Environ Sci Pollut Res (2015) 22:2187–2195
DOI 10.1007/s11356-014-3488-1

RESEARCH ARTICLE

Morphoanatomical responses induced by excess iron in roots of two tolerant grass species

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Received: 30 April 2014 / Accepted: 20 August 2014 / Published online: 31 August 2014

Abstract We aimed to verify whether morphoanatomic alterations occur in response to excess iron, in roots of *Setaria parviflora* and *Paspallum urvillei* (Poaceae), and to localize the presence of the sites of iron accumulation. Plants were subjected to 0.009, 1, 2, 4, and 7 mM Fe-EDTA in nutrient solution. Both species presented iron contents in the roots above the critical toxicity level. The presence of iron plaque on roots of the two species was confirmed, and it may have reduced iron absorption by the plants. Roots from the two species showed typical visual symptoms of stress by excess iron: change in color and mucilaginous and flaccid appearance. Anatomical damage was observed in both species: aerenchyma disruption, alterations in endodermal cells, and irregular shape of both vessel and sieve tube elements. The metal was histolocalized in the cortex and in protoxylem and metaxylem cell walls in both species, which suggests a detoxification strategy for the excess iron. Phenolic compounds were not histolocalized in roots. Microscopic analyses were therefore effective in evaluating the real damage caused by excess iron.

Keywords *Setaria parviflora* · *Paspallum urvillei* · Poaceae · Symptomatology · Histochemistry · Energy dispersive X-ray (EDX)

Responsible editor: Elena Maestri

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Introduction

Iron (Fe) is an essential nutrient for plant growth and development, taking part in several biochemical processes in plant cells (Stephan 2002). Despite being abundant in soil, iron originates iron hydroxides, which are highly insoluble, thus being generally unavailable to plants (Nozoye et al. 2011).

Fe can affect cellular homeostasis and cause toxicity to plants (Williams et al. 2000; Ducic and Polle 2005). However, environments with high levels of metals in the soil may be propitious to the selection of tolerant plants, which in turn may possess strategies that allow them to survive under this kind of stress (Macnair 1993; Milner and Kochian 2008).

Grass species *Setaria parviflora* (Poir.) Kerguélen and *Paspalum urvillei* Steudel are commonly found spontaneously at the margins of a decantation pond, in an iron ore pelletizing industry located in the community of Ubu, Anchieta city, state of Espírito Santo, Brazil. Besides tolerating high Fe concentrations in the substrate, both species accumulate in their tissue iron contents above the toxicity limit, which according to Marschner (1995) is 500 mg kg^{-1}, without altering their growth (Araújo et al. 2014). Species exposed to high iron concentrations may exhibit characteristic visual toxicity symptoms (Santana et al. 2014), even when not presenting changes in their growth (Araújo et al. 2014).

Visual analysis of shoots is a quick alternative that assists in the perception of plant stress (Fontes 2006). Other species accumulating high pollutant concentrations in their tissues might not exhibit visual damage, yet present structural damage in root and shoot tissues (Hecht-Buchholz 1983). Thus, microscopic analyses may assist in injury prognosis and in the assessment of the real damage caused by excessive pollutants (Silva et al. 2000; Sant'Anna-Santos et al. 2006; Sant'Anna-

Santos and Azevedo 2007; Giampaoli et al. 2012; Gomes et al. 2012).

The absorption of high iron concentrations causes both an increased production of reactive oxygen species (ROS) and oxidative stress to cells (Becana et al. 1998; Tsai and Huang 2006). Improving antioxidant systems (either enzymatic or nonenzymatic), which would neutralize such stress, is one of the strategies used by tolerant plants (Sinha et al. 1997; Sytar et al. 2013). An example of a nonenzymatic mechanism is increased production of phenolic compounds. These antioxidants tend to chelate Fe, thus preventing the formation of free radicals (Michalak 2006).

