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Phytoremediation potential of *Salvinia molesta* for arsenite contaminated water: role of antioxidant enzymes

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Abstract Antioxidant enzymes are important components in the defense against arsenic (As) stress in plants. Here, we tested the hypothesis that *Salvinia molesta*, an aquatic fern, counteracts the harmful arsenite (As^{III}) effects by activating scavenging reactive oxygen species (ROS) enzymes. Thus, our objective was to investigate the role of the superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), and ascorbate peroxidase (APX) in *S. molesta* tolerance to As^{III} and indicate the use of this plant in remediation of contaminated water. Plants were grown in nutrient solution at pH 6.5 and exposed to 0, 5, 10, or 20 μM As^{III} for 96 h (analyses of As absorption, mineral nutrient content, and relative growth rate) and for 24 h (analyses of oxidative stress indicators and enzymatic antioxidant defenses). In the floating leaves, there was a greater basal activity of the antioxidant enzymes and less accumulation of As than in submerged leaves. The submerged leaves, which function as roots in *S. molesta*, accumulated more As than floating leaves, and SOD and CAT activities were inhibited. Thus, there was a greater production of ROS and oxidative stress. Our results show that *S. molesta* presents enzymatic antioxidant defenses to alleviate As^{III} toxicity and are more effectives in the floating leaves. These results are important to elucidate the As^{III} tolerance mechanisms in *S. molesta* and the possibility of their use in contamined water phytoremediation. Additional studies exposing plants to more prolonged stress and using As^{III} concentrations closer to those found in contaminated environments will confirm this claim.

Keywords Arsenic · Macrophytes · Phytoremediator species · Water contamination

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1 Introduction

Arsenic (As) is an environmental pollutant and its concentrations have increased in freshwater sources around the world, through both geological processes and anthropogenic activities such as mining, burning fossil fuels, and agricultural use of fertilizers, pesticides, and herbicides containing As. The most common inorganic As forms in the aquatic environment

are arsenate (As^{V}) and arsenite (As^{III}) (Fazi et al. 2016). The latter is considered more toxic and also more present in groundwater sources. In many regions of the world, water intake with high As concentrations represents a serious threat to the health of human populations because of the pollutant toxicity and carcinogenic potential (Hettick et al. 2015; Kumar et al. 2016). This scenario makes it urgent to solve or mitigate this social and environmental problem.

There are many conventional technologies based on physical and chemical processes that are available to remove As from the aquatic environment, but most of them combine costly and complex processes of implantation and maintenance (Fazi et al. 2016; Nicomel et al. 2016). The phytoremediation appears in this scenario as an alternative technique for the removal of pollutants from air, soil and water, with the benefits of having low cost and ease of implantation, as well as being environmentally sustainable. Phytoremediation takes advantage of the ability of some plants to absorb and accumulate toxic elements in their tissues and includes several processes namely, phytoextraction, phytostabilization and rhizofiltration (Jasrotia et al. 2017; Sarwar et al. 2017). Usually aquatic plants perform rhizofiltration, where contaminants are removed by absorption and adsorption being accumulated in the roots (Rahman and Hasegawa 2011; Newete and Byrne 2016).

The effective results using phytoremediation depends on plant ability to absorb, accumulate and tolerate the deleterious effects of the pollutant. The primary As^{III} toxic effect its to promote overproduction of reactive oxygen species (ROS) within plant cells (Sharma 2012). ROS are highly reactive molecules capable of oxidizing membrane lipids, nucleic acids and proteins (Das and Roychoudhury 2014). Oxidative stress is the main deleterious As^{III} effect in plants (Talukdar 2013; Singh et al. 2015a; Farooq et al. 2015), although it may also inhibit the catalytic function of enzymes by binding to their sulfhydryl groups, leading to metabolic damage (Sharma 2012; Farooq et al. 2016a).

To avoid and mitigate oxidative damage promoted by toxic metals, plants have many defense mechanisms such as antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and peroxidases, which eliminate ROS in different subcellular compartments. These enzymes play recognizable role on macrophyte As tolerance (Farnese et al. 2014; Andrade et al. 2016; Leão et al. 2017; da-Silva et al. 2018).

Salvinia molesta is a floating and free-living pteridophyte. Each plant is composed of two floating oblong-shaped green leaves and a set of submerged long and filiform brown leaves, covered with hairs that absorb water and nutrients, and act as a root (Miranda and Schwartsburd 2016). *S. molesta* is native to Brazil, although it is currently found in many other countries. It is considered one of the worst 100 invasive species in the world (Luque et al. 2014), and presents rapid biomass production and reproduction rates (Barros and Henares 2015). These latter *S. molesta* features are important for the phytoremediation and support the relevance of researches to find a use for the species.

