



Lead tolerance of water hyacinth (*Eichhornia crassipes* Mart. - Pontederiaceae) as defined by anatomical and physiological traits

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ABSTRACT

This study aimed at verifying the lead tolerance of water hyacinth and at looking at consequent anatomical and physiological modifications. Water hyacinth plants were grown on nutrient solutions with five different lead concentrations: 0.00, 0.50, 1.00, 2.00 and 4.00 mg L⁻¹ by 20 days. Photosynthesis, transpiration, stomatal conductance and the Ci/Ca rate were measured at the end of 15 days of experiment. At the end of the experiment, the anatomical modifications in the roots and leaves, and the activity of antioxidant system enzymes, were evaluated. Photosynthetic and Ci/Ca rates were both increased under all lead treatments. Leaf anatomy did not exhibit any evidence of toxicity effects, but showed modifications of the stomata and in the thickness of the palisade and spongy parenchyma in the presence of lead. Likewise, root anatomy did not exhibit any toxicity effects, but the xylem and phloem exhibited favorable modifications as well as increased apoplastic barriers. All antioxidant system enzymes exhibited increased activity in the leaves, and some modifications in roots, in the presence of lead. It is likely, therefore, that water hyacinth tolerance to lead is related to anatomical and physiological modifications such as increased photosynthesis and enhanced anatomical capacity for CO₂ assimilation and water conductance.

Key words: heavy metals, ecophysiology, plant anatomy, macrophytes.

INTRODUCTION

Environmental contamination by lead (Pb) is a worldwide problem (Gratão et al. 2005). Lead is one of the most dangerous pollutants and its deposition in soil and water is related to effluents, fuels, industries and agronomical pesticides and fertilizers (Sharma and Dubey 2005).

Traditional techniques for lead removal are expensive and often produce new dangerous effluents. Phytoremediation is an alternative with low cost that has been utilized for soil and water decontamination (Gratão et al. 2005, Rahman and Hasegawa 2011). *Eichhornia crassipes* showed a hyper-accumulation capacity for chromium (Faisal and Hasnain 2003), cadmium (Oliveira et al. 2001) and arsenic (Dhankher et al. 2002, Pereira et al. 2011). The hyperaccumulation

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capacity of this plant is related to its large biomass and the characteristics such as pH and temperature have little influence on the process (Schoenhals et al. 2009).

There is little information on how lead may affect photosynthesis, but it can reduce chlorophyll content and photosynthetic rate affecting the photosystem II (Pinchasov et al. 2006, Cenkci et al. 2010). In rice plants lead promotes an increase in membrane peroxidations and in the activity of antioxidant enzymes (Verma and Dubey 2003). In *Phaseolus vulgaris* lead promoted an increase in oxidant compounds such as phenols (Hamid et al. 2010).

There are few studies of anatomical modifications in *E. crassipes* as a consequence of environmental stresses. Mahmood et al. (2005) reported that in the presence of textile industry effluents, *E. crassipes* plants exhibited a reduction in cell size in leaf tissues, whereas Pereira et al. (2011) found no deleterious effects in anatomy of leaves and roots of this species in the presence of arsenic. In *Plantago major*, lead reduced stomatal density as well as stomatal conductance and vascular bundle size (Kosobrukhov et al. 2004). In wheat, plants under lead contamination exhibited increased antioxidant enzymes activity (Liu et al. 2010). In this study, we aimed to evaluate the lead tolerance of *E. crassipes* plants as related to modifications in its anatomy, gas exchange and antioxidant enzymes activities.

MATERIALS AND METHODS

PLANT MATERIALS AND EXPERIMENTAL DESIGN

Water hyacinth plants (*Eichhornia crassipes* Mart.) were collected and cultivated in a greenhouse at the Biology Department of the Federal University of Lavras, state of Minas Gerais, Brazil. Plants were cultivated in Hoagland and Arnon nutrient solution (Hoagland and Arnon 1940) at 40% of ionic force for 30 days in order to obtain individuals free of endogenous lead and homogeneous clones.

