Phytoremediation of arsenic-contaminated water: the role of antioxidant metabolism of *Azolla caroliniana* Willd. (Salviniales)

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ABSTRACT

Phytoremediation has proven to be an efficient technology for removing arsenic (As) from water, but the plants used in this process need to be tolerant to the damage caused by As. The toxic effect of As on growth and functioning of the antioxidant system was studied in individual plants of *Azolla caroliniana* exposed to five concentrations of As (0.0, 0.25, 0.5, 1.0 and 1.5 mg L⁻¹) for the course of five days. Growth, As absorption, enzymatic activity, total and non-protein thiols and anthocyanin content were assessed. *Azolla caroliniana* was able to take up large amounts of the pollutant, reaching As concentrations of 386.1 µg g⁻¹ dry weight without saturating the absorption mechanism. The tolerance index and the growth of *A. caroliniana* decreased with the increased As uptake. Superoxide dismutase, peroxidases, catalases and glutathione reductase activities increased at lower doses of As and subsequently declined with higher concentrations, whereas ascorbate peroxidase activity was reduced in all treatments. Unlike the enzymatic defence system, anthocyanin and thiol content increased consistently in all treatments and showed a positive correlation with As concentration. Therefore, the increased synthesis of non-enzymatic antioxidants is most likely the main factor responsible for the high As tolerance of *A. caroliniana*.

Keywords: anthocyanin, antioxidant enzymes, aquatic plant, thiol compounds, water remediation

Introduction

Arsenic (As) is a toxic carcinogenic metalloid ubiquitous in the environment with both anthropogenic and geogenic origins. Potential sources of As contamination include mining activity and chemicals used extensively in agriculture as pesticides, insecticides, defoliants, wood preservatives and soil sterilants (Fayiga & Saha 2016). Water polluted with As requires special attention because contaminated water used for drinking, food preparation and irrigation of food crops poses one of the greatest threats to public health (Karn 2015; Palácio *et al.* 2016; Sadee *et al.* 2016). There are several methods to remove As from water, including physical, chemical and biological methods. Among them, the use of aquatic plants, or phytoremediation, is an efficient, viable and low-cost technology (Farnese *et al.* 2014). Plants used in phytoremediation should be able to remove the pollutant from the environment and be tolerant to damage caused by it (Podder & Majumder 2016). One of the most damaging effects of As in plants is oxidative stress caused by an increase in the production of reactive oxygen species (ROS), which may alter the normal metabolism.
of plants and damage cell membranes, causing inhibition of photosynthesis and growth (Silveira et al. 2015) and eventual cell death (Sharma et al. 2012). However, plants have developed mechanisms to mitigate these effects using enzymatic and non-enzymatic antioxidants, such as superoxide dismutase (SOD), peroxidases (POXs) and catalases (Gusman et al. 2013), as well as anthocyanins (Srivastava et al. 2016) and non-protein thiols (Leão et al. 2014b).

_Azolla caroliniana_ is an aquatic plant with high potential for phytoremediation programs due to its capacity to accumulate metals such as cadmium, chromium, nickel and zinc (Benniceli et al. 2004). Within the genus _Azolla_, _A. carolina_ has the highest capacity to accumulate toxic levels of As, however, little information is available about the mechanisms underlying the As tolerance of this plant (Zhang et al. 2008). Based on these facts, this paper aims to verify the role of the antioxidant system in the As tolerance of _A. carolina_ exposed to five concentrations of the pollutant.

**Materials and methods**

**Plant exposure to arsenic**

Specimens of _Azolla carolina_ L. collected in non-polluted dams at the Federal University of Viçosa, Viçosa, Minas Gerais State, Brazil (20°45'25.0"S 42°52'25.5"W) were used in all experiments (average ambient temperature between 20.6 and 25.2 °C and average annual precipitation of 1229 mm). Plants were surface sterilized with 1% sodium hypochlorite for 1 min and extensively rinsed with running tap water and deionized water. Next, the plants were transferred to polyethylene pots with 10 L of Clark’s nutrient solution (pH 6.5) (Clark 1975) and maintained in a growth room with controlled temperature and irradiance (25 ± 2 °C; 230 µmol m⁻² s⁻¹) under a photoperiod of 16 hours for a acclimation period of 3 days. After the acclimation period, plants (approximately 0.3 g of fresh matter were grounded in liquid nitrogen and stored at -80 °C.

