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Involvement of glutathione metabolism in *Eichhornia crassipes* tolerance to arsenic

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Involvement of glutathione metabolism in *Eichhornia crassipes* tolerance to arsenic

Abstract

1. Aquatic macrophytes are potential useful for phytoremediation programs in environments contaminated by arsenic (As). Biochemical and physiological modification analyses in different plant parts are important to understand As tolerance mechanisms. **2.** The objective was to evaluate glutathione metabolism in leaves and roots of Eichhornia crassipes (Mart.) Solms plants subjected to As. Specimens of *Eichhornia crassipes* plants were cultured for three days in Clark's nutrient solution containing 7 μM of As. The enzymes ATP sulfurylase (ATPS), glutathione reductase (GR), glutathione peroxidase (GSH-Px), glutathione sulfotransferase (GST) and γ-glutamylcysteine (γ-ECS) synthetase activity, the glutathione contents, total proteic and non-proteic thiols were evaluated. **3.** The ATPS activity increased in roots. The GR activity in leaves and GSH-Px in roots were lower. GST activity was higher in roots and lower in leaves and γ-ECS activity was higher in this plant's leaves. Glutathione levels were lower, total thiols levels were higher and non proteic levels presented no change

in E. crassipes leaves and roots. Exposure to As increased the enzyme activity involved with sulfur metabolism, such as ATPS. Higher GR activity and lower GSH-Px indicates a greater glutathione conjugation to As, due to the greater GSH availability. The greater GST activity indicates its participation in As detoxification and accumulation, through As GSH conjugation. Changes in the glutathione and thiol levels suggest high phytochelatin synthesis. **4.** In conclusion, the increment in ATPS, GR, GST and γ-ECS activity indicates that these enzymes are involved in GSH metabolism and are part of the E. crassipes As detoxification mechanism.

Keywords: Phytoremediation, thiols, toxicity, water hyacinth.

Introduction

Arsenic (As) is a toxic metalloid affecting biochemical, physiological and morphological plant processes (Silva et al. 2016). High concentrations of this compound in aquatic environments are mainly due to anthropic sources (Kumar et al. 2015). The removal of As from the environment can be accomplished by different techniques, including phytoextraction by aquatic macrophytes (Bernardino et al. 2016), which must tolerate this metalloid.

Tolerance to high metals and metalloids concentrations includes defense mechanism activation against oxidative stress and chelating substances to prevent biological damage (Silveira et al. 2015), since exposure to these pollutants increases the reactive oxygen species (ROS) production. Arsenate to arsenite reduction increased chelating substance synthesis such as glutathione (GSH) and phytochelatins, and the arsenite-bound compounds compartmentation in vacuole may increase tolerance to As (Song et al. 2014). Chelation with sulfur-containing binders, such as GSH and phytochelatins, is the main detoxification route to As (Silva et al. 2017). Thus, exposure to this metalloid stimulates the enzyme activity related to sulfur metabolism (Dixit et al. 2015). Glutathione, synthesized by the γ-glutamylcysteine synthetase and GSH synthetase action, is the main transport form and reduced sulfur storage (Yadav, 2010), thus protecting membranes against free radicals (Farnese et al. 2016). The GSH conjugation to toxic compounds, including As protects plants against pollutants through the enzyme GSH sulfotransferase, a phytochelatins precursor (Singh et al. 2015).

Biochemical and physiological modification analyses in different plant parts are important to understand As tolerance mechanisms. Many aquatic macrophytes are potential useful for phytoremediation programs in environments contaminated by this metalloid. *Eichhornia crassipes* (Mart.) Solms is efficient in removing metals from aquatic environments (Malar et al. 2014, Ting et al. 2018) increasing the importance of studying its As absorption capacity and the toxic effects of this metalloid on this plant. The objective of this study was to analyze GSH metabolic variability in *E. crassipes* plants subjected to As.

Material and methods

Application of As treatments

Eichhornia crassipes specimens were collected at the Fishery Station of the Universidade Federal de Viçosa in Viçosa, Minas Gerais, Brazil (20°45'25.0"S 42°52'25.5"W) and maintained for 24 hours in deionized water. They were transferred to polyethylene containers with 10 L of Clark nutrient solution (Clark, 1975), pH 6.5, and placed in growth room with controlled temperature and light (25 ± 2 ^{o}C, 230 μmol s^{-1} m^{-2}, respectively), under a photoperiod of 16 hours, for three days.

