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RESEARCH ARTICLE

Leaf morphoanatomy of species tolerant to excess iron and evaluation of their phytoextraction potential

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Abstract *Setaria parviflora* (Poir.) Kerguélen and *Paspalum urvillei* Steudel are grasses that grow naturally in a soil with high iron contents. This study aimed to characterize morphoanatomically and histochemically the iron phytotoxicity on leaves and evaluate the phytoextraction potential of these grasses. Saplings were cultivated in hydroponic solution with and without excess Fe-EDTA. Regarding measurements taken on leaves, reduction was observed among treatments of Fe-EDTA on height values of abaxial epidermis and bundle sheath in both species. As for iron histolocalization, stronger reaction was observed in leaves of *S. parviflora*, in comparison with *P. urvillei*. Anatomical damage, such as protoplast retraction, irregular xylem, changes in cell volume, and cell collapse, and visual symptoms, like leaf bronzing, chlorosis, and necrosis, were similar in both species when exposed to excess iron; however, *P. urvillei* showed more severe damage. This species accumulated more iron in shoots than *S. parviflora* and therefore is more favorable for use in phytoextraction. The root system of both species accumulated higher iron concentrations in relation to shoots.

Keywords Histochemistry · Leaf micromorphometry · *Paspalum urvillei* · Phytoremediation · Poaceae · *Setaria parviflora*

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Introduction

Although iron is a vital micronutrient for plant growth and development (Connolly et al. 2003; Connolly and Guerinot 2002), its high content in the substrate can affect cellular homeostasis and cause toxic effects (Williams et al. 2000; Ducic and Polle 2005) like oxidative stress in cells, which may result in tissue damage (Becana et al. 1998; Tsai and Huang 2006).

Most iron ore is extracted in opencast mines, carried to ports by rail and then shipped to steel plants around the world. Currently, steel use has become indispensable, which is demonstrated by the ever-increasing demand for this alloy and consequently for iron ore production (Yellishetty et al. 2010).

Brazil is the world's second biggest producer of iron ore (IBRAM 2011). With the high Brazilian exportation demand, the activity of iron ore mining and beneficiation factories has increased, which augmented the emission of pollutants to the environment (Kuki et al. 2009). Among the main atmospheric pollutants produced are sulfur dioxide (SO_2) and iron ore particulate matter. The latter is insoluble, therefore being unavailable to plants. However, SO_2 contributes to decreasing soil pH and consequently to increasing iron availability to vegetation, which may cause ecological problems and even loss of biodiversity (Kuki et al. 2008).

Setaria parviflora and *Paspalum urvillei* are two perennial grasses native to South America, but that occur widely in warm regions of the world (Scheffer-Basso et al. 2002; Wehtje et al. 2008; Verloove and Sánchez Gullón 2008). These species develop in a decantation pond of a pelletizing industry at Ubu city, state of Espírito Santo, Brazil. This pond is supplied with water from the mining system and plumbing of the iron ore pelletizing industry, therefore being rich in iron particles. *S. parviflora* and *P. urvillei* tolerate high amounts of this pollutant in the substrate without altering their growth (Araújo 2012).

Some circumstances may propitiate the installation of a great metal availability in the soil. High levels of soil

contamination may lead to a scenario of toxicity to plants. Such levels, however, as well as the presence of iron toxicity symptoms in plants, depend on some factors, such as the amount of soluble Fe^{+2} in the soil, the species analyzed, soil type, and soil pH (Becker and Asch 2005).

Some plants can tolerate high levels of metals and accumulate such metals in specific organs. These plants have received attention because of the possibility of their use in the remediation of contaminated environments (Pilon-Smits 2005; Coutinho and Barbosa 2007; Rascio and Navari-Izzo 2011). Phytoextraction is among the bioremediation techniques currently described and refers to the uptake and translocation of metal contaminants in the soil by plant roots to above-ground components of the plant (Padmavathiamma and Li 2007).

Hyperaccumulator plants accumulate unusually high tissue concentrations of an element or its ions (Brooks et al. 1977) and have efficient metal compartmentalization. This allows the accumulation of high concentrations of heavy metals in their tissues without the production of toxic effects. In leaves, this compartmentalization is crucial since it takes place where the photosynthetic apparatus is located (Rascio and Navari-Izzo 2011). Some species can accumulate metal excess in trichomes, cuticle or epidermal common cells, which avoids larger damage to photosynthetic machinery (Küpper et al. 2000; Freeman et al. 2006; Robinson et al. 2003).

A typical visual symptom of iron toxicity is leaf bronzing, which begins in completely expanded leaves and is caused by polyphenol oxides accumulation (Becker and Asch 2005). An essential characteristic of hyperaccumulator plants, however, is the absence of visible toxicity symptoms (Verbruggen et al. 2009). Nevertheless, some species can accumulate high concentrations of pollutants in their tissues with damages not yet macroscopically visible. For this reason, microscopic analyses are of great importance, since they may show structural damage and assist in injury prognosis (Silva et al. 2000; Sant'Anna-Santos et al. 2006a, 2007; Sant'Anna-Santos and Azevedo 2007).

Microscopic analyses can be either quantitative or qualitative. Quantitative analysis (micromorphometry) contributes to the assessment, in numerical terms, of changes in the structure of plant organs caused by external agents (Aguiar et al. 2007). These analyses are usually carried out on the leaf, which is the most sensitive organ to pollution (Dickison 2000).

Depending on the iron concentration in the medium and on the sensitivity of the species studied, some qualitative anatomical alterations may be observed. There can be changes on cell division pattern of root apical meristem, alterations on cortex cells morphology, protoplast retraction, and formation of a cicatrization meristem. In the vascular cylinder, changes in the organization and shape of pericycle cells, and incomplete differentiation of metaxylem have been observed by Siqueira-Silva et al. (2011) studying *Ipomoea pes-caprae* and *Canavalia rosea* subjected to excess iron.

The hypotheses tested were as follows: 1) anatomical analyses show the real extension of the damage caused by excess iron in leaves of *P. urvillei* and *S. parviflora*, on a microscopic level; and 2) these species accumulate high levels of this metal in their tissues, favoring their use in phytoremediation.

Considering the presence of *S. parviflora* and *P. urvillei* in an environment with high iron levels, this study aimed to evaluate the phytotoxicity caused by excess iron through macroscopic and microscopic analyses on the leaf, localize the presence of iron in leaf tissues, and evaluate the phytoextraction potential of these species by examining iron concentration in their roots and shoots.

Material and methods

Cultivation conditions and treatment application

The experiment was carried out in a greenhouse at Universidade Federal de Viçosa (UFV), in Viçosa city, state of Minas Gerais, Brazil (649 m high, 20°45′20″S and 42°52′40″N). Individuals of *S. parviflora* (Poir.) Kerguélen and *P. urvillei* Steudel (Poaceae) were collected at the decantation pond margins of an iron ore pelletizing industry located in Ubu city, state of Espírito Santo (20°46′21, 0″S and 40°34′52, 3″W), Brazil. These individuals were taken to Viçosa city and kept in a greenhouse for plant multiplication. The species identity was confirmed by specialist Hilda Maria Longhi Wagner and specimens were deposited in Herbarium VIC with numbers 36801 and 36802 for *P. urvillei* and *S. parviflora*, respectively.

