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Detoxification of Cr(VI) in *Salvinia minima* is related to seasonal-induced changes of thiols, phenolics and antioxidative enzymes

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HIGHLIGHTS

- Cr(VI) induces oxidative stress in *Salvinia* leaves.
- Cr(VI) is mostly accumulated in submerged leaves.
- MDA increases in Cr-treated leaves.
- Antioxidative enzymes increase by Cr(VI).
- Phenolics and thiols vary inversely under Cr(VI).

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ABSTRACT

In this study, protein- and non-protein-thiol-containing compounds (THCC), soluble phenolics (SP), proline (Pro), proteins and malondialdehyde (MDA) contents, and antioxidative enzyme activities were analyzed in floating and submerged leaves of *Salvinia minima* to establish their role against Cr-induced oxidative stress. We analyzed relationships among biochemical responses to different Cr(VI) concentrations to explore underlying mechanisms of Cr detoxification in plants growing under field conditions during summer and winter seasons. Significant increases in THCC were observed in submerged leaves from both seasons, while in floating leaves THCC increased only in summer being decreased in winter. Contrarily SP increased in floating leaves and decreased in submerged ones. MDA increased significantly in winter-leaves, but in summer-leaves remained unchanged. Antioxidative enzymes, *i.e.* guaiacol peroxidase (G-POD), superoxide dismutase (SOD) and catalase (CAT) showed different activity patterns. G-POD significantly increased in Cr-treated leaves from both seasons, while SOD increased in submerged leaves only, remaining practically unchanged in floating ones. CAT activity increased in floating leaves from both seasons, whereas in submerged ones was decreased or increased. Proteins increased in both leaf types during summer whereas decreased or remained unchanged in winter. Pro increased in winter-submerged leaves only. Results show that seasonal-induced changes occur in all measured parameters.

1. Introduction

Chromium (Cr) is one of the most toxic pollutants commonly occurring in the environment. Anthropogenic release of Cr from leather, electroplating, chromic acid production and refractory steel industries constitutes the main sources of Cr pollution [1]. Cr is not destroyed by degradation and for long time accumulates in the environment giving an increased contamination of soils and water bodies, which has raised severely the menace of this toxic element in the last few decades. Most widespread forms of Cr are trivalent [Cr(III)] and hexavalent [Cr(VI)], which are sufficiently stable to occur naturally. Both Cr(III) and Cr(VI) have different physicochemical properties, the first one being positively charged and less soluble in water than the highly soluble and negatively charged anionic Cr(VI) [2]. Cr(VI) is more mobile than Cr(III) and has a long residence time in surface water and groundwater. It is considered the most toxic form of Cr and poses health risk to humans and animals and impairs the development and growth of plants [3]. Cr(VI) acts as strong oxidizing agent on biological systems and affects several metabolic processes through the induction of oxidative stress, that is the main process underlying Cr toxicity in plants [4]. Plants respond in a variety of different ways to Cr(VI)-induced oxidative stress to avoid its detrimental effects. Plants mainly combat oxidative stress with an array of antioxidative enzymes such

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as guaiacol peroxidase (G-POD), catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and ascorbate reductase (AR) [5]. However, ability of plants to cope with Cr(VI) toxicity also depends of their capacity to accumulate the metal into their tissues and to develop an optimum tolerance for accumulated metal. Plants can reduce toxic levels of Cr(VI) through different mechanisms including cell wall binding, exclusion through ion-selective transporters, and vacuolar compartmentalization [6]. Recent research advances have shown that Cr(VI) detoxification in plants can also be achieved by chelation through active participation of protein-thiol-containing compounds such as phytochelatins (PC) and metallothioneins (MT) [7]. Despite their roles in Cr complexation, THCC also participate in other physiological functions of plants such as metal trafficking and reactive oxygen species (ROS) scavenging [8].

According with Sharma and Dietz [9] carbon and nitrogen allocation pattern, hormonal balance and climatic conditions affect the selection of a determined mechanism against heavy metal toxicity. However, scant information is available on seasonal effects on relationships of THCC with other metabolites and antioxidative enzymes in the mitigation of the oxidative stress induced by Cr(VI). Therefore, we assumed as hypothesis of this work that different seasonal-dependant metabolic events occur in Cr-stressed aquatic plants. The aim of this study was to analyze seasonal relationships between Cr(VI) accumulation and levels of THCC, SP, free Pro, proteins, MDA and antioxidative enzymes (SOD, CAT and G-POD), to explore underlying mechanisms of Cr detoxification occurring in the aquatic fern *Salvinia minima* exposed to different Cr(VI) concentrations under field conditions during summer and winter seasons.

