REVISTA DE CIÊNCIASAGRÁRIAS
Amazonian Journal
of Agricultural and Environmental Sciences
www.ajaes.ufra.edu.br

http://dx.doi.org/10.4322/rca.2013.086

ORIGINAL ARTICLE

Mechanisms of the internal structure and operation of *Panicum aquaticum* in response to arsenic

Mecanismos da estrutura interna e funcionamento de Panicum aquaticum em resposta ao arsênio

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KEYWORDS
Anatomy
Macrophytes
Physiology
Tolerance
Toxic elements

PALAVRAS-CHAVE
Anatomia
Macrófitas
Fisiologia
Tolerância
Elementos tóxicos

ABSTRACT: Due to the high toxicity of arsenic, many studies have attempted to improve the techniques for its removal from the environment, using methods such as phytoremediation by tolerant species. To evaluate the tolerance of *Panicum aquaticum* to contamination by arsenic, both physiological and anatomical evaluations were conducted. The plants were grown in Hoagland and Arnon nutrient solution in a greenhouse for 30 days under six concentrations of arsenic: 0.00, 0.25, 0.50, 1.00, 2.00, and 4.00 mM. Analyses of growth, gas exchange, the anatomy of leaves and roots and of the activity of antioxidant system enzymes were conducted. The relative growth rate, specific leaf area and leaf area ratio were modified in the presence of arsenic. Gas exchange was not affected. The leaf anatomy showed reductions in the epidermal thicknesses on the abaxial face, on the blade and on the chlorenchyma; increases in the set of bulliform cells, in the cuticle thickness and in the area of the sclerenchyma; reductions in the number and distance of vascular bundles; an increase in the stomatal index; an increase in the stomatal functionality only on the adaxial face of the epidermis; and reductions in the number and density of stomata. The roots presented reductions in the thicknesses of the epidermis, endodermis and exodermis and modifications in the Carlquist vulnerability index. Only the catalase activity was affected, showing an increase at the lowest concentrations followed by a decrease at higher concentrations. *P. aquaticum* proved partially tolerant to arsenic at the lowest concentrations and presented evidence of toxicity at the highest concentrations.

RESUMO: *Devido à alta toxicidade do arsênio, muitos estudos têm tentado aprimorar as técnicas para a sua remoção do ambiente, como a fitorremediação por espécies tolerantes. Para avaliar a tolerância de Panicum aquaticum à contaminação por arsênio, realizaram-se análises anatômicas e fisiológicas. As plantas foram cultivadas em solução nutritiva de Hoagland e Arnon em casa de vegetação por 30 dias, sob seis concentrações de arsênio: 0,00; 0,25; 0,50; 1,00; 2,00; 4,00 mM. Foram realizadas análises de crescimento, de trocas gasosas, anatômicas de folhas e raízes, e da atividade de enzimas do sistema antioxidante. A taxa de crescimento relativo, a área foliar específica e a razão de área foliar foram modificadas na presença de arsênio. As trocas gasosas não foram afetadas. A anatomia foliar mostrou redução nas espessuras da epiderme na face abaxial, no limbo e no clorênquima; aumento na área de conjunto de células buliformes, na espessura da cutícula e na área de esclerênquima; redução no número e na distância dos feixes vasculares; aumento do índice estomático; aumento da funcionalidade estomática apenas na face adaxial da epiderme, e redução no número e na densidade de estômatos. As raízes apresentaram uma redução na espessura de epiderme, endoderme e exoderme, e houve modificação do índice de vulnerabilidade de Carlquist. Apenas a atividade da catalase foi afetada, mostrando um aumento nas concentrações mais baixas de arsênio, seguido de uma redução em concentrações mais elevadas. P. aquaticum demonstrou-se parcialmente tolerante ao arsênio nas menores concentrações e apresentou evidências de toxicidade nas maiores concentrações.*

Received: 11/04/2013
Accepted: 12/31/2013

1 Introduction

Arsenic (As) is a naturally occurring metalloid in the environment that is mobilized through weathering, biological activities and volcanic emissions. Human activities represent an important contribution to this process through mining, the burning of fossil fuels, and the use of pesticides and herbicides (SRIVASTAVA et al., 2011). Thus, arsenic contamination has become a serious threat to biota.

Due to the high toxicity of arsenic, many studies have attempted to improve environmental decontamination techniques using tolerant organisms, especially plants (SINGH et al., 2007), for phytoremediation.