This study is different from the other studies with *P. urvillei* and *S. parviflora*, because we focus in the root, which is the first organ of the plant to get in contact with excess metals in the substrate, and in response, it may present anatomical alterations and consequently several effects in the plant body (Lux et al. 2010). Furthermore, in this organ, there could occur the formation of iron plaque, which is the consequence of oxidation of ferrous ion to ferric ion, followed by precipitation of iron oxide hydroxide on the root surface (Taylor and Crowder 1983). The presence of the iron plaque can either reduce or increase the absorption of certain nutrients (Zhang et al. 1999). Thus, to understand the mechanisms of iron absorption and accumulation, it is essential to conduct a structural analysis on the formation process of the iron plaque on the roots, as well as to know the main sites of iron accumulation in plant tissues (Siqueira-Silva et al. 2012).

The hypothesis tested was that *S. parviflora* and *P. urvillei* would not present severe structural damage in the roots in response to excess iron, due to their natural survival in an iron-rich environment. We aimed to verify whether morphoanatomical alterations would occur in the species roots in response to high iron levels in nutrient solution and to localize the sites of iron accumulation.

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 the community of Ubu, Anchieta city, state of Espírito Santo, Brazil (20° 46′ 21.0″ S and 40° 34′ 52.3″ W). This pond is supplied with water from the iron ore pelletizing industry system and from the iron ore pipeline, and therefore is rich in iron particles. Species identity was confirmed by specialist Hilda Maria Longhi Wagner, and specimens were deposited in Herbarium VIC (UFV) with numbers 36801 and 36802 for *P. urvillei* and *S. parviflora*, respectively.

Individuals were transplanted and cultivated in 8-L plastic pots containing conventional substrate (clay soil/sand/bovine manure—3:1:1) 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 culms (leaving approximately 4 cm of both roots and shoots). Clumps were transferred to 5-L polystyrene boxes and kept in Hoagland nutrient solution (Hoagland and Arnon 1950), at half ionic strength, with pH 5.0 (adjusted every 2 days using NaOH 1 N and HCl 1 N) and constant aeration. Solution volume was completed with deionized water whenever necessary in order to reset transpiration demand, and the solution was renewed on a weekly basis. During 40 days, plants were pruned twice at culm height for homogenization, as described above, before treatment application.

In order to avoid undesirable stress due to sudden exposition to high iron doses in the nutrient solution, an acclimatization was carried out, by gradually increasing iron content in the solution until reaching the final concentrations of 1, 2, 4, and 7 mM. During the acclimatization period, the solution was renewed every 12 h. Initially, an increment of 0.25 mM Fe-EDTA was performed until all treatments reached 1 mM Fe-EDTA (except for the control treatment). Then, the final concentration values of each treatment were established with the increment of 2 mM (on treatments 2, 4, and 7 mM Fe-EDTA), 4 mM (on treatments 4 and 7 mM Fe-EDTA), and 7 mM Fe-EDTA (on treatment 7 mM Fe-EDTA only). Time period for plant acclimatization to iron concentrations was 84 h (3.5 days). These iron concentrations were based on Pereira et al. (2013), who worked with another grass species, *Oryza sativa*.

After establishment of the final concentrations, the nutrient solution was renewed every 3 days, and pH was adjusted daily to 5.0 as previously described. Plants remained exposed to treatments during 14 days for anatomical and visual analyses (n=5).

Visual characterization

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

Structural characterization in light microscopy

Root samples with visible symptoms were collected at 5 cm from root apex from all treatments. These samples were fixed in 2.5 % glutaraldehyde and 4 % paraformaldehyde in a sodium phosphate buffer 0.1 M (pH 7.0) (Karnovsky 1965,

modified) for 48 h, and stored in 70 % alcohol. Then, they were dehydrated in an ethyl series and embedded in glycol methacrylate (Leica Historesin, Nussloch/Heidelberg, Germany). Transverse sections of roots (6 μm thick) were obtained with an automatic rotary microtome (model RM2155 Leica Microsystems Inc., Deerfield, IL, USA), stained with 0.05 % toluidine blue (pH 4.0) (O'Brien and McCully 1981), and mounted in Permount.

