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Differential Effects of Cr(VI) on the Ultrastructure of Chloroplast and Plasma Membrane of *Salvinia minima* Growing in Summer and Winter. Relationships With Lipid Peroxidation, Electrolyte Leakage, Photosynthetic Pigments, and Carbohydrates

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Abstract Seasonal variations of chloroplast thylakoids and plasma membrane ultrastructure and changes in some biochemical parameters (e.g., metal accumulation, photosynthetic pigments, carbohydrates, lipid peroxidation, and electrolyte leakage) were studied in fronds of *Salvinia minima* plants exposed to increasing concentrations of Cr(VI) in both winter and summer. Disorganization of stacked (grana) and unstacked (stroma lamellae) thylakoids was greater in winter chloroplasts than in summer chloroplasts. Plasma membrane was less affected than thylakoids. Photosynthetic pigments, lipid peroxidation, soluble sugars, and starch were affected differently in winter and summer. Our results suggest that much greater ultrastructural alterations and changes in metabolite levels occurring in winter fronds are produced by higher oxidative stress resulting from the interactive effect between low temperature, low solar irradiance, and Cr(VI) toxicity, rather than from metal accumulation per se. Seasonal differences occurring in chloroplast ultrastructure and metabolite concentrations were discussed in relation to metabolic implications. Evaluated parameters represent a relevant approach to enhance knowledge on performance and fitness of plants exposed to heavy metals under fluctuating environmental conditions. This work also indicates that selection of suitable macrophytes to remove Cr(VI) requires an additional analyzing focus on structural and metabolic interactions that occur in plants exposed to heavy metals in contrasting seasons.

Keywords Chloroplast ultrastructure · Chlorophyll · Cr(VI) · Electrolyte leakage · Lipid peroxidation · Starch

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1 Introduction

Chromium (Cr) pollution is increasing exponentially around the world due to increased man-made releases into soils, water, and atmosphere. It is one of the most toxic heavy metal pollutants occurring in the environment and is not destroyed by natural degradation (Oliveira 2012). Cr occurs in the environment as trivalent [Cr(III)] and hexavalent [Cr(VI)] oxidation states, the latter being the most toxic for both animals and plants (Zayed and Terry 2003). The toxicity of Cr(VI) is attributed to its high oxidizing capacity that generates reactive oxygen species (ROS) which induce oxidative stress, and the ability to cross biological membranes (Pandey et al. 2009). Toxic effects of Cr(VI) on plant leaves include physiological, biochemical, and morpho-anatomical alterations such as mineral nutrient imbalance, decrease of enzyme activity, disturbance of

stomatal conductance, degradation of photosynthetic pigments, reduction of CO_2 assimilation, brownish-red coloration with necrotic spots, size reduction, and ultramorphological modifications at cellular and organelle levels (Santos and Rodriguez 2012; Daud et al. 2014). It has been pointed that deleterious effects of Cr(VI) have been extensively studied in leaves of terrestrial plants (Singh et al. 2013), whereas aquatic ones have received less attention (Chandra and Kulshreshtha 2004). Among aquatic macrophytes able to accumulate Cr(VI), a select number of floating species (e.g., *Salvinia minima*, *Salvinia herzogii*, *Sphaerotilus natans*, *Salvinia auriculata*, *Pistia stratiotes*, *Eichhornia crassipes*, *Lemna minor*, *L. trisulca*, *L. gibba*, *Spirodela polyrrhiza*, *Azolla caroliniana*, *Limnanthemum cristatum*, *Wolffia globosa*, *and Ipomea aquatica*) have the ability to accumulate high concentrations of Cr(VI) without suffering severe damages (Prasad 2007).

Floating aquatic macrophytes absorb Cr(VI) from a surrounding solution, being mainly accumulated in submerged roots (Marbaniang and Chaturvedi 2014). However, Cr(VI) accumulation in aerial parts (shoot and leaves) also occurs (Sinha et al. 2002). Uptake, translocation, and accumulation of Cr(VI) are dependent upon plant growth characteristics and environmental conditions (Prado et al. 2010). Cr-induced effects on leaves are a critical point to establish the suitability of aquatic macrophytes to remove heavy metals from polluted waters (Rai 2008). Although it is well-known that Cr(VI) affects both ultrastructure and functionality of the photosynthetic apparatus (Rodriguez et al. 2012), there is still much to be done in order to fully understand as the climatic conditions (e.g., seasonal oscillations of ambient temperature and solar radiation) influence the photosynthetic performance of aquatic plants exposed to different levels of Cr(VI). Thus, the aim of this work was targeted to study the effect of increasing Cr(VI) concentrations on *S. minima* plants grown under field conditions in both winter and summer, regarding ultrastructural alterations on chloroplast and plasma membrane, as well as in relation to electrolyte leakage (EL) and accumulation of photosynthetic pigments, starch, soluble sugars, malondialdehyde (MDA), and Cr(VI) in fronds of both Cr-treated and Cr-untreated plants. In this regard, we hypothesized that seasonal differences observed in plant photosynthetic performance, based on photosynthetic pigments and carbohydrate accumulation, are closely related to much greater ultrastructural alterations occurring in winter chloroplasts induced by an interactive synergistic effect between low temperature, low solar irradiance, and Cr(VI) toxicity rather than the metal accumulation per se.

2 Materials and Methods

2.1 Chemicals

Potassium dichromate ($K_2Cr_2O_7$), ACS reagent, ≤99.0 %, was obtained from Sigma-Aldrich (St. Louis, USA). All other chemicals were of analytical grade and were purchased from standard commercial suppliers.