S. molesta has the ability to absorb toxic elements from water, such as lead, mercury (Kumari et al. 2017), and As (Hariyady et al. 2013). In this work we evaluated the *S. molesta* potential to absorb, accumulate and tolerate As^{III}. We tested the hypothesis that *S. molesta* can counteract the harmful As^{III} effects by antioxidant enzymes and ROS removal. Our objective was to evaluate the role of enzymatic antioxidant defenses in *S. molesta* tolerance to As^{III} in a short-term exposure and indicate the use of this plant in remediation of contaminated water.

2 Material and methods

2.1 Plant material and treatment conditions

Specimens of *Salvinia molesta* D. S. Mitchell (Salviniaceae) were collected in an artificial lake in the Botanical Garden of Universidade Federal de Viçosa, Viçosa, Minas Gerais State, Brazil (20°45′24″S 42°52′23″W). Plants were surface sterilized with 1% sodium hypochlorite for 1 min and then extensively rinsed with running tap water and maintained in demineralized water for 24 h. Then, they were transferred to polyethylene flasks with 10 L of Clark's nutrient solution pH 6.5 (Clark 1975), and maintained in a growth room for an acclimation period of 7 days with controlled temperature (25 ± 2 °C), irradiance (250 µmol m^{-2} s^{-1}) and a 16-h light photoperiod.

Plants with similar sizes were then subjected to the following treatments: control (Clark nutrient solution 1/4 strength pH 6.5) and three As concentrations (5, 10 and 20 µM), provided as sodium arsenite ($NaAsO_2$) in

a nutrient solution (Clark nutrient solution 1/4 strength pH 6.5). Experiments were set up with three replicates, consisting of 5 g of fresh weight plants in glass pots with 0.5 L of solution.

The exposure time to treatments was 96 h to analyzes of arsenic absorption, mineral nutrients and relative growth rate (RGR), and 24 h for the other evaluations, under the same conditions described above. At the end of the exposure, plants were harvested and floating and submerged leaves were divided, washed in deionized water and immediately analyzed or stored at − 80 °C.

2.2 Determination of arsenic and nutrients concentration

The floating and submerged leaves of *S. molesta* were washed in deionized water, and placed in a conventional oven at 80 °C, until constant dry mass was obtained. The dry plant material was crushed and digested in a nitric-perchloric acid mixture (2:1 v/v) (Marin et al. 1993) and the concentrations of arsenic (As), calcium (Ca), magnesium (Mg), phosphorus (P) and sulfur (S) were determined through inductively coupled plasma emission spectroscopy (ICP-AES) (Optima 3300 DV, Perkin-Elmer, Norwalk, CT, USA). The accuracy of the method was verified by analysis of certified reference materials (*Lemna minor* (BCR-670)), from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

2.3 Arsenic translocation factor (TF)

The As translocation factor was calculated by the equation; TF = [M]shoots/[M]roots where [M]shoots is the As concentration in floating leaves (μg g^{-1} dry weight (DW)) and [M] roots is the As concentration in submerged leaves in (μg g^{-1} DW).

2.4 Determination of relative growth rate (RGR)

The relative growth rate (RGR) of plants was calculated using the equation proposed by Hunt (1978): $Rw = (\ln w_1 - \ln w_0) \times 1000/(t_1 - t_0)$, where Rw represents relative growth rate; ln w_1 and ln w_0 represents neperian logarithm of the mass at the end and beginning of the experiment, respectively; and $t_1 - t_0$ represents duration of the experiment (days).

2.5 Concentration of reactive oxygen species

To measure the concentration of the superoxide anion radical ($O_2^{\cdot -}$), 50 mg of floating leaves and submerged leaves were incubated in an extraction medium consisting of 100 μM ethylenediaminetetraacetic acid (EDTA) disodium salt, 20 μM NADH and 20 mM sodium phosphate buffer, pH 7.8 (Mohammadi and Karr 2001). The reaction was initiated by adding 100 μL of 25.2 mM epinephrine in 0.1 N HCl. The samples were incubated at 28 °C under stirring for 5 min. The absorbance was read at 480 nm for 5 min. Superoxide anion radical production was assessed by determining the accumulated adenochrome, using the molar absorption coefficient of 4.0 × 103 M^{-1} (Boveris et al. 2002).

