metals from polluted effluents in a process termed rhizofiltration [27]. Among metal-accumulating plants, aquatic macrophytes possess better ability to metal absorption than terrestrial species. Macrophytes are known to assimilate heavy metals by surface adsorption and/or absorption and incorporate them directly inside their tissues [28,29]. Additionally, they also act as catalysts for redox reactions in the rhizosphere [24,30]. Among aquatic macrophytes, *Salvinia* species show high growth rates and accumulate high amounts of heavy metals [28–31]. *Salvinia minima*, a fast growing free-floating fern found in tropical and temperate regions, was chosen for the present study based on its ability to tolerate high hexavalent chromium concentrations [31]. *S. minima* consist of floating leaves (frond) joined in central nodes to another modified submerged leaves (root-like) [32]. Thereby, it is possible to hypothesize that in leaves of *S. minima* exposed to heavy metals take place different physiological events. The aim of this work was to examine the effect of Cr on mitochondrial respiration, soluble acid invertase and soluble carbohydrates in leaves of *S. minima*, to test the hypothesis that different relationships between mitochondrial respiration and sucrose metabolism occur during Cr exposure.

2. Materials and methods

2.1. Plant material

S. minima plants were collected according to uniform size from a non-polluted artificial pond located in the Campus of the School of Natural Sciences (26°50′N, 65°12′W, Tucumán, Argentina). After collection, plants were immediately transferred to 1 L polystyrene pots containing 800 mL of tap water for 3 days under outdoor conditions (*recuperation period*). Healthy plants with fully expanded leaves and uniform weight were selected, thoroughly rinsed with running tap water in order to eliminate any remains of sediment and microalgae and transferred to clean plastic boxes. Boxes were made with polycarbonate that has a low Cr adsorption capacity (San Diego Plastics, CA, USA). Ten plants (25.0 ± 1.1 g wet plant biomass) were floated in each box ($20 \times 15 \times 5$ cm) containing 1 L of 1/32 diluted Hoagland's nutrient solution [33]. Plants were grown in a temperature controlled chamber with a 14/10 h (day/night) photoperiod, 25/20 °C (day/night) temperature regime, 60% relative humidity, and 190 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) (measured with a quantum sensor LI-COR, Lincoln, NE, USA) at the plant level) (*acclimation period*) [34]. PAR irradiation was providing by 12 white fluorescent lamps (Philips TL40, Argentina). After 2 days, acclimated plants were rinsed with running tap water and transferred to clean polycarbonate boxes containing either tap water corresponding to the control (Cr-untreated plants), or 2, 5, and 10 mg L^{-1} Cr concentrations (Cr-treated plants) (*treatment period*). Cr was provided as potassium dichromate ($K_2Cr_2O_7$) and Cr solutions were prepared in tap water. Tap water was used in order to get a similar aquatic medium than the pond where *Salvinia* plants were collected. In addition, there was no difference in plant growth using a distilled water Cr solution [23]. Plants under Cr treatment were not cultivated in Hoagland's solution to avoid chelation and/or cation competition between Hoagland salts and potassium dichromate. Tap water physico–chemical characteristics according to regulation of the SEPAPYS (Servicio Provincial de Agua Potable y Saneamiento, Tucumán, Argentina) were: pH 7.3; EC (μS cm^{-1}) 200; DO (mg L^{-1}) 3.0; TDS (mg L^{-1}) 100; Turbidity (NTU) < 1; As (μg L^{-1}) 0.20; 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}) 9.0; PO_4^{2-} (mg L^{-1}) < 0.2; HCO_3^- (mg L^{-1}) 50.0; SO_4^{2-} (mg L^{-1}) 10.0; Cl^- (mg L^{-1}) 12.0; Ca^{2+} (mg L^{-1}) 10.0; Mg^{2+} (mg L^{-1}) 5.0; Na^+ (mg L^{-1}) 20.0; K^+ (mg L^{-1}) 5.0; 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 L^{-1}) 212. DO = dissolved oxygen; EC = electrical conductivity; TDS = total dissolved solutes; NTU = nephelometric turbidity unit). During the experiment, tap water was added to reach initial volume level at definite intervals (2 days) to compensate water loss by transpiration and evaporation. Because Cr remotion from water by aquatic macrophytes is a fast process [3], the Cr solution was renewed at 3 days to avoid excessive changes in Cr concentration. The pH of Cr solution and control tap water ranged between 6.8 and 7.0 during the experimental period and it was daily measured using a glass combination pH-sensitive electrode coupled to a digital pH meter (Hanna Instrument, Germany). At this pH value, spontaneous reduction of Cr(VI) to Cr(III) and/or Cr(III) precipitation were not detected [35]. Plants were grown for 6 days under previously described conditions. It is worth noting that we chose as experimental period 6 days because preliminary experiments carried out during 9 days showed slightly symptoms of chlorosis and necrosis in Cr-treated plants. For chemical determinations and respiration measurements plants were harvested, rinsed in distilled water and separated in floating and submerged leaves. In order to minimize any diurnal effect on the carbohydrate content all samples were collected at noon.