The *E. crassipes* plants were acclimatized and selected according to their fresh mass, size uniformity and leaf numbers, keeping 40 plants in 20 pots with 0.5 L of Clark solution (two plants per pot), 10 of them containing 7 μM As in the form of $Na_2HAsO_4.7H_2O$ and 10 containing nutrient solution only. After three days, the plants were washed with deionized water and leaf and root samples frozen in liquid nitrogen and stored in a freezer at -80 °C.

The experiment was conducted as a completely randomized factorial design with five replicates. The data were analyzed using ANOVA, and the means were calculated using Tukey's test at 5 % probability. The statistical analyses were conducted using the software SAS (Cary, NC, United States).

Assessment of Enzymes Activity of the Thiol Metabolism

The ATP sulfurylase (ATPS), glutathione reductase (GR), glutathione peroxidase (GSH-Px), glutathione sulfotransferase (GST) and γ-glutamylcysteine synthetase (γ-ECS), glutathione and thiols were extracted from 0.5 g of *E. crassipes* fresh roots and leaves. The samples were macerated in liquid nitrogen and homogeneized in the following reaction media: a) two mL of 0.1 M Tris-HCl buffer, pH 8.0, containing 2 mM $MgCl_2$, 0.1 M KCl and 10 mM DTE (dithioerythritol), plus 0.1 mg of PMSF (phenylmethylsulfonyl fluoride) for

ATPS extraction; b) two mL of 1 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA (ethylene diamine tetraacetic acid), 0.02% triton, 2 mM DTT (dithiothreitol), 1 mM PMSF and PVPP (polyvinyl pyrrolidone) 1% for GR extraction (Carlberg and Mannervik, 1985); c) two mL of 0.1 M Tris-HCl buffer, pH 7.5, 1 mM EDTA and 10 mM $MgCl_2$ for GSH-Px extraction (Nagalakshimi and Prasad, 2001); d) two mL of 0.2 M Tris-HCl buffer, pH 7.8, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and PVPP 5% (w/v) for GST extraction (Habig et al. 1974); e) two ml of 0.1 M Tris-HCl buffer, pH 8.0, 5 mM EDTA and 1% PVPP for γ-ECS extraction (Nagalakshmi and Prasad, 2001); f) two mL of 0.1 M HCl and 1 mM EDTA for glutathione extraction (Anderson, 1985); g) six mL of 0.1 M Tris-HCl, pH 8.0, 1 mM EDTA and 1% ascorbic acid (w/v) to extract the total, non-proteic and proteic thiols (Meuwly and Rauser, 1992).

Following the specific buffer addition, they were flitrated in cheesechoth and centrifuged at 10,000 *g*, for 15 min, for the ATPS enzyme, at 12,000 *g*, 15 min, for GR, GSH-Px, GST and glutathione, at 30,000 *g*, 10 min for γGCS and at 10,000 *g* during 10 min for thiols, always at 4 °C. Enzyme activities and thiols were evaluated in the supernatant.

The ATPS activity was determined by the addition of 0.8 mL of the reaction medium containing 10 μM Tris-HCl, pH 8.0, 2.5 μM pCMB (p-chloromercuribenzoate) and two pyrophosphate units to 0.2 mL of the enzyme extract, followed by incubation at 37 °C for 5 min (Adams and Johnson, 1968) and the inorganic phosphate content was determined in the supernatant by the phospho-molybdate method (Lindeman, 1958). The ATP sulfurylase activity was expressed in nmol of Pi min^{-1} mg^{-1} protein.

The GR activity, expressed in nmol of oxidized glutathione min^{-1} mg^{-1} protein, was determined with the 0.1 mL crude enzyme extract added to 0.9 mL of reaction medium containing Tris-HCl 0,1 M, pH 7.5, GSSG 20 mM and NADPH 2 mM (Carlberg and Mannervik, 1985) and calculated using the molar extinction coefficient 6.22 mM^{-1} cm^{-1}.

The GSH-Px activity, expressed in nmol of NADPH min^{-1} mg^{-1} protein, was determined with the addition of 100 μL of the crude enzyme extract of *E. crassipes* to 0.9 mL of a reaction medium containing 50 mM potassium phosphate, pH 7, 0.1 mM EDTA, 0.114 M NaCl, 1 mM GSH, 0.2 mM NADPH, 0.25 mM H_2O_2 and one unit of glutathione reductase (Nagalakshimi and Prasad, 2001) and calculated using the molar extinction coefficient 6.22 $mM^{-1}cm^{-1}$ (Anderson and Davis, 2004).

