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Research article

Effect of seasonality and Cr(VI) on starch-sucrose partitioning and related enzymes in floating leaves of *Salvinia minima*

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ABSTRACT

Effects of seasonality and increasing Cr(VI) concentrations on leaf starch-sucrose partitioning, sucrose- and starch-related enzyme activities, and carbon allocation toward leaf development were analyzed in fronds (floating leaves) of the floating fern *Salvinia minima*. Carbohydrates and enzyme activities of Cr-exposed fronds showed different patterns in winter and summer. Total soluble sugars, starch, glucose and fructose increased in winter fronds, while sucrose was higher in summer ones. Starch and soluble carbohydrates, except glucose, increased under increasing Cr(VI) concentrations in winter fronds, while in summer ones only sucrose increased under Cr(VI) treatment. In summer fronds starch, total soluble sugars, fructose and glucose practically stayed without changes in all assayed Cr(VI) concentrations. Enzyme activities related to starch and sucrose metabolisms (e.g. ADPGase, SPS, SS and AI) were higher in winter fronds than in summer ones. Total amylase and cFBPase activities were higher in summer fronds. Cr(VI) treatment increased enzyme activities, except ADPGase, in both winter and summer fronds but no clear pattern changes were observed. Data of this study show clearly that carbohydrate metabolism is differently perturbed by both seasonality and Cr(VI) treatment in summer and winter fronds, which affects leaf starch-sucrose partitioning and specific leaf area (SLA) in terms of carbon investment.

1. Introduction

Among environmental stresses, pollution by heavy metals is an increasing concern worldwide due to increasing anthropogenic contamination (Motuzova et al., 2014; Kabata-Pendias and Szteke, 2015). The modulation of growth rate and primary metabolism in response to environmental cues is essential for survival of plants (Yang and Chu, 2011). Plants respond and adapt to heavy metals through various biochemical and physiological processes that allow them cope with metal-induced stress (Fodor, 2002; Emamverdian et al., 2015). Like to drought, cold and salinity, heavy metals affect the flux of photosynthetic fixed carbon in leaves (Gupta and Kaur, 2005; Prado et al., 2011). Although important progresses were made to understand physiological and biochemical mechanisms related to responses of plants to abiotic stress (Koyro et al., 2012). Knowledge on carbohydrate metabolism under heavy metal stress remains limited (Jha and Dubey, 2004; Devi et al., 2007; Prado et al., 2010a). Carbohydrates play pivotal roles as energy source, structural components and signaling molecules during the growth and development of plants under normal and stressed conditions (Stitt and Zeeman, 2012; Tiessen and Padilla-Chacon, 2013; Lastdrager et al., 2014). Sucrose, glucose, fructose and starch are the most frequently reported carbohydrates in the context of stress responses (Rosa et al., 2009a; Ruan, 2014). In fact, many abiotic stresses often involve changes in sucrose and starch contents due to alterations of the allocation pattern of photosynthetic fixed carbon (Rosa et al., 2004; Higuchi et al., 2015; Pilkington et al., 2015). This becomes important because in most plants, including mono and dicotyledonous species, sucrose and starch are the most important photosynthetic products (Tetlow et al., 2004).

Sucrose is the main form in which the photosynthetic fixed carbon is exported from leaves, being synthesised from

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photosynthetic fixed carbon outside the chloroplasts. Starch represents the main carbon reserve stored in plants and can serve as an overflow, when the synthesis of sucrose exceed sucrose consumption by cells (Tetlow et al., 2004). Plants contain two types of starch named: "transitory starch" and "reserve starch". They are synthesised inside plastids, but its function will depend on both plastid type and tissue localization. Transitory starch occurs in photosynthetically competent cells, is synthesised inside chloroplasts during the day and degraded at night to provide carbon to non-photosynthetic metabolism (Lunn, 2007). Reserve starch is synthesised inside amyloplasts and serves as carbon reserve in seeds and storage tissues (Tetlow et al., 2004). It is degraded during both growth and germination processes (Ball and Morell, 2003). Leaf starch-sucrose partitioning is a well regulated process within carbohydrate metabolism and the amount of starch synthesised appears to be coordinated to the length of dark period (Börnke and Sonnewald, 2011). Decreased flux of carbon through sucrose synthesis may lead to lower export and higher accumulation of starch in leaves during the daytime. Starch-sucrose partitioning is affected by both plant development and sink activity, as well as by environmental factors including light intensity, temperature and photoperiod length (Pilkington et al., 2015). Abiotic and biotic stresses also affect the starch-sucrose partitioning in plant leaves (Biemelt and Sonnewald, 2006; Lemoine et al., 2013). Among abiotic stresses, heavy metals strongly affect both photosynthesis and respiration. In previous works we demonstrated that Cr(VI) affect both cytochrome c and alternative pathways of mitochondrial respiration, as well as the accumulation pattern of soluble sugars in floating (fronds) and submerged (lacinias) leaves of *S. minima* plants (Prado et al., 2010a,b). We also demonstrated by TEM microscopy Cr-induced alterations of both the shape and number of starch granules inside the chloroplasts of *S. minima* fronds grown in summer and winter seasons (Prado et al., 2015).

Alterations in leaf carbon partitioning can affect the availability of translocates (i.e. sucrose), day/night export patterns, and even the growth and development (Leonardos et al., 2006). To get new insights regarding to the effects of heavy metals on leaf starch-sucrose partitioning, in the present study we analyse seasonal variations of photosynthetic carbon investment based on specific leaf area (SLA), starch, soluble carbohydrates, and key enzymes of starch and sucrose metabolism induced by Cr(VI) in fronds of *Salvinia minima*, a fast growing aquatic fern used for phytoremediation of heavy metals (Dhir, 2009).