2. Materials and methods

2.1. Plant material

Plants of *S. minima* were collected from a heavy metal non-polluted fresh water pond located at 500 m asl (26°50′S, 65°12′W, Tucumán, Argentina). Plant collection was performed in summer (December to February) 2009/2010 and winter (July to August) 2010. More detailed cultivation conditions and Cr(VI) treatment have been published earlier [10].

2.2. Climatic data

Air temperature and sunlight duration at the experimental site were monitored continuously using an automatic meteorological station (Pegasus EP1000, Argentina). Temperature of Cr(VI) solution was measured with a portable underwater thermistor (-20 ± 1 to $+70 \pm 1$ °C) connected to a Hobo Temp Logger (Onset Computer Corp., Bourne, MA, USA). Photosynthetic active radiation (PAR) and ultraviolet-B radiation (UV-B) were measured using a Quantum Sensor (Li-190SA, Li-Cor, Lincoln, NE, USA) coupled to a Data-Logger (Li-1000, Li-Cor, Lincoln, USA), and a silicon photoelectric cell coupled to a photometer/radiometer (PMA2100, Version 1.17, Solar Light Company, Inc., PA, USA), respectively. PAR was measured at midday when solar radiation reaching earth's surface is higher, while UV-B was recorded daily between 8:00 a.m. and 6:00 p.m. at 1-h interval including sunny and cloudy days to get a better approach of the solar radiation reaching plants.

2.3. Cr(VI) accumulation

Cr(VI) was determined in oven-dried submerged and floating leaves after digestion with HNO_3 at 115 °C by atomic absorption spectrophotometry. Chromium content was expressed as $\mu g\, g^{-1}$ DW. To ascertain that Cr(VI) was the only form of Cr presents in the treatment solutions prior to *S. minima* transplantation and after finishing the experiment, we measured Cr(VI) concentration using the colorimetric 1.5-diphenylcarbazide assay. In acid medium (pH 1–2) Cr(VI) ions develops a purple–violet color with 1.5-diphenylcarbazide as a complexing agent [11]. The absorbance of the purple–violet colored solution was read at 540 nm after 10 min at room temperature. To oxidize Cr(III) produced by a possible reduction of Cr(VI), reaction mixture was added with 4–5 drops of $KMnO_4$ solution. Permanganate in acid medium is able to oxidizing completely Cr(III) to Cr(VI) [12]. To calculate deviation of the analytical method of Cr(VI) a calibration curve was prepared from the standard solution. Standard deviation obtained for the calibration curve was 0.0044, which indicated a good fit of data and within the error limit <2%. This ensures high confidence limits of the experimental measurements.

2.4. G-POD, SOD and CAT extraction and assay

G-POD, SOD and CAT activities were measured in a crude enzyme extract prepared by homogenizing leaves (1 g FW) either in 3 mL of 25 mM Tris–HCl buffer (pH 7.2) containing 5 mM cysteine and 5 mM EDTA for G-POD or in 3 mL of 10 mM potassium phosphate buffer (pH 7.0) and 0.1 mM EDTA for SOD and CAT. After centrifugation at $15{,}000 \times g$ for 20 min at 4 °C, the supernatant was used as enzyme extract. To measure G-POD activity, an aliquot of supernatant (200 μL) was added to 2 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 6.0), 1% guaiacol and 0.4% H_2O_2. Guaiacol oxidation was measured at 420 nm against a blank without H_2O_2. G-POD activity was expressed as UE mg prot^{-1}. The unit of G-POD was defined as the amount of enzyme that causes the formation of 1 μmol tetraguaiacol per min using a molar extinction coefficient of 26.6 mM^{-1} cm^{-1}. SOD activity was assayed following the photoreduction of nitroblue tetrazolium (NBT). A supernatant aliquot (200 μL) was added to 4 mL reaction mixture containing 50 mM sodium carbonate buffer (pH 10.2), 32 mM methionine, 57 μM NBT, 1.33 μM riboflavin and 0.1 mM EDTA. Reaction was initiated by adding riboflavin under a 40 W fluorescent lamp and the photoreduction of NBT (blue formazan production) was measured at 560 nm against a blank without riboflavin. SOD activity was expressed as UE mg prot^{-1}. One unit of SOD activity was defined as the amount of enzyme required to inhibit the photoreduction of NBT by 50% in a 4-mL reaction volume per min. For determination of SOD activity each assay was repeated for five volumes of extract. To calculate SOD unit the following equation was used: $1\ \text{unit} = V/v - 1$, where V and v are the slope of absorbance changes in absence and in presence of the enzyme extract, respectively [13]. To measure CAT activity, an aliquot of supernatant (300 μL) was added to 2.9 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1% Triton X-100 and 10.6 mM H_2O_2. CAT activity was measured at 240 nm against a blank without H_2O_2 and expressed as UE mg prot^{-1}. One CAT unit was defined as the enzyme activity that decomposed 1 μmol of H_2O_2 per min using a molar extinction coefficient of 39.4 mM^{-1} cm^{-1}.