Initially, plants were cultivated in 8-L plastic pots containing conventional substrate (soil/sand/humus) for approximately 90 days. Then, clumps of each species were carefully extracted from the substrate, using running water to separate the roots from it. After a visual standardization based on clump volume, a pruning was carried out on roots and at culm height, and the clumps were transferred to 5-L polystyrene boxes. Clumps were kept in Hoagland nutrient solution (Hoagland and Arnon 1950), at half ionic strength, with constant aeration, at pH 5.0, which was adjusted every 2 days using NaOH (1N) and HCl (1N). The hydroponic solution was composed of 0.5 mM $NH_4H_2PO_4$, 3 mM KNO_3, 2 mM $Ca(NO_3)_2.4H_2O$, 1 mM $MgSO_4.7H_2O$, 23.13 μM H_3BO_3, 4.57 μM $MnCl_2.4H_2O$, 0.382 μM $ZnSO_4.7H_2O$, 0.16 μM $CuSO_4.5H_2O$, 0.0695 μM MoO_3, and 9 μM Fe-EDTA. This solution was renewed weekly, and its volume was completed with deionized water whenever necessary. During 40 days, plants were pruned twice at culm height for homogenization, before application of treatments.

Plants were gradually submitted to different Fe^{2+} concentrations in nutrient solution in the form of Fe-EDTA (0.009; 1, 2, 4, and 7 mM). During this period, the solution was renewed

every 12 h, with an increment of 0.25 mM Fe-EDTA until reaching 1 mM Fe-EDTA. After that, the increment was 2, 4, and 7 mM Fe-EDTA, until reaching the final iron concentrations.

After reaching the final concentrations, the nutrient solution was renewed every 3 days, and pH was adjusted on a daily basis to 5.0, as previously described. Plants remained exposed to treatments during 14 days for anatomical and visual analyses, and during 18 days for iron quantification in dry matter and in hydroponic solution.

Iron quantification in dry matter of shoots and roots, and in nutrient solution

Plants were separated in shoots and roots, the latter being washed with dithionite–citrate–bicarbonate to eliminate the iron adhered to root surface (Taylor and Crowder 1983). This material was placed in a drying oven at 75 °C until constant dry weight, ground in grinder (model TE048, Tecnal Marconi, Piracicaba, São Paulo, Brazil), and sifted into dimensions smaller than 1 mm. Iron content was determined according to the methodology described by Malavolta et al. (1989), in which sample extracts were obtained by nitroperchloric digestion and determined by atomic absorption. Iron contents in the Hoagland solution freshly prepared for all treatments of Fe-EDTA were determined by atomic absorption (model FS Spectra 220, Varian, Australia).

Translocation factor and bioconcentration factor

After quantification of iron accumulated in shoots and roots, translocation factor (TF) and bioconcentration factor (BCF) were calculated according to the methodology proposed by Bao et al. (2009). TF is defined as the ratio between iron concentration in shoots and iron concentration in roots. BCF is the ratio between iron concentration in the plant and iron concentration in the nutrient solution.

Visual characterization

After 14 days of experiment, visual symptoms in leaves of *S. parviflora* and *P. urvillei* caused by excess iron were photographed with a digital camera (model Cyber-Shot DSC-W310, Sony Corporation, Japan).

Structural characterization and micromorphometric analysis in light microscopy

Samples were collected from the margin and midrib of five fully expanded leaf blades in regions with symptoms for structural characterization and without symptoms for micromorphometric analysis, located on the third to the fifth nodes, of all individuals ($n=5$). They were fixed in glutaraldehyde (2.5 %) and paraformaldehyde (4 %) in a sodium phosphate buffer 0.1 M (pH 7.0;

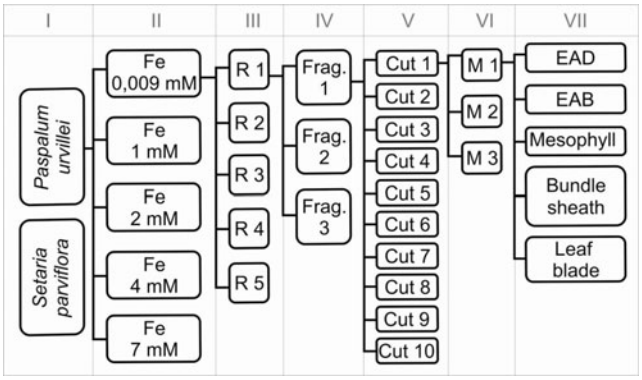

Fig. 1 Sampling scheme adopted in the study of the influence of five iron concentrations on leaf micromorphometry of *Setaria parviflora* and *Paspalum urvillei*. The figure exemplifies the sampling performed on the first level of each column. *I* species, *II* treatments, *III* plant repetitions, *IV* leaf fragments, *V* cuts, *VI* measurements, *VII* parameters evaluated. *Fe* iron concentration in hydroponic solution, *R* plant repetition, *Frag.* leaf fragment embedded in historesin, *M* measurements, *EAD* epidermis of leaf adaxial surface, *EAB* epidermis of leaf abaxial surface

Karnovsky 1965) for 48 h and stored in 70 % alcohol. Samples were then dehydrated in an ethyl series and embedded in glycol methacrylate (Leica Historesin, Nussloch/Heidelberg, Germany). Transverse sections of leaves (5 μm thick) were made using an automatic rotary microtome (model RM2155 Leica Microsystems Inc., Deerfield, IL, USA), stained with toluidine blue pH 4.0 and mounted between microscopy glass slides and coverslips in Permount.

Height of epidermal cells from both adaxial and abaxial leaf surfaces, leaf blade thickness, and mesophyll and vascular bundle sheath height were measured in cross sections. For each repetition, in all treatments, three leaf fragments were used, ten cuts being obtained out of each fragment, and one image being captured in each cut. In each image, three measurements were performed for each parameter, totalizing 450 images per treatment per parameter in each species (Fig. 1). Images were obtained in fields on the histological cuts that allowed visualization of three groups of bulliform cells and three vascular bundles. All measurements were performed close to the vascular bundles. In the epidermis of adaxial surface, the bulliform cell measured was the biggest one above the bundles. Measurements were performed using image analysis software Anati Quanti version 2.0 for Windows® (Aguiar et al. 2007).

Histochemical analysis

In order to detect iron presence in leaf tissues on Karnovsky fixed samples, cross sections were performed on leaf blades of plants from treatments 0.009 and 7 mM Fe-EDTA. Sections were obtained in a table microtome (model LPC, Rolemberg e Bhering Comércio e Importação LTDA, Belo Horizonte, Minas Gerais, Brazil) and rinsed with distilled water. Then,

they were submitted to Perls' method (Stevens and Chalk 1996) adapted by Silva et al. (2006), being exposed to a solution containing 4 % potassium ferricyanide and 4 % hydrochloric acid for 48 h. This test shows the presence of iron by a blue staining called Prussian Blue. A negative control, in which cuts were not exposed to the reaction, was conducted in parallel. After distilled water rinsing, sections were mounted in water.