2. Materials and methods

2.1. Plant culture and Cr(VI) treatment

Healthy plants of *S. minima* were collected from a heavy metals unpolluted fresh water pond located at 500 m asl (26°50′S, 65°12′W, Tucumán, Argentina). Plants were collected in winter (July–August) 2015 and summer (December–February) 2015/2016. Uniform plants with fully expanded fronds and similar size were transferred to plastic pots (15 plants per pot, ~35 g FW) containing 1000 mL of Cr(VI) solution of different concentrations. Metal solutions were obtained from a stock Cr(VI) solution (500 mg L^{-1}) prepared in tap water using analytical grade potassium dichromate ($K_2Cr_2O_7$). All Cr(VI) treatment solutions were prepared using tap water in order to get a similar aquatic media where plants come from. Plants were cultivated in Hoagland's solution to avoid chelation and/or ionic competition between Hoagland anions and dichromate anions ($Cr_2O_7^{2-}$) for plant cell wall-binding sites. Addition of mineral nutrients may also result in microalgae growth that can interfere with heavy metal uptake. Cr(VI) concentrations were 0 (control), 5, 10 and 20 mg L^{-1}. Plants were cultivated outdoor during 7 days under weather conditions prevailing at the Campus of the Faculty of Natural Sciences (Tucumán, Argentina). We chose 7 days as treatment period because preliminary tests carried out in our laboratory showed that *S. minima* plants are able to grow and stay healthy in tap water for at least 9 days without nutrient supply. Climatic parameters (air temperature, relative humidity and daylength) were monitored using and automatic meteorological station (Pegasus EP1000, Argentina. Photosynthetically active radiation (PAR) was measured at noon with a Quantum Sensor (Li-190SA, Li-Cor, Lincoln, USA) coupled to a Data-Logger (Li-1000, Li-Cor, Lincoln, USA). The temperature of treatment solutions was measured with a portable underwater thermistor (-20 ± 1 °C to +70 ± 1 °C) connected to a Hobo Temp Logger (Onset Computer Corp., Pocasset, USA) (Table 1).

After Cr(VI) treatment, plants were harvested, rinsed in distilled water and cut to obtain fronds. For chemical and enzymatic determinations fronds were stored at -20 °C. To avoid diurnal changes in the concentrations of starch and soluble sugars, as well as in enzyme activities, plants were always collected at noon.

2.2. Cr(VI) accumulation

Oven-dried fronds (80 °C for 48 h) of Cr-untreated and Cr-treated plants were powdered in a mortar with pestle, and then were digested in HNO3 at 115 °C for 15 min following the USEPA 3051 protocol (www.epa.gov/epaoswer/hazwaste/test/pdfs/3051.pdf). Cr(VI) in digested samples was determined using an atomic absorption spectrophotometer (Perkin–Elmer 373, England), and the concentration expressed as μg g^{-1} DW. A blank of HNO_3 was also measured to ensure the correctness of metal quantification. Accuracy of metal determination was ascertained by addition of a known metal concentration. Overall recovery of Cr(VI) associated with digestion process was in the 90–95% range. Data were from three independent measurements.

2.3. Specific leaf area (SLA)

To obtain the specific leaf area, SLA, twenty frond discs (0.08093 cm^2) were punched out from fully developed fronds using a cork borer (3.21 mm i.d.). Next, discs were oven-dried for 48 h at 80 °C and then weighted to obtain the DW. Specific leaf area was calculated as the ratio of leaf area to corresponding leaf dry biomass and was expressed as cm^2 g^{-1}.

2.4. Soluble sugars and starch content

Soluble sugars (sucrose, glucose and fructose) were extracted from 1 g FW of floating leaves by homogenisation in a mortar and pestle with 2 mL of 80% ethanol (v/v). Resulting homogenate was heated in a water bath at 75 °C for 10 min, centrifuged at 5000 × *g* for 10 min, and collected the supernatant. Resulting precipitate was

Table 1
Climatic data recorded in summer and winter seasons during the experimental period.

Climatic parameter	Summer	Winter
Air maximum temperature	31.5 ± 1.5 °C	13.5 ± 1.5 °C
Air minimum temperature	20.2 ± 1.3 °C	6.3 ± 1.0 °C
Solut. maximum temperature	32.3 ± 1.7 °C	13.4 ± 1.3 °C
Solut. minimum temperature	20.2 ± 1.3 °C	5.1 ± 1.0 °C
Relative humidity	67.3 ± 6.5%	50.4 ± 5.0%
Daylength	>13.0 h	9.0 h
PAR solar irradiance (μmol m^{-2} s^{-1})	1926 ± 89	1015 ± 59
Cloudy and/or rainy days	0	0

homogenized twice with 2 mL of 80% v/v ethanol, heated in water bath, and centrifuged again. Supernatants were pooled and dried under a stream of hot air. Dry residue was resuspended in 1 mL of distilled water and desalted by filtration through an ion-exchange column (Amberlite MB3, BDH). Total soluble sugars were determined according to Dubois et al. (1956). Sucrose was determined by the protocol of Cardini et al. (1955) and fructose by the method of Roe and Papadopoulos (1954). Glucose was determined using a glucose oxidase-peroxidase coupled assay according to Jorgensen and Andersen (1973). Total and individual soluble sugar contents were expressed as mg g^{-1} FW.

For starch determination, insoluble fraction from ethanolic extraction of soluble sugars was resuspended in 2 mL of 2.5 M NaOH and boiled for 5 min to starch gelatinization. After cooling the pH of gelatinized starch was adjusted to 4.5 using 2 M HCl, and the resulting suspension was hydrolysed with buffered amyloglucosidase (15 UE mL^{-1} of *Rhizopus* mold amyloglucosidase in 0.1 M sodium acetate buffer, pH 4.5). After 10 min incubation at 50 °C, aliquots were taken from reaction mixture and soluble reducing sugars released were assayed by the Nelson's method (1944). Starch content was expressed as mg maltose equivalent released g^{-1} FW.