Histochemical analysis

In order to detect the presence of iron in tissues from roots, after sample fixation in Karnovsky, cross sectioning was performed on roots of plants from treatments 0.009 mM (control) and 7 mM Fe-EDTA. Sections were obtained in a table microtome (model LPC, Rolemberg e Bhering Comércio e Importação LTDA, Belo Horizonte, MG, Brazil). After distilled water rinsing, sections were set in a solution containing 4 % potassium ferricyanide and 4 % hydrochloric acid for 48 h (Stevens and Chalk 1996, adapted by Silva et al. 2006). This test shows the presence of iron by a blue staining called Prussian Blue. A negative control, in which sections 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 roots 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, samples were dehydrated in an ethyl/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 with samples previously stored in methanol for 72 h under vacuum for removal of the phenolic compounds.

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

Structural characterization in scanning electron microscopy and energy dispersive X-ray microanalysis (EDX)

For analysis of the root surface, root samples of both species were collected at 1 cm from root apex, in plants from treatments 0.009 and 7 mM Fe-EDTA, and fixed in Karnovsky (Karnovsky 1965, modified). Samples were dehydrated in an ethyl series and critical point dried with liquid CO_2 (model CPD 030, Bal-Tec, Liechtenstein). Dry fragments were fixed on metal supports. Some fragments from each treatment were sputter-coated with gold (model FDU 010, Balzers, Liechtenstein) for analysis in scanning electron microscopy, while other fragments were sputter-coated with carbon for microanalysis by EDX. Both are qualitative analyses. Photographic documentation was carried out in a SEM (Leo, model 1430VP, Cambridge, England) operated at 20 kV with an X-ray probe coupled.

Statistical analysis

The experiment was mounted in a factorial scheme and randomized block design, with two species (*S. parviflora* and *P. urvillei*), five treatments (0.009, 1, 2, 4, and 7 mM Fe-EDTA), and five repetitions.

Results

Visual characterization

Visual alterations were observed in roots of both *S. parviflora* and *P. urvillei* in all excess iron concentration. As iron concentration in the solution increased, we noticed an intensification of the yellowish color in *P. urvillei* roots (Fig. 1a, b, c, e) and of the orange color in *S. parviflora* roots (Fig. 1f, g, h, j). The organ presented, in both species, a mucilaginous and flaccid appearance. In *P. urvillei* roots, necrotic spots could also be observed (Fig. 1e).

Structural characterization in light microscopy

Roots of *S. parviflora* and *P. urvillei* present an uniseriate epidermis. In the root cortex of both species, there is an aerenchyma and an uniseriate endodermis composed of compact cells with an "U"-shaped cell wall thickening. In *P. urvillei*, there is an exodermis, which is composed of one layer of tabular cells. Root is polyarch with an exarch xylem and the presence of a pith. Pericycle is also uniseriate (Fig. 2a, e).

In both studied species, anatomical alterations were observed, a greater intensity of damage taking place with increasing Fe-EDTA concentrations. *S. parviflora* and *P. urvillei* presented aerenchyma disruption (Fig. 2f), alterations in endodermal cells (Fig. 2g), and alterations in the shape of both vessel and sieve tube elements (Fig. 2f, g). *P. urvillei* presented protoplast retraction in cortical and endodermal cells (Fig. 2c), formation of a cicatrization tissue in the cortex (Fig. 2b), and collapse of pith cells (Fig. 2d). In *S. parviflora*, irregularities in the shape of pericycle cells were also observed (Fig. 2g).