Poaceae have shown potential for use in the recovery of areas degraded by toxic elements due to their ease of growth and rapid and thick coverage of the environment, providing a physical structure that mitigates erosion and adds organic matter to the system (CARNEIRO; SIQUEIRA; MOREIRA, 2001). Accordingly, *Panicum aquaticum*, belonging to the subfamily Panicoideae (Poaceae), may present characteristics favorable to the tolerance to arsenic.

The aim of this study was to assess the tolerance of the macrophyte *P. aquaticum* to increasing concentrations of arsenic by characterizing its anatomical and physiological responses.

2 Materials and Methods

Plants collected in a pond free of contamination by toxic elements were grown in a greenhouse in modified Hoagland and Arnon nutrient solution (1950) with a 40% total ionic strength. The uniform daughter plants were transferred to plastic trays with a capacity of 4.0 L. Each tray contained 2.0 L of washed sand and 2.0 L of Hoagland and Arnon nutrient solution (1950) with increasing concentrations of arsenic (0.00, 0.25, 0.50, 1.00, 2.00 and 4.00 mM) in the form of $Na_2HAsO_4.7H_2O$, selected on the basis of the allowed maximum value (0.5 mg L^{-1}) of total As for effluent discharges into bodies of freshwater (BRASIL, 2011).

For the growth analyses, collections were made at the beginning of the experiment and after 30 days, at the end of the experiment. The plants were separated into leaf, stem and root. The leaf area was obtained from photographs of all of the leaves from each plant and the measurement and summation of the areas of these leaves using the image analysis software Image Tool version 3.0 (UTHSCSA, 2002). Then, all of the material was placed in a forced circulation oven at 60 °C to a constant weight. The data for the dry leaf, stem and root masses were obtained on an analytical balance. All of the data were used for determining the growth parameters in accordance with the software proposed by Hunt et al. (2002).

After 20 days, the gas exchange characteristics of the plants were evaluated through an infrared gas analyzer (IRGA) model LCA4 - ADC - INSTRUMENTAL. The analyses were performed in the morning between 9 and 11 h.

At the end of the experimental period, the plants were collected for anatomical analyses, fixed in 70% FAA (formaldehyde p.a, glacial acetic acid and 70% ethanol) and then preserved in 70% ethanol (KRAUS, ARDUIN, 1997). Paradermic sections of the leaves and transversal sections of the roots and leaves were collected by standard procedures of plant microtechniques according to Johansen (1940). All of the slides were photographed by a 2500 Moticam camera attached to the microscope, model Olympus CX41, and the analyses were performed using the image analysis software Image Tool version 3.0 (UTHSCSA, 2002) .

For the analyses of the antioxidant enzyme system, samples were collected in the morning, stored in liquid nitrogen and preserved in a freezer at –80 °C. For extraction, 0.2 g of leaves and roots macerated in liquid nitrogen with the addition of 1.5 mL of the extraction buffer were utilized. The extract was centrifuged, and the supernatant was collected and stored at –20 °C until analysis in enzyme assays using superoxide dismutase (SOD) (according to Giannopolitis and Ries (1977)), catalase (CAT) (as proposed by Havir and McHale (1987)) and ascorbate peroxidase (APX) (according Nakano and Asada (1981)).

The experiment was conducted in a completely randomized design with six treatments and five replicates. The data were first tested for normality by the Shapiro-Wilk test and then subjected to analysis of variance and comparison of means by the Scott - Knott test for $p < 0.05$ or to regression with the aid of the statistical software Sisvar (FERREIRA, 2007).

3 Results and Discussion

Among the growth variables, only the ratio root/shoot and the net assimilation rate (NAR) were not affected by the different concentrations of arsenic. The other characteristics evaluated presented a decrease at the highest concentrations (Table 1).

High concentrations of arsenic are toxic to a majority of plants because arsenic interferes with metabolic processes and inhibits their growth and development. This decrease in growth is ascribed often to the reduction in carbon assimilation by photosynthesis (MATEOS-NARANJO; ANDRADES-MORENO; REDONDO-GÓMEZ, 2012). However, the rate of photosynthesis (Fc = 0.478 and P = 0.7894), with a mean of 0.03 mol m^{-2} s^{-1}; the stomatal conductance (Fc = 0.2 and P = 0 .9593), with a mean of 0.06 mmol m^{-2} s^{-1}; the ratio Ci/Ca (Fc = 2.205 and P = 0.0871), averaging 0.26; and the rate of transpiration (Fc = 0.444 and P = 0.8131), with a mean of 0.007 mmol m^{-2} s^{-1}, were not affected at any of the concentrations of arsenic.