2.5. THCC measurement

THCC in submerged and floating leaves was determined using a modification of the method of Linde and García-Vazquez [14]. It is based on the determination of —SH groups by spectrophotometry using the Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid, DTNB). Briefly, 1 g FW of leaf sample was homogenized in 3 mL of 20 mM Tris–HCl buffer (pH 8.6), containing 0.5 M sucrose and 0.01% β-mercaptoethanol to maintain reduced thiols. After centrifugation at $15{,}000 \times g$ for 20 min at 4 °C, the supernatant was collected and used to THCC determination. An aliquot of supernatant (400 μL) was added with 400 μL of Ellman's reagent

Table 1
Climatic data recorded in summer and winter during field Cr-treatment period. Data are average of three independent experiments, $p < 0.05$ ($n = 20$).

Climatic parameter	Summer	Winter
Temperature (air)	30.8 ± 1.7 °C	12.6 ± 1.5 °C
Temperature (Cr(VI) sol.)	32.5 ± 1.8 °C	12.4 ± 1.4 °C
Sunlight duration	>13 h	9 h
PAR (μmol m^{-2} s^{-1})	1786 ± 72	1010 ± 47
UV-BBE (kJ m^{-2} s^{-1})	28.3 ± 3.1	11.8 ± 2.8
Cloudy days	2	1

(5 mM DTNB 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 incubate at 37 °C for 60 min, absorbance was read at 412 nm against a blank without supernatant. THCC concentration was calculated from a calibration curve prepared with pure reduced glutathione (GSH), and expressed in mg g^{-1} FW.

2.6. Soluble protein and Pro measurements

Free Pro was extracted from 1 g FW leaf sample and determined with ninhydrin according to Ting and Rouseff [15]. Soluble proteins were extracted and determined as described by Prado et al. [10].

2.7. SP and MDA measurements

SP and MDA contents in floating and submerged leaves of *S. minima* plants were determined as described [16].

2.8. Statistics

All analyses were performed in triplicate. Data presented are means ± SD of three independent experiments. Data were subjected to analysis of variance and differences were significant at $p < 0.05$ level.

3. Results

3.1. Climatic data

Seasonal means of air and Cr(VI) solution temperatures, PAR and UV-B radiations and sunlight duration occurring during the experiment are presented in Table 1. Maximum values of air and Cr(VI) solution temperatures did not vary significantly in both seasons, but were much higher in summer than in winter. Air and Cr solution temperatures were 30.8 and 32.5 °C for summer, and 12.6 and 12.4 °C for winter, respectively. Sunlight duration (day-length) was over 13 h in summer and 9 h in winter. Maximum mean values of PAR and UV-B (expressed as UV-BBE) were 1786 μmol m^{-2} s^{-1} and 28.3 kJ m^{-2} s^{-1} for summer, and 1010 μmol m^{-2} s^{-1} and 11.8 kJ m^{-2} s^{-1} for winter, respectively. In addition, one and two cloudy days were recorded for winter and summer, respectively.

3.2. Cr(VI) accumulation

Seasonal accumulation of Cr(VI) in leaves of *S. minima* is shown in Fig. 1. Leaf Cr accumulation was found to be correlated with metal concentration occurring in growth solution. Highest Cr(VI) accumulations were observed in summer, with values of 3388 and 637 μg g^{-1} DW for submerged and floating leaves, respectively. In winter Cr accumulation strongly decreased in both leaf types with values of 710 and 189 μg g^{-1} DW for submerged and floating leaves, respectively. Cr(VI) values in submerged and floating leaves from summer were 4.8- and 3.4-fold higher than values found in leaves from winter. Moreover, summer and winter Cr(VI) concentrations were 5.3- and 3.8-fold higher in submerged leaves than in floating ones. Cr(VI) was not detected in control plants. Though in principle, there is a possibility of reduction of Cr (VI) into Cr(III) by submerged leaves of *S. minima* [17]. However, colorimetric determination of residual Cr(VI) in the treatment solution with and without $KMnO_4$ in acid medium did not show any differences (data not shown). Therefore, in this study Cr(VI) was the only form accumulated in *Salvinia* leaves.