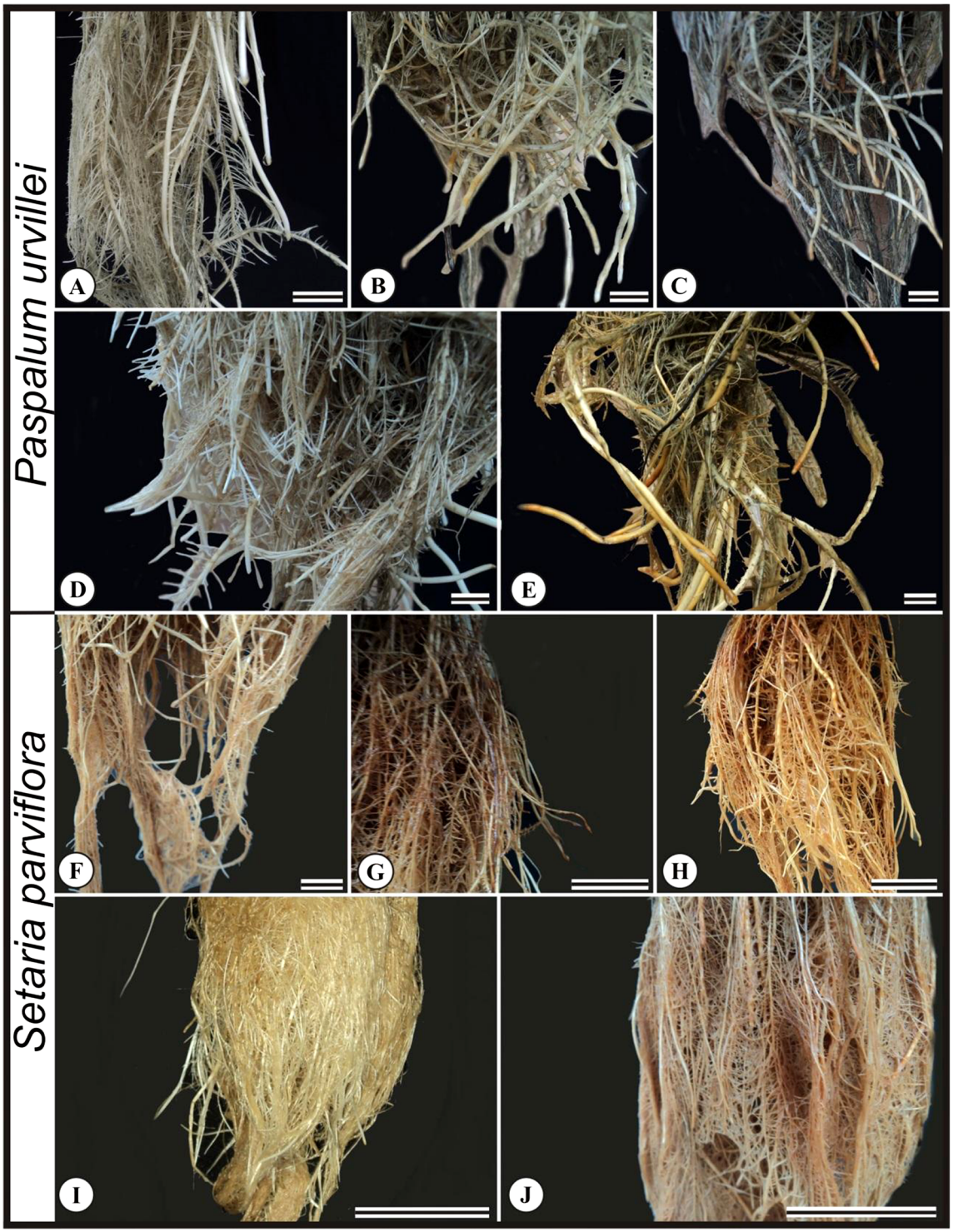

Fig. 1 Root system of *Paspalum urvillei* (**a**–**e**) and *Setaria parviflora* (**f**–**j**), subjected to 1 mM (**a**, **f**), 2 mM (**b**, **g**), 4 mM (**c**, **h**), and 7 mM (**e**, **j**) Fe-EDTA. Control treatment with 0.009 mM Fe-EDTA (**d**, **i**). *Bars* (**a**–**e**, **g**–**j**) 20 mm. **i** 100 mm