2.2 Plant Material and Cr(VI) Treatment

Study was carried out outdoor in winter (July and August) 2013 and summer (December and February) 2013/2014, southern hemisphere. We choose *S. minima* as plant material due to its fast growth and large biomass production, being able to assimilate Cr(VI) through root and leaf uptake (Maine et al. 2004). Healthy *S. minima* plants with uniform size were collected from an unpolluted 50-year-old man-made pond (~3000 m^2, 26° 50′ S, 65° 12′ W, 500 m a.s.l., Tucuman, Argentina). Plants were cultivated for 7 days in Cr(VI)-containing tap water solutions (0, 2, 5, 10, and 20 mg L^{-1}) prepared from a $K_2Cr_2O_7$ stock solution (500 mg L^{-1}) as described previously (Prado et al. 2010). To avoid excessive changes in the Cr(VI) concentration of treatment solutions, 3 days after cultivation start were renewed totally. The pH of freshly prepared Cr(VI) solution was 6.7, ranging between 6.6 and 6.8 during the cultivation period in both seasons. The mean values of air and Cr(VI) solution temperatures were 33.8±1.7 and 33.5±1.8 °C in summer and 12.6±1.5 and 12.3±1.4 °C in winter, respectively. After Cr(VI) treatment, plants were harvested, rinsed in distilled water, and cut to obtain fronds for both transmission electron microscopy (TEM) analysis and metabolite determinations. In order to minimize any diurnal change in photosynthetic pigments and carbohydrate concentrations, sample fronds were collected at noon.

2.3 Transmission Electron Microscopy (TEM)

Fronds with similar size and without visual damage symptoms were selected for TEM analysis (three plants per each Cr(VI) treatment, per each season). Small

sections (2×2 mm) were cut from the middle part of fronds, prefixed in 4 % glutaraldehyde in 100 mM potassium phosphate buffer, pH 7.2, postfixed in 1 % OsO_4 in the same buffer, and then dehydrated and embedded in Spurr's epoxy resin. Ultrathin sections were stained with 2 % uranyl acetate and subsequently with 0.4 % lead citrate to observe chloroplast ultrastructure. For plasma membrane analysis, samples were taken from control (Cr-untreated) and 20 mg L^{-1} Cr(VI) concentration (Cr-treated) plants. TEM observations were performed with a LEO 906E transmission electron microscope equipped with a CCD camera (Mega View III, Germany).

2.4 Chlorophyll and Carotenoids

Chlorophyll and carotenoids were extracted and determined as described by Prado et al. (2010). Concentrations of total chlorophyll (total Chl), chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and carotenoids (Car) were expressed as micrograms per gram FW.

2.5 Soluble Sugars and Starch

Total soluble sugar concentration was determined by the phenol-sulphuric acid method (Dubois et al. 1956) as described by Prado et al. (1998). Soluble sugars (glucose, fructose, and sucrose) were extracted and measured as described by Rosa et al. (2004). Starch was determined by measuring reducing sugars released after enzymatic hydrolysis according to Prado et al. (1998). Soluble sugars and starch contents were expressed as micrograms per gram FW and micrograms maltose equivalent per gram FW.

2.6 Electrolyte Leakage

Electrolyte leakage (EL) was determined by measuring the electrical conductivity according to Singh et al.'s (2007) method with minor modifications. EL was calculated using the formula EL (%)=$(E_1/E_2)\times 100$ and expressed as percentage.

2.7 Malondialdehyde (Lipid Peroxidation)

Lipid peroxidation was estimated in terms of malondialdehyde (MDA) accumulation by using the thiobarbituric acid reagent (Du and Bramlage 1992). MDA concentration was determined using the molar extinction coefficient 155×10^{-3} M^{-1} cm^{-1} and expressed as nanomoles per gram FW.

2.8 Cr(VI) Accumulation

At the end of the experiment, fronds were harvested, dried to dryness, and ground to fine powder. Frond Cr content was determined by atomic absorption spectrometry according to USEPA 3051A protocol, (http://www.epa.gov/wastes/hazard/testmethods/sw846/online/3_series.htm). The concentration of Cr in samples was expressed as micrograms per gram dry weight. The overall recovery of Cr associated with digestion process was in the 90–95 % range. In Cr-untreated fronds (control), Cr(VI) content was below the detection limit. Data were from two independent measurements.

2.9 Statistical Analyses

For all determinations, at least three replicates were analyzed and two independent experiments were performed. Results were analyzed by one-way analysis of variance (ANOVA) using the Sigma Stat program for Windows, version 3.5. Significance of differences in numerical results from different treatments was tested using the Tukey's multiple comparison test. Differences were accepted as significant if $P<0.05$. Values are given as means±SD.

3 Results

3.1 Chloroplast and Plasma Membrane Ultrastructure

TEM micrographs of control and Cr-treated chloroplasts from plants grown in summer and winter are shown in Fig. 1. Control summer chloroplasts showed an abundant well-organized inner membrane system with numerous well-developed grana and few stroma lamellae. Starch grains and plastoglobuli were scarce (Fig. 1a). Control winter chloroplasts showed a denser stroma and many stroma lamellae. Grana were smaller and less abundant than in summer chloroplasts. Starch grains and plastoglobuli were abundant. Many plastoglobuli were present in aggregated forms (Fig. 1b). Chloroplasts of both seasons showed lenticular shape (insets in Fig. 1a, b). Summer chloroplasts exposed to 2 mg L^{-1} Cr(VI) concentration practically did not show ultrastructural alterations, with large well-organized grana and scarce plastoglobuli (Fig. 1c). By contrast, in winter