3.3. G-POD, SOD and CAT activities

Soluble G-POD activity in summer and winter significantly increased ($p < 0.05$) in Cr-treated leaves, being higher in submerged leaves than in floating ones. Highest values of G-POD activity in floating and submerged leaves from summer were 13.7 and 14.8 UE mg prot^{-1}, which were 108% and 78% higher than values found in Cr-untreated leaves. In similar winter leaves, highest G-POD values were 16.3 and 175.6 UE mg prot^{-1}, which were 30% and 94% higher than control values. SOD activity in summer and winter significantly increased in Cr-treated submerged leaves. Highest values of SOD activity in submerged leaves of both summer and winter were 1188.2 and 473.6 UE mg prot^{-1} that were 6- and 16-fold higher than values found in the control. In floating leaves from the two seasons, practically, there were not significant changes in SOD activity. CAT activity was dissimilarly affected by Cr(VI) in both seasons. Enzyme activity increased in Cr-treated floating leaves, reaching values of 26.3 and 25.8 UE mg prot^{-1} at 20 mg L^{-1} Cr(VI) concentration in summer and winter, respectively. Interestingly, in winter the highest CAT activity (33.9 UE mg prot^{-1}) was found under lowest Cr(VI) concentration. Contrary, in Cr-exposed submerged leaves from both seasons, CAT activity was either decreased or increased, but there were no great changes (Table 2).

3.4. THCC

THCC concentration showed significant interseasonal differences between floating and submerged leaves. In summer-floating leaves, THCC content showed a significant initial increase up to 2 mg L^{-1} Cr concentration reaching a maximum value of 0.196 mg g^{-1} FW that was 42% higher than that observed in control leaves. From this Cr(VI) concentration, THCC remained without significant changes ($p < 0.05$) until the end of the experiment. In Cr-treated submerged leaves, THCC concentration showed a progressive increase reaching a maximum value (0.228 mg g^{-1} FW) at 20 mg L^{-1} Cr(VI) concentration. This THCC concentration was 137% higher than concentration observed in Cr-untreated leaves. Contrary, winter-floating leaves showed a progressive initial decrease of THCC reaching at 5 mg L^{-1} Cr(VI) concentration a minimum content of 0.299 mg g^{-1} FW that was 51% lower than value observed in Cr-untreated leaves. From this point on, THCC increased up to 10 mg L^{-1} Cr(VI) concentration reaching a value of 0.457 mg g^{-1} FW, and then it returned to concentration observed at 5 mg L^{-1} Cr(VI). By contrast, THCC in submerged leaves showed a sustained increase until the end of the experiment, reaching a maximum concentration of 0.15 mg g^{-1} FW that was 3.5-fold higher than that found in control leaves (Table 3).

3.5. Soluble proteins and Pro

Soluble proteins were differentially affected by Cr(VI) treatment in both seasons. In summer the protein content increased under Cr exposure reaching values of 0.63 and 0.49 mg g^{-1} FW at 20 mg L^{-1} Cr(VI) concentration in floating and submerged leaves, respectively. These values were 59% and 342% higher than values found in control leaves. In winter-floating leaves, no significant differences ($p < 0.05$) between Cr-treated and Cr-untreated plants

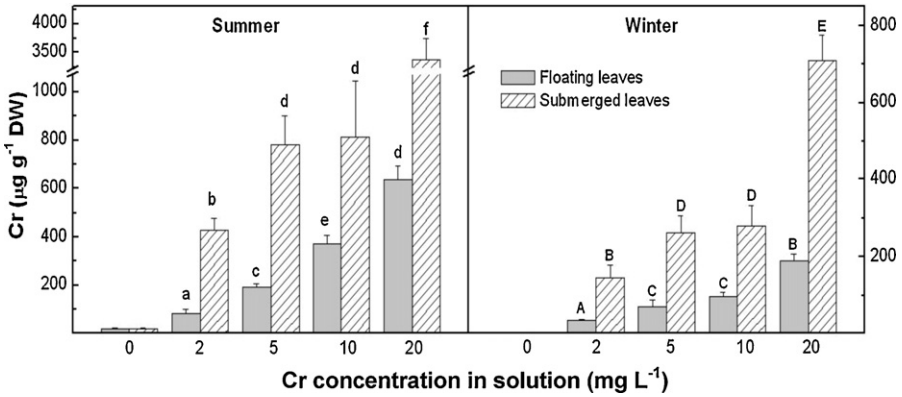

Fig. 1. Seasonal variation in Cr(VI) accumulation in floating and submerged leaves of *S. minima* exposed to different Cr(VI) concentrations. Different letters on bars indicate significant differences at $p < 0.05$ ($n = 3$). Lowercase letters are used to denote significance between summer values. Uppercase letters are used to denote significance between winter values.

were observed. In winter-submerged leaves, maximum protein content (0.073 mg g^{-1} FW) was found at 5 mg L^{-1} Cr(VI) concentration, but from this point on a progressive decrease until the end of the experiment was observed. Comparing with Cr-untreated leaves, highest protein decrease was 60%. Pro concentration was also affected by Cr(VI) treatment in both seasons. Metal treatment decreased Pro content of summer-floating leaves by 21% compared with Cr-untreated leaves; while in summer-submerged leaves no significant changes were found. In winter-floating leaves, there were no significant changes in Pro content, but in submerged leaves it was increased by 75% compared with control leaves (Table 3).