The hydrogen peroxide (H_2O_2) concentration was determined using 200 mg of floating leaves and submerged leaves that were homogenized in an extraction medium consisting of 50 mM potassium phosphate buffer, pH 6.5, containing 1 mM hydroxylamine and centrifuged at 10,000×*g* for 15 min at 4 °C. Subsequently, 50 μL aliquots of the supernatant were added to a reaction medium containing 100 μM ammonium iron (II) sulfate ($Fe^{(II)}NH_4SO_4$), 25 mM sulfuric acid, 250 μM xylenol orange, and 100 mM sorbitol (Gay and Gebicki 2000). The samples were kept in the dark for 30 min, and the absorbance was read at 560 nm. The H_2O_2 concentrations were estimated based on a calibration curve prepared with H_2O_2 standards.

2.6 Rate of electrolyte leakage (EL)

The cell damage was evaluated through an assessment of membrane integrity, quantifying electrolyte leakage according to Faria et al. (2013). Floating leaves discs and submerged leaves apices were obtained after the treatments, rinsed thoroughly in distilled water and maintained in 10 mL of distilled water in sealed vials for 6 h at room temperature. The electrolyte leakage was estimated from the electrical conductivity in the solution containing the plant tissues samples using an electrical conductivity meter (DM31, Digimed, Brazil). The conductivity was expressed as a percentage of the total conductivity measured after incubating the vials at 90 °C for 2 h.

2.7 Assessment of the antioxidant enzymes activity: superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APX)

To assess enzyme activity, 0.3 g fresh weight (FW) of floating leaves and submerged leaves were homogenized in extraction medium containing 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 (PVP). The homogenate was centrifuged at 12,000 $\times g$ for 15 min. at 4 °C and the supernatant was used to assess superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) and ascorbate peroxidase (APX) activities.

The SOD activity (SOD, EC 1.15.1.1) was measured according to the inhibition of *p*-nitroblue tetrazolium (NBT) photoreduction, according to the method of Giannopolitis and Ries (1977). The enzymatic activity was expressed in SOD units corresponding to the amount of enzyme required to inhibit 50% of of NBT photoreduction per minute (Beauchamp and Fridovich 1971).

The CAT activity (CAT, EC 1.11.1.6) was estimated as the rate of decomposition of H_2O_2 during the first minute of the reaction at 240 nm (Havir and McHale 1987) and calculated using a molar extinction coefficient of 36 mol^{-1} L cm^{-1}. The enzymatic activity was expressed in micromoles H_2O_2 min^{-1} g^{-1} FW.

The POX activity (POX, EC 1.11.1.7) was estimated as the rate of production of purpurogallin at 420 nm according to the proposed by Nakano and Asada (1981) with a molar extinction coefficient of 2.47 $mmol^{-1}$ L cm^{-1} (Chance and Maehley 1955). The enzymatic activity was expressed in micromoles purpurogallin min^{-1} g^{-1} (FW).

The APX activity (APX, EC 1.11.1.11) was assessed as the rate of ascorbate oxidation at 290 nm (Nakano and Asada 1981) using a molar extinction coefficient of 2.8 $mmol^{-1}$ L cm^{-1}. The enzymatic activity was expressed in micromoles ascorbate min^{-1} g^{-1} FW.

2.8 Data analysis

The experiment was performed using a completely randomized design. Analysis of variance was performed and the means of the arsenite concentrations and types of plant leaves (floating and submerged) were compared using Tukey's test at 5% significance. The statistical analyses were performed with the statistical software SAS 9.1 (SAS Institute Inc. 2004).

3 Results

3.1 Concentration of arsenic, translocation factor of arsenic, and relative growth rate

The As concentration in floating leaves increased according to the increment of the pollutant in solution and reached 103 µg g^{-1} DW following treatment with 20 µM As^{III}. The highest As concentration in submerged leaves was observed for 10 µM As^{III}, but it decreased for 20 µM. The As concentration was higher in the submerged leaves compared to the floating leaves at all concentrations (Table 1). The As translocation factor (TF) in *S. molesta* ranged from 0.28 to 0.68 and it was higher in plants exposed to 20 µM As^{III} compared to 5 and 10 µM As^{III} (Table 1).