chloroplasts, disorganized grana and dilated thylakoids were clearly visible. Grana were smaller than in summer chloroplasts (Fig. 1d). Under 5 mg L^{-1} Cr(VI) concentration, summer chloroplasts showed a less number of well-organized grana with increasing interthylakoidal spaces, no disruption of the outer chloroplast envelope was observed (Fig. 1e). Winter chloroplasts showed a generalized disarrangement of the inner membrane system with increasing dilated thylakoids and disorganized grana (Fig. 1f). Summer chloroplasts exposed to 10 mg L^{-1} Cr(VI) concentration exhibited an increased number of stroma lamellae with slightly disorganized grana and large interthylakoidal spaces, no disruption of the outer envelope was observed (Fig. 1g). Under 10 mg L^{-1} Cr(VI) concentration, winter chloroplasts appeared swollen with disorganized grana and numerous dilated thylakoids, plastoglobuli, and large starch grains were also observed (Fig. 1h). At the highest Cr(VI) concentration, summer chloroplasts showed a decreased number of grana with increased stroma lamellae and slightly dilated thylakoids. Starch grains and large plastoglobuli were also present; no disruption of the outer chloroplast envelope was observed (Fig. 1i). Winter chloroplasts showed a strong decrease of the stroma lamellae system, and many thylakoids appeared greatly distended with vesicular appearance; large starch grains with unusual forms were also observed. A less number of plastoglobuli compared with summer chloroplasts were observed. The outer envelope showed disruption points at several places (Fig. 1j). Figure 2 shows the plasma membrane of control and 20 mg L^{-1} Cr-treated fronds. Summer and winter Cr-untreated plasma membranes were smooth, continuous, and tightly clung to the cell wall with uniform matrix (Fig. 2a, b). The plasma membrane of Cr-treated summer fronds appeared continuous, distorted, and clung to the cell wall (Fig. 2c). Cr-treated winter plasma membrane appeared greatly rough, shrunken, and withdrawn of the cell wall (Fig. 2d).

3.2 Photosynthetic Pigments

Concentrations of photosynthetic pigments after 7 days Cr(VI) exposure are shown in Table 1. In general, there were no marked seasonal variations in concentration patterns of total Chl, Chl *a*, and Chl *b*. The total Chl of Cr-treated fronds from both seasons decreased when comparing with Cr-untreated fronds, but it was more affected in winter. In both seasons, the lowest concentrations of total Chl, Chl *a*, and Chl *b* were observed at 20 mg L^{-1} Cr(VI) concentration. Chl *b* was more affected than Chl *a* in Cr-treated fronds in both seasons. The Chl *a*/Chl *b* ratio was lower in Cr-treated winter fronds compared with summer fronds. It ranged between 3.03 and 4.71 in the former and between 4.01 and 5.50 in the latter. Car concentration decreased with increasing Cr(VI) concentrations in both seasons, but was less affected in summer

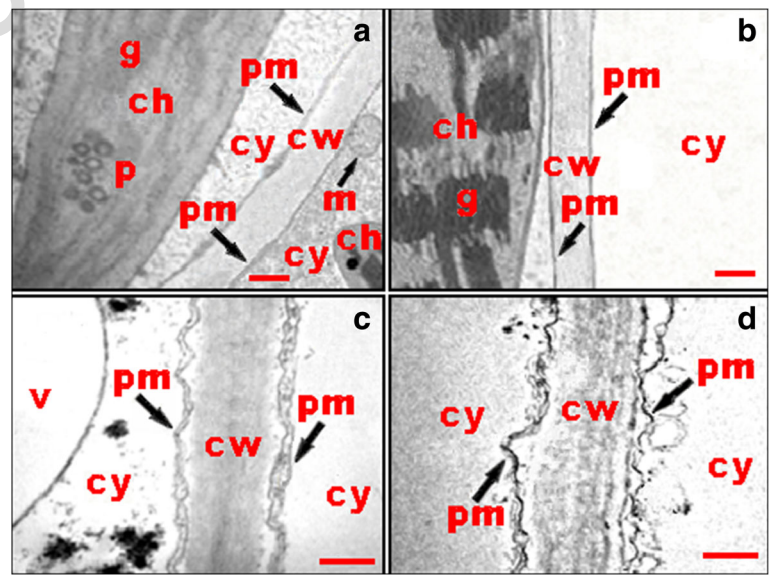

Fig. 2 Effects of 20 mg L^{-1} Cr(VI) concentration on plasma membrane ultrastructure of *S. minima* frond cells. Smooth and continuous plasma membrane tightly clung to the cell wall of Cr-untreated **a** summer and **b** winter cells. **c** Distorted and continuous plasma membrane clung to the cell wall of Cr-treated summer cells. **d** Plasma membrane highly rough, shrunken, and withdrawn of the cell wall with a nonuniform cell wall matrix of Cr-treated winter fronds; *ch*, chloroplast, *v*, vacuole. Other abbreviations are given in Fig. 1. *Bar*=0.5 μm