3.6. SP and MDA

SP concentration in floating leaves of both seasons strongly increased by Cr(VI) treatment, reaching increases over 3- and 4-fold compared with Cr-untreated leaves at 20 mg L^{-1} Cr(VI) concentration. Maximum SP concentrations for summer and winter were 1906 and 4589 μmol phenol equivalent g^{-1} FW, respectively. In submerged leaves of both seasons an opposite trend was observed being SP content significantly decreased ($p < 0.05$) by Cr(VI) treatment. Summer and winter MDA concentrations were higher in floating leaves than in submerged ones. MDA concentration in summer season did not vary significantly by comparing Cr-treated with Cr-untreated leaves. By contrast, winter MDA content significantly increased ($p < 0.05$) in Cr-treated leaves reaching values of 0.549 and 0.415 nmol g^{-1} FW in floating and submerged leaves, respectively. These values were 45% and 310% higher than values observed in control leaves (Table 3).

4. Discussion

Oxidative stress induced by heavy metals disturbs strongly the cellular energy balance and has a negative impact on both physiological performance and survival of plants [18]. To combat Cr(VI) toxicity, plants need to maintain the metal in a non-reactive compartment (*e.g.* vacuole) and/or immobilize it through selective binding to specific molecules [19]. Different strategies against Cr toxicity have been characterized in aquatic macrophytes under laboratory-controlled conditions, but not under field conditions [20]. It is well-known that under field conditions the responses of plants to heavy metals are upon depending of metal concentration and environmental factors [21]. Thus, it is expected that different mechanisms against Cr(VI) toxicity are occurring in *S. minima* growing under different climatic conditions. In this study, we analyze the effects of two contrasting climatic seasons, *i.e.* summer and winter on Cr(VI) accumulation and as thiols, phenolics and antioxidative enzymes complement each other during Cr detoxification in both floating and submerged leaves. Our results clearly showed that Cr(VI) accumulation in both leaf types was dependent upon metal concentration and growth season. Highest metal accumulation in floating and submerged leaves was found in summer (Fig. 1). Predominant accumulation of Cr(VI) in submerged leaves may reflect the ability of *S. minima* to withstanding Cr-induced oxidative stress on metabolic functions [16]. Oxidative stress in aquatic and terrestrial plants is avoided *via* increase of enzymatic (SOD, CAT, peroxidase [POD], glutathione reductase [GR]) and non-enzymatic (GSH, ascorbate [AS], tocopherol [TCP]) antioxidative metabolites, as well as by enhanced accumulation of phenol-derived compounds

Table 2
Summer and winter activities of antioxidative enzymes (G-POD, SOD and CAT) in floating and submerged leaves of *S. minima* exposed to different Cr(VI) concentrations. Values followed by the same letter within a column are not significantly different at $p < 0.05$ ($n = 9$). Lowercase letters are used to denote significance between summer values. Uppercase letters are used to denote significance between winter values.