2.5. Enzyme activities

2.5.1. Cytosolic fructose 1,6 bisphosphatase (cFBPase)

cFBPase activity was extracted according to Prado et al. (1991). Briefly, fronds (1 g FW) were homogenized in a chilled mortar and pestle with 3 mL of 100 mM sodium phosphate buffer, pH 7.5, containing 10 mM dithiothreitol, 2 mM EGTA and 5% (w/v) polyvinylpolypyrrolidone. After centrifuging at 15000 × *g* for 10 min at 4 °C the supernatant was dialysed for 3 h against 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM dithiothreitol. Dialysed supernatant was used as enzyme extract. cFBPase activity was estimated by determining enzyme-released Pi from FBP. Briefly, a reaction mixture (200 μL) containing 100 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, 4 mM FBP and 100 μL of enzyme extract was incubated at 28 °C for 60 min and then stopped by adding 250 μL of 10% (w/v) trichloroacetic acid (TCA). After centrifuging at 12000 × *g* for 5 min the released Pi was determined colorimetrically as described by Chen et al. (1956). A reaction mixture without $MgCl_2$ was incubated to eliminate the interference of any non-specific phosphatase activity. Enzyme activity was expressed as μmol Pi min^{-1} mg^{-1} protein. Additionally, cFBPase activity was also determined by a continuous spectrophotometric assay using a reaction mixture (500 μL) containing 100 mM Hepes-NaOH, pH 7.5, 100 mM KCl, 4 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM NADP, 2 UE of phosphoglucoisomerase, 1 UE of glucose-6-phosphate dehydrogenase and 100 μL of enzyme extract according to Prado et al. (1991).

2.5.2. Sucrose phosphate synthase (SPS), sucrose synthase (SS) and ADP-glucose pyrophosphorylase (AGPase)

SPS, SS and AGPase activities were extracted according to Rosa et al. (2009b). Briefly, fronds (1 g FW) were homogenized in a chilled mortar and pestle with 3 mL of 100 mM Tris-HCl buffer, pH 7.6, containing 10 mM $MgCl_2$, 5 mM EDTA, and 1 mM β-mercaptoethanol. The homogenate was centrifuged at 15000 × g for 15 min at 4 °C, and the supernatant dialysed for 2 h against 10 mM Tris-HCl buffer, pH 8.0, containing 10 mM $MgCl_2$, and 1 mM β-mercaptoethanol. Dialysed supernatant was used as enzyme extract. SPS and SS activities were measured as described by Batta and Singh (1986) with minor modifications. Briefly, SPS reaction mixture (100 μL) containing 40 mM Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol, 8 mM UDP-glucose, 16 mM F6P, 8 mM G6P, and 20 μL of enzyme extract was incubated at 37 °C for 30 min. SS was assayed using fructose instead of F6P. Reaction products i.e. sucrose-6-phosphate (S6P) or sucrose (suc) were determined by the Cardini et al. (1955) procedure. Enzyme activities were expressed as μmol S6P min^{-1} mg^{-1} protein and μmol sucrose min^{-1} mg^{-1} protein, respectively. AGPase activity was measured following the production of NADPH according to Sowokinos (1976). *First step:* reaction mixture (500 μL) containing 40 mM Hepes-NaOH buffer, pH 8.0, 5 mM $MgCl_2$, 10 mM ADP-glucose, 20 mM 3-phosphoglycerate, 10 mM sodium pyrophosphate, and 100 μL of enzyme extract was incubated at 37 °C for 20 min. After incubation, tubes were heated in a boiling water bath for 3 min, cooled in an ice bath, centrifuged at 5000 × *g* for 5 min, and collected the supernatant. *Second step:* reaction mixture (1 mL) contained 40 mM Hepes-NaOH buffer, pH 7.4, 12 mM NADP, 2.25 UE phosphoglucomutase (PGM), 3 UE glucose-6-phosphate dehydrogenase (G6PDH) and 450 μL of first step supernatant. NADPH production was monitored at 340 nm and 37 °C using a double beam spectrophotometer (Hitachi U-2800A, Japan). Absorbance was recorded until the loss of initial linear reaction rate. Enzyme substrates and components of coupled assay were in excess and thus the rate of NADPH production stayed linear regarding to enzyme concentration and time. All assays were run with the corresponding controls without ADP-glucose to detect any non-AGPase-dependant NADPH production. Enzyme activity was expressed as μmol NADPH min^{-1} mg^{-1} protein.

2.5.3. Soluble acid invertase (AI)

AI activity was extracted and measured according to Prado et al. (1985) with minor modifications. Briefly, fronds (1 g FW) were homogenized in a chilled mortar and pestle with 3 mL of 50 mM sodium phosphate buffer, pH 7.4, containing 1 mM β-mercaptoethanol and 5 μM $MnSO_4$. Resulting homogenate was centrifuged at 15000 × *g* for 15 min at 4 °C, and supernatant was collected. Dialysed supernatant (2 h against 10 mM sodium acetate buffer, pH 5.5, containing 1 mM β-mercaptoethanol) was used as enzyme extract. Because extraction buffer did not contain detergents, chelating and chaotropic agents or high salt concentrations, the probability of extracting cell wall invertase activity was unlikely. To measure AI activity, a reaction mixture (100 μL) containing 200 mM sodium acetate buffer, pH 5.5, 60 mM sucrose, 1 mM β-mercaptoethanol, and 10 μL of enzyme extract was incubated at 37 °C for 30 min. Reducing sugars released were determined by the Nelson's method (1944) using glucose as standard. AI activity was expressed as μmol reducing sugars min^{-1} mg^{-1} protein.

2.5.4. Total amylase (TA)

Extraction and determination of total (α + β) amylase activity was performed as described by Rosa et al. (2004). Briefly, fronds (1 g FW) were homogenized in a chilled mortar and pestle with 3 mL of 100 mM sodium acetate buffer, pH 5.5, containing 1 mM β-mercaptoethanol and 3 mM $CaCl_2$. After centrifuging at 15000 × g for 10 min at 4 °C, collected supernatant was dialysed for 2 h against 10 mM sodium acetate buffer, pH 5.5, containing 1 mM β-mercaptoethanol and 3 mM $CaCl_2$, and used as enzyme extract. To measure TA activity a reaction mixture (100 μL) containing 10% soluble starch, 200 mM sodium acetate buffer, pH 5.5, 1 mM β-mercaptoethanol and 3 mM $CaCl_2$ and 20 μL of enzyme extract was incubated at 37 °C for 60 min. Reducing sugars released were determined by Nelson's method (1944) with maltose as standard. Total amylase activity was expressed as μmol maltose equivalent min^{-1} mg^{-1} protein.