Table 1. Analysis of the growth of *Panicum aquaticum* plants under increasing concentrations of arsenic.

As	RGR	LAR	SLA	LA
(mM)	g $g^{-1}d^{-1}$	cm^2 g^{-1}		cm^2
0.00	0.08 a	76.35 a	220.75 c	34.35 a
0.25	0.07 b	42.56 d	248.87 b	37.03 a
0.50	0.08 a	55,57 c	330.28 a	27.01 b
1.00	0.05 b	18.73 e	189.32 c	24.66 b
2.00	0.01 c	09.92 f	084.86 d	26.03 b
4.00	0.09 a	57.96 b	237.16 b	25.68 b

The means followed by the same letter in the column do not differ from one another (Scott-Knott 5%). A = Relative Growth Rate (RGR), B = Leaf Area Ratio (LAR), C = Specific Leaf Area (SLA), D = Leaf Area (LA).

It is known, however, that plants under stress usually have high respiration rates, causing changes in growth with greater carbon loss and reduction in biomass accumulation. According to Flexas et al. (2006), of the total carbon assimilated by photosynthesis, usually more than half is lost in respiration processes needed for growth and the maintenance of plant metabolism, but this balance can change under stress. This could be the cause of the modification in the growth of *P. aquaticum* in the presence of arsenic.

Although the rate of photosynthesis was maintained, the decrease in the LAR and in the LA and the modification in the SLA likely affected the growth because the LAR represents one of the components responsible for the increase of biomass, measured as RGR, and undergoes variations on the basis of the SLA (PINZÓN-TORRES; SCHIAVINATO, 2008). Considering that the NAR describes the efficiency of net production of the assimilation apparatus, namely, the photosynthesis per unit leaf area (PINZÓN-TORRES; SCHIAVINATO, 2008), it is likely that the lack of change across the treatments was favored by maintaining photosynthesis, despite the reduction in the LAR and the SLA.

The maintenance of photosynthesis in *P. aquaticum* in the presence of arsenic contradicts several studies that have shown a decline in the rate of photosynthesis (MATEOS-NARANJO; ANDRADES-MORENO; REDONDO-GÓMEZ, 2012) as a result of the toxicity of this metalloid. According to Stoeva and Bineva (2003), arsenic stress can lead to the formation of reactive oxygen species (ROS), cause oxidative stress and damage the membranes of chloroplasts. However, the response of the photosynthetic apparatus found in *P. aquaticum* most likely reflects the action of the antioxidant system because no effect of the treatment on these processes was observed, suggesting that the photochemical phase of photosynthesis may not have experienced any inhibition (PEREIRA et al., 2011).

The majority of the features of the leaf tissues in the cross section were affected by the concentration of arsenic (Table 2). Only the number of bulliform cells was not affected by the treatments.

An important function of the leaf epidermis is gas exchange (CASTRO; PEREIRA; APIA, 2009). Thus, changes in the thickness of the epidermis as well as in the cuticle can affect transpiration (GRISI et al., 2008) as well as resistance to the diffusion of CO_2 (MIYAZAWA; TERASHIMA, 2001). However, as noted above, arsenic treatment did not affect the rate of transpiration or the ratio Ci/Ca. Likewise, the changes in the thickness of the blade and in the chlorophyll parenchyma presented by *P. aquaticum* were not sufficient to affect the availability of CO_2 in the leaf, despite their important role in gas exchange (CASTRO; PEREIRA; PAIVA, 2009).

The increase in the sclerenchyma under higher metal concentrations can be attributed to the adsorption of the metal by the cell walls, constituting an alternative pathway for immobilizing these ions and preventing translocation to the photosynthetic tissues (GOMES et al., 2011). In fact, the distribution between the tissues of the leaf tends to minimize the metal concentration in the chlorophyll parenchyma, preventing damage to photosynthesis (GOMES et al., 2011). This hypothesis could explain the observed changes in the leaf tissues of *P. aquaticum*.

Although they varied in number, the sets of bulliform cells in *P. aquaticum* increased in area on the leaf blade. This suggests an increase in cell volume and consequently a greater opening of the leaf, allowing greater exposure of the leaf area and compensating for the reduction in leaf area.

A study of the anatomical characteristics of the leaves of *P. aquaticum* in paradermic sections showed larger changes in the adaxial epidermis resulting from arsenic treatment (Table 3).