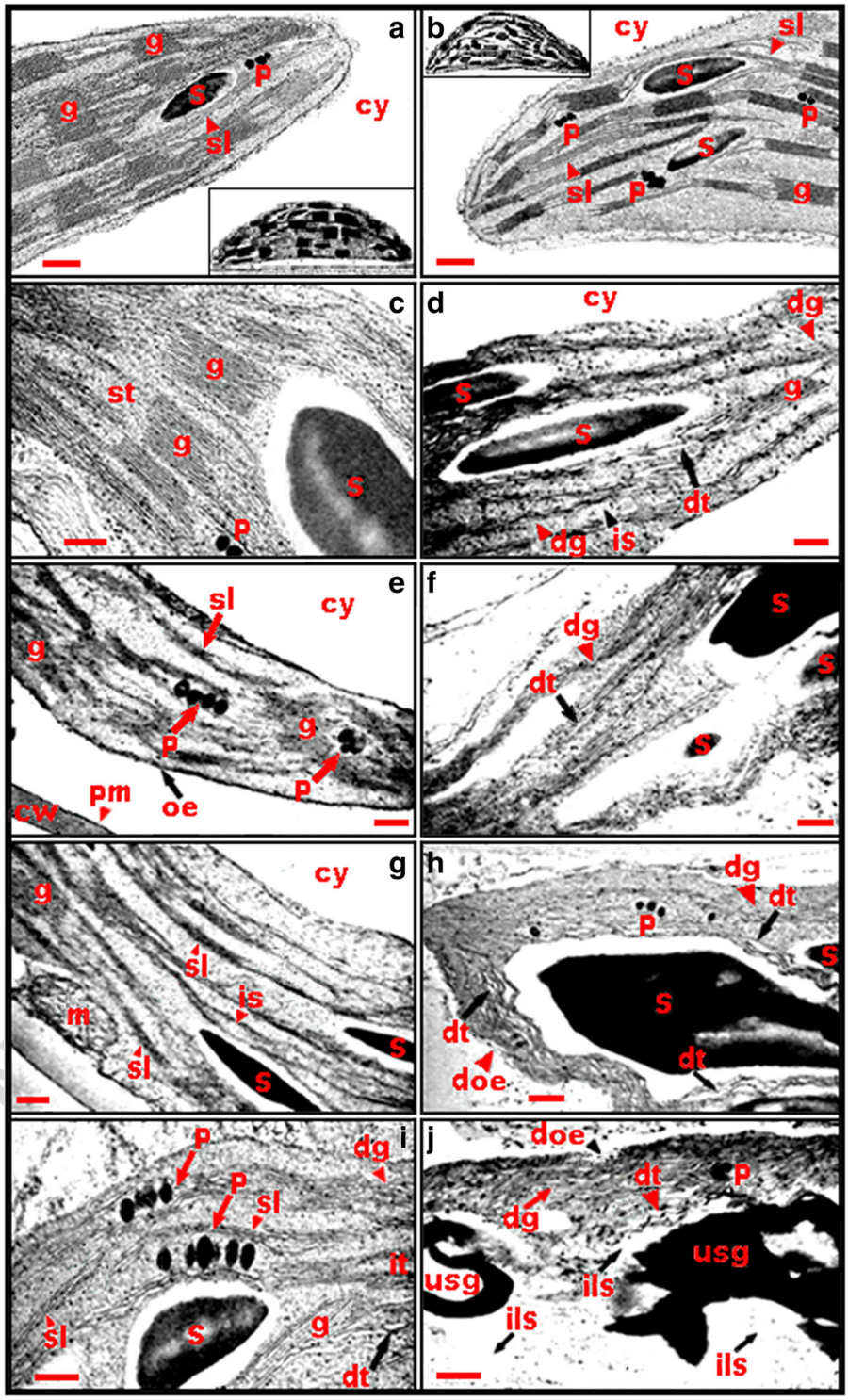

Fig. 1 TEM micrographs of Cr-untreated and Cr-treated chloroplasts of *S. minima* fronds following treatment of plants during 7 days with increasing Cr(VI) concentrations in summer (**a**, **c**, **e**, **g**, **i**) and winter (**b**, **d**, **f**, **h**, **j**). Concentrations of 0 mg L^{-1} (control) (**a**, **b**); (*insets*) whole chloroplasts; 2 mg L^{-1} (**c**, **d**); 5 mg L^{-1} (**e**, **f**); 10 mg L^{-1} (**g**, **h**); 20 mg L^{-1} Cr(VI) concentration (**i**, **j**); *cw*, cell wall; *cy*, cytoplasm; *dg*, disorganized grana; *doe*, disrupted outer envelope; *dt*, dilated thylakoids; *g*, granum; *ils*, interthylakoidal lightly stained space; *is*, interthylakoidal space; *m*, mitochondrion; *oe*, outer envelope; *p*, plastoglobuli; *pm*, plasma membrane; *s*, starch grain; *sl*, stroma lamellae; *st*, stroma; *usg*, unusual starch grain. *Bar*= 1 μm

fronds. At the end of the experiment, Car was decreased by 17.6 % in summer and 34.4 % in winter, respectively. In Cr-treated fronds, decreases of Car concentrations were lower than decreases of total Chl giving decreasing total Chl/Car ratios in both seasons.

t1.1 **Table 1** Effect of Cr(VI) on total Chl, Chl *a*, Chl *b*, and Car concentrations, as well as on the Chl *a*/Chl *b* ratio and total Chl/Car ratio in fronds of *S. minima* growing under field conditions during 7 days in summer and winter

t1.2 t1.3 Cr(VI) (mg L^{-1})	Total Chl (μg g^{-1} FW)	Chl *a*	Chl *b*	Car	Chl *a*/Chl *b*	Total Chl/Car
t1.4 Summer						
t1.5 0	$423.5\pm41.6_{aA}$	$321.3\pm31.7_{aA}$	$102.2\pm20.0_{aA}$	$94.7\pm9.3_{aA}$	$3.14\pm0.18_{dD}$	$4.47\pm0.4_{cA}$
t1.6 2	$311.1\pm43.1_{bB}$	$258.9\pm32.2_{bB}$	$62.2\pm15.3_{bB}$	$83.0\pm6.8_{aA}$	$4.01\pm0.16_{cC}$	$3.75\pm0.2_{dB}$
t1.7 5	$335.6\pm42.6_{bB}$	$277.8\pm35.6_{abA}$	$57.8\pm15.7_{bB}$	$85.3\pm7.1_{aA}$	$4.81\pm0.18_{abB}$	$3.93\pm0.2_{dcB}$
t1.8 10	$337.8\pm46.1_{bB}$	$282.2\pm30.8_{abA}$	$55.6\pm17.8_{bB}$	$88.0\pm7.3_{aA}$	$5.07\pm0.23_{aB}$	$3.84\pm0.4_{dcB}$
t1.9 20	$288.8\pm48.4_{cB}$	$244.4\pm27.8_{bB}$	$48.4\pm16.8_{bB}$	$78.0\pm7.9_{abB}$	$5.50\pm0.33_{aA}$	$3.70\pm0.3_{dB}$
t1.10 Winter						
t1.11 0	$495.6\pm41.9_{aA}$	$366.7\pm36.9_{aA}$	$128.9\pm20.0_{aA}$	$70.4\pm6.1_{bA}$	$2.84\pm0.15_{dC}$	$7.04\pm0.5_{aA}$
t1.12 2	$391.1\pm43.1_{aA}$	$293.3\pm11.1_{aA}$	$97.8\pm19.5_{aA}$	$57.6\pm7.3_{cB}$	$3.03\pm0.16_{dC}$	$6.79\pm0.7_{aA}$
t1.13 5	$300.1\pm30.3_{bB}$	$240.1\pm22.2_{bB}$	$60.0\pm8.9_{bB}$	$58.0\pm6.5_{cB}$	$4.00\pm0.14_{cB}$	$5.17\pm0.6_{bcB}$
t1.14 10	$237.8\pm31.3_{cdC}$	$191.1\pm13.2_{cC}$	$46.7\pm5.2_{cC}$	$51.0\pm7.2_{cB}$	$4.09\pm0.13_{cB}$	$5.40\pm0.6_{bB}$
t1.15 20	$220.9\pm26.3_{cdC}$	$182.2\pm18.2_{cC}$	$38.7\pm4.5_{dC}$	$46.2\pm4.1_{cdB}$	$4.71\pm0.16_{bA}$	$5.71\pm0.5_{bB}$