Wet plant biomass (FW) was immediately determined after harvesting whereas the dry plant biomass (DW) was determined by drying weighed wet samples at 80 °C in a hot air oven for 4 days and weighed again.

2.2. Extraction and quantification of photosynthetic pigments

Chlorophyll and carotenoids were extracted from 10 mg (wet plant biomass) of floating leaves with 2 mL of dimethyl sulfoxide. Samples were incubated in darkness for 12 h at 45 °C. Chlorophyll *a*, *b* and carotenoids were determined spectrophotometrically at wavelengths of 665, 649 and 480 nm. Pigment concentrations were calculated using Wellburn's formulae [36], and expressed as μg g^{-1} FW.

2.3. Respiration measurement and determination of AOX capacity

Total respiration, and cytochrome and alternative respiratory activities of *S. minima* leaves were measured polarographically with the Clark electrode (Yellow Springs Instrument Co. USA) and recorded in a Gilson oxygraph (Gilson Medical Electronics, Inc.

USA), at 28 °C in a thermostatted cell. Floating and submerged leaves from both Cr-untreated (control) and Cr-treated plants were harvested in the sixth day and sliced with a razor blade at approximately 1 mm strips to yield 70 and 100 mg (wet plant biomass) of floating and submerged leaves, respectively. Strips were submerged in 2 mL of an air saturated solution (initial concentration of O_2 was considered to be 240 μM) containing 50 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.0. This pH value was selected because it is similar to pH of the artificial pond. The oxygen consumption was measured using 1 mM KCN as inhibitor of the cytochrome pathway and 3 mM SHAM (salicylhydroxamic acid) as inhibitor of the alternative oxidase. These concentrations of KCN and SHAM had no apparent side effect in our system and produced maximal inhibition, as indicated by titration curves using different concentrations of each inhibitor in the presence and absence of the other. Steady rates of respiratory O_2 consumption were determined after about 20 min under assay conditions. Alternative oxidase capacity (AOX capacity) defined as the SHAM-sensitive O_2 uptake in presence of KCN [37], was determined by subtracting the rate of O_2 uptake in presence of both KCN and SHAM from the rate of O_2 uptake in presence of only KCN. The O_2 uptake in presence of SHAM and KCN is called residual respiration. Although the role of residual respiration is unclear it has been suggested that it is apparently related to the activity of supplementary terminal oxidases, including Cu-containing oxidases and/or other non-mitochondrial oxidases [38,39]. Respiration data were expressed as nmol O_2 mg^{-1} FW min^{-1}.

2.4. Extraction and quantification of soluble carbohydrates

Soluble carbohydrates were extracted from 1 g (wet plant biomass) of floating and submerged leaves by homogenization in 2 mL of 80% (v/v) ethanol with a mortar and pestle. The homogenate was heated in a water bath at 75 °C for 10 min and the insoluble fraction removed by centrifugation at 5000 × *g* for 10 min. The precipitate was resuspended in 2 mL of 80% (v/v) ethanol and centrifuged again. Supernatants were pooled and dried under a stream of hot air. Resulting residue was resuspended in 1 mL of distilled water and desalted by filtration through an ion-exchange column (Amberlite MB3, England). Sucrose was determined by the protocol of Cardini et al. [40] and fructose by the method of Roe and Papadopoulos [41]. Glucose was determined using a glucose–oxidase–peroxidase coupled assay according to Jorgensen and Andersen [42]. Soluble sugars were expressed as mg g^{-1} FW.

2.5. Extraction and assay of soluble acid invertase

All extraction steps were carried out at 4 °C. Soluble acid invertase was extracted from 1 g (wet plant biomass) of floating and submerged leaves by homogenization in a chilled mortar with 3 mL of extraction solution containing 50 mM NaH_2PO_4/Na_2HPO_4 buffer, pH 7.4, 1 mM β-mercaptoethanol and 5 μM $MnSO_4$. Resulting homogenate was centrifuged at 12,000 × *g* for 15 min and the supernatant dialysed against 10 mM sodium acetate buffer, pH 5.5, containing 1 mM β-mercaptoethanol for 60 min. Because extraction solution did not contain detergent, chelating and chaotropic agents or high salt concentrations, the probability of extracting cell wall invertase activity was very low. Invertase activity was measured spectrophotometrically as previously described by Prado et al. [43]. Briefly, 50 μL of extract was added to reaction mixture containing 80 mM sodium acetate buffer, pH 5.5, 60 mM sucrose, 1 mM β-mercaptoethanol, and water in a final volume of 200 μL. The reaction was incubated at 37 °C for 60 min in a water bath and stopped by addition of 0.5 mL copper-alkaline reagent. Reducing sugars released were estimated by the Somogyi-Nelson method [44]. Enzyme activity was expressed as μmol reducing sugars g^{-1} FW min^{-1}.