Detection of phenolic compounds was performed on leaves without visual symptoms, of plants from treatments 0.009 and 7 mM Fe-EDTA. Samples were fixed in a solution of formalin with ferrous sulfate (4 % formaldehyde and 10 % ferrous sulfate) for 24 h under vacuum. After distilled water rinsing, the samples were dehydrated in ethanol/buthyl series (Johansen 1940) and embedded in histological paraffin. Samples were then cut on a rotary microtome (8 μm thick), deparaffinized, and microscopic glass slides were mounted in Permount. The control test was performed on samples previously stored in methanol for 72 h under vacuum in order to remove the phenolic compounds.

All cited analyses were registered in a photomicroscope (model AX70RF, Olympus Optical, Tokyo, Japan) equipped with an U-photo system and coupled to a camera (model AxioCam, Carl Zeiss, Jena, Germany), and image acquisition was performed using software AxioVision LE (Carl Zeiss) and a microcomputer at the Laboratory of Plant Anatomy of UFV.

Statistical analysis

The experimental design adopted was randomized block, with five treatments and five repetitions per treatment on the micromorphometry, and four repetitions per treatment on the other analyses. Data were submitted to analysis of variance and means were compared by Tukey's test at 5 % probability by statistical software SAEG 9.0 UFV (Euclydes 2004).

Results

Iron quantification in dry matter of shoots and roots, and in nutrient solution

Iron concentration in dry mass of shoots and roots from both species significantly increased in relation to metal availability in the nutrient solution (Fig. 2). Iron content in *S. parviflora* in relation to the freshly prepared nutrient solution was c.a. 3 times greater in shoots and c.a. 25 times greater in roots when exposed to the last treatment (7 mM Fe-EDTA). In *P. urvillei*, at the same concentration, shoots accumulated over 4 times and roots c.a. 24 times more iron, in relation to the iron available in the freshly prepared nutrient solution, confirming that most of the metal was retained in the roots (Fig. 2).

The root system of both species accumulated higher iron concentrations in relation to shoots. At 7 mM Fe-EDTA, *P.*

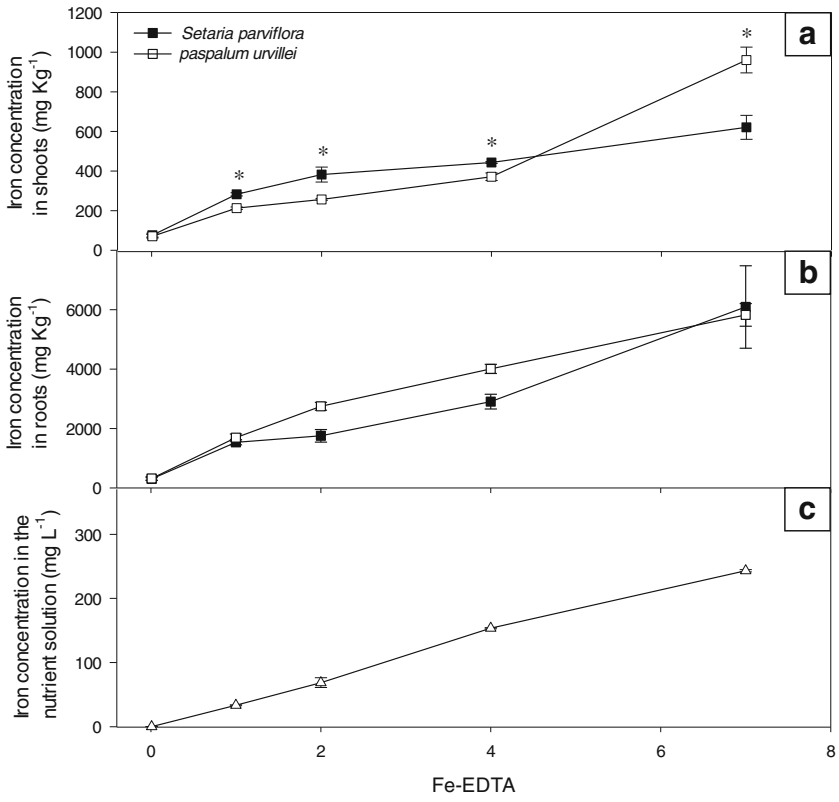

Fig. 2 Iron quantification in dry matter of shoots (*a*) and roots (*b*) of *Setaria parviflora* and *Paspalum urvillei* after 18 days of exposure to excess iron, and in the nutrient solution (*c*). *Asterisks* significant differences between species at different Fe-EDTA treatments

urvillei retained c.a. six times more iron in roots when compared to shoots and *S. parviflora* c.a. ten times more iron in roots than in shoots. Furthermore, *P. urvillei* accumulated higher iron concentrations in the root system at all treatments in relation to *S. parviflora*, except at 7 mM Fe-EDTA, in which the latter accumulated 266.25 mg/kg more. The opposite occurred in shoots of the two species: *S. parviflora* retained greater iron concentrations at treatments 0.009, 1, 2, and 4 mM Fe-EDTA, while *P. urvillei* presented about 339.5 mg/kg more iron than *S. parviflora* at 7 mM Fe-EDTA (Fig. 2).

TF and BCF

The two studied species showed TF with significant difference among treatments and between species, but not in the interaction between these two factors (Table 1). Both species showed highest TF values at the control treatment.

In shoots, the bioconcentration factor showed significance among treatments, between species and in the interaction between them. *S. parviflora* bioconcentrated approximately three times more iron at treatment with 1 mM Fe-EDTA than at 7 mM Fe-EDTA concentration. *P. urvillei* showed the lowest BCF value at 4 mM Fe-EDTA concentration, which was c.a. two times lower than at treatment with 1 mM Fe-EDTA (Table 1).

Table 1 Iron translocation factor (TF) and bioconcentration factor (BCF) in *Setaria parviflora* and *Paspalum urvillei* after 18 days of treatment with different Fe-EDTA concentrations in the nutrient solution

Species	Fe-EDTA (mM)	TF	BCF (L kg^{-1})	
			Shoots	Roots
Setaria parviflora	0.009	0.25		
	1	0.18	6.16 Aa	36.78
	2	0.22	4.45 Aa	21.10
	4	0.15	2.39 Ab	16.94
	7	0.10	2.32 Bb	24.69
Paspalum urvillei	0.009	0.22		
	1	0.12	4.22 Ba	41.05
	2	0.09	2.70 Ab	35.34
	4	0.09	1.96 Ac	24.03
	7	0.17	3.80 Aa	23.49
ANOVA				
Fe-EDTA (Fe)		*	*	*
Species (Sp)		*	*	*
Fe X Sp		n.s.	*	n.s.
Block		n.s.	n.s.	n.s.