Values followed by the same lowercase letter for each determined parameter and for each Cr(VI) concentration within a column are not significantly different when comparing between seasons. Values followed by the same uppercase letter within a column for each determined parameter and for each season are not significantly different according to Tukey's multiple comparison test (n=6, P<0.05)

3.3 Total Soluble Sugars, Sucrose, Glucose, Fructose, and Starch

Concentrations of total soluble sugars were higher in both Cr-untreated and Cr-treated winter fronds compared with summer ones, but no seasonal differences in accumulation patterns were observed (Table 2). When analyzing concentrations of individual sugars, significant seasonal differences were found. Glucose and fructose contents were much higher in winter (9- and 4-folds approximately), while sucrose content was significantly higher in summer fronds. In general, sucrose and

t2.1 **Table 2** Seasonal effect of different Cr(VI) concentrations on total soluble sugars (TSS), sucrose, glucose, fructose, and starch concentrations in fronds of *S. minima* growing under field conditions during 7 days in summer and winter seasons

t2.2 t2.3 Cr(VI) (mg L^{-1})	TSS (μg g^{-1} FW)	Sucrose	Glucose	Fructose	Starch (μg malt eq. g^{-1} FW)
t2.4 Summer					
t2.5 0	$850.9\pm77.2_{fC}$	$143.7\pm16.2_{eE}$	$46.49\pm4.67_{dB}$	$584.6\pm42.8_{eC}$	$1214.6\pm109.6_{dA}$
t2.6 2	$893.2\pm74.7_{fC}$	$216.2\pm21.8_{dD}$	$26.35\pm2.24_{fD}$	$610.5\pm45.3_{eB}$	$1101.2\pm107.6_{dA}$
t2.7 5	$1009.7\pm92.5_{eB}$	$275.0\pm26.5_{cC}$	$35.13\pm2.86_{eC}$	$660.8\pm54.7_{dB}$	$1089.6\pm100.4_{dA}$
t2.8 10	$1223.1\pm114.2_{dA}$	$343.7\pm31.1_{bB}$	$97.30\pm4.78_{cA}$	$743.6\pm57.8_{dA}$	$1091.8\pm112.4_{dA}$
t2.9 20	$1108.4\pm107.5_{eB}$	$431.3\pm38.4_{aA}$	$32.43\pm3.78_{eC}$	$569.2\pm46.8_{eC}$	$1152.4\pm109.9_{dA}$
t2.10 Winter					
t2.11 0	$2152.7\pm198.3_{cC}$	$107.4\pm11.9_{fB}$	$466.7\pm36.9_{aA}$	$1515.9\pm120.0_{cC}$	$1473.3\pm126.5_{cC}$
t2.12 2	$2249.3\pm231.6_{cC}$	$180.5\pm13.1_{dA}$	$474.0\pm41.1_{aA}$	$1468.2\pm139.5_{cC}$	$1871.8\pm182.1_{bB}$
t2.13 5	$2786.7\pm256.8_{bB}$	$115.8\pm13.3_{fB}$	$269.6\pm23.2_{bB}$	$2261.9\pm188.9_{bB}$	$2046.1\pm198.5_{bB}$
t2.14 10	$3376.7\pm298.9_{aA}$	$153.7\pm15.3_{eA}$	$240.1\pm25.4_{bB}$	$2916.7\pm227.5_{aA}$	$2697.4\pm234.4_{aA}$
t2.15 20	$2868.7\pm277.7_{bB}$	$169.4\pm14.3_{dA}$	$291.8\pm28.2_{bB}$	$2341.3\pm197.5_{bB}$	$2589.7\pm275.2_{aA}$

Values followed by the same lowercase letter for each determined carbohydrate and for each Cr(VI) concentration within a column are not significantly different when comparing between seasons. Values followed by the same uppercase letter within a column for each determined carbohydrate and for each season are not significantly different according to Tukey's multiple comparison test (n=6, P<0.05)

fructose increased with increasing Cr(VI) concentrations in both seasons, but at highest metal concentration, winter and summer fructose concentrations decreased slightly when comparing with values found at 10 mg L^{-1} Cr(VI) concentration. Glucose concentration decreased significantly in Cr-treated winter fronds, but in summer ones was not observed a defined tendency and even a sharp increase occurred at 10 mg L^{-1} Cr(VI) concentration. Starch content was clearly higher in winter fronds than in summer ones. There were no statistically significant changes of the starch content in Cr-treated summer fronds. By contrast in winter fronds, a significant and sustained increase was observed (Table 2).

3.4 Electrolyte Leakage (EL)

Electrolyte leakage from *S. minima* fronds exposed to increasing Cr(VI) concentrations increased significantly in winter only. The highest increase (27.5 %) was found at 20 mg L^{-1} Cr(VI) concentration. By contrast, there were no significant changes of EL in Cr-treated summer fronds (Fig. 3).

3.5 Malondialdehyde (MDA)

Lipid peroxidation in *Salvinia* fronds, measured as MDA concentration, is shown in Fig. 4. MDA concentration increased under Cr(VI) exposure in both winter and summer fronds when comparing with control ones. In summer fronds, MDA increased up to 5 mg L^{-1} Cr(VI) concentration (26.4 %) and then it remained practically unchanged. In winter fronds, MDA increased strongly, reaching a maximum increase of 72.4 % at 20 mg L^{-1} Cr(VI) concentration. Interseasonal comparison of MDA content showed significantly higher values in winter fronds for all used Cr(VI) concentrations.