For all assayed enzymes, control reactions without substrate were run in parallel to evaluate endogenous activities. Assays to determine linear correlation between enzyme amount and reaction time were also performed.

2.6. Protein

Protein concentration of enzyme extracts was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

2.7. Statistics

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

3. Results

3.1. Cr(VI) accumulation

Cr(VI) accumulation in *S. minima* fronds for both seasons is shown in Fig. 1. Accumulated metal significantly increased with increasing Cr(VI) concentration. Highest metal accumulation was found in the summer. Maximum Cr(VI) concentrations in summer and winter fronds (665 μg g^{-1} DW and 190 μg g^{-1} DW) occurred at 20 mg L^{-1} Cr(VI) concentration. Maximal seasonal difference in metal accumulation between summer and winter fronds was 3.6 fold and was observed at 10 mg L^{-1} Cr(VI) concentration. Values of Cr_{summer}/Cr_{winter} ratio for each Cr(VI) concentration were 3.62, 3.69 and 3.50, respectively (data not shown). Cr(VI) was not detected in Cr-untreated samples.

3.2. Specific leaf area (SLA)

SLA was higher in summer control plants compared with winter ones, but in presence of increasing Cr(VI) concentrations it was affected differently in both seasons. SLA of Cr-treated summer fronds was always lower than that of Cr-untreated fronds. Contrarily, in winter plants treated with Cr(VI) an increase of the SLA occurred. Under 5 mg L^{-1} Cr(VI) concentration a significant decrease (-23.6%) of SLA in summer fronds was observed, while in winter fronds a significant increase (32.9%) was found. A slight decreasing tendency of SLA in summer and winter fronds was observed from 5 mg L^{-1} Cr(VI) concentration (Fig. 2).

3.3. Starch, total soluble sugars, sucrose, glucose and fructose

Patterns of carbohydrates in Cr-untreated and Cr-treated fronds showed significant differences between summer and winter seasons (Fig. 3 and Fig. 4). Starch content of both control and Cr-exposed summer fronds was significantly lower ($p < 0.05$) than in winter fronds. In these fronds starch increased under Cr(VI) treatment. Maximum increase was 83.1% at 10 mg L^{-1} Cr(VI) concentration. By contrast in summer fronds there were no significant changes in starch content of Cr-treated plants compared with control ones (Fig. 3). Total soluble sugars was higher in winter fronds compared with summer ones. In Cr-treated winter fronds total soluble sugars increased strongly reaching a maximum increase of 61.9% under 10 mg L^{-1} metal concentration. Contrarily, total soluble sugars of summer fronds increased slightly in Cr-treated plants being only significant at 10 mg L^{-1} Cr(VI) (Fig. 4A). In Cr-treated fronds sucrose concentration increased significantly with increasing Cr(VI) concentration in both seasons. Under 20 mg L^{-1} Cr(VI) concentration, sucrose accumulation in sumer and winter fronds was 269% and 71% higher than in Cr-untreated fronds (Fig. 4B). Of interest, in summer fronds sucrose accumulation was more affected by Cr(VI) treatment than starch accumulation whereas no significant differences were observed in winter fronds (Figs. 3 and 4B). Maximal glucose concentration in summer fronds occurred at 10 mg L^{-1} Cr(VI) concentration whereas in winter fronds decreased significantly. Maximal winter decrease was 50% and was observed at 10 mg L^{-1} Cr(VI) concentration (Fig. 4C). Fructose concentration increased in Cr-treated summer and winter fronds, but was stronger in the latter. Maximum increases were 191% (winter) and 24% (summer) and occurred at 10 mg L^{-1} Cr(VI). However, under 20 mg L^{-1} Cr(VI) fructose concentration decreased in both seasons being more pronounced in summer fronds (Fig. 4D).

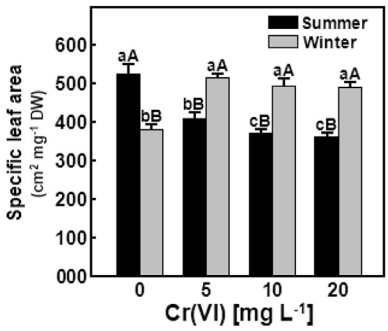

Fig. 2. Specific leaf area of summer and winter fronds exposed to increasing Cr(VI) concentrations. Data are mean ± SE of 3 replications (n = 10). Different lowercase letters on bars indicate significant differences for each season. Different uppercase letters indicate significant differences between seasons, for each Cr(VI) concentration.

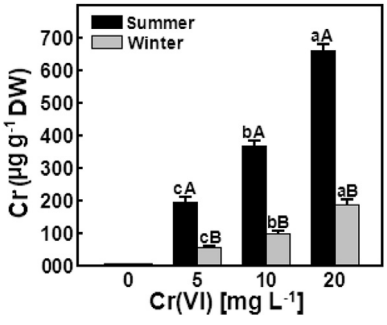

Fig. 1. Cr(VI accumulation in fronds of S. minima plants grown during 7 days in both summer and winter seasons. Data are mean ± SE of 3 replications (n = 6). Different lowercase letters on bars indicate significant differences for each season. Different uppercase letters indicate significant differences between seasons, for each Cr(VI) concentration.