The increase in the stomatal index (SI) on the abaxial epidermis suggests the influence of arsenic in the differentiation of cells for the formation of stomata. Pereira et al. (2011) found similar results in water hyacinth plants in the presence of arsenic. On the adaxial face, modifications in the number and size of the stomata determined the changes in their density and functionality. This agrees with the findings of Castro, Pereira and Paiva (2009), who stated that environmental conditions affect the size and density of stomata, allowing the plant to adapt to its environmental conditions.

In the anatomy of the roots of *P. aquaticum*, all evaluated features were affected by the presence of arsenic (Figure 1 and Table 4). The reductions in the thicknesses of the endodermis and exodermis of *P. aquaticum* indicate the toxicity of the arsenic treatment, as these features are considered adaptations to stress factors, such as heavy metals, because they function as apoplastic barriers (MARQUES et al., 2011). This reduction most likely favored the translocation of arsenic to the shoot.

Table 2. The characteristics of the leaf tissue in cross sections of *Panicum aquaticum* at various concentrations of arsenic**.**

As	EAB	EAD	LM	PC	DF	CT	ECL	ACB	NF
(mM)				(μm)				(μm^2)	
0.00	14.37 b	12.48 a	188.28 b	141.12 b	56.06 b	5.72 b	1134.60 d	2210.61 b	07 a
0.25	15.89 a	13.12 a	203.57 a	153.84 a	60.97 b	5.71 b	1437.55 c	2894.53 b	06 b
0.50	16.24 a	12.44 a	208.86 a	156.01 a	64.17 b	5.61 b	1709.72 b	3231.18 a	06 b
1.00	13.97 b	11.35 b	196.11 b	147.28 b	58.57 b	5.84 b	1802.73 b	2681.97 b	07 a
2.00	14.94 b	12.28 a	196.97 b	148.71 b	82.71 a	5.82 b	1596.22 b	3112.76 a	06 b
4.00	15.33 b	12.95 a	192.65 b	145.22 b	65.36 b	6.26 a	1999.99 a	3066.93 a	06 b

Means followed by the same letter in a column do not differ from one another (Scott-Knott 5%). EAB = thickness of the epidermis of the abaxial face, EAD = thickness of the epidermis of the adaxial face, LM = blade thickness, PC = thickness of the chlorenchyma, DF = distance between the vascular bundles, NF = number of vascular bundles (per mm^{-1} of leaf), CT = cuticle thickness, ECL = sclerenchyma area.

Table 3. The characteristics of the leaf epidermis in paradermic sections of *Panicum aquaticum* at increasing concentrations of arsenic.

ABAXIAL FACE					
As	SD	SI	POL	EQU	FUN
(mM)		(%)	(µm)		
0.00	100 a	0.21 c	34.46 a	21.95 a	1.58 a
0.25	091 a	0.22 c	34.69 a	21.69 a	1.60 a
0.50	096 a	0.23 b	34.81 a	23.04 a	1.52 a
1.00	089 a	0.23 b	34.56 a	22.13 a	1.56 a
2.00	098 a	0.25 a	34.94 a	22.45 a	1.56 a
4.00	089 a	0.24 b	35.94 a	21.93 a	1.64 a
ADAXIAL FACE					
As	SD	SI	POL	EQU	FUN
(mM)		(%)	(µm)		
0.00	145 a	0.27 b	29.87 b	20.21 a	1.49 b
0.25	126 b	0.27 b	31.45 a	19.69 a	1.60 a
0.50	148 a	0.29 a	29.88 b	19.51 a	1.54 a
1.00	132 b	0.27 b	31.75 a	19.99 a	1.59 a
2.00	153 a	0.30 a	29.52 b	20.42 a	1.45 b
4.00	154 a	0.30 a	29.31 b	19.83 a	1.48 b

Means followed by the same letter in a column do not differ from one another (Scott-Knott,%). SD = stomatal density (per mm^2 of leaf), SI = stomatal index, POL = polar diameter of the stomata, EQU = equatorial diameter of the stomata, FUN = stomatal function (ratio POL/EQU).

Although the thickness of the cortex decreased during treatment, the ratio between the area of the vascular cylinder and the total area was only slightly affected. In this case, the ratio among the root tissues only changed slightly**,** contradicting other authors who stated this to be a consequence of continuous exposure to heavy metals (VACULÍK et al., 2012).

Aerenchymas provide a low resistance path to the internal movement of O_2 (and other gases) between the shoot and root ends in plants in flooded environments (LI et al., 2011). Accordingly, the observed reduction in the proportion of aerenchymas may have affected the growth of the plants.