2.6. Estimation of total Cr content

Cr content of *S. minima* tissues was determined from 0.5 g (dry plant biomass) of floating and submerged leaves by HNO_3 wet-digestion following the USEPA 3051 method [45]. The digestion was carried out at 115 °C for 15 min and the Cr content determined by atomic absorption spectrophotometry (Perkin-Elmer 373, England). Cr content was expressed as μg g^{-1} DW.

2.7. Statistical analysis

Differences among treatments were analyzed by one-way ANOVA, taking $p < 0.05$ as significant according to Tukey's Multiple Range Test. Results are the average of three independent experiments and are represented as mean ± SD.

3. Results

3.1. Dry plant biomass (DW) and photosynthetic pigments

Table 1 shows the effect of Cr on DW and photosynthetic pigments of *S. minima* plants after 6 days of metal exposure. Dry plant biomass of the whole plant and both floating and submerged leaves were significantly reduced ($p < 0.05$) at 10 mg L^{-1} Cr concentration. Submerged leaves were more affected than floating leaves with 38.2% DW reduction for the former and 23.2% for the latter. Chlorophyll and carotenoid contents, and Chl *a*/*b* ratio in floating leaves were not affected by Cr treatment (Table 1). Data of photosynthetic pigments based on leaf DW showed a similar pattern (not shown). In submerged leaves of both control and Cr-treated plants, photosynthetic pigments were not detected. Values in Table 1 indicate means ± SD.

3.2. Respiration measurements and AOX capacity

After exposure of *S. minima* plants to Cr solution, total respiration and AOX capacity were differentially increased in both floating and submerged leaves. At 5 mg L^{-1} Cr concentration values of total respiration and AOX capacity of floating leaves were 2.53 and 0.346 nmol O_2 mg^{-1} FW min^{-1} respectively, being increased by

Table 1
Dry plant biomass (DW) of the whole plant and both floating and submerged leaves as well as chlorophyll (*a* and *b*) and carotenoid concentrations, and Chl *a*/*b* ratio of *S. minima* plants exposed to different Cr concentrations during a 6 days period. Data of DW are per plant. Values are mean ± SD of three independent experiments ($n = 10$). Values in each column followed by the same letter are not significantly different at $p < 0.05$ (Tukey's Multiple Range Test).

Cr (mg L^{-1})	Whole plant	Floating leaves	Submerged leaves	Chl *a*	Chl *b*	Chl *a*/*b*	Carotenoids
	(mg DW)			(μg g^{-1} FW)			(μg g^{-1} FW)
0	65.5 ± 2.9a	50.0 ± 3.1a	15.2 ± 1.5a	596.1 ± 62a	237.4 ± 17a	2.51 ± 0.12a	96.33 ± 4.81a
2	64.1 ± 2.2a	48.9 ± 2.0a	13.4 ± 1.0a	576.4 ± 39a	240.4 ± 26a	2.40 ± 0.11a	99.52 ± 5.35a
5	60.3 ± 2.0a	49.2 ± 2.2a	10.3 ± 1.1b	578.0 ± 46a	242.2 ± 23a	2.39 ± 0.15a	94.86 ± 4.80a
10	48.3 ± 1.9b	38.4 ± 3.0b	9.4 ± 1.8b	608.5 ± 56a	246.3 ± 17a	2.47 ± 0.08a	99.72 ± 6.16a