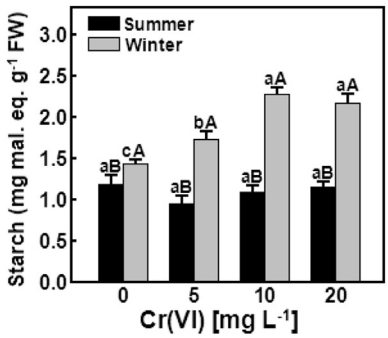

Fig. 3. Starch content of summer and winter fronds exposed to increasing Cr(VI) concentrations. Data are mean ± SE of 3 replications (n = 6). Different lowercase letters on bars indicate significant differences for each season. Different uppercase letters indicate significant differences between seasons, for each Cr(VI) concentration.

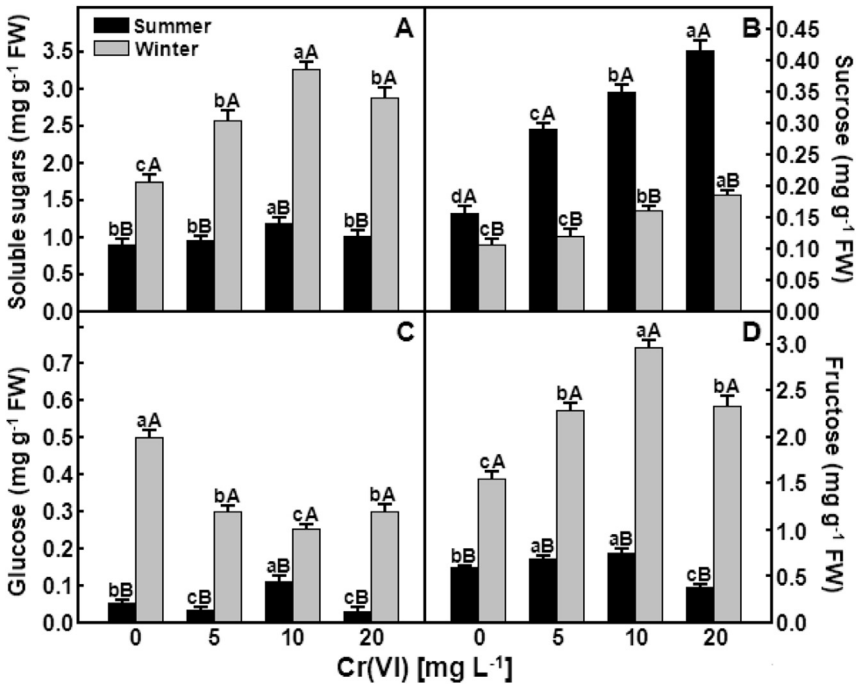

Fig. 4. Contents of soluble carbohydrates (total sugars [**A**], sucrose [**B**], glucose [**C**] and fructose [**D**]) of summer and winter fronds exposed to increasing Cr(VI) concentrations. Data are mean ± SE of 3 replications (n = 6). Different lowercase letters on bars indicate significant differences for each season and each measured sugar. Different uppercase letters indicate significant differences between seasons for each Cr(VI) concentration and each measured sugar.

3.4. Enzyme activities

3.4.1. ADP-glucose pyrophosphorylase (AGPase) and total amylase (TA)

AGPase activity in winter fronds was significantly higher than in summer fronds, but was affected negatively by Cr(VI) in both seasons. Summer and winter enzyme patterns were similar, but enzyme activity was decreased by 58.6% (summer) and 42.7% (winter) under 10 and 20 mg L^{-1} Cr(VI) concentrations, respectively (Fig. 5A). In Cr-exposed summer and winter fronds, total amylase activity increased significantly until reach maximum increase values of 114.7% (summer) at 10 mg L^{-1} Cr(VI) concentration and 58.7% (winter) under 5 mg L^{-1} Cr(VI), respectively. At the highest Cr(VI) concentration amylase activity decreased in both seasons (Fig. 5B).

3.4.2. Cytosolic fructose 1,6 bisphosphatase (cFBPase) and sucrose phosphate synthase (SPS)

Cytosolic FBPase activity was higher in summer than in winter, but the presence of increasing Cr(VI) concentrations in growth solutions increased enzyme activity in both seasons. Maximum increases 40.1% (summer) and 46.4% (winter) occurred at 5 mg L^{-1} Cr(VI) concentration. From this point on cFBPase activity decreased until control value at 20 mg L^{-1} Cr(VI) concentration (Fig. 5C). SPS activity increased by Cr(VI) treatment in both seasons, but was higher in winter fronds. Maximum increases were 75.3% (summer) and 344% (winter) and occurred at the highest Cr(VI) concentration (Fig. 5D).

3.4.3. Sucrose synthase (SS) and soluble acid invertase (AI)

Activities of SS and AI were affected differently by Cr(VI) treatment in both seasons. Enzyme activities were higher in winter fronds compared with summer ones, excepting SS at 10 mg L^{-1} Cr(VI) concentration (Fig. 5E and F). In summer fronds, SS activity showed a sustained increase up to 10 mg L^{-1} Cr(VI), but from this point on decreased strongly until 20 mg L^{-1} Cr(VI) concentration. In winter fronds, SS activity decreased significantly at 5 and 10 mg L^{-1} Cr(VI) concentrations, but increased markedly at 20 mg L^{-1} Cr(VI). Maximum increases were 277.6% (summer) and 60% (winter), respectively (Fig. 5E). AI activity was higher in winter fronds than in summer ones, but was less affected by Cr(VI) treatment. In both seasons significant increases of enzyme activity were observed at the highest Cr(VI) concentration. Maximum increases of AI activity were 71.9% (summer) and 26.1% (winter) (Fig. 5F).