The relative growth rate (RGR) of floating leaves did not vary at 5 µM As^{III} compared to the control, but it was 34% and 60% lower than the control at 10 and 20 µM As^{III}, respectively. The growth of submerged leaves was affected in all As^{III} treatments. There was an intense deterioration of the submerged *S. molesta* leaves and tissue residues were accumulated in the culture solution at the end of the exposure period, resulting in negative values at the two highest As^{III} concentrations. The RGR was higher in floating leaves for all As^{III} treatments (Table 1).

3.2 Mineral nutrient concentration

The calcium (Ca) concentration did not vary in the floating leaves, while it increased in the submerged leaves of the plants exposed to 10 and 20 µM As^{III}. A higher concentration of Ca was recorded in the submerged leaves than in the floating leaves for all treatments (Table 2).

There was a reduction in the magnesium (Mg) concentration in floating leaves exposed to 20 µM As^{III} compared to the control. In the submerged leaves, the Mg concentration decreased after plants were exposed to As^{III}, but did not vary in the different

Table 1 Arsenic concentration [As], translocation factor (TF), and relative growth rate (RGR) of *S. molesta* after 4 days of As^{III} exposure

As^{III} (μM)	[As] (μg g^{-1} DW)		TF	RGR (mg day^{-1} g^{-1} DW)	
	Floating leaves	Submerged leaves		Floating leaves	Submerged leaves
0	nd[a]	nd[a]	–	66.94 ± 5.40Aa	67.86 ± 12.65Aa
5	39.45 ± 3.77Cb	110.50 ± 3.16Ca	0.36 ± 0.02B	67.02 ± 1.17Aa	41.12 ± 8.80Bb
10	56.71 ± 2.70Bb	200.25 ± 4.72Aa	0.28 ± 0.02C	43.93 ± 3.23Ba	− 27.02 ± 1.45Cb
20	103.04 ± 2.66Ab	148.63 ± 5.55Ba	0.68 ± 0.03A	26.28 ± 3.06Ca	− 29.68 ± 2.41Cb

Values are the mean of three replicates ($n = 3$) ± SD. Means followed by different capital letters for the same column and lowercase letters for the same row, show significant difference at $P < 0.05$, according to Tukey's test

DW dry weight

[a]Not detected

concentrations. In all As^{III} treatments, the Mg concentration was higher in the floating leaves than in the submerged leaves (Table 2).

The phosphorus (P) concentration did not vary in the floating leaves of plants exposed to arsenite compared to the control, but there was a reduction in 20 μM, compared to 5 and 10 μM As^{III}. In submerged leaves, the P concentration decreased after exposure to 10 and 20 μM As^{III}. A higher P concentration was observed in the floating leaves than in the submerged leaves for all treatments (Table 2).

The sulfur (S) concentration did not differ from control in the floating leaves. In the submerged leaves, S was reduced in all As^{III} treatments, but did not differ among them. A higher S concentration was observed in the submerged leaves than in the floating leaves of the control and 20 μM As^{III} treatments (Table 2).

3.3 Reactive oxygen species concentration and cell membrane damage

The superoxide anion radical ($O_2^{\cdot-}$) concentration increased in *S. molesta* floating leaves exposed to As^{III}, but did not differ between 10 and 20 μM treatments. In submerged leaves, the increase in $O_2^{\cdot-}$ concentration occurred according to the increase in the As^{III} concentration (Table 3).

In floating leaves, the hydrogen peroxide (H_2O_2) concentrations increased only at 20 μM As^{III}. In the submerged leaves, the H_2O_2 concentration decreased at 20 μM compared to 10 μM As^{III}. The $O_2^{\cdot-}$ and H_2O_2 concentrations were higher in the floating leaves in all treatments compared to submerged leaves (Table 3).

The rate of electrolyte leakage (EL) did not vary in floating leaves. In submerged leaves, there was an increase of 142% and 198% at 10 and 20 μM As^{III}, respectively, compared to control. The EL was higher in submerged leaves compared to floating leaves in the two largest As^{III} concentrations (Table 3).

3.4 Antioxidant enzyme activity

All As^{III} treatments promoted an increase in SOD activity in *S. molesta* floating leaves. In the submerged leaves, there was no difference between control and As^{III} concentrations. SOD activity was reduced at 20 μM As^{III} compared to 5 and 10 μM treatments. Higher SOD activity was recorded in the floating leaves for all As^{III} treatments compared to submerged leaves (Fig. 1a).

CAT activity increased in the floating leaves only at 5 μM and 20 μM As^{III} compared to contol. In the submerged leaves, there was no difference among treatments. There was higher CAT activity in the floating leaves for all treatments compared to submerged leaves (Fig. 1b).