Cr(VI) (mg L^{-1})	G-POD (UE mg $prot^{-1}$)		SOD (UE mg $prot^{-1}$)		CAT (UE mg $prot^{-1}$)	
	Floating	Submerged	Floating	Submerged	Floating	Submerged
Summer						
0	$6.6 \pm 0.5_a$	$8.3 \pm 0.9_a$	$178.1 \pm 14.4_a$	$185.9 \pm 14.5_a$	$17.3 \pm 1.7_a$	$49.2 \pm 4.5_a$
2	$3.5 \pm 0.3_b$	$10.0 \pm 1.1_a$	$162.5 \pm 15.2_a$	$169.5 \pm 13.7_a$	$20.2 \pm 2.0_a$	$42.6 \pm 4.8_a$
5	$13.7 \pm 1.2_c$	$10.0 \pm 0.9_a$	$159.9 \pm 13.0_a$	$445.9 \pm 23.6_b$	$15.6 \pm 1.3_{a,b}$	$33.1 \pm 3.0_b$
10	$13.2 \pm 1.1_c$	$14.8 \pm 1.3_b$	$165.1 \pm 14.8_a$	$1188.2 \pm 98.9_c$	$22.8 \pm 2.0_{a,c}$	$32.3 \pm 3.4_b$
20	$12.2 \pm 1.2_c$	$13.9 \pm 1.1_b$	$166.4 \pm 17.1_a$	$835.9 \pm 85.7_d$	$26.3 \pm 2.6_{a,c}$	$44.4 \pm 4.5_a$
Winter						
0	$12.5 \pm 0.9_A$	$90.3 \pm 5.3_A$	$32.5 \pm 2.9_A$	$28.6 \pm 3.0_A$	$16.1 \pm 1.3_A$	$34.6 \pm 3.5_A$
2	$15.1 \pm 1.3_B$	$85.3 \pm 4.1_A$	$37.7 \pm 3.2_A$	$473.6 \pm 36.8_B$	$33.9 \pm 2.9_B$	$22.4 \pm 2.1_B$
5	$16.1 \pm 1.4_B$	$130.2 \pm 8.2_B$	$43.7 \pm 4.5_{A,B}$	$292.5 \pm 26.4_C$	$18.9 \pm 1.8_A$	$27.1 \pm 2.5_B$
10	$16.3 \pm 1.4_B$	$175.6 \pm 14.8_C$	$43.5 \pm 4.0_{A,B}$	$380.9 \pm 34.1_D$	$27.1 \pm 2.6_C$	$38.0 \pm 4.1_A$
20	$16.0 \pm 1.5_B$	$156.6 \pm 12.4_C$	$32.5 \pm 2.1_{A,B}$	$449.6 \pm 35.9_{D,B}$	$25.8 \pm 2.2_C$	$35.3 \pm 3.1_A$

Table 3
Seasonal concentrations of THCC, soluble proteins, Pro, SP and MDA, in floating and submerged leaves of *S. minima* exposed to different Cr(VI) concentrations. Values followed by the same letter within a column are not significantly different at $p < 0.05$ ($n = 9$). Lowercase letters are used to denote significance between summer values. Uppercase letters are used to denote significance between winter values.

Cr(VI) (mg L^{-1})	THCC (mg GSH g^{-1} FW)		Protein (mg g^{-1} FW)		Pro (μg g^{-1} FW)		SP (μmol ph. eq. g^{-1} FW)		MDA (nmol g^{-1} FW)	
	Floating	Submerged	Floating	Submerged	Floating	Submerged	Floating	Submerged	Floating	Submerged
Summer										
0	$0.138 \pm 0.011_{a}$	$0.096 \pm 0.008_{a}$	$0.397 \pm 0.023_{a}$	$0.144 \pm 0.02_{a}$	$75.0 \pm 3.2_{a}$	$15.5 \pm 1.3_{a}$	$0.561 \pm 0.04_{a}$	$0.187 \pm 0.022_{a}$	$0.427 \pm 0.04_{a}$	$0.335 \pm 0.026_{a}$
2	$0.196 \pm 0.009_{b}$	$0.133 \pm 0.01_{b}$	$0.548 \pm 0.046_{b}$	$0.281 \pm 0.018_{b}$	$57.5 \pm 3.4_{b}$	$13.7 \pm 1.6_{a}$	$0.514 \pm 0.035_{a}$	$0.147 \pm 0.019_{a}$	$0.439 \pm 0.034_{a}$	$0.355 \pm 0.03_{a}$
5	$0.193 \pm 0.02_{b}$	$0.140 \pm 0.009_{b}$	$0.486 \pm 0.052_{b}$	$0.342 \pm 0.026_{c}$	$63.3 \pm 4.7_{b}$	$14.8 \pm 1.4_{a}$	$0.603 \pm 0.05_{b}$	$0.061 \pm 0.008_{b}$	$0.469 \pm 0.03_{a}$	$0.311 \pm 0.031_{a}$
10	$0.184 \pm 0.017_{b}$	$0.189 \pm 0.02_{d}$	$0.555 \pm 0.031_{b}$	$0.370 \pm 0.04_{c}$	$67.2 \pm 3.7_{b,c}$	$11.7 \pm 1.2_{b}$	$1.491 \pm 0.11_{c}$	$0.047 \pm 0.005_{c}$	$0.473 \pm 0.05_{a}$	$0.318 \pm 0.03_{a}$
20	$0.194 \pm 0.013_{b}$	$0.228 \pm 0.018_{c}$	$0.630 \pm 0.052_{c}$	$0.493 \pm 0.038_{d}$	$57.0 \pm 4.1_{b}$	$13.3 \pm 1.4_{a}$	$1.906 \pm 0.18_{d}$	$0.047 \pm 0.004_{c}$	$0.476 \pm 0.043_{a}$	$0.317 \pm 0.032_{a}$
Winter										
0	$0.579 \pm 0.041_{A}$	$0.043 \pm 0.006_{A}$	$0.135 \pm 0.02_{A}$	$0.052 \pm 0.003_{A}$	$55.0 \pm 4.3_{A}$	$13.8 \pm 1.1_{A}$	$1.111 \pm 0.12_{A}$	$0.456 \pm 0.032_{A}$	$0.378 \pm 0.04_{A}$	$0.134 \pm 0.011_{A}$
2	$0.402 \pm 0.029_{B}$	$0.056 \pm .0005_{B}$	$0.146 \pm 0.017_{A}$	$0.073 \pm 0.007_{B}$	$53.5 \pm 3.7_{A}$	$18.3 \pm 2.0_{B}$	$1.767 \pm 0.14_{B}$	$0.289 \pm 0.03_{B}$	$0.433 \pm 0.04_{A}$	$0.366 \pm 0.04_{B}$
5	$0.299 \pm 0.032_{C}$	$0.071 \pm 0.006_{C}$	$0.169 \pm 0.02_{A}$	$0.035 \pm 0.004_{C}$	$50.2 \pm 5.1_{A}$	$13.3 \pm 1.1_{A}$	$2.433 \pm 0.32_{C}$	$0.622 \pm 0.053_{C}$	$0.560 \pm 0.047_{B}$	$0.360 \pm 0.031_{B}$
10	$0.457 \pm 0.037_{B}$	$0.136 \pm 0.02_{D}$	$0.175 \pm 0.015_{A}$	$0.021 \pm 0.001_{D}$	$52.5 \pm 4.9_{A}$	$17.0 \pm 2.0_{B}$	$4.322 \pm 0.43_{D}$	$0.244 \pm 0.02_{B}$	$0.549 \pm 0.05_{B}$	$0.335 \pm 0.034_{B}$
20	$0.304 \pm 0.031_{CB}$	$0.150 \pm 0.018_{D}$	$0.176 \pm 0.02_{A}$	$0.029 \pm 0.002_{D}$	$52.7 \pm 5.1_{A}$	$24.2 \pm 2.2_{C}$	$4.589 \pm 0.49_{D}$	$0.328 \pm 0.04_{B}$	$0.549 \pm 0.042_{B}$	$0.415 \pm 0.04_{C}$