4. Discussion

4.1. Cr(VI) accumulation

Accumulation of heavy metals in aerial parts of aquatic macrophytes varies through seasons as result of plant growth dynamics and also in response to variations of metal bioavailability in surrounding water (Rai, 2008). However, most of results obtained with both floating and submerged species are controversial. Some indicate that highest metal concentrations occur during the autumn, while others show highest metal contents during spring or summer (Duman et al., 2006; Bragato et al., 2009; Grudnik, 2010). Agreeing with these last, our data also showed the highest content of Cr(VI) in fronds of summer-grown plants (Fig. 1). Since the bioaccumulation of Cr(VI) is an energy-dependant process (Emamverdian et al., 2015), one may assume that lower metal content found in winter fronds is produced by both decreased metal uptake and reduced root-frond translocation induced by low temperatures occurring during the winter. In support of this assumption Cr_{summer}/Cr_{winter} ratio did not show significant differences under all assayed Cr(VI) concentrations. This may indicate that in our experimental conditions, temperature is the major factor controlling both root metal uptake and foliar metal accumulation.

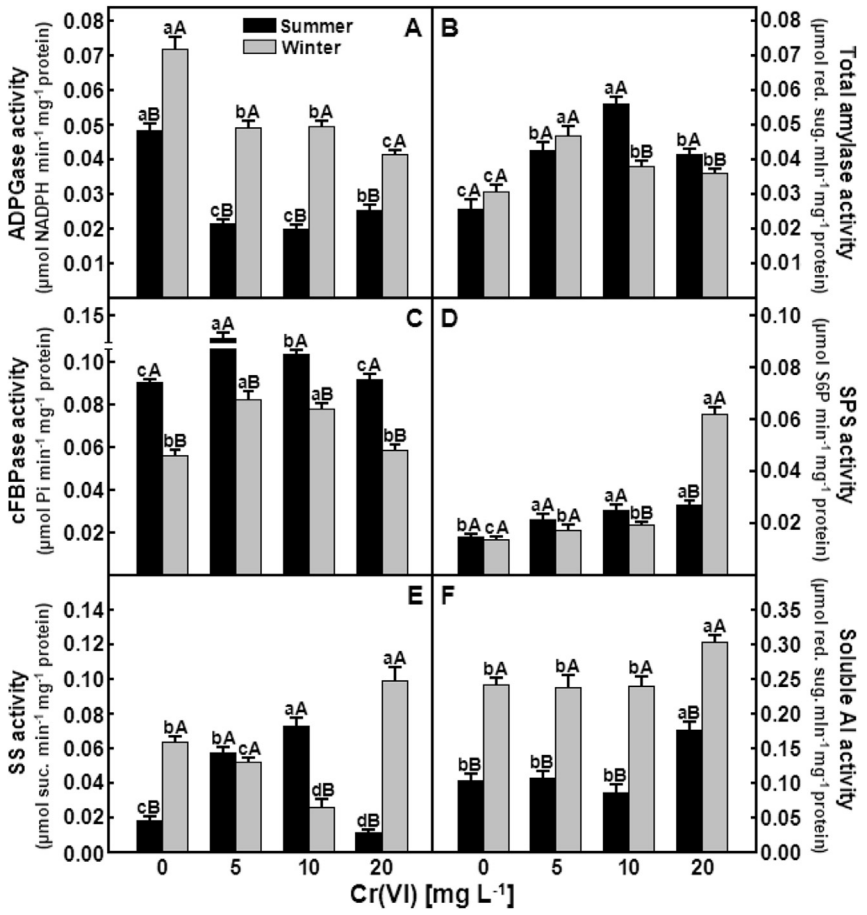

Fig. 5. Starch and sucrose metabolizing enzymes (ADPGase [**A**], total amylase [**B**], cFBPase [**C**], SPS [**D**], SS [**E**], soluble AI [**F**]) of summer and winter fronds exposed to increasing Cr(VI) concentrations. Data are mean ± SE of 3 replications (n = 6). Different lowercase letters on bars indicate significant differences for each season and each measured enzyme activity. Different uppercase letters indicate significant differences between seasons for each Cr(VI) concentration and each measured enzyme activity.

4.2. Specific leaf area (SLA)

Leaves are organs sensitive to environmental changes and may exhibit phenotypic plasticity to stressful conditions (Poorter et al., 2009). Under abiotic stress (e.g. drought, salinity, mineral deficiency, UV-B radiation, low and high temperature, heavy metals), leaves alter their physiology, morphology and development in response to environmental changes (Alaoui-Sossé et al., 2004; Xu et al., 2009). Photosynthesis, relative growth rate (RGR), and SLA are within the group of leaf parameters more affected by heavy metals (Alaoui-Sossé et al., 2004; Vernay et al., 2007; Prado et al., 2010b; Borowiak and Fidler, 2014). SLA is an important leaf morphological parameter related to carbon investment and represents the integration of leaf area and leaf dry mass being also correlated with leaf thickness and photosynthetic capacity (Xu et al., 2009). SLA can act as indicator of leaf source-sink balance because changes in SLA are correlated with changes in photosynthate allocation (Valantin et al., 1998; Shipley, 2002). In fact, a small SLA is correlated with increased leaf dry matter accumulation (high leaf sink activity and higher carbohydrate consumption) whereas a large SLA is correlated with a low dry matter accumulation (low leaf sink activity and lower carbohydrate consumption). Agreeing with these assumptions, our data show lower values of SLA and lower accumulation of soluble sugars in Cr-treated summer fronds compared with winter ones (Figs. 2 and 4A). Furthermore in a previous study we demonstrated higher biomass accumulation in Cr-treated summer fronds when comparing with Cr-treated winter fronds (Prado et al., 2010b). In line with our findings it has also been reported that the high level of soluble sugars, preferably hexoses, observed in leaves of the aquatic macrophyte *Lobelia dortmanna* during the winter season is due to less production of leaf material (Farmer and Spence, 1987).