The POX activity in floating leaves increased only in plants exposed to 20 μM As^{III}. Increase of 61% were observed in the submerged leaves at 20 μM As^{III} compared to control. The POX activity was always higher in floating leaves compared to submerged leaves (Fig. 1c).

APX activity was increased at 5 μM and 10 μM As^{III} in the floating leaves. In submerged leaves it increased with the pollutant dose. Higher APX activity

was recorded in floating leaves than in submerged leaves (Fig. 1d).

4 Discussion

Except for hyperaccumulator species, As translocation is restricted in most plants, including macrophytes. This supports As accumulation in submerged leaves rather than in floating leaves of *S. molesta* (Zhao et al. 2009; Rahman and Hasegawa 2011; Freitas-Silva et al. 2016). This response is mainly due to the formation of complexes among As^{III}, phytochelatins (PCs), and glutathione (GSH) in root cells. The complexes are then retained in vacuoles, which decreases As reactivity and cell damage (Liu et al. 2010; LeBlanc et al. 2013; Farooq et al. 2016a). This defense mechanism may explain the low TF and the highest As accumulation in *S. molesta* submerged leaves.

Although *S. molesta* did not present characteristics to tell its As phytoremediation capacity, such as As accumulation higher than 1000 μg g^{-1} dry weight and a TF > 1, its use should not be excluded. These characteristics are found in some terrestrial and hyperaccumulating plants (Kumar et al. 2015; Fahyiga and Saha 2016). Therefore, it is not appropriate to use them to define the phytoremediation potential of macrophytes. In floating aquatic species absorption occurs on roots surface and in the aerial part that is in contact with the environment (Rezania et al. 2016). Thus, the whole plant contributes to pollutant stabilization, absorption and its removal from the water.

It is not possible to distinguish between As absorbed and adsorbed by *S. molesta*. The adsorption of As on the root surface in some plant species requires the formation of an iron plaque in the presence of oxygen (Yamaguchi et al. 2014; Fresno et al. 2016). This iron plaque has a much higher affinity for As^{V} ion sequestration than As^{III} and acts as a barrier to the metalloid transport into the plant (Chen et al. 2005; Rahman and Hasegawa 2011; Hu et al. 2015). *S.molesta* plants was exposed to As^{III} in a non-oxygenated nutrient solution. Thus, we can believe that the adsorption was minimal compared to the As absorption by the submerged leaves.

The As^{III} can be transported from the inside of the plant to the environment via efflux (Li et al. 2016; Han et al. 2016). The aquaporins are intrinsic membrane proteins able to bidirectional transport in some plant

Table 2 Mineral nutrient (Ca; Mg; P; S) concentrations in *S. molesta* floating and submerged leaves after 4 days of As^{III} exposure

As^{III} (μM)	Mineral nutrient concentration (mg g^{-1} DW)							
	Calcium (Ca)		Magnesium (Mg)		Phosphorus (P)		Sulfur (S)	
	Floating leaves	Submerged leaves	Floating leaves	Submerged leaves	Floating leaves	Submerged leaves	Floating leaves	Submerged leaves
0	5.75 ± 0.27Ab	7.52 ± 0.72Ca	2.85 ± 0.09Aa	3.12 ± 0.32Aa	2.44 ± 0.06ABa	2.16 ± 0.08Ab	2.21 ± 0.07Ab	3.09 ± 0.23Aa
5	5.56 ± 0.09Ab	7.60 ± 0.47Ca	2.80 ± 0.05ABa	1.90 ± 0.33Bb	2.79 ± 0.17Aa	2.19 ± 0.24Ab	2.18 ± 0.07Aa	2.36 ± 0.21Ba
10	5.70 ± 0.44Ab	9.69 ± 0.60Ba	2.92 ± 0.09Aa	1.99 ± 0.28Bb	2.72 ± 0.16Aa	1.65 ± 0.06Bb	2.25 ± 0.05Aa	2.19 ± 0.30Ba
20	6.67 ± 0.77Ab	13.45 ± 0.70Aa	2.37 ± 0.04Ba	1.90 ± 0.02Bb	2.32 ± 0.17Ba	1.54 ± 0.14Bb	1.94 ± 0.02Ab	2.23 ± 0.02Ba

Values are the mean of three replicates ($n = 3$) ± SD. Means followed by different capital letters for the same column and lowercase letters for the same row, show significant difference at $P < 0.05$, according to Tukey's test