Cloned plants selected by size and the number of leaves, were transplanted to plastic pots containing 4 L of Hoagland and Arnon nutrient solution at 20% of ionic force and the following lead concentrations: 0, 0.50, 1.00, 2.00 and 4.00 mg L⁻¹. The experiment was conducted for 20 days, after this period, plants were harvested and subsequently divided into shoots and roots. The experimental design was completely randomized with five treatments and five replicates. Data were submitted to one-way Anova and Scott-Knott test at P<0.05 in sisvar statistical software.

GAS EXCHANGE EVALUATION

After 15 days, experimental plants were evaluated for: net photosynthesis (A), stomatal conductance (g_s), transpiratory rate (E) and the internal and atmospheric carbon rate (Ci/Ca). Measurements were conducted with the infrared gas analyzer (IRGA) model LI-6400 (Li-COR Biosciences, Lincoln-USA). These evaluations were made with fully expanded and pathogen-free leaves, in five replications. Measurements were made at 10 hours and the photon flux of photosynthetic radiation was standardized at 1000 μmol m⁻² s⁻¹ in the equipment chamber.

ANATOMICAL EVALUATIONS

Anatomical evaluations were conducted with clonal plants at the end of the 20 day experimental period. Whole Plants were fixed in F.A.A._{70%} solution (formaldehyde, acetic acid and ethanol 70%) for 72 hours, and stored in ethanol 70%. Paradermal sections were prepared for abaxial and adaxial faces of the leaves. Sections were cleared with 50% sodium hypochlorite solution, washed in distilled water for 2 ten-minute periods, stained with a 1% safranin aqueous solution, and mounted in 50% glycerol (Johansen 1940). Leaf portions were removed from the median region and transverse sections were made with a bench-top hand microtome (LPC type). Sections were

cleared with 50% sodium hypochlorite solution, washed twice in distilled water, and stained with safrablau solution (safranin 1% and astra blau 0.1% in the proportion of 7:3), and mounted in glycerol 50% (Johansen 1940). Slides were observed and photographed with an Olympus light microscope (BX 60 model – Olympus, Tokyo, Japan) and with a digital camera (Canon A630 – Canon Inc., Tokyo, Japan). Photomicrographs were evaluated in UTHSCSA-Imagetool software and quantitative analyses of the tissues and structures were performed in five sections by statistical replicate and five fields per section. The IVC (Carlquist vulnerability index) was calculated (Carlquist 1975), as well as the aerenchyma proportion of the root cortex, as described by Pereira et al. (2008), and the stomatal density and stomatal index according to Castro et al. (2009).

ANTIOXIDANT ENZYMES ACTIVITY

For biochemical analyses assays, roots and leaves were collected at 20 days from clones that were fully developed in the lead solutions. These organs were frozen in liquid nitrogen and stored at -80°C . For protein extractions, 0.5 g of roots and leaves were ground in 2.0 mL of extraction buffer (1.924 μL of potassium phosphate buffer 0.1 M at pH 7; 20 μL of EDTA 0.1 M; 8 μL of DTT 0.5 M; 16 μL of PMSF 0.1 M and 40 mg of PVPP) modified from Bor et al. (2003). The extracts were centrifuged at 14000 g at 4°C for 20 minutes, and the supernatant was used for the enzymatic analysis of catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD). APX activity evaluations were performed following Nakano and Asada (1981), CAT activity analysis as described by Madhusudhan et al. (2003), and the SOD activity evaluated following Giannopolitis and Ries (1977).

RESULTS

Gas exchange characteristics in *E. crassipes* were modified by the lead treatments. Concentrations at 0.50 and 1.0 mg L^{-1} increased the photosynthetic

rate of plants by 13.95% and 11.29%, respectively, compared to the control group; but at higher concentrations, only small and non-significant variation occurred (Figure 1A). The stomatal conductance was reduced in concentrations of 1.0 mg L^{-1} and above; the reduction was of 57.83% in comparison to the control (Figure 1B). Likewise, the transpiratory rate was increased by 9.1% only at the highest concentration (4.0 mg L^{-1}) (Figure 1C). The Ci/Ca rate was increased in the 0.50 mg L^{-1} and maintained this level in all higher concentrations (Figure 1D).