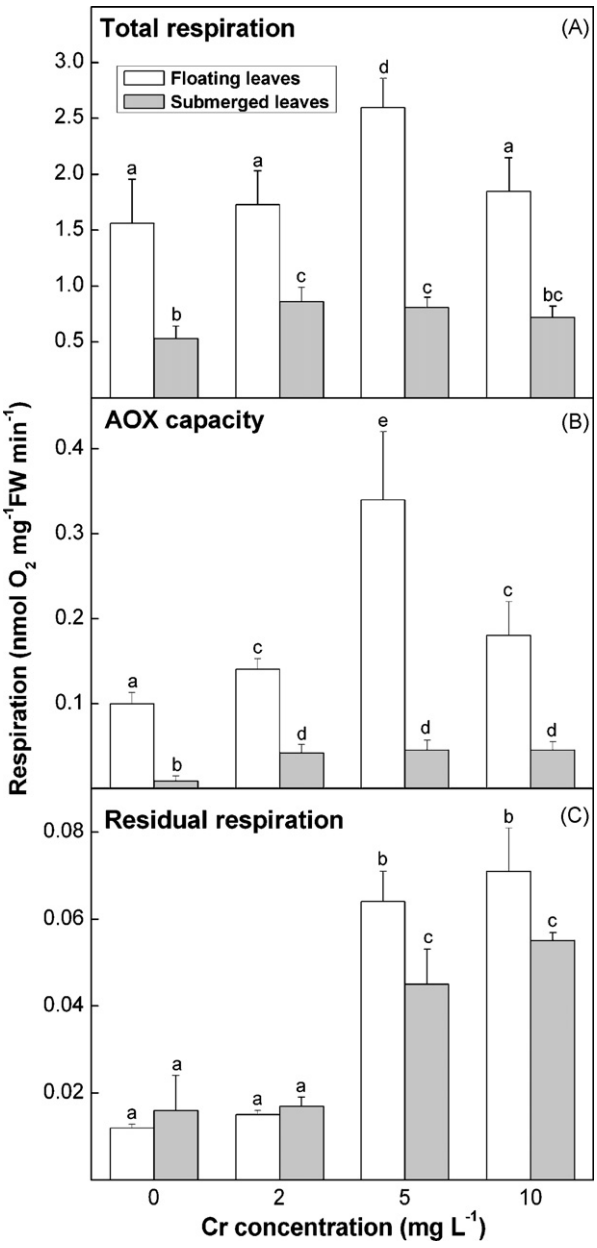

Fig. 1. Respiration of floating and submerged leaves of *S. minima* after 6 days of exposure to different Cr concentrations. (A) Total respiration, (B) AOX capacity, (C) Residual respiration. Values are mean ± SD of three independent experiments ($n = 10$). Lines on top of the bars represent standard deviation (SD). Bars with different letters are significantly different at $p < 0.05$ (Tukey's Multiple Range Test).

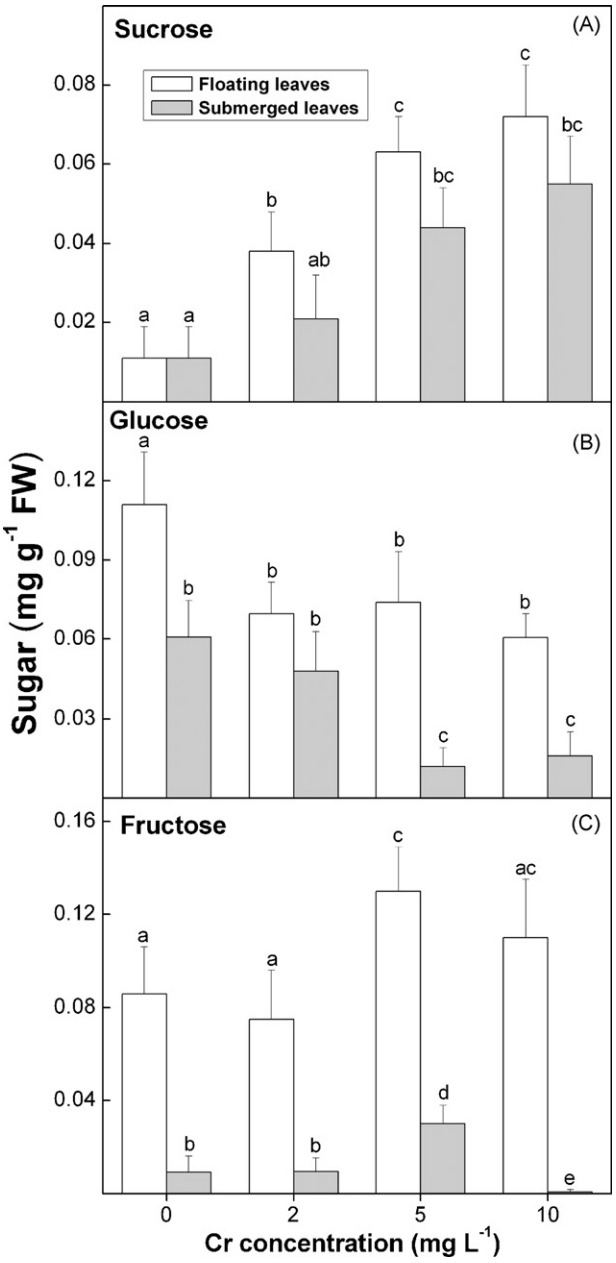

Fig. 2. Content of soluble carbohydrates in floating and submerged leaves of *S. minima* after 6 days of exposure to different Cr concentrations. (A) Sucrose, (B) Glucose, (C) Fructose. Values are mean ± SD of three independent experiments ($n = 10$). Lines on top of the bars represent standard deviation (SD). Bars with different letters are significantly different at $p < 0.05$ (Tukey's Multiple Range Test).