[5,22]. Uptake of heavy metals depends of their availabilities that are controlled by physicochemical factors such as solubility, pH and water temperature [23]. According to Zumdahl [24], seasonal variations in water temperature have no direct effect on metal solubility whereas increases of pH are generally accompanied by decreases of metal solubility. In our study, no seasonal changes in pH values of Cr(VI) solutions were observed, and then we assume that lowest content of Cr(VI) found in winter and its different interseasonal distribution pattern observed in floating leaves, could be related to a reduced Cr(VI) uptake and/or to changes in metal detoxification mechanisms induced by a different season-dependant metabolic activity occurring in Cr-treated leaves [10,16]. In agreement with our findings, seasonal variations in heavy metal accumulation were also reported in other aquatic macrophytes [23]. To explain seasonal differences observed in Cr(VI) distribution patterns of *Salvinia* leaves, it is necessary to take into account that metal accumulation also depends upon capacity of metal chelation and/or ability of cells to vacuolar sequestration of metal [6]. In this context, thiol-containing compounds deserve a great attention due to their ability of heavy metal complexation [7,8]. Chemical complexation of Cr(VI) by THCC compounds such as metallothioneins, glutathione an other thiols have been communicated [25–27]. Moreover, induction of the synthesis of metallothioneins and phytochelatins by Cr(VI) were also communicated for plants in recent years [7,28–30]. In agreement with these findings, our data showed that content of THCC in submerged leaves significantly correlated with accumulation of Cr(VI) in both seasons (Table 3), being lowest THCC content found in winter, probably due to a water-temperature-induced metabolic decrease. However, concentration of THCC in summer-submerged leaves was 52% higher than content in winter-submerged leaves, but summer accumulation of Cr(VI) was 477% higher than winter accumulation. According with these results, THCC would not seem to play a main role in Cr(VI) chelation in submerged leaves of *Salvinia* plants. Likewise in a previous experiment carried out in our laboratory we demonstrated that submerged leaves of *S. minima* accumulate high amounts of Cr(VI) onto their cell walls through adsorption processes [16]. Then, it can be assume that accumulation of Cr(VI) in submerged leaves occurs through a combination of two coordinated mechanisms: (a) binding (adsorption) of Cr(VI) to leaf surfaces, (b) uptake of Cr(VI) by leaf cells. By contrast, no relationship between THCC content and Cr accumulation was observed in floating leaves from both seasons (Table 3 and Fig. 1). Since GSH represents the major pool of THCC in plants [8], observed reduction of THCC in winter-floating leaves could indicate an increased reduction of —SH groups from GSH pool, either *via* complexation with Cr(VI) (GSH–Cr complex) [27], or by reversible thiol-disulphide exchange process ($GSH \leftrightarrow GSSG$) [31]. However, decreased THCC could also indicate an enhanced transport of these compounds to submerged leaves to act against higher Cr-induced oxidative stress occurring in these leaves. To support this assumption, MDA concentration (an indicator of lipid peroxidation) significantly increased in winter-submerged leaves, but not in floating ones (Table 3). Moreover, in a previous study we found an increased lipoxygenase (LOX) activity in Cr-treated winter-submerged leaves [10]. Long-distance transport of THCC has been communicated for several plant species exposed to different heavy metals [32,33]. THCC also play a prominent role in scavenging of ROS generated by heavy metal-induced oxidative stress [3,8]. Similarly, soluble phenolics (SP) have been also recognized as efficient antioxidative molecules due to their ability to act as metal chelators [34,35] and/or ROS scavenger through their ability to donate electrons or hydrogen atoms [36]. Thus, it is expected that relationships between THCC and SP may be occur in Cr-exposed leaves. In the present study, SP concentration seem to be correlated with THCC content in summer-floating leaves, and then it could be assumed that both compounds are involved in antioxidative mechanism