DW dry weight

Table 3 Concentration of superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and electrolyte leakage (EL) in *S. molesta* floating and submerged leaves after 4 days of As^{III} exposure

As^{III} (μM)	O_2^- (nmol mg^{-1} FW)		H_2O_2 (nmol mg^{-1} FW)		EL (%)	
	Floating leaves	Submerged leaves	Floating leaves	Submerged leaves	Floating leaves	Submerged leaves
0	54.49 ± 1.37Ca	7.01 ± 1.21Db	387.17 ± 2.28Ba	268.95 ± 8.83ABb	20.86 ± 1.28Aa	14.22 ± 2.00Cb
5	77.69 ± 2.67Aa	27.84 ± 2.08Cb	388.82 ± 3.50Ba	253.39 ± 17.78ABb	21.13 ± 1.99Aa	17.82 ± 1.34Ca
10	72.52 ± 2.92Ba	41.05 ± 0.27Bb	390.84 ± 15.02Ba	276.73 ± 17.73Ab	19.73 ± 1.21Ab	34.51 ± 3.74Ba
20	73.20 ± 1.99Ba	64.44 ± 0.92Ab	438.63 ± 21.35Aa	238.35 ± 3.16Bb	21.57 ± 0.93Ab	42.29 ± 3.35Aa

Values are the mean of three replicates ($n = 3$) ± SD. Means followed by different capital letters for the same column and lowercase letters for the same row, show significant difference at $P < 0.05$, according to Tukey's test