164.3% and 349.5% over control values (1.54 and 9.90×10^{-2} nmol O_2 mg^{-1} FW min^{-1}, respectively). AOX was less affected by 2 and 10 mg L^{-1} Cr concentrations, while total respiration was not significantly ($p < 0.05$) affected. By contrast, in submerged leaves maxima values of both parameters were observed at 2 mg L^{-1} metal concentration (0.805 and 3.98×10^{-2} nmol O_2 mg^{-1} FW min^{-1}, respectively), being increased by 161.0% and 251.9% over control values (0.50 and 1.58×10^{-2} nmol O_2 mg^{-1} FW min^{-1}, respectively). Thereafter they remained relatively stable; however, the highest values of total respiration and AOX capacity were observed in floating leaves (Fig. 1A and B). It is necessary to point out that AOX measurements do not reflect actual AOX activity during respiration in uninhibited conditions, because the addition of inhibitors may modify the partitioning of electron flux over the cytochrome respiratory pathway and alternative respiratory pathway [46]. Thus, AOX capacity shows activity only under the given set of conditions used. Residual respiration ranged between 1.7% and 6.5% in floating leaves and 2.0–9.8% in submerged leaves of total O_2 uptake (Fig. 1C). Values in Fig. 1 indicate means ± SD.

3.3. Soluble carbohydrates

Soluble sugars were affected by Cr treatment in both floating and submerged leaves (Fig. 2). Results showed that the content of sucrose and fructose at both 10 mg L^{-1} and 5 mg L^{-1} Cr concentrations was higher in Cr-treated than in control leaves (Fig. 2A and C). By contrast, the glucose content was highest in control leaves (Fig. 2B). Although each sugar showed, in general, a similar distribution pattern in both leaves, the highest values were

Table 2
Cr concentration in floating and submerged leaves of *S. minima* plants after treatment with different Cr concentrations during a 6 days period. Values are mean ± SD of three independent experiments ($n = 4$).). Values in each column followed by the same letter are not significantly different at $p < 0.05$ (Tukey's Multiple Range Test).

Cr (mg L^{-1})	Floating leaves	CF	Submerged leaves	CF	Submerged/floating ratio
	(μg g^{-1} DW)		(μg g^{-1} DW)		
0	ND	ND	ND	ND	ND
2	92.4 ± 7.3a	46 ± 5a	498.1 ± 13.0a	249 ± 8a	5.39 ± 0.11a
5	243.2 ± 12.0b	49 ± 3a	1163.3 ± 32.3b	233 ± 7b	4.78 ± 0.15b
10	484.5 ± 20.2c	48 ± 4a	2210.1 ± 33.6c	221 ± 8b	4.56 ± 0.08c

ND = not determined.

always observed in floating leaves. Carbohydrate data based on leaf DW showed a similar trend (not shown). Values in Fig. 2 indicate means ± SD.

3.4. Cr content

Analysis of Cr content in *S. minima* revealed a higher metal accumulation in submerged leaves compared with floating leaves, but it was not found in control plants. Cr accumulation increased in both floating and submerged leaves with the increase of Cr concentration in the growth solution, but the accumulation was higher in submerged than in floating leaves. Maxima accumulation values for floating and submerged leaves were 484.5 and 2210.1 μg g^{-1} DW at 10 mg L^{-1} Cr concentration (Table 2). The concentration factor (CF) defined as [Cr concentration (mg g^{-1} DW) in leaves/Cr concentration (mg mL^{-1}) in the solution at equilibrium] ratio [47], ranged in submerged leaves from 249 to 221 whereas in floating leaves from 46 to 49, depending on initial Cr concentration. The submerged/floating ratio ranged from 5.39 to 4.56 between 2 and 10 mg L^{-1} Cr concentrations (Table 2). Values in Table 2 indicate means ± SD.