The glutathione sulfotransferase activity was determined after addition of 0.1 mL of crude enzyme extract to 0.9 mL of a reaction medium containing 0.2 M potassium phosphate, pH 6.5, 20 mM GSH and 0.1 mM CDNB (1-chloro 2,4-dinitrobenzene). The activity was

calculated using the molar extinction coefficient 9.6 mM^{-1} cm^{-1} and expressed in nmol min^{-1} mg^{-1} protein.

The γ-glutamylcysteine synthetases activity was determined with the 100 μL crude enzyme extract added to 0.9 mL of a reaction medium containing 10 μM sodium glutamate, 10 μM aminobutyrate, 2 μM Na_2EDTA, 0.2 mg BSA, 20 μM $MgCl_2$, 5 μmol ATP disodium salt, 150 mM KCl and 100 μM Tris-HCl, pH 8.2. The inorganic phosphate content in the supernatant determined by the phosphomolybdate method (Lindeman, 1958). The γ-ECS activity was expressed in nmol Pi mg^{-1} protein.

The protein content of the enzyme extracts was determined with BSA as standard to express the enzyme activity (Lowry et al. 1951).

Total glutathione content (GSH + GSSG)

Aliquots with 200 μL of the crude enzyme extract were added to 200 μL of sodium phosphate 125 mM, pH 7.5, containing EDTA 6.3 mM, 500 μL of NADPH 0.3 mM and 100 μL of DTNB [5,5'-dithiobis acid (2-nitrobenzoic)] 6 mM. After incubation at 30 °C for 5 min, 10 μL of glutathione reductase (50 U mL^{-1}) was added and absorbance was determined at 412 nm for 1 min. The concentration of glutathione was determined based on the calibration curve using reduced glutathione standards. The results were expressed as nmol g^{-1} fresh weight (FW).

Total, proteic and non-proteic thiols content

Total thiols content was determined in 0.5 mL of the supernatant with addition of 1.5 mL of potassium phosphate 0.2 M, pH 8.2, 0.1 mL of Ellman´s reagent [5,5 '-dithiobis acid (2-nitrobenzoic)] 0.01 M and 7.9 mL of methanol.

The non-proteic thiols content were determined in 5.0 mL of the supernatant with addition of 1.0 mL of 50% (w/v) TCA, 4.0 mL of H_2O and centrifuged at 10,000 *g* for 15 min. At two mL of the supernatant were added 4.0 mL of potassium phosphate 0.4 M, pH 8.9 and 0.1 ml of Ellman´s reagent 0.01 M. The absorbance was read at 412 nm and the total and non-proteic thiol content calculated with the molar extinction coefficient of 13100 M^{-1} cm^{-1} and expressed as nmol SH g^{-1} FW (Sedlak et al. Lindsay, 1968).

The proteic thiols content was calculated by the difference between the total soluble thiols and the non-proteic thiols and expressed in nmoles of SH g^{-1} FW.

Results

The ATPS activity was 18% higher in the *E. crassipes* plant roots maintained in nutrient solution with 7 μM As when compaired to the control and similar in the leaves of this treatment to those in the control (Figure 1A). The GSH-Px activity in roots and leaves treated with As decreased by 43 and 22%, respectively (Figure 1B).

The GR activity in *E. crassipes* roots and leaves subjected to As increased 45 and 17%, respectively (Figure 1C). GST activity was 51% higher in the roots and 24% lower in the leaves while γ-ECS activity in roots was similar to the control but increased 16% in leaves (Figure 1E).

The total and non-proteic thiol contents increased 24 and 48% in the roots and 42 and 58% in *E. crassipes* plant leaves exposed to As, respectively, (Figure 2A and B) but total GSH levels in roots and leaves were lower in plants exposed to As (Figure 2C).

Discussion

Exposure to high concentrations of Pb, Cd and As stimulates the enzymatic activity involved in sulfur metabolism for plant detoxification. This metabolism demands cysteine and GSH, involved in enzyme biosynthesis of the ascorbate-glutathione cycle (Silva et al. 2017).