3.6 Cr(VI) Accumulation

Cr(VI) accumulation in summer and winter fronds is shown in Table 3. Metal content increased significantly with increasing Cr(VI) concentrations. Maximum Cr contents were 713.4±57 μg g^{-1} DW (summer fronds) and 212.6±11 μg g^{-1} DW (winter fronds), respectively. Cr(VI) accumulation in summer fronds was 3.4-folds higher than that in winter fronds. The content of Cr(VI) in control fronds was negligible in both seasons. Cr_{sum}/Cr_{win} ratio did not show significant changes under increasing Cr(VI) concentrations.

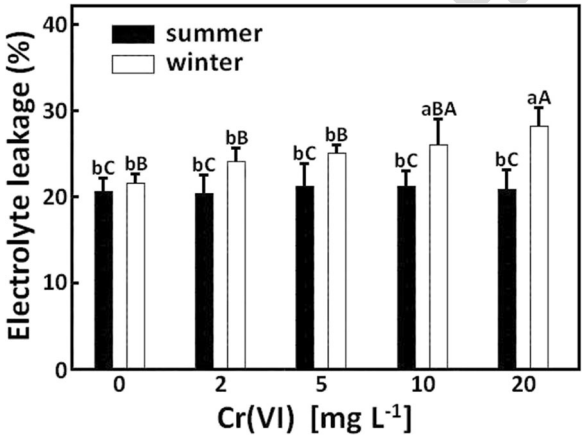

Fig. 3 Electrolyte leakage in fronds of *S. minima* grown during 7 days under field conditions in winter and summer, subject to different Cr(VI) treatments. *Bars* represent SD. For each season, same *lowercase letters* are not significantly different. For each Cr(VI) concentration, same *uppercase letters* are not significantly different according to Tukey's multiple comparison test (n=6, P<0.05)

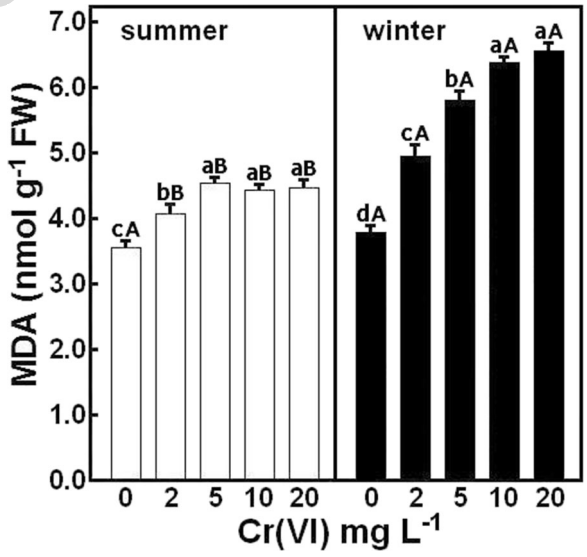

Fig. 4 Malondialdehyde (MDA) accumulation in fronds of *S. minima* grown during 7 days under field conditions in winter and summer in the presence of different Cr(VI) concentrations. *Bars* represent SD. For each season, same *lowercase letters* are not significantly different. For each Cr(VI) concentration, same uppercase letters are not significantly different according to Tukey's multiple comparison test (n=6, P<0.05)

t3.1 **Table 3** Seasonal Cr(VI) accumulation and Cr_{sum}/Cr_{win} ratio in fronds of *S. minima* growing under increasing Cr(VI) concentrations in the field during 7 days in summer and winter

t3.2 t3.3 Cr(VI) (mg L^{-1})	Summer (μg g^{-1} DW)	Winter	Cr_{sum}/Cr_{win}
t3.4 0	ND	ND	
t3.5 2	$85.8\pm9_{dA}$	$25.1\pm5_{dB}$	$3.42\pm0.5_{a}$
t3.6 5	$197.5\pm22_{cA}$	$59.6\pm6_{cB}$	$3.31\pm0.3_{a}$
t3.7 10	$375.1\pm23_{bA}$	$97.5\pm8_{bB}$	$3.85\pm0.4_{a}$
t3.8 20	$673.4\pm57_{aA}$	$191.0\pm11_{aB}$	$3.53\pm0.5_{a}$

ND not detectable

Values followed by the same lowercase letter for each season and Cr_{sum}/Cr_{win} ratio are not significantly different. Values followed by the same uppercase letter for each Cr(VI) concentration are not significantly different according to Tukey's multiple comparison test (n=4, P<0.05)

4 Discussion

4.1 Chloroplast Ultrastructure

Differences in sunlight intensity and maximum and minimum temperature values occurring between summer and winter seasons affect dynamically and reversibly the morphology and ultrastructure of chloroplasts of plants growing in natural ecosystems (Nevo et al. 2012; Kirchhoff 2013). Winter chloroplasts of many plants species have less number of grana and stroma lamellae than summer chloroplasts. Thylakoids are often swollen, and the chlorophyll is reduced (Lütz 2010). Nonetheless in some species, season-dependant structural differences are scarcely visible (Oquist and Huner 2003). In agreement with this finding, the transmission electron microscopy (TEM) analysis did not show major seasonal differences in the shape, size, and structural organization of thylakoids between Cr-untreated summer and winter chloroplasts. Minor seasonal differences were also observed in the shape and size of starch grains and plastoglobuli (Fig. 1a, b). By contrast in Cr-treated fronds, chloroplast ultrastructural alterations occurred in both seasons but were much more evident in winter (Fig. 1c–j). Ultrastructural alterations occurring in winter chloroplasts include swelling, disorganization of thylakoids, loss of grana and stroma lamellae, and disruption of chloroplast outer envelope (Fig. 1d, f, h, j), while summer chloroplasts showed slight and scarce ultrastructural alterations only under high Cr(VI) concentrations (Fig. 1c, e, g, i). Since ultrastructural changes were more evident in Cr-treated winter chloroplasts, it can be assumed that a temperature-dependant metal tolerance mechanism can be operating during the summer season to protect the chloroplast structure against Cr-induced damage. Besides different occurring structural features, winter and summer chloroplasts can also exhibit differences in photosynthetic pigments, photosystem (PSI, PSII) functionality, and thylakoid membrane integrity (Lütz 2010). Season-dependant changes in the content of certain metabolites such as starch and soluble sugars as well as in the activity of several carbohydrate- and oxidative stress-related enzymes can also occur (Savitch et al. 2000; Karuppanapandian et al. 2009).