FW fresh weight

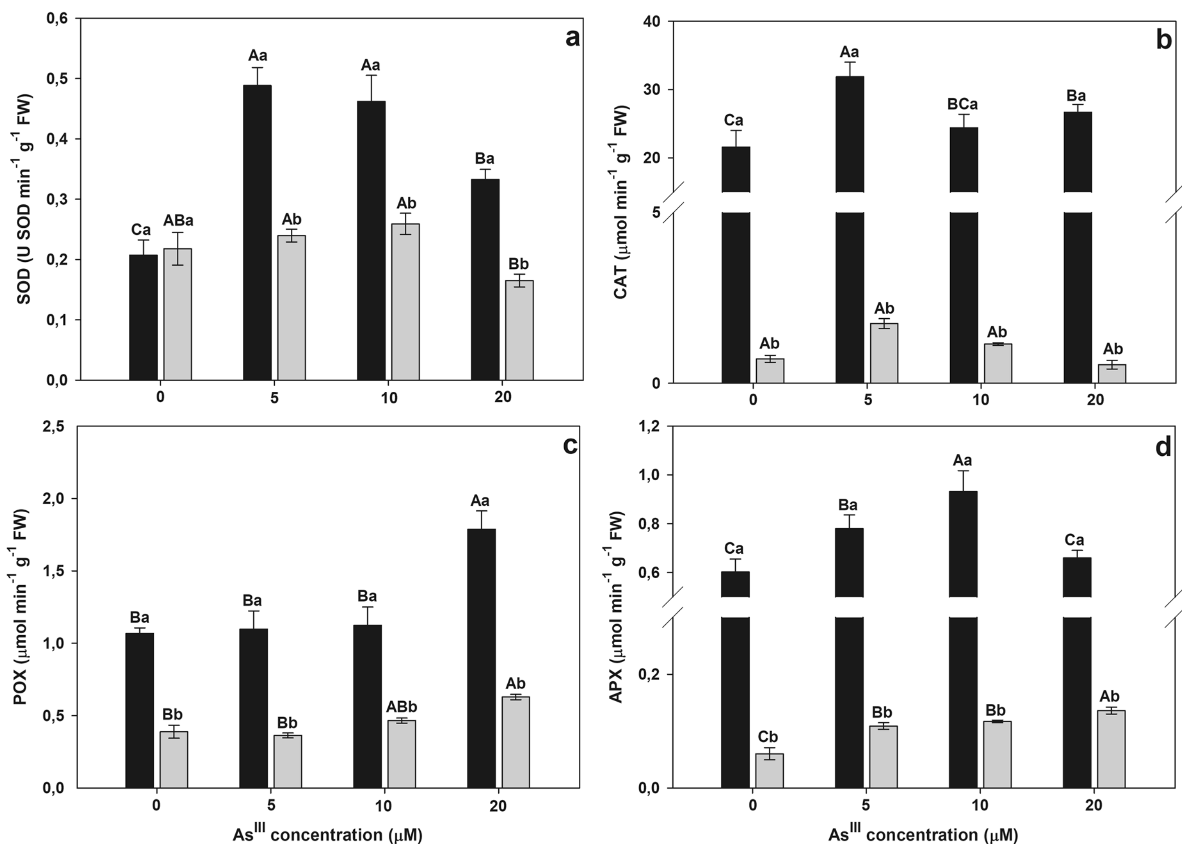

Fig. 1 Activity of antioxidant enzymes: **a** superoxide dismutase (SOD), **b** catalase (CAT), **c** peroxidase (POX), **d** ascorbate peroxidase (APX) in *S. molesta* floating leaves (black bar) and submerged leaves (grey bar) after 24 h of As^{III} exposure. Values are the mean of three replicates ($n = 3$) ± SD. Means followed by different capital letters for the same plant part and lowercase letters between plant parts, show significant difference at $P < 0.05$, according to Tukey test. *FW* fresh weight

species and can perform As^{III} symplast efflux, as a defense mechanism (Pommerrenig et al. 2015; Xu et al. 2015; Afzal et al. 2016). Efflux and translocation to floating leaves may be the reasons why pollutant accumulates in *S. molesta* submerged leaves and does not follow the increases in As^{III} concentration in solution. Damage caused by As^{III} in the cell membranes can inhibit the function of aquaporins and

affect the polutant's absorption, as well as the acquisition of water and nutrients by plants (Hoffman and Schenk 2011). Reductions of Mg, P, and S concentrations in *S. molesta* support this hypothesis of stress-induced cell membrane damage.

Nutritional deficiency during stress can impair the photosynthetic process and plant energetic metabolism, as well as compromise antioxidant system defenses (Finnegan and Chen 2012; Dixit et al. 2015; Shen et al. 2016). Moreover, a reduction in root growth and size in plants exposed to As decreases the availability of essential nutrients in leaves and roots (Reed et al. 2015). However, increases in Ca may be a defense mechanism, because the nutrient acts as a secondary messenger in response to stress factors and improves the plant antioxidant defenses (Ahmad et al. 2015; Rahman et al. 2015; Edel et al. 2017). Calcium also participates in the cell wall and membrane stabilization and regulates the metabolic process related to other nutrients uptake (White and Broadley 2003; Hepler 2005). Additionally, Ca and P ions are able to form complexes with toxic elements and immobilize them in the cell wall (Parrota et al. 2015), reducing their potential damage.

The severe reduction in biomass observed in submerged leaves prove the high As^{III} toxicity. The ROS increase can cause intense oxidative damage and promote plant tissue necrosis, affecting plant biomass (Talukdar and Talukdar 2013; Upadhyaya et al. 2014). The same happened with macrophytes *Ceratophyllum demersum* (Khang et al. 2012) and *Nasturtium officinale* (Ozturk et al. 2010) in which As^{III} caused biomass loss. Thus, it is possible that As^{III} damage to submerged leaves induced a decrease in water absorption and contributes to inhibiting plant growth. In addition, the reduction of photosynthesis promoted by As also impairs the biomass gain (Sharma 2013, Gusman et al. 2013, Farooq et al. 2016b).

It should be considered that the As^{III} concentrations in this study are higher than most of those naturally found in in countries with remarkable levels of water contamination (Chakraborti et al. 2016). Therefore, a high amount of pollutant increased the toxic effects on plant growth.

Corroborating the study hypothesis, the As^{III} induced oxidative stress in *S. molesta* submerged leaves as evidenced by the increases in $O_2^{\cdot-}$ and electrolyte leakage. Membranes are the primary ROS targets in plant cells and when its structure is disturbed, electrolyte leakage occurs. Thus, membrane damage can function as a biomarker of As toxicity (Finnegan et al. 2012; Anjum et al. 2015). The membrane integrity of the floating leaves denote lesser As^{III} effects in the plant shoots. This may be the result of lower As accumulation coupled with a greater activity of antioxidant enzymes.

The ROS generation in plants exposed to toxic metals can trigger the expression of antioxidant enzyme genes, as well as increase the activity of these enzymes for stress acclimatization (Ahammed et al. 2013; Rout and Sahoo 2013; Ahmad et al. 2015; Farooq et al. 2015). SOD is the first line of antioxidant enzymatic defense in plants because it controls the $O_2^{\cdot-}$ removal in cell (Gill et al. 2015). Increases in SOD activity and greater As tolerance have been reported in different plants (Mishra et al. 2011; Gupta et al. 2013; Kanwar et al. 2015; Dixit et al. 2015). In *S. molesta* floating leaves, SOD activity may have helped to protect cells from oxidative damage.