3.5. Acid invertase activity

Invertase activity was differently affected by Cr treatment in floating and submerged leaves of *S. minima* (Fig. 3). Exposure to 2 and 10 mg L^{-1} Cr concentrations significantly reduced ($p < 0.05$) invertase activity in floating leaves, while in submerged leaves it was only affected by the last concentration. The highest value of enzyme activity in Cr-treated floating leaves was observed at 5 mg L^{-1} Cr concentration, but there was no significant difference ($p < 0.05$) with 0 mg L^{-1} (control). Submerged leaves also showed the highest value of enzyme activity at 5 mg L^{-1} Cr concentration, but it was 5.6-fold higher than control. However, when activity is compared between Cr-treated leaves the highest values were observed in floating leaves. Since in Cr-treated leaves metal could bind to enzyme, 0.92 μg of Cr was added to reaction mixture of Cr-untreated leaves. No change in enzyme activity was observed (data not shown). The amount of Cr added was calculated from the value of 2694.6 μg g^{-1} DW that it is the Cr content found in the whole plant (floating and submerged leaves) exposed to 10 mg L^{-1} Cr concentration. Invertase activity based on leaf DW showed a similar trend (not shown). Values in Fig. 3 indicate means ± SD.

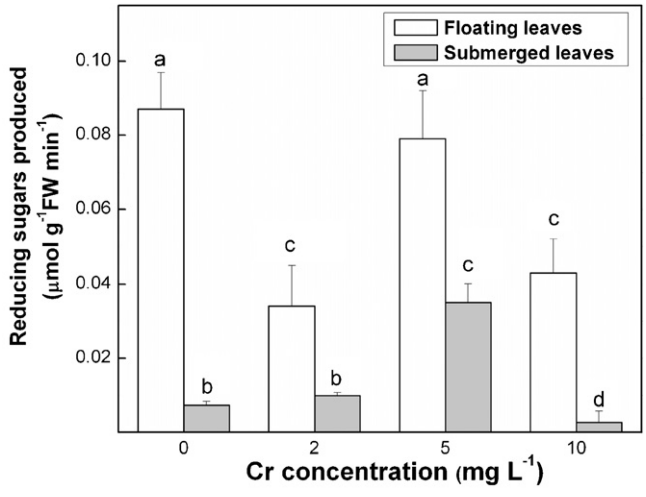

Fig. 3. Soluble acid invertase activity in floating and submerged leaves of *S. minima* after 6 days of exposure to different Cr concentrations. Values are mean ± SD of three independent experiments ($n = 10$). Lines on top of the bars represent standard deviation (SD). Bars with different letters are significantly different at $p < 0.05$ (Tukey's Multiple Range Test).

4. Discussion

Results show that submerged leaves of *S. minima* are capable of accumulating substantial concentration of Cr, up to 2.21 mg g^{-1} DW from a 10 mg L^{-1} Cr solution. The concentration factor (CF) of submerged leaves was lower than that reported for other *Salvinia* species [28,30,31]; however, with time-exposure longer than 6 days, the values of CF significantly increased [23]. Floating leaves did not show significant variations in CF values at all assayed Cr concentrations, but the submerged/floating ratio decreased from low to high Cr concentration. According to Maine et al. [3] this fact set out the hypothesis of whether Cr translocation to aerial parts (floating leaves) was a fast process or if increase in the Cr content in aerial parts was due to Cr uptake by leaves in contact with metal solution. These authors demonstrated that floating leaves of *Salvinia herzogii* directly absorb a high percentage of trivalent chromium from growth solution and that this process is the main cause of the increase of Cr in aerial parts. In that context, they demonstrated that Cr concentration of floating leaves in contact with Cr solution was approximately 5-fold higher than in non-contact leaves [3]. Despite that Maine's study was carried out with Cr(III), it has been demonstrated that several aquatic macrophytes such as *Salvinia auriculata*, *Pistia stratiotes* and *Eichhornia crassipes* reduce Cr(VI) to one that is less toxic Cr(III) during metal biosorption process at the roots [15,24]. In addition, it has been demonstrated that for hexavalent chromium there is an equilibrium between $Cr_2O_7^{2-}$ and CrO_4^{2-} depending on solution pH according to the reaction:

$$2CrO_4^{2-} + 2H^+ \leftrightarrow Cr_2O_7^{2-} + H_2O$$

Then, in concentrations ranging from 0.018 to 184 mg L^{-1} more than 80% of $K_2Cr_2O_7$ is found as CrO_4^{2-} at pH 7.2 whereas $HCrO_4^-$ is found in a less percentage [34]. Because of structural similarity between CrO_4^{2-} and SO_4^{2-} the uptake of hexavalent chromium is a metabolically mediated process via sulphate-transport system located in the cell membrane, thereby Cr(VI) is readily incorporated inside the plant [48,49]. Hence, based on these findings we believe that floating leaves of *S. minima* absorb substantial amounts of Cr directly from metal solution, then is expected that submerged/floating ratios decrease from low to high Cr concentration of growth solution. Nevertheless, we do not measure the uptake