Averages followed by the same letter do not differ by Tukey's test at 5 % probability. Upper case letters compare species at the same Fe-EDTA concentration, when there is interaction between iron concentrations and species; lower case letters compare Fe-EDTA treatments in the species

n.s. nonsignificant

In roots, *P. urvillei* showed higher BCF values comparatively with *S. parviflora*. The highest BCF values were found at treatment with 1 mM Fe-EDTA (Table 1).

Visual characterization

Control plants of the two studied species exhibited no symptoms of iron toxicity (Fig. 3a, f). After exposure to excess iron, changes were observed on the external morphology of leaves from both *P. urvillei* and *S. parviflora* (Fig. 3b–e, g–j).

In *P. urvillei*, symptoms of iron toxicity began to appear at 1 mM Fe-EDTA concentration, on completely expanded leaves (Fig. 3b). Punctual bronzing reaching small extensions of the leaf blade was observed in few leaves (Fig. 3b–e). Leaf bronzing evolved according to the increase in iron concentration. In *P. urvillei*, at 2 mM Fe-EDTA, bronzing occurred in larger portions of the leaf blade (Fig. 3c); at 4 mM Fe-EDTA, it reached a larger number of leaves and a larger length of each leaf blade, occupying almost the entire leaf margin, which was curled (Fig. 3d); at 7 mM Fe-EDTA concentration, bronzing was observed in almost all leaves and largely expanded throughout the leaf blade (Fig. 3e). Leaves from the last treatment showed less rigid aspect, besides necrosis, wrinkled appearance at apical region, and curled appearance at leaf margin close to bronzing (Fig. 3e).

S. parviflora, at 1 mM Fe-EDTA, showed no symptoms of iron toxicity (Fig. 3g). Starting at 2 mM Fe-EDTA, leaf bronzing was observed (Fig. 3h). At 4 mM Fe-EDTA, this symptom reached a higher number of leaves and a greater extension of the leaf blade (Fig. 3i); at 7 mM Fe-EDTA concentration, bronzing was followed by small necrosis and chlorosis at leaf blade margin (Fig. 3j). Symptoms appeared more discreetly in *S. parviflora* in relation to the ones observed in *P. urvillei*. Leaf wilting was not observed in neither studied species.

Structural characterization in light microscopy

Leaves of *P. urvillei* and *S. parviflora* present a uniseriate epidermis with bulliform cells grouped in numbers of 3–5 at the adaxial surface. Both species present Kranz anatomy, with vascular bundles surrounded by a parenchymatous bundle sheath and mesophyll radially disposed, similar to a crown. In the major vascular bundles, there is a fiber cap above xylem and below phloem (Figs. 4a, b and 5a, b). In *P. urvillei*, stomata occur both on abaxial and adaxial leaf surfaces, and bulliform cells are bulky with quadrangular contour (Fig. 4b). In *S. parviflora*, stomata and unicellular trichomes are found on both leaf surfaces, and bulliform cells are oval (Fig. 5b).

After exposure to excess iron, *P. urvillei* presented, at concentration 1 mM Fe-EDTA, derangement of mesophyll cells, presence of hypertrophied cells, and hyperplasia of the

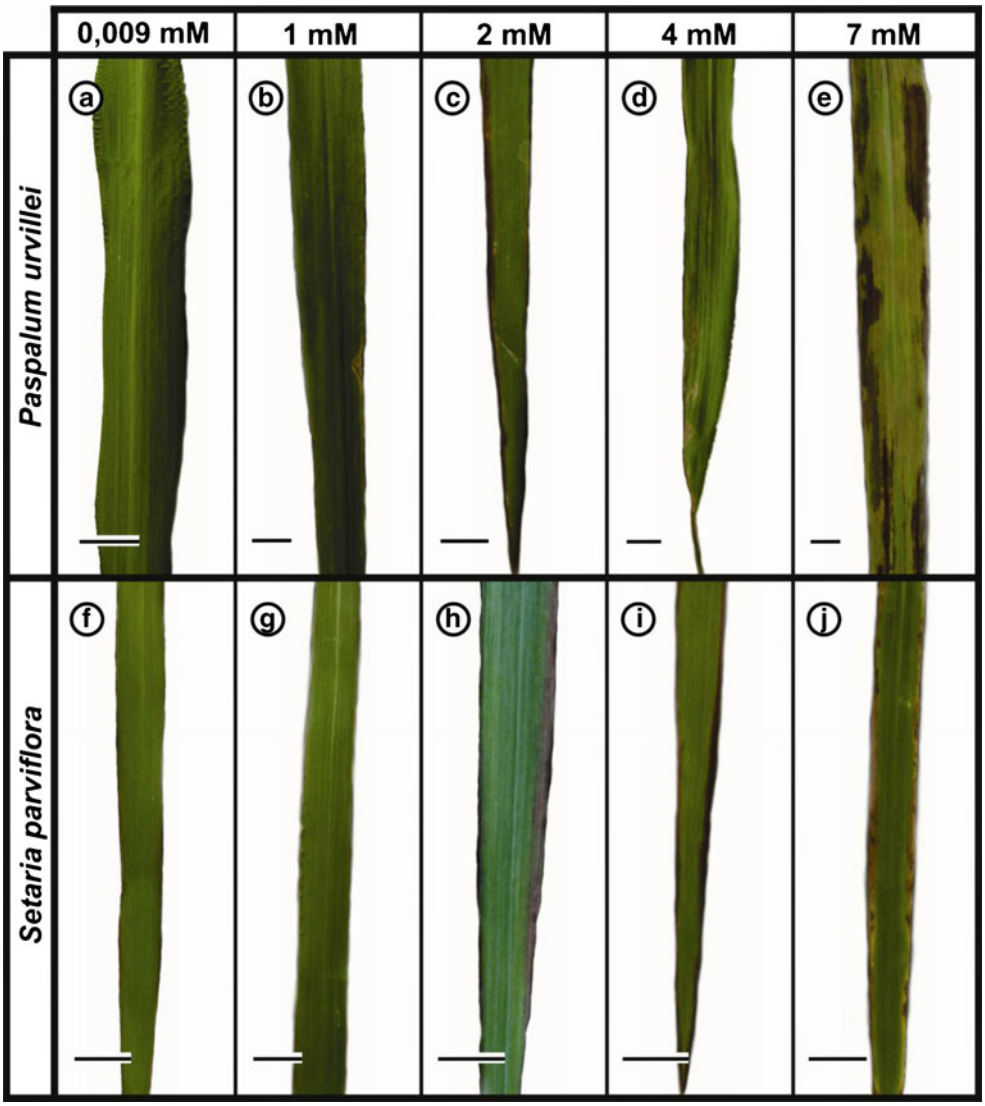

Fig. 3 Leaves of *Paspalum urvillei* (*a*–*e*) and *Setaria parviflora* (*f*–*j*), subjected to 1 mM (*b*, *g*), 2 mM (*c*, *h*), 4 mM (*d*, *i*), and 7 mM (*e*, *j*) Fe-EDTA. *a*, *f* Control treatment, subjected to 0.009 mM Fe-EDTA. *Bars* 10 mm

mesophyll cells located beneath bulliform cells (Fig. 4c). This species, at 2 mM Fe-EDTA concentration, showed protoplast retraction; abrupt alteration on the shape of epidermal, mesophyll, and bundle sheath cells; and cell collapse (Fig. 4d, e). At 4 mM Fe-EDTA concentration, protoplast retraction was observed (Fig. 4f). At 7 mM Fe-EDTA treatment, necrotic areas, alteration on wall shape and differentiation of metaxylem elements, decreased volume of bulliform cells, and disorganization and collapse of mesophyll cells could be visualized (Fig. 4g, h).