The different lead concentrations promoted modifications in the leaf anatomy of *E. crassipes*. Leaf thickness was increased by 19% in the 1.0 mg L^{-1} in comparison to the control (Table I and Figure 2), but there were no significant modifications to the leaf epidermis, palisade and spongy parenchyma, or the palisade/spongy parenchyma rate, in the presence of Pb (Table I). The distance between the vascular bundles was reduced in 32.05% in the 0.50 mg L^{-1} lead concentration and in all higher concentrations (Table I). The proportion of leaf aerenchyma did not exhibit any differences related to lead treatments Pb (Table I).

The abaxial leaf surface showed an increase of 15% in stomatal density in the 0.5 mg L^{-1} , and an increase of 8.69% in the 1.0 mg L^{-1} and in all higher concentrations (Table II). The number of regular epidermal cells and the stomatal dimensions were not modified by lead (Table II). The stomatal polar/equatorial diameter rate (stomatal functionality) increased by 17.71% in the 1.0 mg L^{-1} and in the higher concentrations (Table II). The stomatal index increased 13.33% in the 1.00 mg L^{-1} lead concentration and this was unaltered in the higher concentrations (Table II).

On the leaf adaxial surface, the stomatal density increased by 19.51% in the 0.50 mg L^{-1} lead concentration, and this was unaltered with higher concentrations (Table II). There were no changes in the number of regular epidermal cells, or in the stomatal polar diameter, in the presence of

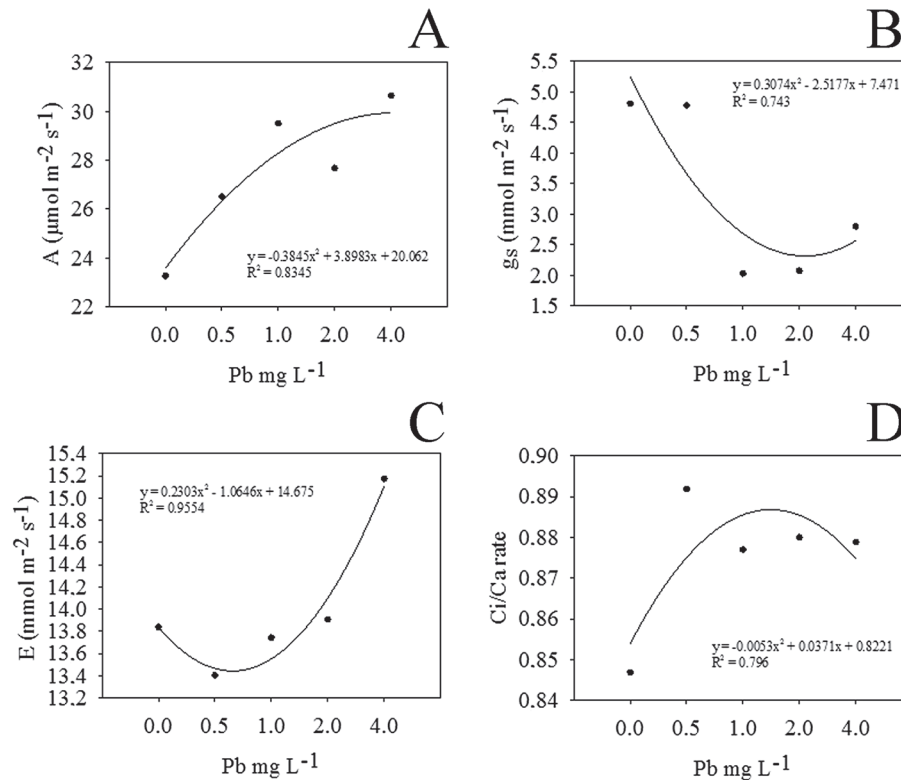


Figure 1 - Gas exchanges characteristics of *Eichhornia crassipes* grown in nutrient solutions under different lead concentrations. A = photosynthesis, B = stomatal conductance, C = transpiratory rate, D = Ci/Ca rate. bars= standard error.

TABLE I
Leaves quantitative anatomical characteristics in cross sections of water hyacinth (*Eichhornia crassipes*) grown under different lead concentrations (mg L⁻¹).