4.3. Starch and soluble sugars

Accumulation of carbohydrates i.e. starch and soluble sugars in leaves, is a highly environmentally dependent multifaceted process, but is also influenced by stressful conditions such as heavy metals (Prado et al., 2011). Starch accumulation in Cr-exposed leaves of aquatic macrophytes has been studied in a few species such as *Lemna minor*, *Pistia stratiotes*, *Salvinia minima*, *Azolla caroliniana* and *Spirodela polyrhiza* (Bassi et al., 1990; Wilson and Al-Hamdani, 1997; Nichols et al., 2000; Appenroth et al., 2003; Reale et al., 2016). However, there are no reports on the effect of seasonality on starch-sucrose partitioning. Under subtropical climates plants are generally subjected to cooler temperatures and lower solar radiation during the winter regarding to prevailing climatic conditions in the warm season (summer) (Ribeiro et al., 2009). Thus, it can be expected that climatic parameters (e.g. air/water temperature, daylength and PAR solar irradiance), also affect the carbon allocation at both leaf and whole plant level.

According to Stitt and Zeeman (2012) the partitioning of photosynthetic fixed carbon at the whole plant level requires a complex control to supply immediate carbon demand during the

diurnal period through sucrose synthesis and also to anticipate nocturnal demand through starch accumulation in leaves (transitory starch). Although starch synthesis is enhanced by both raising temperature and increasing daylength (Geigenberger, 2011; Pilkington et al., 2015), our results showed higher starch content in Cr-untreated winter fronds when comparing with Cr-untreated summer fronds (Fig. 3). Starch accumulation in leaves under reduced sink demand has been recognized as the major regulatory process involved in direct feedback modulation of leaf photosynthesis (Zhou and Quebedeaux, 2003; Fan et al., 2010). Thus, lowest metabolic activity (low sink demand) induced by winter low temperature probably triggers the higher starch content found in winter fronds. Supporting our assumption, it has been reported that aquatic macrophytes growing at low temperatures and also in presence of heavy metals exhibit an altered sink activity (Pilon and Santamaría, 2001; Paiva et al., 2009). Furthermore, it has also been demonstrated that leaves accumulated more starch in short days than in long days (Mugford et al., 2014). In consonance with these findings, our data showed that Cr-exposed summer and winter fronds have different starch accumulation patterns. Starch accumulation in winter fronds depends on Cr(VI) concentration, but in summer fronds no correlation is observed (Fig. 3). This may indicate that in Cr-treated summer and winter fronds the starch accumulation is regulated differently by environmental factors, which, in turn, affect the starch-sucrose partitioning (Lemoine et al., 2013). Since reduced metabolic activity of sink tissues induces soluble sugars accumulation in source leaves (Iglesias et al., 2002; Rosa et al., 2004), our results showed higher levels of total soluble sugars in winter fronds (Fig. 4A).

In many plants, photosynthetic fixed carbon is mostly channelized toward the synthesis of sucrose (Tetlow et al., 2004). Thus, highest sucrose concentration found in summer fronds may be partly due to increasing photosynthesis derived from increasing temperature and longer daylength. Metabolic imbalance imposed by heavy metals also leads to sucrose accumulation in leaves (Alaoui-Sossé et al., 2004; Devi et al., 2007; Dias et al., 2016). In fact, our data showed an increased sucrose accumulation in both summer and winter Cr-exposed fronds, but was more pronounced in the first ones (Fig. 4B). Contrarily, very low glucose level observed in Cr-untreated (control) and Cr-treated summer fronds (Fig. 4C), probably reflects a higher temperature-dependant hexose consumption to sustain both increased cellular respiration and increased biomass production like was observed in plants growing in warm environments (Klõšeiko and Mandre, 2001). In fact, in previous study we observed both increased oxygen consumption (respiration) and increased number of new fronds in control and Cr-treated *S. minima* plants growing in summer when comparing with winter-grown plants (Prado et al., 2010a,b). By contrast, higher fructose accumulation in winter fronds under increasing Cr(VI) concentrations can be related to both low metabolic demand and higher activity of sucrose-hydrolysing enzymes. However, differences observed in glucose and fructose accumulation patterns of Cr-treated fronds probably are produced by disturbs in photosynthetic carbon allocation induced by Cr(VI) exposure (Smith and Stitt, 2007; Geigenberger, 2011; Pilkington et al., 2015).

Sugar accumulation decreases photosynthesis rate through a feedback inhibition mechanism (Paul and Pellny, 2003). It can also stimulate respiration and concomitantly the over-reduction of some electron transport chain components increasing reactive oxygen species (ROS) generation (Møller, 2001). In this context, accumulated sucrose could act as an effective sink for electron excess through the alternative oxidase (AOX) pathway avoiding over-reduction of the mitochondrial electron chain (Gandin et al., 2009). Activation of the alternative respiratory pathway allows plants limit the increase of harmful ROS derived from heavy metal-induced oxidative stress (Saha et al., 2016). In a previous study we found an increased oxidase alternative capacity in fronds of *S. minima* exposed to increasing Cr(VI) concentrations (Prado et al., 2010a).

4.4. Enzymes of starch and sucrose metabolism

To elucidate the influence of winter and summer climatic conditions on starch-sucrose partitioning in Cr-exposed fronds, we analyzed activities of starch- and sucrose-related enzymes i.e. ADP-glucose pyrophosphorylase (AGPase), total amylase (TA), cytosolic fructose 1,6 bisphosphatase (cFBPase), sucrose phosphate synthase (SPS), sucrose synthase (SS) and soluble acid invertase (AI), under increasing Cr(VI) concentrations. AGPase activity in winter fronds was higher than in summer ones (Fig. 5A), but Cr treatment decreased enzyme activity in both seasons being more pronounced in summer. Decreased enzyme activity patterns showed close similarities between seasons, indicating a similar damage at enzyme level induced by Cr(VI). According with Neuhaus and Stitt (1990), under low irradiances the control of leaf starch synthesis only reside in AGPase catalysed reaction, while under high irradiances this control is redistributed toward other enzymes of the biosynthetic pathway. However, it has also been demonstrated that the length of daily photosynthetic period itself is responsible of starch accumulation, independently of PAR irradiance (Chatterton and Silvius, 1981). This could indicate that different season-dependant metabolic scenarios are acting in both synthesis and accumulation of starch in fronds of *S. minima* plants. In consonance with this assumption the pattern of starch accumulation in Cr-treated fronds was different in both seasons (Fig. 3). Thus, it can be assumed that different regulatory mechanisms are operating on starch metabolism in winter and summer fronds.