S. *parviflora* showed no symptoms of iron toxicity at 1 mM Fe-EDTA concentration (Fig. 5c). At 2 mM Fe-EDTA, alteration on the shape of metaxylem cells was observed (Fig. 5d). At 4 mM Fe-EDTA treatment, apparent reduction on leaf blade thickness due to cell collapse, irregular xylem, and protoplast retraction were observed, as well as changes in the shape of mesophyll cells and epidermal cells from both abaxial and adaxial leaf surfaces (Fig. 5e, f). At 7 mM Fe-EDTA concentration, greenish and brownish content could be observed in mesophyll cells on the toluidine blue-stained leaf sections. Changes in cell shape were also verified at this concentration (Fig. 5g, h).

Micromorphometric analysis

Regarding measurements carried out on leaves, significant difference was observed between species in height of epidermis of both adaxial and abaxial leaf surfaces, mesophyll height, bundle sheath height and leaf blade thickness. *P*. *urvillei* presented higher values for height of epidermis of adaxial surface, mesophyll and bundle sheath, as well as for leaf blade thickness (Table 2).

Significant reduction was observed among treatments of Fe-EDTA on height values of epidermis of abaxial surface and bundle sheath, in the two species. For both parameters, tissue thickness was statistically equal at the

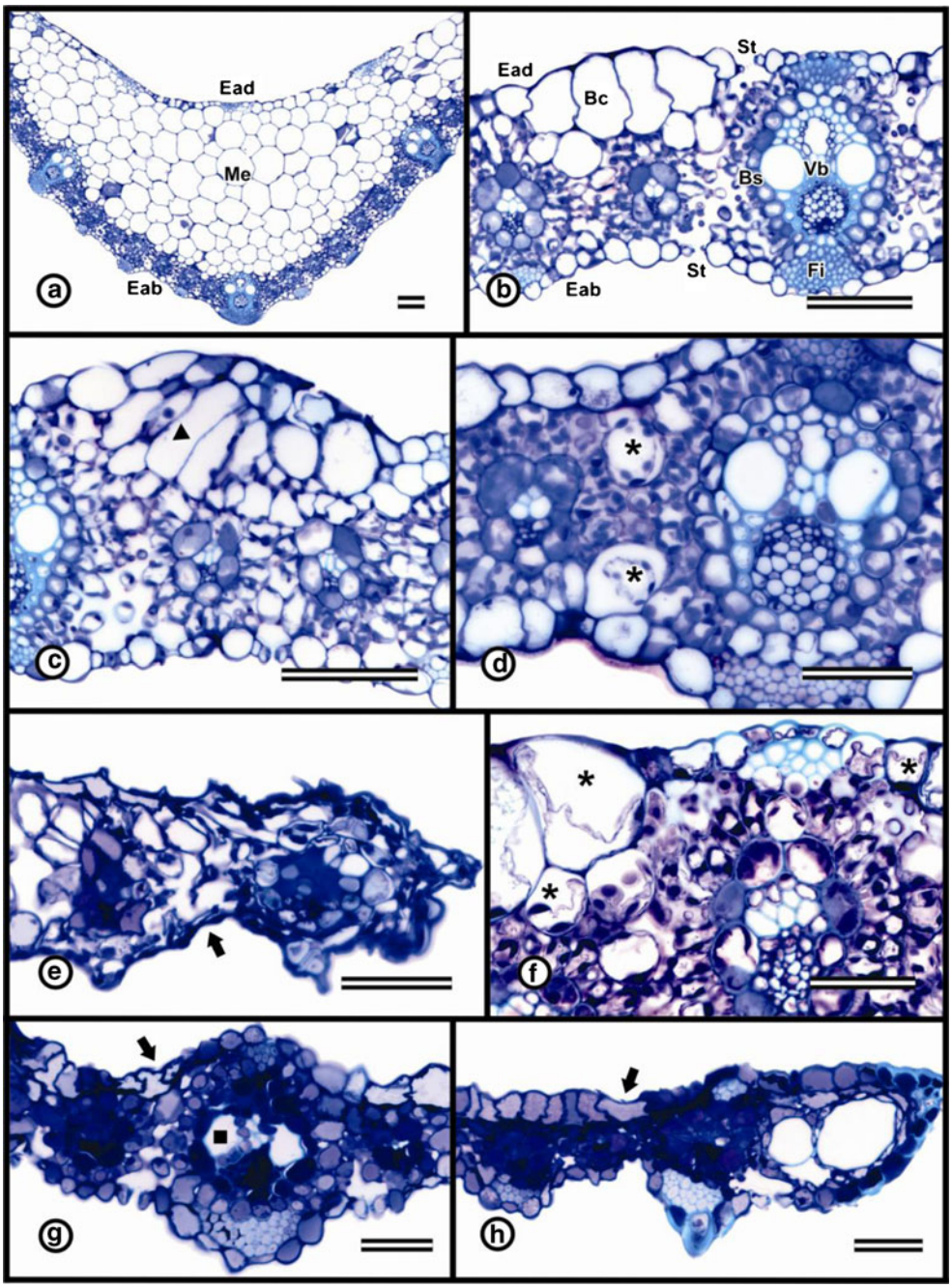

Fig. 4 Leaves of *Paspalum urvillei* under different iron concentrations in transversal sections. *a*, *b* Control treatment—0.009 mM Fe-EDTA; *c* 1 mM Fe-EDTA; *d*, *e* 2 mM Fe-EDTA; *f* 4 mM Fe-EDTA; *g*, *h* 7 mM Fe-EDTA. *Ead* epidermis of adaxial surface, *Eab* epidermis of abaxial surface, *Vb* vascular bundle, *Me* mesophyll, *Bc* bulliform cell, *Fi* fibers, *St* stomata, *Bs* bundle sheath. *Asterisk* protoplast retraction, *arrow* alteration in cell shape, *triangle* hyperplasia, *square* alteration in cell shape of a vessel element. *Bars*—*a*, *b*, *c* (100 μm); *d*, *e*, *f*, *g*, *h* (50 μm)

control treatment (0.009 mM) and at treatment with 1 mM Fe-EDTA, and significant reduction on such thickness took place at 2, 4, and 7 mM Fe-EDTA treatments, among which no statistical difference could be observed (Table 2).