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Plants were washed with deionized water and placed into a conventional oven at 80 °C until constant dry weight was achieved. Plants were then digested (Marin et al. 1993), and the As concentration was analyzed using an hydride generation atomic absorption spectrophotometer (Shimadzu®, AA6701F). The relative growth rate (RGR) of the plants was calculated using the equation proposed by Hunt (1978):

\[
R_w = \frac{(\ln w_1 - \ln w_e) \times 1000}{(t_1 - t_o)^
\]

where \(R_w\) represents relative growth rate; \(\ln w_e\) and \(\ln w_1\) represents neperian logarithm of the mass at the end and beginning of the experiment, respectively; and \(t_1 - t_o\) represents duration of the experiment (days).

The As tolerance was estimated by calculating the tolerance index (TI) (%), as proposed by Wilkins (1978):

\[
TI (\%) = \frac{(Rw^*/Rw) \times 100}{
\]

where \(Rw^*\) is the relative growth rate of plants in solution with As, and \(Rw\) is the relative growth rate of plants in solution without arsenic.

**Effects of arsenic on enzymatic activity**

To assess the activity of antioxidant enzymes, approximately 0.3 g of fresh matter were grounded in liquid nitrogen and homogenized in extraction medium comprising 0.1 M potassium phosphate buffer, pH 6.8, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1% (w/v) polyvinylpyrrolidone (PVPP) (Peixoto et al. 1999). The samples were centrifuged at 12,000 xg for 15 min at 4 °C and the supernatant was used for superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POX, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.8.1.7) analyses.

SOD activity was determined by the addition of 50 µL of enzymatic extract to 5 mL of reaction solution containing 50 mM potassium phosphate buffer, pH 7.8, 13 mM methionine, 0.1 mM EDTA, 75 mM nitroblue tetrazolium (NBT) and 2 mM riboflavin. The reaction was conducted at 25 °C in a reaction chamber under a 15W fluorescent lamp for 5 min. The amount of blue formazan produced by NBT photoreduction was measured by absorbance at 560 nm. A reaction solution identical to the solution described above but kept in the dark for the same amount of time was prepared, and the absorbance at 560 nm of this solution was subtracted from the absorbance of the sample that was illuminated (Giannopolitis & Ries 1977). One unit of SOD was defined as the quantity of enzyme required to inhibit NBT photoreduction by 50% (Beauchamp & Fridovich 1971).

CAT activity was determined by the addition of 0.1 mL of enzymatic extract to 2.9 mL of reaction solution consisting of 50 mM potassium phosphate buffer, pH 7.0 and 12.5 mM H₂O₂ (Havir & McHale 1987). The decrease in the absorbance during the first minute of the reaction was measured at 240 nm at 25 °C. The enzymatic activity
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### Determination of thiols component content

Samples of plants (0.5 g) were macerated in liquid nitrogen and then added to 6 mL of reaction solution containing 0.1 M Tris-HCl buffer (pH 8.0), 1 mM EDTA and 1% ascorbic acid. The homogenized extract was centrifuged at 10,000 xg for 10 min at 4 °C (Meuwly & Rausser 1992), and the supernatant was used for the determination of the total soluble and non-protein thiols content.

The total thiols content was determined in 0.5 mL of supernatant added to 1.5 mL of potassium phosphate buffer (0.2 mol L⁻¹, pH 8.2), 0.1 mL Ellman's reagent [5,5' - dithiobis-(2-nitrobenzoic acid)] (0.01 mol L⁻¹) and 7.9 mL of methanol. After 15 min of reaction at 37 °C, the absorbance at 412 nm was determined. Using a molar extinction coefficient of 13,100 mol⁻¹ L cm⁻¹, the non-protein thiols content was calculated, and the results are expressed as nmoles of SH g⁻¹ FW (Sedlak & Lindsay 1968).

The non-protein thiols content was determined in 5.0 mL aliquots of supernatant added to 1.0 mL of trichloroacetic acid 50% (w/v) and 4.0 mL H₂O and maintained for 1 h on ice. The samples were centrifuged at 10,000 xg for 15 min, and 2.0 mL aliquots were added to 4.0 mL of potassium phosphate buffer (0.4 mol L⁻¹, pH 8.9) and 0.1 mL of Ellman's reagent (0.01 mol L⁻¹). After 5 min at room temperature, the absorbance at 412 nm was determined. Using a molar extinction coefficient of 13,100 mol⁻¹ L cm⁻¹, the non-protein thiols content was calculated, and the results are expressed as nmoles of SH g⁻¹ FW (Sedlak & Lindsay 1968).