Transitory starch can be broken down by both hydrolytic and phosphorolytic pathway being the former predominant in plant cells (Weise et al., 2006). In hydrolytic pathway both α- and β-amylase play key roles producing maltose and glucose that are exported to the cytoplasm to sustain sucrose synthesis during the night (Asatsuma et al., 2005; Weise et al., 2006). Beyond well-known chloroplastic amylases, leaves also contain less characterized extrachloroplastic amylases that also participate in degradation of starch granules (Lin et al., 1988; Ghiena et al., 1993). In addition to their role in starch-sucrose partitioning, both chloroplast and extrachloroplast amylases also participate in defence mechanisms against abiotic and biotic stresses (Doyle et al., 2007). For example it has been demonstrated that β-amylase degrades transitory starch to sustain proline biosynthesis under drought stress (Zanella et al., 2016). However, increased starch degradation in Cr-exposed fronds could also serve to provide fuel (soluble sugars) and carbon skeletons to sustain both enhanced respiration and synthesis of different protective compounds (e.g. phenolics, thiols, proline, lignin, suberin) needed to counteract the metal toxicity. In support of this assumption in previous studies we demonstrated both enhanced respiration and increased accumulation of soluble phenolics in Cr-exposed *Salvinia* fronds (Prado et al., 2010a, 2012). Thus increased total amylase activity occurring in summer and winter fronds can be related to defence mechanism against Cr-induced stress. Besides its possible role in stress response, higher total amylase activity in Cr-exposed summer fronds may also explain the higher accumulation of sucrose occurring in these fronds compared with winter ones (Fig. 4B).

During the light period both cFBPase and SPS are responsible of sucrose synthesis in leaves (Börnke and Sonnewald, 2011). Our results show that cFBPase and SPS were affected differently by both seasonality and Cr(VI) treatment. In Cr-unexposed fronds, cFBPase activity was higher in summer while SPS activity did not show

significant differences between seasons. Under Cr(VI) exposure enzyme activities increased in both seasons, but activity patterns were different between enzymes (Fig. 5C and D). Beyond that increased activities of cFBPase and SPS certainly contribute to increased sucrose accumulation in winter and summer fronds, a question still remains to be clarified. If sucrose accumulation patterns in summer and winter Cr-exposed fronds are similar but enzyme activity patterns are not, one might assume that under Cr(VI) exposure other unknown balancing mechanisms underlying carbohydrate metabolism are present which lead to similar sucrose accumulation patterns in both seasons. This fact could be important to ensure survival of *S. minima* plants in Cr-polluted aquatic systems in all seasons. In support of this assumption it has been observed that *S. minima* have a wide range of acclimation ability to various light intensities and temperature values (Al-Hamdani and Ghazal, 2009). Further studies are needed to get new insights on this topic. There are no reports on the effect of Cr(VI) on cFBPase activity in *Salvinia* species and then this study represents the first report regarding to Cr(VI) effect on this enzyme.

Synthesised sucrose is translocate to sink-sites to be hydrolysed either by invertase (acid and neutral types) or sucrose synthase (SS) to produce free hexoses (fructose and glucose) the former and fructose and UDP-glucose the latter, to sustain both energetic and synthesizing metabolism (Koch, 2004). AI and SS activities, in general, were higher in winter fronds than in summer ones in both Cr-unexposed and Cr-exposed *S. minima* plants (Fig. 5E and F). In Cr-exposed winter fronds enzyme activities increased strongly under highest metal concentrations. Although this fact could lead to apparent wasteful hydrolysis of sucrose during winter as a result of decreased plant growth (Prado et al., 2010b), released hexoses could be metabolized to produce NADPH. Accumulated NADPH could be utilized to sustain both NADPH-dependant biosynthetic reactions and NADPH-dependant ROS-detoxifying enzymes (Møller, 2001). Furthermore, higher activities of AI and SS in Cr-exposed winter fronds may also contribute to increase the free fructose pool which can be channelized via erythrose-4-phosphate (E4P) toward the synthesis of phenolic compounds (Mustafa and Verpoorte, 2007). Agreeing with this assumption, in a previous study we observed much higher content of soluble phenolics in Cr-exposed winter fronds compared with summer ones (Prado et al., 2012). On the other hand, increased SS activity in winter fronds can also increase ADP-glucose concentration which, in turn, can be imported inside the chloroplasts, and then incorporated to the novo synthesis of transitory starch, as was stated by Muñoz et al. (2005). Then, higher SS activity of winter fronds could also contribute to higher starch content found in these fronds (Prado et al., 2015). However, according to Gibon's statement, highest hexose levels found in Cr-exposed winter fronds can also upregulate the leaf source function to sink storage activity, leading to higher starch accumulation (Gibon et al., 2004).

5. Conclusions

Our results revealed that carbohydrate metabolism in term of starch and soluble sugars accumulation under contrasting seasons is affected by Cr(VI) exposure. Cr(VI) stress causes alterations in the content of transitory starch, sucrose glucose and fructose, as well as in activities of starch and sucrose metabolizing enzymes. Data obtained suggest that changes occurring in carbohydrate metabolism of *S. minima* fronds exposed to increasing Cr(VI) concentrations, depend of interactive effects among ambient temperature, day-length and solar irradiance rather than metal toxicity *per se*. However, additional studies on seasonality and Cr(VI) effects on other enzymes of carbohydrate metabolism including Calvin cycle enzymes are expected to shed further light on starch-sucrose partitioning in aquatic macrophytes used in phytoremediation processes.

Conflict of interest

Authors declare that they have no conflict of interest.

Contributions

MR and CP initiated de project and performed most of experiments. SCP contributed experimental cultivation of plants. EP measured Cr content in fronds. FP wrote the manuscript and drew figures. All authors proofread the manuscript.

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