Histochemical analysis

At control treatment, in leaves of both *P. urvillei* and *S. parviflora*, a weak reaction for iron (low intensity blue color) was observed (Fig. 6a, b). In the two species, iron could be histolocalized in epidermal cells of both adaxial and abaxial leaf surfaces, as well as in trichomes in *S. parviflora* (Fig. 6b). At 7 mM Fe-EDTA concentration, after 24 h of reaction, *P. urvillei* leaves showed positive staining only in bundle sheath cells (Fig. 6c), whereas in *S. parviflora* leaves, such staining could be seen in mesophyll cells, bundle sheath cells, and stomata (Fig. 6d). After 48 h of exposure to the reagent, the two species showed a positive reaction in epidermal common cells of both adaxial and abaxial leaf surfaces, in stomata, trichomes (only in *S. parviflora*), bulliform cells and in mesophyll, bundle sheath, xylem, and phloem cells (Fig. 6e, f). In

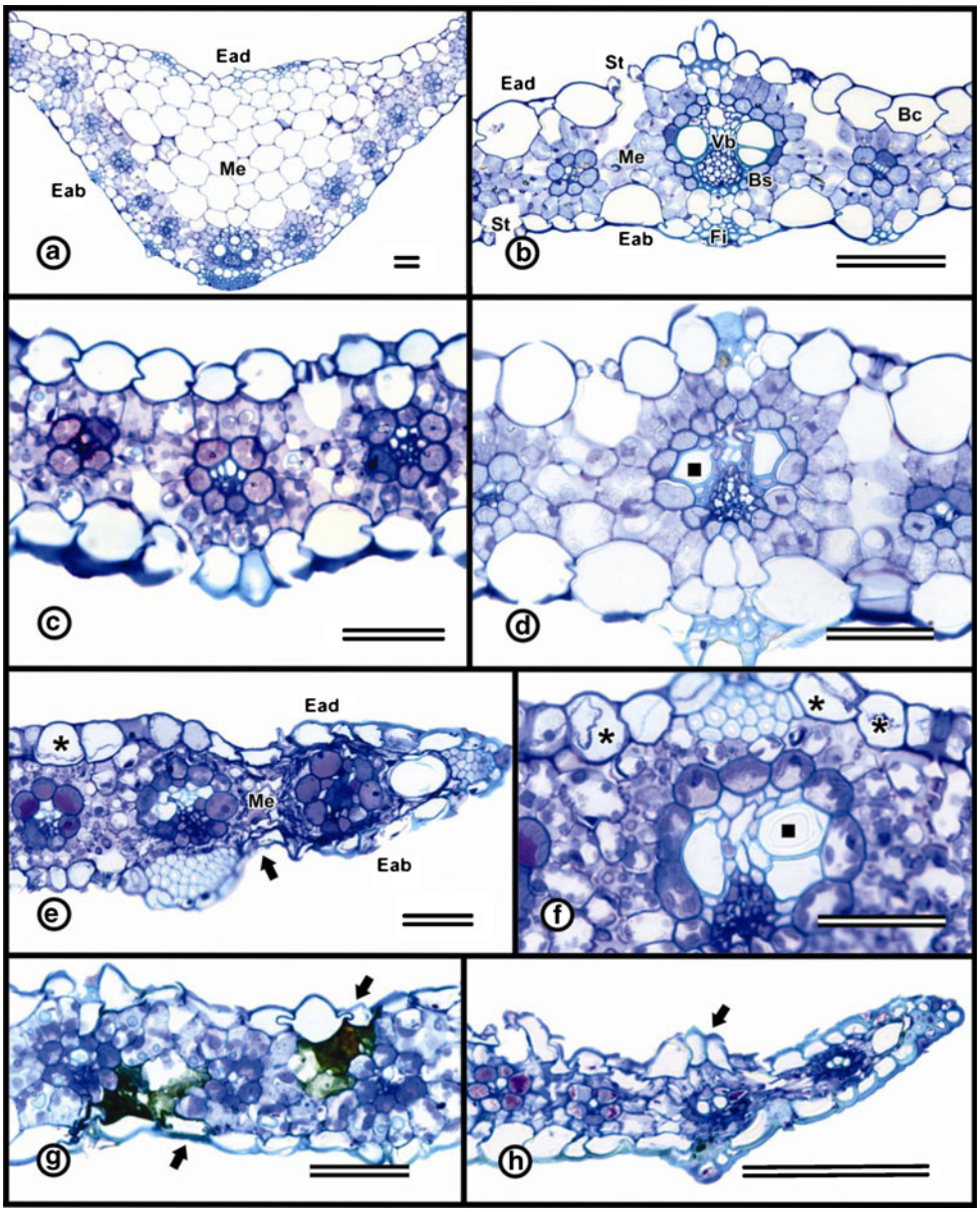

Fig. 5 Leaves of *Setaria parviflora* under different iron concentrations in transversal sections. *a*, *b* Control treatment—0.009 mM Fe-EDTA; *c* 1 mM Fe-EDTA; *d* 2 mM Fe-EDTA; *e*, *f* 4 mM Fe-EDTA; *g*, *h* 7 mM Fe-EDTA. *Ead* epidermis of adaxial surface, *Eab* epidermis of abaxial surface, *Vb* vascular bundle, *Me* mesophyll, *Bc* bulliform cell, *Fi* fibers, *St* stomata, *Bs* bundle sheath. *Arrow* alteration in cell morphology, *square* alteration in cell shape of a vessel element. *Bars*—*a*, *b*, *h* (100 μm); *c*, *d*, *e*, *f*, *g* (50 μm)

S. parviflora leaves, a reaction stronger (high intensity blue color) than in *P. urvillei* leaves was observed.

The presence of phenolic compounds could not be detected by means of histochemical test in leaf tissues of plants from 0.009 and 7 mM Fe-EDTA treatments in either species.

Discussion

S. parviflora and *P. urvillei* accumulated, at 7 mM Fe-EDTA treatment, high iron levels in both shoots and roots. According to Vose et al. (2000), some features are essential for species so that they can be used in phytoremediation processes. These features include tolerating high-pollutant concentrations in the medium, retaining the pollutant in the root system or translocating it to shoots, high growth rate and biomass production, easy propagation, and natural occurrence in polluted areas. Araújo (2012), studying the same species at the same concentrations of Fe-EDTA, stated that there was no reduction in plant growth after 18 days of experiment, which is the time period necessary for both species to complete their life cycle. These results favor the use of the two grasses in phytoremediation, since plants capable of accumulating and tolerating high heavy metal concentrations can be used in such process (Raskin et al. 1997; Porebska and Ostrowska 1999; Dahmani-Muller et al. 2000; Ogundiran and Osibanjo 2008). However, longer duration experiments, as well as experiments with soil as substrate, are necessary to better elucidate the species behavior in iron-rich environments.