The CVI can be influenced by environmental conditions, making it an index that reflects the vulnerability of the xylem to embolism (CASTRO; PEREIRA; PAIVA, 2009). Thus, *P. aquaticum* showed poorer efficiency in hydraulic conductivity in the presence of arsenic, suggesting toxicity, causing damage to the translocation of nutrients to the shoot and contributing to the reduction in the LAR and the RGR.

Regarding the enzymes of the antioxidant system, only the catalase activity (CAT) in the leaf was affected by the different concentrations of arsenic (Figure 2). In this case, increased activity was found at the lowest concentrations, with the highest activity achieved at a 1.0 mM arsenic concentration, followed by a decrease to activity values equal to the control in the two highest concentrations of the metalloid.

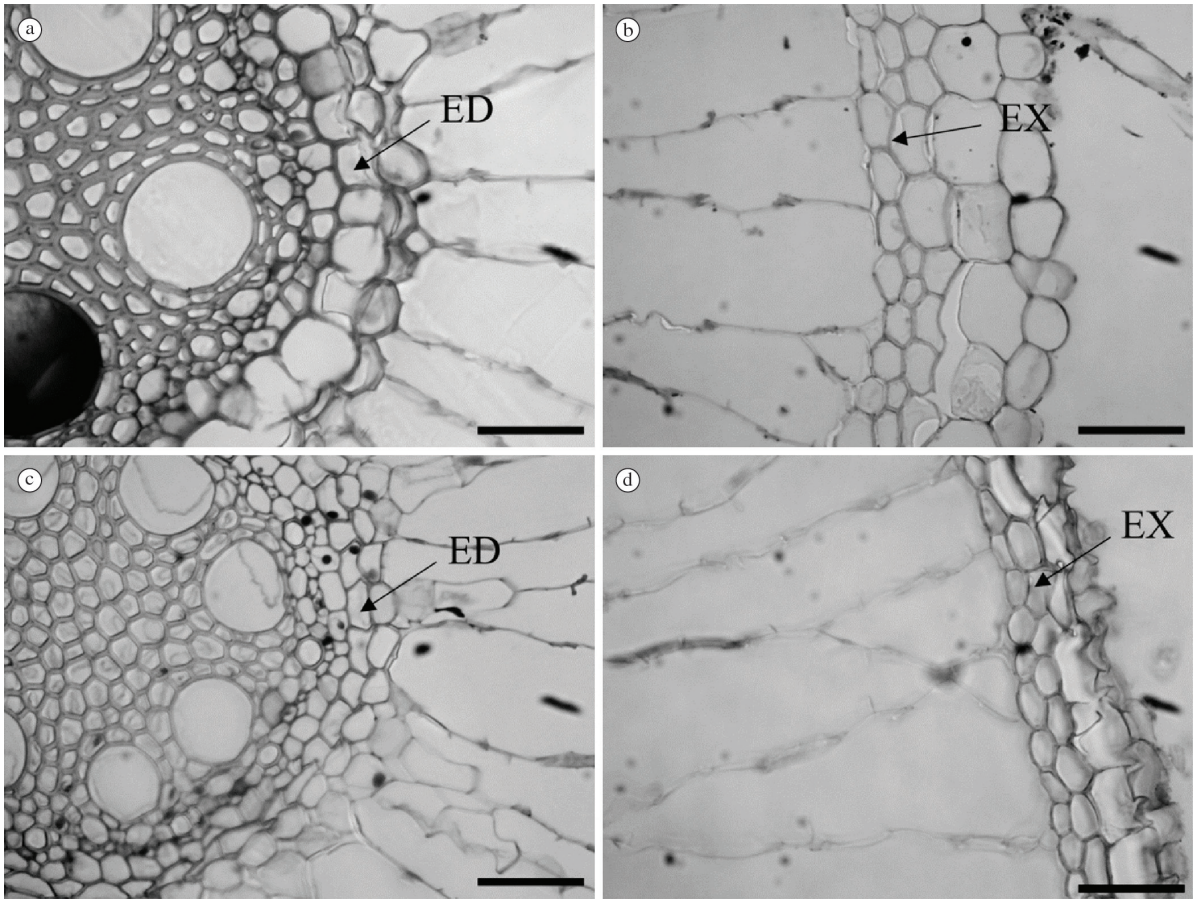

Figure 1. Detail of the endodermis (a and c) and exodermis (b and d) of the root of *Panicum aquaticum* under arsenic contamination. a and b = 0.0 mM, c and d = 4.0 mM. Bars = 50 µm.