4.2 Photosynthetic Pigments and Malondialdehyde

Fronds of Cr-treated *Salvinia* plants showed decreased concentrations of chlorophyll (Chl) [*a*, *b*, and total (*a*+*b*)] and carotenoids (Car) and also an increased concentration of malondialdehyde (MDA) when comparing with Cr-untreated fronds. Although summer and winter chloroplasts exhibited a similar pattern of changes, they were higher in the latter (Table 1 and Fig. 4). Chl *a* was less affected than Chl *b* by Cr(VI), giving higher values of Chl *a*/Chl *b* ratio. Since heavy metals trigger a ROS-induced oxidative stress in plant chloroplasts (Dubey 2011), a higher decrease of Chl *b* may indicate that it is more sensitive than Chl *a* to Cr-induced oxidative degradation of Chl molecule (Cuello and Lahora 1993). Chl *a* and Chl *b* are present in both photosystem I (PSI) and photosystem II (PSII), but their relative contents are quite different (Taiz and Zeiger 2006). Although positive linear correlations between Chl *a*/Chl *b* and PSI/PSII ratios have been observed in many aquatic and terrestrial plants (Pfündel and Pfeffer 1997), the PSII contains more Chl *b* and is more sensitive to oxidative damage than the PSI (Vass 2012). According to Lage-Pinto et al. (2008), higher PSI/PSII ratios represent an adaptative mechanism to sustain the photosynthetic activity of metal-stressed plants. Unfortunately, the analysis of PSI and PSII was not carried out in this study and then Lage-Pinto's assumption cannot be confirmed. However, since unstacked thylakoids (stroma lamellae) contain proportionally more PSI than stacked thylakoids (grana) (Rojdestvenski et al. 2002), we assume that a decreased number of grana could act as aleatory adaptive mechanism against heavy metal toxicity. In agreement with this assumption, TEM micrographs of Cr-treated chloroplasts revealed a progressive substitution

of grana by stroma lamellae in summer fronds (Fig 1c–j). Thus, higher Chl *a*/Chl *b* ratios found in Cr-treated fronds could be associated to high Cr(VI) tolerance that exhibits *S. minima*. Decreases of Chl can also be produced by other mechanisms such as inhibition of biosynthetic enzymes and disturbance of mineral uptake (Liu et al. 2008). Thus, great caution should be always taken when interpreting the results of studies aimed at the dissection of chlorophyll concentration as affected by Cr(VI), particularly in case such studies are made analyzing only a few biochemical parameters. Carotenoid content also decreased in Cr-treated plants but was less affected in summer fronds (Table 1). Besides their role as accessory photosynthetic pigments, Car also play an important role in metal-stressed plants by protecting the Chl molecule against photooxidative destruction mediated by singlet oxygen ($^1O_2$) whatever the initial production of ROS (Choudhury and Behera 2001). Thus, $^1O_2$ seems to be the major ROS ultimately involved in Cr-induced oxidative damage (Triantaphylidès et al. 2008). Hence, less reduction of Car under excess of Cr(VI) occurring in summer fronds compared with winter fronds, 17.6 and 34.4 % respectively, might also be a reason of the lower content of Chl found in winter chloroplasts. Furthermore, Car also protect the structure of photosynthetic apparatus by capturing $^1O_2$ produced in chloroplasts through a thermal energy dissipation process (physical quenching), which can prevent the $^1O_2$-induced peroxidation of thylakoid unsaturated fatty acids (Telfer, 2014). Thus, minor thylakoid disorganization occurring in Cr-treated summer chloroplasts may be related with less Car decreases that occur therein. Agreeing with this finding, the content of MDA, an indicator of lipid peroxidation, was significantly higher in Cr-treated winter fronds compared with summer ones (Fig. 4). Under stressful conditions, Car can also react with $^1O_2$ (chemical quenching), which produces the direct oxidation of Car (Ramel et al. 2012). In these conditions can be expected that a higher reduction in the level of Car occurs in winter fronds. In this regard, the total Chl/Car ratio, an indicator of environmental stress, was significantly higher in winter fronds compared with summer fronds (Table 1). According to Fargašová (2008) under continuous heavy metal exposure, the total Chl/Car ratio usually shows values between 4.0 and 3.5 in summer and higher than 5.0 in winter due to shady leaf condition. Agreeing with this finding, the total Chl/Car ratio ranged between 3.70 and 3.93 in Cr-treated summer fronds and between 5.17 and 6.79 in Cr-treated winter fronds. Hence, Car may be indubitably considered as functional components of Cr(VI) tolerance mechanism operating in *Salvinia* plants growing in contrasting seasons.

4.3 Starch and Soluble Sugars

Chloroplast starch metabolism is strongly affected by both heavy metal toxicity and fluctuating environmental conditions, particularly solar irradiance (day length) and temperature (Shanker et al. 2005; Prado et al. 2010; Geigenberger 2011; Mahajan et al. 2013). Data on the effect of heavy metals, day length, and low temperatures on chloroplast starch grains are controversial. Increases, decreases, and even no changes in starch grains have been reported for plants exposed to heavy metals (Solymosi and Bertrand 2010). In this work, chloroplast starch grains were differently affected by increasing Cr(VI) concentrations in winter and summer seasons. Depending on Cr(VI) concentration, winter chloroplasts showed a progressive change in the shape and number of starch grains (Fig. 1), but in the presence of 20 mg L^{-1} Cr(VI) concentration, starch grains were unusually large and also irregularly shaped (Fig. 1h, j). In summer chloroplasts, no significant changes in the number and size of starch grains were observed under increasing Cr(VI) concentrations (Fig. 1c, g, i). When the starch content of the whole frond was chemically determined, higher contents were observed in Cr-treated summer and winter fronds compared with Cr-untreated fronds. Although there were no significant differences between Cr-untreated and Cr-treated summer fronds, in winter, the starch content showed significant differences between Cr-untreated and Cr-treated fronds (Table 2). Decreases in photosynthesis and respiration rates and changes in the sink-strength have been found in many aquatic macrophytes exposed to both low temperature and Cr(VI) (Pilon and Santamaría 2001; Vajpayee et al. 2001; Appenroth et al. 2003; Paiva et al. 2009; Prado et al. 2010; Staehr and Borum 2011). Although in this work, the assimilation of CO_2 and sink-source carbon partitioning was not measured, it can be assumed that low temperature- and low solar irradiance-induced metabolic changes occurring during the winter season are responsible of the higher starch content observed in Cr-untreated winter fronds compared with Cr-untreated summer ones. However, when comparing summer and winter starch contents in Cr-treated fronds, a different