PB	ADE (μm)	ABE (μm)	MP (μm)	PP (μm)	SP (μm)	PP/SP	VBD (μm)	AEP (%)
0.00	09.9 a	12.1a	285.8b	67.61a	236.2a	0.3a	103.7 a	38a
0.50	09.7 a	12.2a	273.7b	66.80a	209.9a	0.3a	082.3 b	26a
1.00	09.3 a	13.2a	325.7a	64.13a	267.2a	0.2a	085.2 b	35a
2.00	09.7 a	12.7a	299.9a	68.16a	214.8a	0.3a	076.4 b	87a
4.00	11.1 a	13.9a	324.6a	69.15a	277.8a	0.2a	069.6 b	32a

Means followed by same letters in columns did not differ by Scott-Knott test at P<0.05.

ADE = thickness of adaxial epidermis, ABE = thickness of abaxial epidermis, MP = thickness of mesophyll, PP = thickness of palysade parenchyma, SP = thickness of spongy parenchyma, PP/SP = palysade/spongy parenchyma proportion, VBD = distance between vascular bundles, AEP = aerenchyma proportion on leaves (area/area).

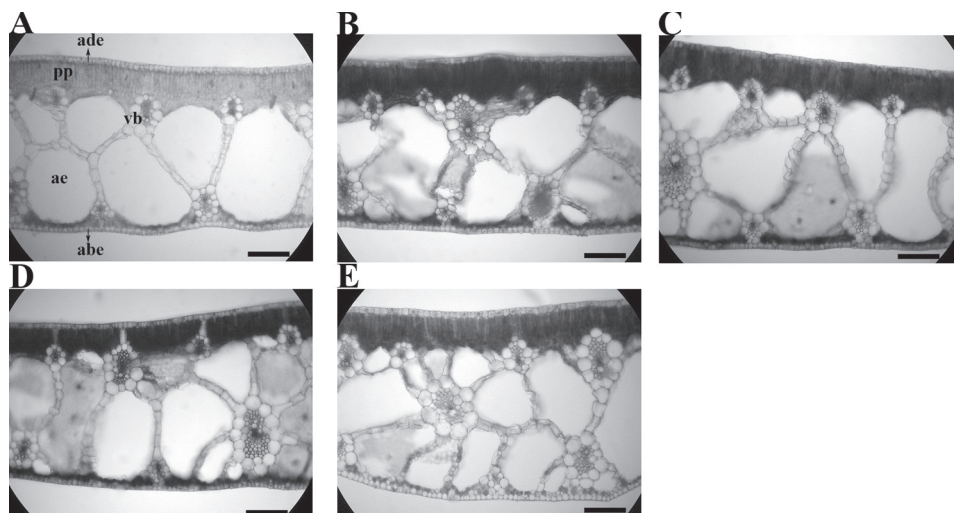


Figure 2 - Leaves anatomical modifications of *Eichhornia crassipes* grown in nutrient solutions under different lead concentrations. ade= adaxial epidermis, abe = abaxial epidermis, pp = palysade parenchyma, ae = aerenchyma chamber, vb = vascular bundle. A = 0.00 mg L⁻¹, B= 0.50 mg L⁻¹, C = 1.00 mg L⁻¹, D = 2.00 mg.L⁻¹, E = 4.00 mg.L⁻¹. bars = 100 µm.

TABLE II
Leaves quantitative anatomical characteristics in paradermal sections of water hyacinth (*Eichhornia crassipes*) grown under different lead concentrations (mg L⁻¹).

Abaxial surface							
Lead	SN	CN	PD (µm)	ED (µm)	SD	SF	SI (%)
0.00	08.0 c	63.0 a	44.38a	25.4 a	102.7 c	1.70 b	13 b
0.50	09.2 b	73.0 a	42.36a	23.4 a	118.2 b	1.80 b	13 b
1.00	10.0 a	66.0 a	46.07a	22.8 a	128.4 a	2.03 a	15 a
2.00	10.0 a	64.4 a	48.34a	23.8 a	128.4 a	2.03 a	16 a
4.00	10.0 a	65.4 a	45.71a	22.2 a	128.4 a	2.06 a	15 a
Adaxial surface							
Lead	SN	CN	PD (µm)	ED (µm)	SD	SF	SI (%)
0.00	8.20 b	65.4 a	44.6 a	26.8 a	105.3 b	1.70 b	13 b
0.50	9.80 a	75.0 a	46.6 a	23.7 b	125.9 a	1.50 b	13 b
1.00	9.60 a	63.4 a	45.0 a	24.6 b	123.3 a	1.80 b	15 a
2.00	9.80 a	65.0 a	45.0 a	21.0 c	125.9 a	2.20 a	15 a
4.00	9.80 a	65.2 a	47.3 a	22.1 c	125.9 a	2.20 a	15 a

Means followed by same letters in columns did not differ by Scott-Knott test at P<0.05.