of Cr by floating leaves, thus this supposition needs further studies for its confirmation. Different plants vary in their ability to absorb and accumulate hexavalent chromium in their tissues [50]. However, most of them accumulate higher Cr concentrations in roots than that aerial parts due to the ability of plant roots to convert CrO_4^{2-} to Cr(III), which is retained in root cortex cells and poorly translocated to aerial parts [3,48–51]. Our results showed that like roots, the submerged leaves of *S. minima* also accumulated high Cr concentrations. At all Cr concentrations studied the concentration of Cr in submerged leaves was nearly to 5-fold higher than in floating leaves (Table 2). These results agree with similar data previously reported for *S. herzogii* under Cr(III) exposure [3]. Therefore, *Salvinia* plants seem to be useful to remove Cr from contaminated environments, independently if hexavalent chromium is reduced or not prior to cell uptake.

Previous studies demonstrated that Cr exposure decreased total dry matter production and yield in *Salvinia* species [30,31,52]. Agreeing with these findings our results demonstrated that the growth of *S. minima*, based on dry biomass production was significantly decreased by 10 mg L^{-1} Cr concentration. According to Nichols et al. [52] the decrease of dry biomass under increasing hexavalent chromium concentrations, can be attributed to decline in CO_2 assimilation due to a metal-induced reduction of photosynthetic pigments. However, in the present study no changes in chlorophyll and carotenoid concentrations were observed (Table 1). In agreement with our data, no change in chlorophyll content was also observed for *S. natans*. Even in this species chlorophyll *b* concentration slightly increased when plants were exposed to Cr solution [30]. Hence, it is evident that Cr may also produce growth reduction by interfering with other cellular processes. However we do not measure CO_2 assimilation, then this supposition cannot be confirmed. Furthermore, the unchanged chlorophyll concentration observed in our study could also be attributed to shorter Cr exposure than that Nichols' work. In support of this assumption, it has been observed a direct correlation between heavy metal exposition and chlorophyll loss [53]. Although the chlorophyll loss has been attributed to decrease of chlorophyllase activity induced by heavy metals [54], it can also be related to membrane oxidative damage produced by the heavy metal-induced ROS generation [12,13]. In that context, Cr produces both oxidative stress and ROS generation [34,55]. Nevertheless, Cr also produces alterations in mitochondrial respiration [8]. Although information on Cr accumulation in mitochondria is lacking, there is evidence that Cr has access to functional elements of the electron transport chain in root mitochondria [8]. Inhibition of respiration in presence of an excess of metal ions, and its increase under mild metal stress was reported that occur in animal and plant mitochondria [56]. Inhibition of electron transport by Cr may be due to its interaction with Cu and Fe ions of electron carriers (non-heme iron–sulphur protein and heme proteins, the cytochromes) undergoing redox changes during electron fluxes. Cytochromes in mitochondria may directly transfer electrons to Cr to reduce it, or alternatively the reduced heme group in cytochromes may serve as a ligand for Cr to block the electronic transfer [8]. Cr-induced blockage of electron transport brings about a significant enhancement in ROS generation [56]. Cyanide-insensitive alternative oxidase in plant mitochondria has been shown to limit ROS production under stress conditions to provide an additional route for dissipating electrons while avoiding single electron-reduced components. Moreover, mitochondrial respiration (through both cytochrome and alternative pathways) optimizes chloroplast photosynthesis by modulating cellular metabolites related to both intracellular redox state and sucrose synthesis [9,38]. Stimulation of the alternative oxidase pathway would also allow consumption of carbon excess, to correct imbalance between carbohydrate supply and demand, thus controlling anabolism and growth [57]. Additionally, it has been demonstrated that heavy metal stress reduces the rate of glycolysis and pyruvate content, being accompanied by an increase in the contribution of oxidative pentose phosphate pathway and residual respiration [58]. In these conditions, plants can switch biochemical carbohydrate pathway from primary to secondary metabolism. Hence, although exact role of residual respiration is unclear it could serve to burn the excess of metabolites toward secondary metabolism to enhance the synthesis of protective metabolites (*e.g.* flavonoids, cinnamic acids, phenolics) [58]. Agreeing with this supposition in a preliminary study carried out in our laboratory was demonstrated that both floating and submerged leaves of Cr-treated *S. minima* plants accumulate high phenolic concentrations (unpublished results). In that context, relationships between Cr accumulation, carbohydrate metabolism and mitochondrial respiration are expected that can take place in *S. minima* exposed to Cr solution. Our results demonstrated that Cr exposure increases both total respiration and alternative respiratory pathway, based on AOX capacity in floating and submerged leaves of *S. minima* plants, being highest values in the former (Fig. 1A and B). Although relationships between carbohydrate metabolism and alternative respiratory pathway have been studied [59–61], they were not completely clarified. Whereas Millenaar et al. [59] showed that relative contribution of the alternative pathway to mitochondrial respiration increased by the decrease of sugar concentration; Padmasree et al. [60] demonstrated that alternative respiratory pathway was directly depending of the soluble sugar level. In contrary, González-Meler et al. [61] reported that functionality of the alternative respiratory pathway was independent of the sugar concentration. Moreover, it has been reported that under stress condition, sucrose synthesis might act as an effective sink for the excess of ATP through the alternative respiratory pathway [62]. Agreeing with this finding our data showed increase of sucrose concentration in Cr-treated leaves (Fig. 2A). Enhancement of sucrose content was also observed in *Azolla caroliniana* in presence of hexavalent chromium [63] and *S. minima* in presence of aluminium [64]. According to these findings we considered that the absorbed Cr might affect activities of enzymes involved in sucrose metabolism. Thereby, our data showed that soluble acid invertase (the main enzyme catalyzing the cleavage of sucrose) was affected in *S. minima* exposed to Cr (Fig. 3). However, the affectation pattern was different between floating and submerged leaves. Because Cr does not inhibit invertase activity *"in vitro"*, this fact could be due to metabolic differences between photosynthetic (floating) and non-photosynthetic (submerged) leaves. Moreover, our results also indicate that invertase activity was significantly ($p < 0.05$) decreased by the highest metal concentration, especially, in submerged leaves (Fig. 3). Additionally, this fact could explain the low level of glucose and fructose observed in such leaves (Fig. 2B and C). Agreeing with this supposition, the inhibition of invertase activity by heavy metals has also been communicated for other plants [65]. Furthermore, previous studies performed in our laboratory have demonstrated that Cr exposure increased the activity of sucrose synthase (SS) (the predominant enzyme catalyzing the first reaction of starch formation) in floating leaves of *S. minima* [66]. Additionally, we demonstrated that floating leaves of *S. minima* plants accumulate significant starch amounts [23]. Increases in SS activity and starch content were also reported for *A. caroliniana* exposed to hexavalent chromium [63]. In fact, soluble acid invertase seems to play a more crucial role than that SS to supply soluble sugars for plant respiration under Cr stress. Agreeing with this assumption the glucose/fructose ratio in floating leaves of *S. minima* ranged between 1.1 and 1.7 indicating that invertase is the enzyme involved in sucrose–hexose conversion. By contrast, in accordance with changes observed in carbohydrate pattern, glucose and fructose were more substantially decreased for Cr treatment in submerged leaves, with glucose/fructose ratios