Table 2 Leaf micromorphometric analysis (μm) in *Setaria parviflora* and *Paspalum urvillei* after 14 days of treatment with different Fe-EDTA concentrations in the nutrient solution

Species	Fe-EDTA (mM)	Epidermis of adaxial surface	Epidermis of abaxial surface	Mesophyll	Vascular bundle sheath	Leaf blade
Setaria parviflora	0.009	91.20	61.15	121.05	23.30	273.40
	1	88.52	53.47	114.60	22.28	256.59
	2	86.71	48.08	111.84	20.82	246.62
	4	85.63	45.86	111.06	20.18	242.55
	7	90.53	47.19	106.57	19.99	244.29
Paspalum urvillei	0.009	139.44	39.96	179.97	32.93	359.37
	1	120.77	38.63	166.14	29.70	325.54
	2	128.78	35.85	178.98	29.34	343.61
	4	134.39	38.28	181.80	29.71	354.48
	7	123.07	37.62	177.76	30.83	338.44
ANOVA						
Fe-EDTA (Fe)		n.s.	*	n.s.	*	n.s.
Species (Sp)		*	*	*	*	*
Fe X Sp		n.s.	n.s.	n.s.	n.s.	n.s.
Block		n.s.	n.s.	n.s.	n.s.	n.s.

n.s. nonsignificant

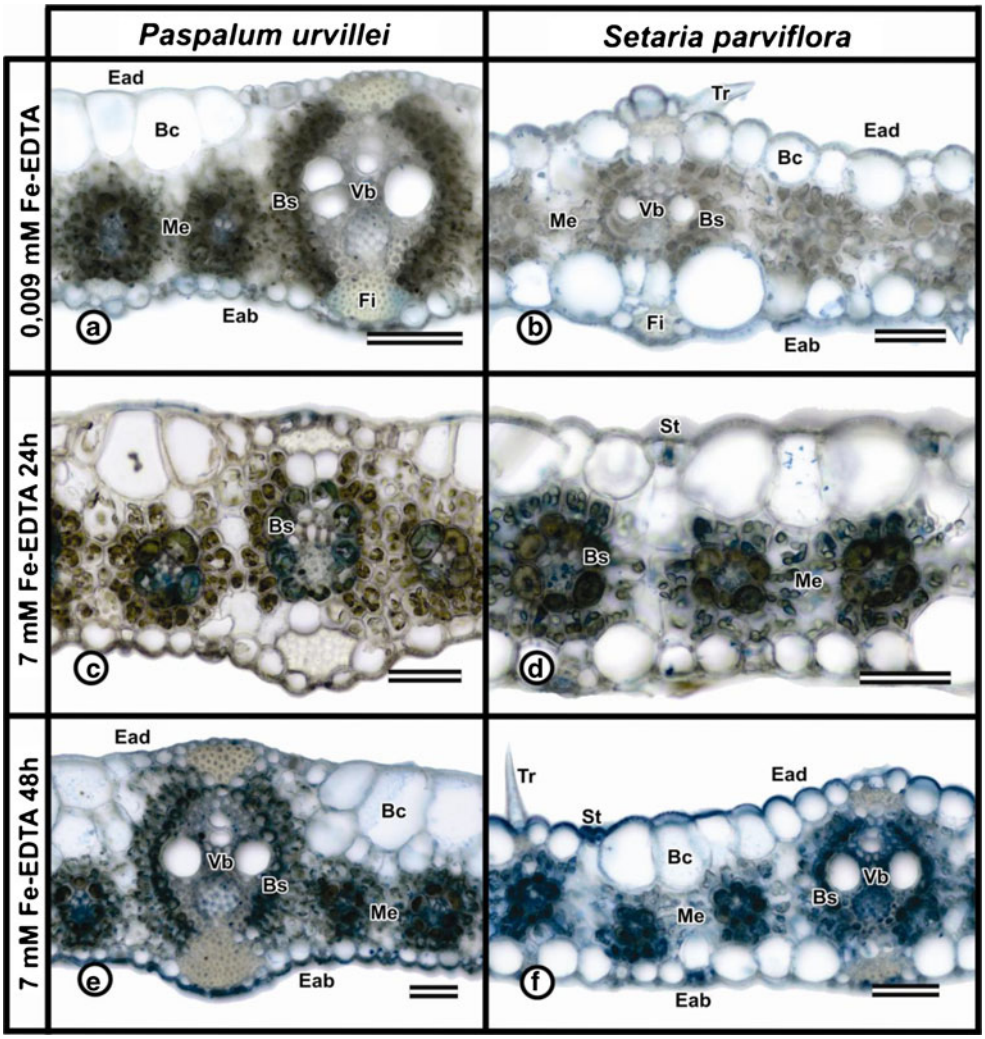

Fig. 6 Leaves of *Paspalum urvillei* (*a*, *c*, *e*) and *Setaria parviflora* (*b*, *d*, *f*) in transversal sections at 0.009 mM (*a*, *b*) and 7 mM Fe-EDTA (*c*, *d*, *e*, *f*) after 24 h (*c*, *d*) and 48 h (*e*, *f*) of exposure to the Prussian Blue reagent. *Ead* epidermis of adaxial surface, *Eab* epidermis of abaxial surface, *Vb* vascular bundle, *Me* mesophyll, *Bc* bulliform cell, *Bs* bundle sheath, *Fi* fibers, *Tr* trichome, *St* stomata. *Bars* 50 μm

Although *P. urvillei* and *S. parviflora* have retained phytotoxic iron concentrations in the shoots (Marschner 1995), the roots were more efficient in the storage process. High BCF values indicate a tendency of *P. urvillei* and *S. parviflora* to accumulate iron in the root system, and low TF values indicate a limited metal translocation to the shoots. The high capacity of absorbing and accumulating the metal in the root system is also described by Yoon et al. (2006), Gomes et al. (2011), and Zancheta et al. (2011). While the higher retention of iron in roots and its consequent lower transportation to shoots may be an important tolerance mechanism, this process is, at the same time, a limiting factor for using the phytoextraction technique. It is, however, extremely favorable to the application of rhizospheric bioremediation through phytofiltration and phytostabilization (Memon et al. 2001; Cheng 2003; Raskin et al. 1997). According to Roccotiello et al. (2010), plants with TF<1 and BCF>1, like *S. parviflora* and *P. urvillei*, are referred to as excluders, able to immobilize metals in roots and considered suitable for phytostabilization.

High iron contents in shoots and roots were directly related to visual symptoms, which were more intense at 7 mM Fe-EDTA. According to Verbruggen et al. (2009), hyperaccumulator species combine three factors: high metal tolerance, metal accumulation in shoots, and absence of visual symptoms. Thus, *P. urvillei* and *S. parviflora* are not hyperaccumulator species, since they showed leaf bronzing and macroscopic necrosis. However, the two grasses are phytoextractors, since they absorbed the metal and accumulated it in the harvestable shoots (Kumar et al. 1995; Prasad and Freitas 2003).

Bronzing, the main visual foliar symptom observed in the two studied species, is a typical symptom of toxicity by excess iron. It is associated with damage on cellular components (Becker and Asch 2005), as it has been observed in the anatomical analyses of this study. Moreover, chlorosis, a symptom associated with nutritional disorder and characterized by leaf yellowing (Guirra et al. 2011), has also been observed at leaf margins in the present work.