SOD activity can be inhibited by As (Du et al. 2017). Because of its affinity for the sulfhydryl (-SH) proteins groups, As^{III} disturbs the catalytic functions of enzymes (Shen et al. 2013). Inhibition of antioxidant enzymes allows hydroxyl radical (OH•) generation, a highly reactive molecule with extreme cellular oxidizing capacity (Halliwell and Gutteridge 2015). If this happens, oxidative stress is intensified. A similar situation may have occurred in *S. molesta* submerged leaves, in which the SOD activity was negligible and there was significant increases in $O_2^{\cdot-}$ concentrations.

The SOD activity produces H_2O_2 (Gill et al. 2015). This helps to explain the insignificant variation in H_2O_2 in the submerged leaves. However, this direct relationship between increase in SOD activity and H_2O_2 concentration was not verified in the floating leaves. The performance of H_2O_2-eliminating enzymes support these results. Thereby, CAT was an important enzyme for H_2O_2 control in floating leaves. CAT is responsive to As mainly in plant leaves (Mishra et al. 2011; Gusman et al. 2013). Thus, CAT activity was lower in *S. molesta* submerged leaves which act as roots, and was not changed in response to As^{III}. Catalase has a lower affinity for H_2O_2 than peroxidases enzymes (Mhamdi et al. 2010; Das and Roychoudhury 2014). Therefore, peroxidases is important in ROS control.

Increases in POX and APX activities suggests an important role for these enzymes in As^{III} tolerance in

S. molesta. POX and APX are recognized members of the antioxidant defense system against As in macrophytes such as *Pistia stratiotes* (Farnese et al. 2014), *Azolla caroliniana* (Leão et al. 2017), and *Spirodela intermedia* (da-Silva et al. 2017). The peroxidases' activity indicates defense mechanisms mediated by antioxidants, which protect plant cell structures and reduce As toxic effects (Dave et al. 2013; Dixit et al. 2015). In addition, APX is an enzyme component of the ascorbate–glutathione cycle, as one of the most efficient ROS elimination system in As stressed plants (Singh et al. 2015b).

In the *S. molesta* floating leaves, while the APX activity increased in the two lowest As^{III} concentrations, POX activity was stimulated only under 20 µM As^{III}. Similarly, APX activity increased in submerged leaves at all As^{III} concentrations, while POX showed a relevant increase only in the most stressed plants.This result suggests the coordinated action of these two enzymes in the peroxide elimination, depending on the As stress intensity. Differences in the enzymes affinity for their substrate may be the cause of this responses. Indeed, it is known that APX has a high affinity for H_2O_2 and acts on its elimination even when it is in low concentrations in the cell (Sofo et al. 2015; Anjum et al. 2016).

The antioxidant enzymes activity seems to act in a coordinated way in order to alleviate As^{III} toxic effects in *S. molesta* and avoided cell damage in the floating leaves. There is also a remarkable difference in the activity level of the enzymes between the plant parts. The CAT, POX and APX activities in the floating leaves are much higher than in submerged leaves, even in the control condition. The higher ROS production in photosynthetic tissues, further increased by photorespiration (Demidchik 2015; Del Río and López-Huertas 2016; Mitler 2017), can explain these results. Even under optimal environmental conditions, the intense ROS production by photosynthesis requires a higher concentration of antioxidant enzymes in leaves (Hajiboland 2014).

It is known that the differences in As compartmentalization in cells can also influence plant defense responses (Shaibur and Kawai 2009). Thus, while sequestration mechanisms using metal chelating molecules such as glutathione and phytochelatin can be more effective in root cells, antioxidant enzymes may be more important in leaf cells (Begum et al. 2016, Silva et al. 2017). Additionally, a higher accumulation of toxic metals in the roots is suggested to be a protective mechanism for the plant's photosynthetic apparatus (Liu et al. 2010; Gomes et al. 2012; Gusman et al. 2013).

The results obtained in this study show the response of enzymatic antioxidant system in *S. molesta* to As^{III} exposure. This defense mechanism has been shown to be important for As tolerance in plants and this trend was also observed in this study. However, further studies assessing the *S. molesta* antioxidant defenses in a longer stress, and the use of equivalent concentrations to those found in contaminated environments are still necessary to establish the plant As^{III} tolerance and its potential for use in contamined water phytoremediation.

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