accumulation pattern was observed. In summer fronds, starch accumulation seems to be independent of Cr(VI) concentrations, whereas in winter, fronds becomes dependent on metal concentration. Thus, we assumed that the starch accumulation that occurs in Cr-treated *Salvinia* fronds during the winter and summer must be regulated differently by environmental factors, which affect the sink-strength intensity for carbohydrate partitioning (Lemoine et al. 2013). Decreases of sink-strength intensity induce the accumulation of soluble sugars and feedback inhibition of photosynthesis (Iglesias et al. 2002). In this regard, our results showed higher levels of total soluble sugars in both Cr-untreated and Cr-treated winter fronds compared with summer ones. Individual sugars, i.e., sucrose, glucose, and fructose showed different accumulation patterns. Glucose and fructose were higher in winter fronds whereas sucrose was higher in summer fronds (Table 2). It was stated that soluble sugars, mainly hexoses, are higher in aquatic macrophytes during the winter season due to less production of leaf material (Farmer and Spence 1987). Consistent with this finding, we observed a less emergence of new ramets in *S. minima* cultivated in winter (a ramet refers to each pair of fronds on the older rhizome) (Prado et al. 2010). Lower sucrose content in winter fronds can also account for an increased sucrose synthase-catalyzed hydrolysis of sucrose to produce fructose and ADP-glucose that is imported into chloroplast to the novo synthesis of transitory starch granules (Muñoz et al. 2005) and/or an enhanced invertase-catalyzed sucrose cleavage to produce free glucose and fructose (Prado et al. 2010). Furthermore, according to Gibon's statement, the higher level of hexoses found in Cr-treated winter fronds could also upregulate the leaf source function to sink storage activity giving a higher starch accumulation (Gibon et al. 2004). Then, it could be assumed that accumulation patterns of starch and soluble sugars occurring in Cr-treated winter fronds as well as the higher number and size of starch grains observed in Cr-treated winter chloroplasts are controlled by a unique carbohydrate cycle triggered by an interactive effect between low temperature, low solar irradiance, and Cr(VI) toxicity.

4.4 Electrolyte Leakage and Plasma Membrane Ultrastructure

Integrity and functionality of plasma membrane are used as indicators of Cr(VI) tolerance in plants (Chandra and Kulshreshtha 2004; Shanker et al. 2005). In this study, significant increases of electrolyte leakage (EL), an indicator of the plasma membrane injury, were observed in Cr-treated winter fronds exposed to 10 and 20 mg L^{-1} Cr(VI) concentrations (Fig. 3). Increased values of EL have also been associated with heavy metal-induced disruptions of thylakoids (Aravind and Prasad 2005). In fact, our results showed greater disorganization of thylakoids and ultrastructural alterations of plasma membrane in Cr-treated winter fronds (Fig. 1h, j and Fig. 2d). In Cr-treated summer fronds, there were no significant changes in EL, and consequently, TEM micrographs showed minor ultrastructural alterations in plasma membrane and scarce thylakoid disorganization (Fig. 1g, i and Fig. 2c). No changes of EL were also communicated for pea plants cultivated in the presence of 20 mg L^{-1} Cr(VI) concentration at 20/25 °C (Pandey et al. 2009). Metal-induced injury of leaf plasma membrane has been associated to high metal accumulation (Dubey 2011). Our data, however, showed an inverse trend, i.e., summer fronds accumulate more Cr, but show less plasma membrane damage; while winter fronds accumulate less metal, but show higher membrane damage (Table 3 and Fig. 2c, d). Since Cr(VI) uptake is an active energy-dependant mechanism (Shanker et al. 2005), it can assume that lower Cr accumulation in winter fronds is produced by both decreased metal uptake and reduced root-shoot translocation induced by winter low temperature. Supporting this assumption, the ratio of summer-accumulated metal to winter-accumulated metal did not show significant seasonal differences under increasing Cr(VI) concentrations (Table 3), indicating that temperature is the major factor that controls both root uptake and leaf accumulation of Cr(VI) in *Salvinia* plants. TEM micrographs also revealed greater ultrastructural alterations of thylakoids, which reinforces our assumption that an interactive effect between low temperature, low solar irradiance, and Cr(VI) toxicity, through a synergistic mechanism, is responsible of structural and metabolic changes that occur in winter chloroplasts, rather than the metal accumulation per se. Further studies will be needed to achieve a better understanding as *S. minima* interactively transduces signals of low temperature, day length, and Cr(VI) toxicity to modulate the accumulation and mobilization of carbohydrates in winter fronds and also to cope with Cr-induced oxidative stress and ultrastructural damages.

5 Conclusions

Data of this work clearly show the interconnectivity between structural and metabolic traits occurring in fronds of *S. minima* exposed to Cr(VI) under two contrasting seasons. Results reveal that much greater ultrastructural alterations observed in thylakoids and plasma membrane as well as in carbohydrate accumulation in winter fronds depend closely of an interactive effect between low temperature, low solar irradiance, and Cr(VI) toxicity. Evaluated parameters represent a relevant approach to enhance the knowledge on the performance and fitness of plants exposed to heavy metals under fluctuating environmental conditions. No doubt, this work will benefit those studies that are conducted to implement the removal of Cr(VI) from contaminated aquatic systems under field conditions.

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