Table 4. The characteristics of the root tissue in cross sections of *Panicum aquaticum* for increasing concentrations of arsenic.

As	EP	EX	ED	ECX	ACAT	PAR	CVI
(mM)			(µm)				
0.00	41.26 **a**	26.62 **a**	15.59 **a**	457.76 **a**	0.06 **a**	0.83 **a**	0.09 **c**
0.25	44.01 **a**	23.38 **b**	11.69 **b**	375.84 **b**	0.04 **b**	0.81 **b**	0.12 **b**
0.50	38.66 **a**	22.63 **b**	11.69 **b**	310.75 **c**	0.05 **a**	0.85 **a**	0.10 **c**
1.00	33.13 **b**	21.05 **b**	11.75 **b**	287.61 **c**	0.06 **a**	0.76 **b**	0.09 **c**
2.00	40.99 **a**	23.66 **b**	12.59 **b**	330.71 **c**	0.06 **a**	0.85 **a**	0.16 **a**
4.00	41.84 **a**	23.94 **b**	11.35 **b**	406.36 **b**	0.06 **a**	0.85 **a**	0.12 **b**

Means followed by the same letter in the same column do not differ from one another (Scott-Knott 5%). EP = thickness of the epidermis, EX = thickness of the exodermis, ED = thickness of the endoderm, ECX = thickness of the cortex, ACAT = ratio between the area of the vascular cylinder and the total area, PAR = proportion of aerenchyma in the cortex, CVI = Carlquist vulnerability index.

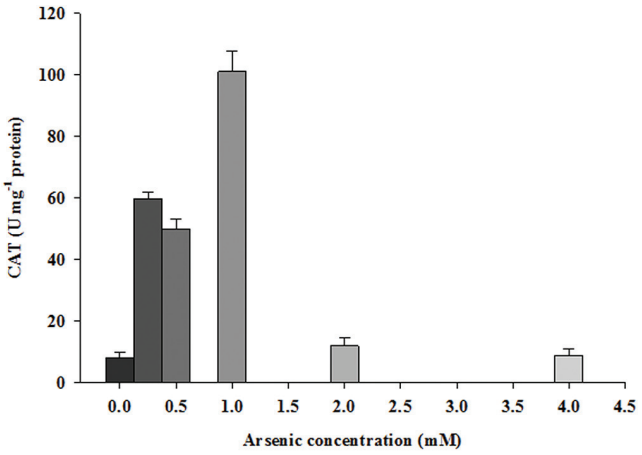

Figure 2. Catalase activity in the leaves of *Panicum aquaticum* at increasing concentrations of arsenic. U = nmol min^{-1} and H_2O_2.

Among the various biochemical and physiological processes in a plant, the behavior of the antioxidant system is fundamental to understanding the tolerance of plants to arsenic. However, the absence of changes in the activities of SOD, CAT and APX in the root at increasing concentrations of the metalloid contradicts this hypothesis in *P. aquaticum*. The observed results provide evidence that such enzymes are weakly responsive to stress due to arsenic, with their activity not being altered in response to increasing concentrations of the metalloid, allowing the action of ROS in crucial processes.

Due to the behavior demonstrated by both SOD and APX, it is possible to infer that the removal of ROS occurred in response to stress and was performed more efficiently by the CAT in the leaf at intermediate concentrations. Most likely, the leaf CAT activity increased to the detriment of APX as a compensatory mechanism. However, a balance in the CAT and APX enzymes is essential to the control of ROS (BOWLER et al., 1991) because they both act on peroxide hydrogens. However, the reduction in CAT activity in the leaf at higher concentrations of arsenic could indicate inhibition due to the limited amount of available enzyme and inhibited enzyme synthesis, demonstrating signs of arsenic toxicity. Thus, most likely, other components of the antioxidant system acted to minimize the damaging action of ROS, especially in relation to photosynthesis.

4 Conclusions

The majority of the surveyed characteristics, such as the modification in the relative growth, the thickness of various tissues and the activity of the CAT, presented negative changes, especially at the highest concentrations of arsenic.

Thus, the species *Panicum aquaticum* can be considered partially tolerant to arsenic at the lowest concentrations of arsenic but exhibited evidence of toxicity at the highest tested concentrations.

Acknowledgements

The authors are grateful to FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) and CNPq (National Counsel of Technological and Scientific Development) for supporting this research and for providing a scholarship to the first author.

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