SN = number of stomata by field; CN = number of regular epidermal cells by field; PD = stomatal polar diameter; ED = stomatal equatorial diameter; SD = stomatal density (stomata by mm²); SF = stomatal functionality (PD/ED rate); SI = stomatal index.

lead (Table II). However, lead promoted reductions on the stomatal equatorial diameter, which was reduced by 8.15% in concentrations of 0.50 and 1.00 mg L⁻¹ and 14.52% in the higher concentrations (Table II). These reductions increased by 40.90% the stomatal functionality in the 2.00 and 4.00 mg L⁻¹ concentrations (Table II). The stomatal index increased by 13.33% in the 1.00 mg L⁻¹ and in concentrations thereafter (Table II).

Root epidermal thickness and proportion of aerenchyma did not exhibit any changes in the presence of lead (Table III). However, the endodermal thickness increased 31.52% in the 1.00 mg L⁻¹ and higher concentrations (Table III), whilst the cortical thickness increased by 52.07% in the 2.0 mg L⁻¹ and higher concentrations (Table III). In 0.50 mg L⁻¹ and higher concentrations the IVC was reduced by 33.91%, this reduction being related to the increase in the number of tracheary elements in the xylem that was observed in all lead concentrations (Table III, Figure 3). The exodermal thickness increased by 24.36% in the 1.00 mg L⁻¹ and higher concentrations, and phloem thickness increased 39.12% (Table III).

The antioxidant system of *E. crassipes* showed some responses related to the presence of lead (Figure 4). The APX activity in leaves and roots was modified only in the 2.00 mg L⁻¹ or higher lead concentrations (Figure 4A). In the roots, the CAT activity did not alter, but in the leaves in all lead concentrations an increased activity was found (Figure 4B). The SOD activity increased in leaves and roots of *E. crassipes* in the 1.00 mg L⁻¹ concentration or higher, but, in roots, a decrease was encountered in the 4.00 mg L⁻¹ concentration.

DISCUSSION

The photosynthetic system response encountered in *E. crassipes* in the presence of lead was different from other species described in literature. According to Pinchasov et al. (2006) lead may promote reductions in the photosynthetic rate. Lead also

reduces the chlorophyll biosynthesis in some plants such as *Brassica rapa* (Cenkci et al. 2010) and *Phaseolus vulgaris* (Hamid et al. 2010). In addition to the damage on chlorophyll biosynthesis, prejudicial effects on photosynthesis can be related to the formation of reactive oxygen species (ROS), which reduce membrane stability in chloroplasts as a consequence of lipid peroxidation (Stoeva and Bineva 2003). Such effects on chloroplast membranes are very common in plants under lead stress (Verma and Dubey 2003). However, *E. crassipes* reduced the effects of lead stress by increasing the antioxidant enzyme activities. This kind of response is essential in lead tolerant plants as described by Verma and Dubey (2003) and Singh et al. (2010). With more active enzymes in the leaves, *E. crassipes* plants were able to cope with lead deleterious effects and maintain the photosynthetic capacity.

E. crassipes plants were not only able to maintain photosynthesis in the presence of lead, but they also increased the photosynthetic rate. This increase in photosynthesis must be related to regulatory factors. The photosynthetic rate is regulated by different factors, but the two most relevant are the radiation and CO₂ (Zhou and Han 2005). Since the radiation was standardized for all treatments in the IRGA chamber at 1000 μmol m⁻² s⁻¹, the main factor that contributed to the increase in photosynthesis was the CO₂ capture capacity of the plants; and the leaf capacity for CO₂ capture is associated with the modifications in leaf anatomical structure, i.e. characteristics such as: stomatal density, index, functionality and the total leaf thickness.