of these leaves. In winter-floating leaves, there were no correlations between THCC and SP contents in Cr-treated plants (Table 3). Although, we cannot explain this finding probably it occurs through unknown season-dependant antioxidative relationships. Contrary to floating leaves, in submerged ones a much less content of SP was found in both seasons (Table 3). This unexpected result could indicate no a reduced SP synthesis, but rather their polymerization, and then it can be correlated with increasing deposition of Cr-induced lignin (polymerized phenolics) found in cell walls of Cr-treated submerged leaves [16]. Polymerized phenolics are not extracted with ethanol 96% that is the solvent used for extraction of SP, and also they are not detected using standard SP colorimetric methods [37]. Therefore a lower content of SP is determined in submerged leaves. According with Polle et al. [38] G-POD is a key enzyme into lignification process and participates in oxidation of monomeric phenolics prior to cross-linking polymerization during the synthesis of lignin and/or suberin. Agreeing with this finding, we demonstrated that G-POD activity increased in Cr-treated leaves from both seasons being higher in submerged leaves than in floating ones (Table 2). Thus, we assume that highest activity of G-POD found in submerged leaves of Cr-treated plants is involved in enhanced deposition of lignin induced by exposure to Cr(VI). Increased synthesis of lignin under heavy metal stress has also been communicated for other aquatic macrophytes [39]. By contrast, increased SP concentration and soluble G-POD activity observed in Cr-treated floating leaves, probably, could be involved in a like vacuolar antioxidative mechanism as described in other plants [40].

Another mechanism by which many plants respond to heavy metals is the accumulation of free amino acids, *i.e.* proline (Pro) and histidine [9]. Amino acids protect cells against heavy metal toxicity by chelation of metal and/or scavenging of ROS [41]. In our study, a significant Pro accumulation induced by Cr(VI) was only observed in winter-submerged leaves at highest Cr(VI) concentration, suggesting that this amino acid may be involved in *S. minima* protection through scavenging of ROS generated by increasing oxidative stress induced at highest metal concentration. In agreement with this assumption both MDA content (in this work) and ROS generation (in Ref. [16]) were strongly increased in winter-submerged leaves under 20 mg L^{-1} Cr(VI) concentration, when compared with Cr-untreated leaves. Besides its direct function as ROS scavenger, Pro can also protect and stabilize ROS scavenging enzymes, and activate other alternative detoxification pathways [42]. Furthermore Pro can improve heavy metal stress tolerance by stabilizing protein turnover machinery and/or by stimulating accumulation of stress-protective proteins [43]. No direct relationship between free Pro concentration and soluble protein content was found in Cr-treated *Salvinia* leaves, indicating that both protein and amino acid metabolisms were differentially affected by Cr(VI). In agreement with our assumption, no positive correlations between Pro accumulation and protein synthesis were also communicated for other heavy metals [42]. Taken together, our results support that both THCC and SP in conjunction with antioxidative enzymes play key roles to alleviate deleterious effects of Cr(VI) in leaves of *S. minima*.

5. Conclusions

Cell functioning under Cr(VI) stress involves combined functions of a large number of biochemical reactions through many metabolic pathways. Interaction and coupling of these metabolic pathways ensure a unified and regulated system of metabolic flows in both cells and whole plant [44]. Thus, we hope that critical examination of concepts given in this study may contribute to improve understanding of metabolic relationships underlying plant responses to Cr(VI) toxicity, a phenomenon that intrigued to plant stress physiologists for many years but still eludes its complete elucidation.

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