ranging from 4.6 to 4.9. This fact could reflect a decrease in invertase/sucrose synthase ratio and concomitantly an increase in the content of phosphorylated glucose that is required to starch synthesis [67]. However, the starch content in submerged leaves of Cr-treated plants is very low and sucrose synthase activity is decreased [23,66]. Then, it is possible to argue that soluble invertase and other enzymatic activities linked to carbohydrate metabolism can be operating in submerged leaves to get an energy conserving balance and thereby to maintain a reduced ATP consumption to cope with the accumulated metal. In support of this hypothesis submerged leaves of Cr-treated *S. minima* plants exhibited percentages of residual respiration higher than that observed in floating leaves (Fig. 1C). Hence, we assumed that different metabolic events related to soluble sugars take place in both floating and submerged leaves of *S. minima*, and soluble acid invertase probably plays an important role against Cr toxicity.

5. Conclusions

S. minima plants seem to have high Cr tolerance and a good metal accumulation capacity. Submerged leaves accumulated most of Cr and this may be an important mechanism for Cr tolerance and accumulation, thereby it can alleviate Cr toxicity in floating leaves. Sustaining the hypothesis that entry of Cr inside leaves may be accomplished by reduction to Cr(III) or mediated by the sulphate- transport system, the absorbed Cr affects physiological and metabolic parameters of *S. minima* plants. Results suggest the existence of interactions between Cr accumulation and mitochondrial respiration, and indicate that Cr-induced variations of soluble acid invertase activity and sucrose concentration seem to be related to changes in the alternative oxidase capacity in both floating and submerged leaves. Given these results, this study represents an important contribution to understand metabolic network between growth, respiration and carbohydrate metabolism in plants exposed to heavy metals. However, since we only used hexavalent chromium as treatment solution, a new experiment using solution containing trivalent chromium is being planned and will be reported elsewhere. Furthermore, our results also suggest that selection of suitable aquatic macrophytes for potential application in phytoremediation might require an additional focus on relationships between heavy metals and the primary metabolism of the considered plant species. Also, this work represents the first communication on the alternative respiratory pathway in the genus *Salvinia*.

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