Histochemical analysis

At control treatment, *P. urvillei* roots showed strong reaction in epidermal and exodermal cells and weak reaction in cortex, pericycle, xylem, and phloem cells (Fig. 3a). As for *S. parviflora*, roots presented strong reaction in epidermal cells and weak reaction in cortex cells (Fig. 3d).

In *P. urvillei*, at concentration 7 mM Fe-EDTA, epidermis, aerenchyma, endodermis, pericycle, phloem, and protoxylem showed strong reaction (Figs. 3b, c). At this concentration,

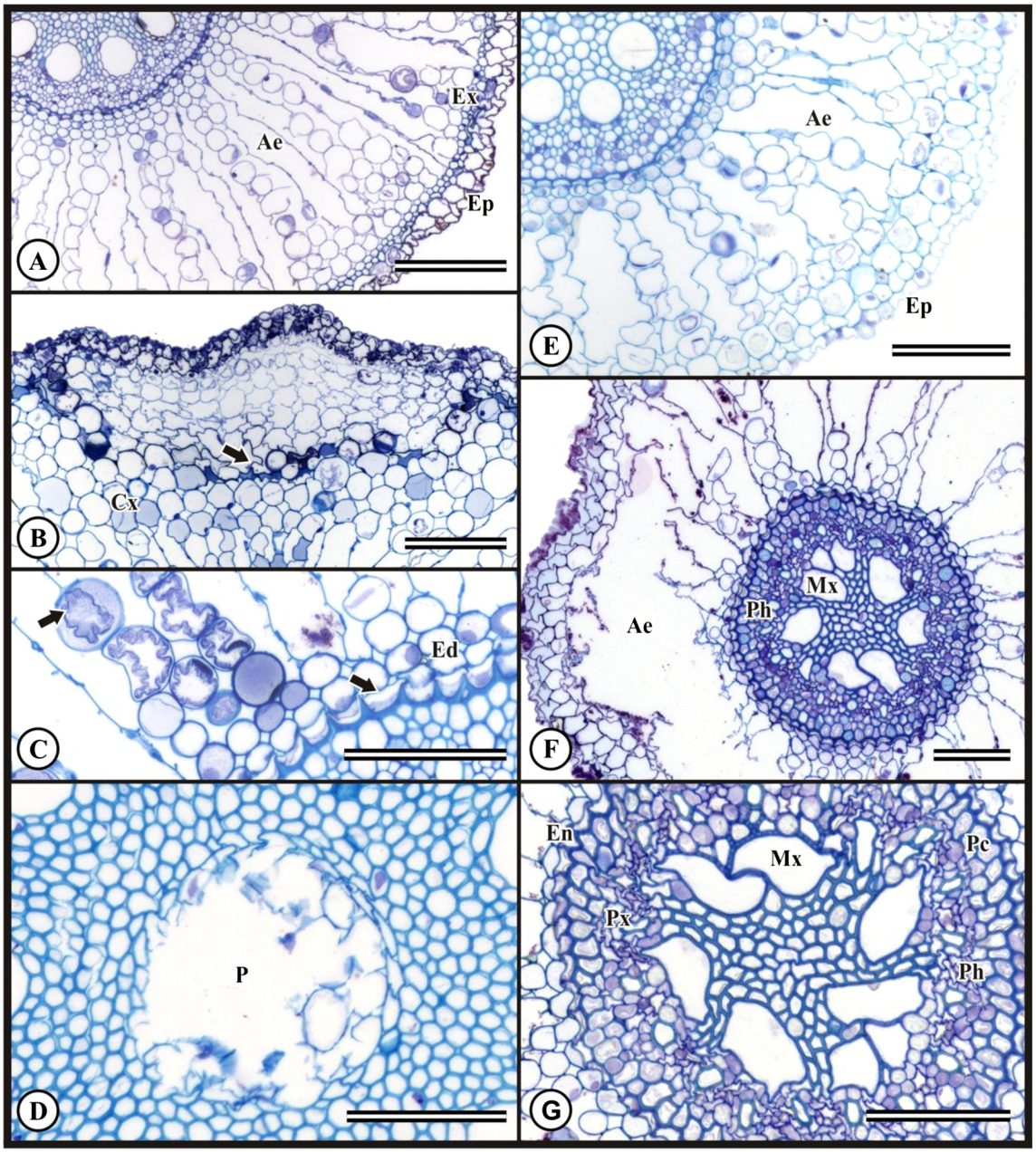

Fig. 2 Anatomical alterations in roots of *Paspalum urvillei* (**a**–**d**) and *Setaria parviflora* (**e**–**g**) in cross sections. **a**, **e** Control treatment, with 0.009 mM Fe-EDTA in nutrient solution. **b**–**d**, **f**, **g** Treatment with 7 mM Fe-EDTA in nutrient solution. **b** Formation of a cicatrization tissue in cortical cells. **c** Protoplast retraction of aerenchyma and endodermis cells. **d** Collapse of pith cells. **f** Aerenchyma disruption. **g** Alteration in endodermis and pericycle cells. **f**, **g** Irregular shape of both vessel and sieve tube elements. *Ep* epidermis, *Ex* exodermis, *Cx* cortex, *Ae* aerenchyma, *Ed* endodermis, *Pc* pericycle, *Mx* metaxylem, *Px* protoxylem, *Ph* phloem, *P* pith. *Bars* **a**, **b** 200 μm, **c**–**g** 100 μm

S. parviflora showed strong reaction in epidermis, phloem, and xylem cells (Fig. 3e, f). The two species showed a positive reaction for iron histolocalization in cortex cells and in protoxylem and metaxylem cell walls (Fig. 3b, e, detail). The presence of iron was not detected in the pith of either species or in the endodermis of *P. urvillei*.

In both species, roots from the treatments analyzed showed a negative result in the histochemical test for detection of phenolic compounds (not shown).

Structural characterization in scanning electron microscopy and energy dispersive X-ray microanalysis

Root surface in plants of both *P. urvillei* and *S. parviflora* from the control treatment in scanning electron microscopy (SEM) showed epidermal cells with normal appearance, with distinguishable delimitations between cells (Fig. 4a, c). At concentration 7 mM Fe-EDTA, roots of the two species presented deposition of an external substance heterogeneously