### Experimental design and statistical analysis

The experiments followed a completely randomized experimental design with six replicates. Data were analyzed by ANOVA and linear regression. The equations were calculated using the software SAS 9.1 (SAS Institute Inc. 2004).

### Results

**Arsenic uptake and effect on growth and tolerance index of *A. caroliniana***

Arsenic uptake by *A. caroliniana* increased with increasing concentrations of the metalloid in the solution, reaching 386.1 µg g⁻¹ DW (dry weight) at the highest dose (Tab. 1). The saturation of the absorption and accumulation mechanisms for this element had not occurred.

The growth of *A. caroliniana* was affected by As in the nutrient solution, occurring a decrease in the RGR with the increment of the concentration of the pollutant. Similarly, tolerance index also decreased in response to increasing concentrations of As (Tab. 1).

**Effect of arsenic on enzymatic activity**

The activity of the antioxidant enzymes indicated that As accumulation induces a strong antioxidant response in *A. caroliniana* (Fig. 1 A-E). The SOD activity was greatly increased at As concentration of 1 mg L⁻¹. At the highest concentration of the metalloid, however, the activity of the enzyme decreased but still remained higher than in the control (Fig. 1A).
Table 1. As uptake, relative growth rate and tolerance index in *Azolla caroliniana* exposed to As during five days.

<table>
<thead>
<tr>
<th>As concentration (mg L⁻¹)</th>
<th>As uptake (µg g⁻¹ DW)</th>
<th>Relative growth rate (mg g⁻¹ FW day⁻¹)</th>
<th>Tolerance index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>25.4 e</td>
<td>109.97 a</td>
<td>100 a</td>
</tr>
<tr>
<td>0.25</td>
<td>209.1 d</td>
<td>76.98 b</td>
<td>70 b</td>
</tr>
<tr>
<td>0.5</td>
<td>271.2 c</td>
<td>72.03 b</td>
<td>65.5 c</td>
</tr>
<tr>
<td>1.0</td>
<td>323.5 b</td>
<td>62.69 c</td>
<td>57 d</td>
</tr>
<tr>
<td>1.5</td>
<td>386.1 a</td>
<td>56.94 c</td>
<td>51.78 d</td>
</tr>
</tbody>
</table>

Means followed by the same letter were not significantly different according to Tukey’s test at 5 % probability.

CAT activity increased in *A. caroliniana* by 26.44 % at 0.25 mg L⁻¹ As. At higher concentrations, however, the enzyme activity was negatively affected by exposure to the pollutant (Fig. 1B).

POX activity was extremely low in the control plants and increased after the exposure of *A. caroliniana* to the pollutant. Indeed, POX activity was increased by 39.72 % in *A. caroliniana* at 1.0 mg L⁻¹ As. Enzymatic activity decreased in plants exposed to the highest As concentration, although POX activity still has remained higher than in the control (Fig. 1 C). A very similar pattern was observed in relation to GR enzyme whose activity increased at 1.0 mg L⁻¹ As and decreased in the subsequent concentration (Fig. 1 E).

APX was the most sensitive enzyme to As toxicity (Fig. 1D). Actually, the activity of this enzyme showed a sharp decrease in plants exposed to concentrations above 0.25 mg L⁻¹ As, indicating that even low pollutant concentrations are able to inactivate the enzyme.

**Effect of arsenic on non-enzymatic antioxidants**

The content of non-enzymatic antioxidants total thiols, non-protein thiols (Fig. 2A, B) and anthocyanins (Fig. 3) had increases in all the As concentrations. Unlike what occurred with the enzymes the concentration of non-enzymatic antioxidants did not decrease in the highest concentration of the pollutant, indicating lower sensitivity of these molecules to damage triggered by the metalloid.

**Discussion**

This study evaluated the effect of As exposure on growth, As accumulation and antioxidant defences of *Azolla caroliniana*. Among all the species of *Azolla*, *A. caroliniana* proved to be able to accumulate the highest As concentration (Mahmud et al. 2008; Sánchez-Viveros et al. 2011; Li et al. 2016), which makes this plant an interesting tool for use in phytoremediation. However, the accumulation of As triggered several types of cell damage (Islam et al. 2015), which can be observed through increasing concentrations of oxidative stress biomarkers, such as enzymes and thiols, and by the decrease in plant growth (Dazy et al. 2012).