The ATPS higher activity in *E. crassipes* roots can be an essential step to supply the sulfur demand (Oliveira et al. 2009) and represents a tolerance mechanism to As. The GSH-Px activity decreases in the *E. crassipes* roots and leaves after exposure to As probably due to the performance of other peroxidases against oxidative stress. This facilitates GSH use in As detoxification mechanisms through the conjugation to the methaloid or the use of GSH in the biosynthesis of phytochelatins (Silva et. al 2017). This result is similar to that observed in Lemna gibba submitted to As (Leão et al., 2014a), in which the arsenite binds to glutathione and phytochelatins due to the high affinity with these compounds.

The increased GR activity in *E. crassipes* roots and leaves subjected to As, which catalyzes the reduction of GSSH to GSH, may be related to the GSH availability (Begum et al., 2016), as reported for *Canavalia ensiformis* exposed to As (Nascimento et al., 2011). In addition, GSH participates in the arsenate reduction to arsenite by arsenate reductase (Huang et al., 2008). The GST activity increasing in the *E. crassipes* roots exposed to As suggests involvement in the detoxification and accumulation of this element, by the GSH conjugation to As (Kumar et al. 2015), as observed in *Arachis hypogaea* (Bianucci et al. al. 2017). The higher activity of γ-ECS in *E. crassipes* leaves exposed to As is due to the GSH pool

maintenance, whose synthesis occurs mainly in the cytosol, chloroplasts and mitochondria, being the first enzyme involved in this process (Noctor et al. 2012). This was also reported for *Ceratophyllum demersum* (Mishra et al. 2008), but this enzyme apparently did not play a key role in the As detoxification in *Sesuvium portulacastrum* (Lokhande et al., 2011). Increased GR activity in roots and leaves and γ-ECS in leaves suggest GSH production mechanisms in *E. crassipes* roots with redox GSH status maintenance (Felipe et al., 2009). The lower activity of this enzyme in the *E. crassipes* leaves than in its roots was due to redox state maintenance and GSH biosynthesis activation.

The increment in total and non-proteic thiol levels in *E. crassipes* exposed to As is associated with the detoxification process, as observed in *Lemna gibba* (Leão et al. 2014b). Similar proteic thiol levels in *E. crassipes* plant roots and leaves exposed to As indicates that these compounds have no effective participation in the tolerance mechanisms for this metalloid in this species. The reduction in total GSH levels suggests that As tolerance mechanisms in *E. crassipes* involved the phytochelatins synthesis, compounds considered to be the main ligands to As (Abbas et al. 2018).

Plasmolysis and collapses in the epidermal cells and spongy and palisade parenchyma presented damage in the *E. crassipes* tissues exposed to As, due to the lower cell wall resistance, as observed in *Pistia stratiotes* (Farnese et al. 2017), but not on young *Pteris vittata* leaves (Li et al. 2006), the latter being a hyperaccumulating species with As tolerance mechanisms.

The absence of damage to the *E. crassipes* plant roots exposed to As indicates a high translocation of this metalloid to their aerial part. Similar root damage absence was observed in *E. crassipes* treated with cadmium (Toppi et al. 2007). This shows that tolerance involves, among other mechanisms, the As differential translocation in the plant.

The enzyme activity related to GSH metabolism is an important As detoxification mechanism component in *E. crassipes*. Thus, although it can not be considered a hyperaccumulating species of As, it has been found that it is able to tolerate moderate concentrations of this metalloid and, therefore, these data may contribute to phytoremediation program in aquatic environments contaminated with As.

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Captions of the figures:

Figure 1. ATP sulfurylase (ATPS) (A), glutathione peroxidases (GSH-Px) (B), glutathione reductase (GR) (C), glutathione sulfotransferases (GST) (D) and synthetases γ-glutamylcysteine (γ-ECS) (E) activity in the roots and leaves of *Eichhornia crassipes* subjected to As. Control (*black*) and As (*gray*) (*) Significant difference ($p<0.05$) between treatments per plant part. Bars represent the standard deviation of the mean.

Figure 2. Total soluble (TT), non-proteic (NPT) and proteic (PT) thiols in roots (A) and leaves (B) and total glutathione (GSH + GSSG) in the roots and leaves (C) of *Eichhornia crassipes* subjected to 7 μM of As. Control (*black*) and As (*gray*) (*) Significant difference ($p<0.05$) between treatments per plant part. Bars represent the standard deviation of the mean.