Iron, despite being an essential micronutrient, may be toxic at high concentrations (Ducic and Polle 2005). Toxicity symptoms of metal stress, such as chlorosis and necrosis, could be observed, and were associated to changes in leaf anatomical structure. In visually affected areas, alterations were observed in both species, such as derangement of the mesophyll cells, cell hypertrophy, protoplast retraction, changes in cell shape and volume, cell death, and apparent reduction in leaf blade thickness. Iron, at high levels in the cell, catalyzes the Fenton reaction, which is characterized by the generation of hydroxyl radicals capable of degrading cellular structures, including the plasma membrane, by inducing lipid peroxidation, which may alter its structure and permeability (Sinha et al. 1997; Connolly and Guerinot 2002; Silva et al. 2006). *S. parviflora* and *P. urvillei* showed high iron contents in their leaf tissues. Future studies evaluating oxidative stress through oxygen-reactive species quantification may explain some of the anatomical alterations observed. Furthermore, malondialdehyde content quantification is considered an indicator of lipid peroxidation (Sinha and Saxena 2006), which could be related to protoplast retraction and cell death. Reduction in leaf blade thickness at visually affected regions was also reported by Silva et al. (2005) and Sant'Anna-Santos et al. (2006a) in response to other pollutants, and leaf wrinkling and curling, which are usually associated to cell hypertrophy and hyperplasia (Sant'Anna-Santos et al. 2006b), were also observed in this work.

Nutritional disorder can also explain the anatomical changes visualized in *P. urvillei* and *S. parviflora*. Araújo (2012) showed that these two grass species, at 7 mM Fe-EDTA, presented nutritional disorder regarding potassium and nitrogen concentrations, as a result of excess iron. Siqueira-Silva et al. (2011) also verified in *I. pes-caprae* reduction in the nitrogen concentration in leaves, indicating that the mechanisms of translocation of this nutrient may have been influenced by the excess iron condition. The decrease in potassium and nitrogen contents may compromise xylem lignification. This refers particularly to nitrogen, since it influences production of lignin in grasses, as it makes part of the amino acid precursors of the synthesis of such compound (Marschner 1995). Thus, the deformation on the shape of xylem cells, found in leaves of *P. urvillei* and *S. parviflora*, may have been caused by problems in the synthesis of lignin as a result of nutritional disorder. Reduction in size and number of xylem vessels elements due to a toxic metal (Cd) has been reported by Sandalio et al. (2001).

According to Zobel (1996), extravasation of phenolic compounds from the vacuole occurs due to disruption of cellular membranes, thereby originating necrosis. In necrotic regions of *S. parviflora* leaves, there was an accumulation of green-colored compounds, seen on sections stained with toluidine blue. This phenomenon has been associated by some authors to the presence of phenolic compounds (Sant'Anna-Santos and Azevedo 2010). However, through histochemical test, the presence of such compounds was not observed in leaves of neither *S. parviflora* nor *P. urvillei*, despite of their importance due to their role as antioxidants (Pasqualini et al. 2003; Haripyaree et al. 2010). Then, it is possible that *S. parviflora* and *P. urvillei* present other compounds playing this role, like phytochelatins (PCs). Due to their high affinity for transition metals, phytochelatins, whose function is related to maintenance of cellular homeostasis, bind to such metals, forming a PC–metal complex. This complex is often sequestered in the vacuole (Sharma and Dietz 2006), thus leading to a greater tolerance to the heavy metal. Similarly to PCs, the nonproteogenic amino acid nicotianamine (NA) may play a role in the tolerance to excess iron. The membrane transport of reduced iron is related to its binding to NA. Pich et al. (2001) indicate the possible importance of NA in vacuolar

sequestration at the detoxification of excess iron. The detoxification ability of *P. urvillei* and *S. parviflora* need to be investigated in future work.

Although both grasses have accumulated iron contents above the critical toxicity level, in leaves of *S. parviflora*, symptoms were visually milder, in comparison with *P. urvillei*, as observed in all concentrations of Fe-EDTA. Many homeostatic mechanisms enable plants to maintain essential metal ions in cellular compartments, thus minimizing the harmful effects of these ions in excess (Clemens et al. 2002; Michalak 2006). Some of the mechanisms employed by *S. parviflora* may be the binding of iron in the cell wall in roots and leaves before it can come into contact with sensitive biomolecules in the cell; metal storage in the vacuole, changes in gene expression, and the induction of chelating proteins within the cell, which increases the tolerance level of the plant (Memon and Schröder 2009; Cosio et al. 2004; Douchkov et al. 2005), avoiding cell damage. However, these mechanisms were not evaluated in this study.

After 24 h of exposure to Prussian Blue, the bundle sheath stained intensely both in *P. urvillei* and *S. parviflora*. Our results show that increasing concentrations of this metal intensify structural damage on bundle sheath cells, corroborating the micromorphometric analysis, which revealed significant reduction in this tissue on both species, on visually asymptomatic regions of the leaf.

After 48 h of exposure to Prussian Blue, iron was histolocalized in all regions of the leaf blade of both *P. urvillei* and *S. parviflora* at concentration 7 mM Fe-EDTA. In plant cells, over 90 % of the iron stored in ferritins is usually located in chloroplasts. Moreover, iron acts as a cofactor and is an efficient catalyst in controlled redox chemistry (Hell and Stephan 2003), being present in different plant tissues, which confirms the results found. Epidermal and bundle sheath cells stained more intensely, showing that the grasses were able to accumulate higher iron content in these cells. In substrates rich in essential metals, in order to maintain cellular homeostasis plants can sequester the excess of these metals in the vacuole (Hall 2002), cell wall (Memon et al. 2001), or cuticle (Robinson et al. 2003), either in trichomes, epidermal common cells, or bundle sheath cells, causing less damage to the photosynthetic machinery (Küpper et al. 2000; Freeman et al. 2006; Robinson et al. 2003).

The reduction in thickness of the epidermis of abaxial surface and bundle sheath, both verified in this study through micromorphometry, may be related to a higher metal accumulation in these tissues, which has already been described by some authors in studies on the cellular compartmentalization of heavy metals (Lombi et al. 2002; Robinson et al. 2003; Cosio et al. 2005; McNear et al. 2005). Most of these studies have reported the localization of such elements in the epidermis (Robinson et al. 2003; Cosio et al. 2005; McNear et al. 2005; Lombi et al. 2002), not only on the abaxial side of the leaf, but also in the adaxial side, as well as in vascular tissues (McNear et al. 2005).

Conclusion

S. parviflora and *P. urvillei* showed similar behavior regarding iron accumulation, i.e., higher metal concentrations were retained in roots than in shoots. However, toxic iron levels were accumulated in leaf tissues, promoting visual and anatomical damage in this organ. Visually, there was bronzing, chlorosis, and necrosis, and, anatomically, there was protoplast retraction, alteration in cell shape and volume, derangement of mesophyll cells, and cell death. Symptoms were similar on both grasses, but more severe in *P. urvillei* at all concentrations of Fe-EDTA. Therefore, both hypotheses tested were confirmed, since the analyses performed evidenced anatomical changes caused by excess iron, providing better visualization and understanding of the damage; and the plants tolerate and accumulate high iron concentrations in their tissues, thus favoring the possible use of these grasses in phytoextraction, but mainly in rhizospheric bioremediation.

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