Decrease in the growth rate is a typical plant response to As exposure (Farooq et al. 2016). Nevertheless, although As exposure reduced the RGR, *A. caroliniana* continued to show growth even after accumulating high concentrations of the pollutant. Usually, the reduction in the growth of plants subjected to As is much more drastic than what was observed in *A. caroliniana* (Farnese et al. 2014). The maintenance of growth is an important parameter to assess the tolerance of plants to toxic chemical pollutants and is an essential feature in plants used in phytoremediation programs (Chen et al. 2016).

The ability of *A. caroliniana* to maintain the growth is probably a result of the activation of defence mechanisms involved in the mitigation of damage triggered by As (Kandziora-Ciupa et al. 2016). Toxic compounds promote an increase in ROS generation, which results in oxidative damage to biomolecules and subcellular structures (Erinle et al. 2016). To prevent these harmful effects, plants develop tolerance mechanisms, such as the activation of enzymatic and non-enzymatic antioxidant systems, which are also indicators of oxidative stress in plants (Brain & Cedergreen 2009; Gusman et al. 2013).

Antioxidant enzymes have an important role in the defence against As toxicity and are considered non-specific biomarkers of oxidative stress (Dazy et al. 2012). The first enzyme involved in the detoxification process is SOD, which promotes the conversion of superoxide radicals ($O_2^{•−}$) to hydroxide peroxide ($H_2O_2$). Therefore, SOD activity results in increased $H_2O_2$ generation and must be accompanied by an increase in the activity of enzymes responsible for scavenging $H_2O_2$, such as CAT and POX. The increase of SOD activity in response to As toxicity observed in this study has also been reported in other plants, such as *Lactuca sativa* (Gusman et al. 2013) and *Eichhornia crassipes* (Andrade et al. 2016). However, when As levels are very high, the extent of the damage caused by the pollutant decreases enzyme activity. The decrease in SOD activity at high heavy metal concentrations may be attributed to the inactivation of the enzyme by $H_2O_2$ or to the existence of other ROS and the inactivation of other enzymes involved in the degradation of these compounds (Khan et al. 2009).

The enzymes CAT and POX act directly to scavenge $H_2O_2$ (Sharma et al. 2012), whereas APX and GR participate in the ascorbato-glutathione cycle that also promotes $H_2O_2$.
Figure 1. Activity of antioxidant enzymes in *Azolla caroliniana* exposed to arsenic during five days. The parameters included Superoxide dismutase activity (SOD) (A); catalase activity (CAT) (B); peroxidase activity (POX) (C); ascorbate peroxidase activity (APX) (D); and glutathione reductase (GR) (E).
decomposition (Caverzan et al. 2012). Accordingly, these enzymes were important in A. caroliniana for defence against ROS at low As concentrations, but in higher concentrations the toxic effects of the pollutant inactivated the enzymes. Similar results were observed in rice (Shri et al. 2009) and aquatic plants (Farnese et al. 2014; Andrade et al. 2016) after exposure to high As concentrations. Given that plant growth was maintained even in the highest concentrations of the metalloid, it is likely that A. caroliniana uses other strategies for survival. In fact, the non-enzymatic antioxidant content (anthocyanins, total thiols and non-protein thiols) was increased in A. caroliniana plants exposed to all As concentrations.

Anthocyanins are a large class of water-soluble pigments in the flavonoid group that are found in all plant tissues. Environmental stresses are well known to stimulate production of anthocyanin, and studies have shown that anthocyanins can quench almost all types of ROS, including \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) (Liu et al. 2016). Moreover, anthocyanins have roughly four times greater antioxidant capacity than \( \alpha \)-tocopherol and ascorbate (Agati & Tattini 2010). The antioxidative properties of anthocyanins arise from their high reactivity as hydrogen or electron donors, their ability to chelate transition metal ions and the ability of the polyphenol-derived radicals to stabilize and delocalize unpaired electrons (Duan et al. 2007). The increase in anthocyanin content might also be related to phosphate deficiency. Phosphate and arsenate are analogues and compete for the same sorption sites in the root apoplast and for the same uptake system in the root plasmalemma. As a consequence, As in the nutrient solution decreases...
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