The stomatal density is one of the most important plant characteristics related to CO₂ capture, and in stress conditions such as water stress, the stomatal density can increase its values in the most efficient plants (Grisi et al. 2008). An increase in the stomatal density in the presence of lead was reported in *Plantago major*, but with reduced stomatal conductivity (Kosobrukhov et al. 2004). The *E. crassipes* plants increased not only the

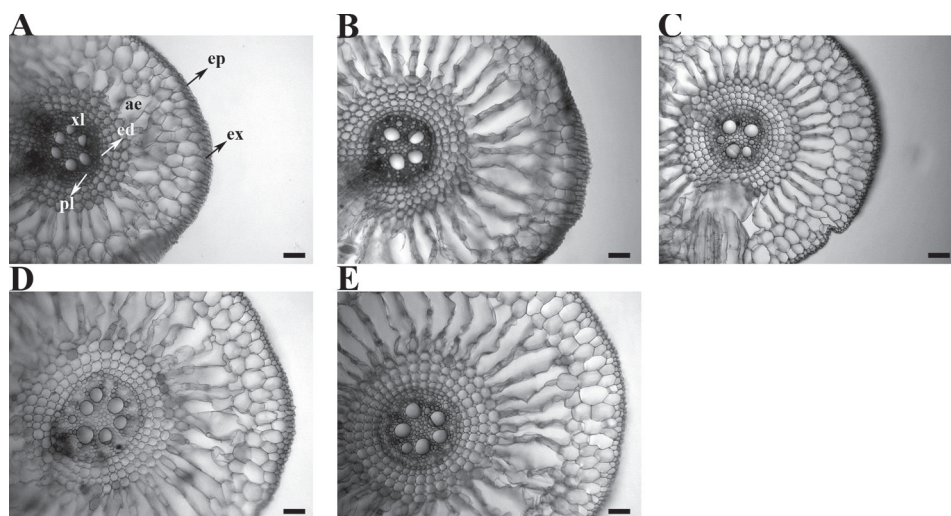


Figure 3 - Root cross sections in *Eichhornia crassipes* grown in nutrient solutions containing different lead concentrations. ep = epidermis, ex = exodermis, er = aerenchyma chamber, ed = endodermis, xl = xylem, pl = phloem. A = 0.00 mg L⁻¹, B = 0.50 mg L⁻¹, C = 1.00 mg L⁻¹, D = 2.00 mg.L⁻¹, E = 4.00 mg.L⁻¹. bars = 100 μm.

TABLE III

Root anatomical characteristics of water hyacinth (*Eichhornia crassipes*) grown under different lead concentrations (mg L⁻¹).

Lead	AEP (%)	EP (μm)	EX (μm)	ED (μm)	IVC	CT (μm)	PL (μm)
0.00	20 a	19.90 a	15.1 b	542.24 b	2.30 a	26.27 b	26.99 b
0.50	19 a	19.95 a	16.8 b	494.23 b	1.68 b	26.62 b	25.23 b
1.00	14 a	18.92 a	17.9 a	555.22 b	1.52 b	31.79 a	30.29 a
2.00	17 a	19.82 a	18.4 a	722.90 a	1.53 b	32.67 a	35.10 a
4.00	18 a	18.89 a	19.8 a	751.56 a	1.59 b	31.71 a	34.85 a

Means followed by same letters in columns did not differ by Scott-Knott test at P<0.05.

AEP = aerenchyma proportion in cortex, EP = thickness of epidermis, EX = thickness of exodermis, ED = thickness of endodermis, IVC = Carlquist vulnerability index (mean tracheary element diameter/number of tracheary elements), CT = thickness of the cortex, PL = thickness of the phloem.

stomatal density but also the stomatal index in the presence of lead. Effectively, our study shows the importance of stomatal characteristics to maintain photosynthetic capacity of lead tolerant plants by increasing the CO₂ capture capacity and thus permit an increase in photosynthesis in the presence of lead.

An increase in mesophyll thickness increases the leaf storage capacity for CO₂. As the aerenchyma proportion in leaves was the same in the presence of lead, with higher leaf thickness, the total aerenchyma was increased. This tissue is directly

related to gas storage in plant organs most likely with the total CO₂ captured by stomata being stored in aerenchyma and slowly utilized by the chlorenchyma in the photosynthetic process. This hypothesis is supported by the increase in the Ci/Ca rate, showing larger proportions of the CO₂ in the *E. crassipes* plants growing under lead influence. According to Zhou and Han (2005) a higher Ci/Ca rate represents a larger amount of CO₂ in the leaves.

Increased stomatal functionality results from more effective stomata. These stomata are more

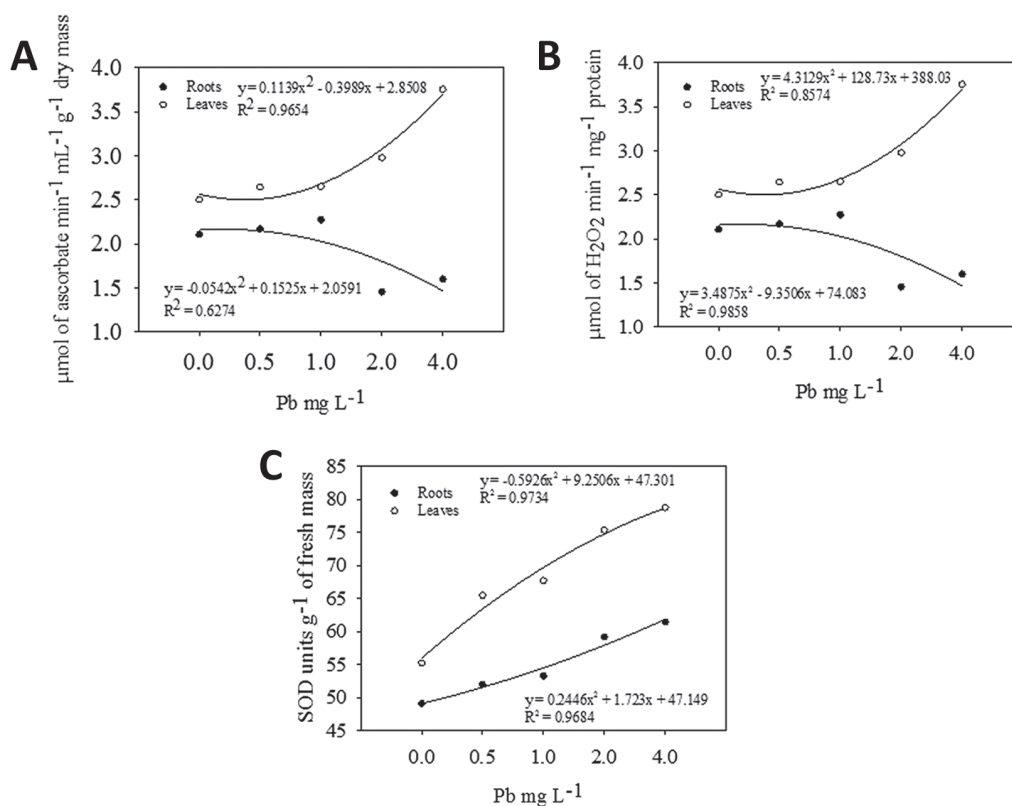


Figure 4 - Antioxidant enzyme activities of *Eichhornia crassipes* grown in nutrient solutions containing different lead concentrations. A = Catalase activity, B = Ascorbate peroxidase activity, C = superoxide dismutase activity. Bars = standard error.

effective for the CO₂ uptake and transpiration control. In our study, *E. crassipes* showed an enhanced capacity to capture and store CO₂, thus increasing the photosynthetic rate. The absence of modifications in the leaf epidermis, palisade and spongy parenchyma in the presence of lead resulted in a good development of the leaf tissues with no evidence of lead toxicity. Smaller distances between the vascular bundles results in increased amounts of vascular tissue and a higher capacity to conduct water and photoassimilates from leaves to the sink organs in plants.

One of the most important anatomical characteristic of the roots of aquatic plants is the proportion of aerenchyma; and the root aerenchyma can increase in stress tolerant plants (Pereira et al. 2008, Souza et al. 2009, 2010). Lead can reduce cell growth in plant roots due to its toxicity (Kozhevnikova et al. 2009), and this can cause deformations of plant tissues and

structures in roots (Xu et al. 2007). As an apoplastic barrier, the epidermis is the first tissue in roots that has to cope with the effects of toxic elements. The absence modifications to the root epidermis in the presence of lead in *E. crassipes* is one of the characteristics related to the tolerance of this species.

In *E. crassipes*, the antioxidant enzymes increased both in the roots and leaves, and this shows the great importance of this system for stress tolerance and the protection of the photosynthetic system. This is a common response in plants growing in the presence of lead, as described for rice by Verma and Dubey (2003) and for wheat, by Liu et al. (2010). But, in the roots of *E. crassipes* this system was only slightly stimulated. The lysigenous aerenchyma is dependent of the production of reactive oxygen species (Seago et al. 2005, Gunawardena 2008), and in water

stress tolerant plants the antioxidant system in the roots can undergo reduction (Pereira et al. 2010). Consequently, a reduced antioxidant enzyme activity in the roots can be related to the maintenance of the aerenchyma proportion.

The capacity for lead hyper-accumulation was reviewed by Schoenhals et al. (2009), and this accumulation is more intense in roots than in the shoots (Gonçalves Júnior et al. 2008). Lead accumulation in roots can be important for plant stress tolerance, because it reduces the effects on the photosynthetic system in the leaves. The endodermis is the most important apoplastic barrier in roots, blocking the translocation of the lead to shoots. In *E. crassipes*, the endodermis thickness increased, thus reducing the lead flux to shoots.

The IVC is related to vascular system efficiency, and a reduction of the IVC increases the water transport in roots, and has been found to increase in stress tolerant plants (Carlquist 1975, Pereira et al. 2008, Souza et al. 2009). *E. crassipes* plants showed a capacity to increase the water and nutrient transport from roots to shoots, and this can be related to stress tolerance. The increase in phloem in the roots under lead effects may also be a stress tolerance mechanism, because it can increase the photoassimilate flux to the roots, leading to higher root growth. Stress tolerance of *E. crassipes* to lead may have similar effects to those reported for arsenic tolerance to this species, as described by Pereira et al. (2011).

Therefore, the water hyacinth can cope with lead stress without damage to its structure or physiology. The presence of lead increased the photosynthetic rate, which was associated with an increase in antioxidant system enzymes and CO₂ capture mechanisms. Leaf structure in *E. crassipes* in the presence of lead increased the CO₂ capture mechanisms and did not show any toxicity stress. Likewise, the roots in *E. crassipes* did not show toxicity stress but rather exhibited favorable characteristics in the presence of lead.

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RESUMO

Este estudo teve como objetivo verificar a tolerância do aguapé ao chumbo e verificar as modificações anatômicas e fisiológicas decorrentes. As plantas de aguapé foram cultivadas em solução nutritiva com cinco diferentes concentrações de chumbo sendo: 0,00; 0,50; 1,00; 2,00 e 4,00 mg L⁻¹ por um período de 20 dias. Foram avaliadas a fotossíntese, transpiração, condutância estomática e a razão Ci/Ca aos 15 dias decorridos do início do experimento. Ao final do experimento, as modificações anatômicas nas raízes e folhas além da atividade das enzimas do sistema antioxidante foi avaliada. A fotossíntese e a razão Ci/Ca aumentaram em todos os tratamentos com chumbo. A análise da anatomia foliar não demonstrou nenhuma evidência de efeitos tóxicos mas demonstrou modificações nos estômatos e na espessura do parênquima esponjoso na presença de chumbo. De forma semelhante, a anatomia das raízes não demonstrou nenhum efeito tóxico mas o xilema e floema, bem como as barreiras apoplásticas demonstraram modificações favoráveis. Todas as enzimas do sistema antioxidante demonstraram aumento na atividade nas folhas e algumas modificações nas raízes em função da presença de chumbo. Portanto, a tolerância ao chumbo do aguapé está relacionada com modificações anatômicas e fisiológicas como o aumento da fotossíntese e modificações anatômica que aumentam a capacidade de captação de CO₂ e a condutividade hidráulica.

Palavras-chave: metais pesados, ecofisiologia, anatomia vegetal, macrófitas.

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