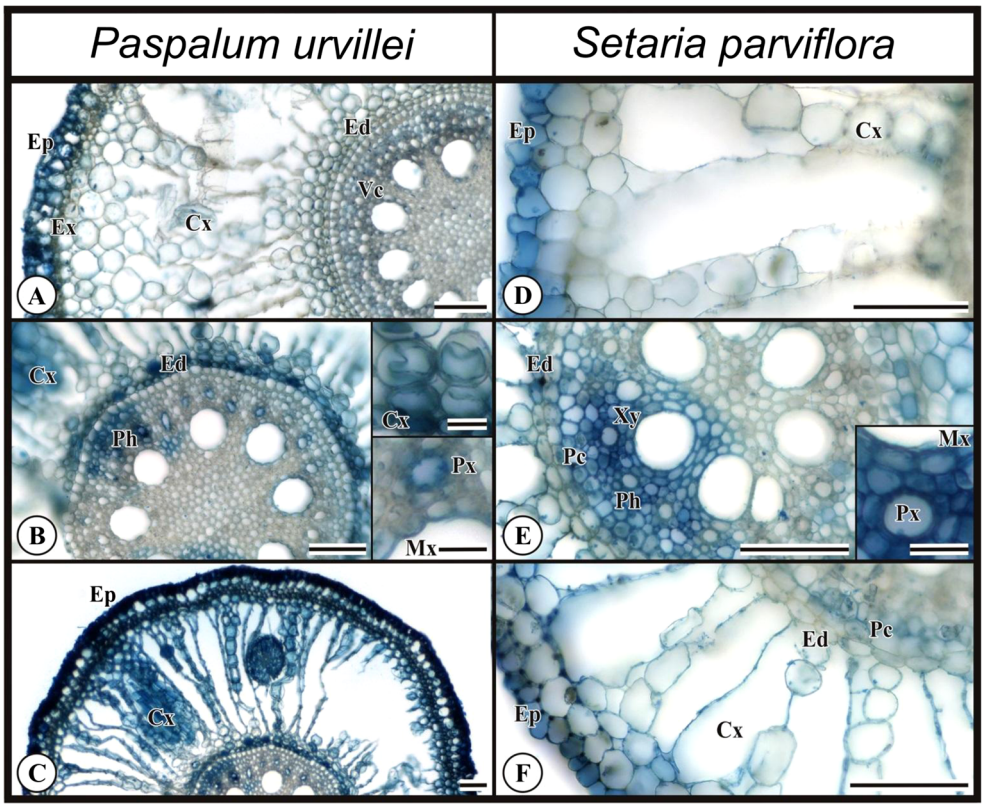

Fig. 3 Iron histolocalization in roots of *Paspalum urvillei* (**a**–**c**) and *Setaria parviflora* (**d**–**f**), cultivated with 7 mM Fe-EDTA (**b**, **c**, **e**, **f**) in nutrient solution, after 48 h of exposure to the Prussian Blue reagent. **a**, **d** Control treatment with 0.009 mM Fe-EDTA. *Ep* epidermis, *Ex* exodermis, *Cx* cortex, *Pc* pericycle, *Vc* vascular cylinder, *Ed* endodermis, *Ph* phloem, *Px* protoxylem, *Mx* metaxylem, *Xy* xylem. *Bars* 100 μm (details at **b** and **e**) 500 μm

distributed over the root surface, i.e., iron plaque, which hampered the visualization of limits between cells (Fig. 4b, d). Additionally, in *P. urvillei*, a cracked appearance was observed in the root surface.

EDX of root surface (Fig. 5a, d) in plants from treatment 7 mM Fe-EDTA (Fig. 5c, f) showed an increase in the amount of superficial iron of 98.82 % in *P. urvillei* and 97.51 % in *S. parviflora*, in relation to plants from the control treatment (Fig. 5b, e).

Discussion

P. urvillei and *S. parviflora* are both species that tolerate and accumulate in their root iron amounts above the toxicity limit (500 mg kg^{-1}) (Marschner 1995), as already verified by Santana et al. (2014), who studied the iron effects on these two species with the same exposure time as the one used in our study. The high iron content caused morphological and anatomical alterations in roots of the two studied species.

SEM and EDX analyses confirmed the presence of iron plaque, which was the cause of the color change in roots of *P. urvillei* and *S. parviflora*. The aerenchyma in roots of both species may have favored the formation of iron plaque, which then resulted in visual changes. Aerenchyma propitiates the development of an oxidation zone on roots (Evans 2003), which contributes to the oxidation of Fe^{2+} from the nutrient solution to Fe^{3+}, and also to the precipitation of iron oxyhydroxides on the root surface (Chen et al. 1980).

The role of the iron plaque on the root surface remains controversial. Some authors state that the plaque may either decrease or increase absorption of certain nutrients and metals (Jiang et al. 2009; Seyfferth et al. 2010; Wang et al. 2010; Zhong et al. 2010; Liu et al. 2011). In a study with the same two species and same Fe-EDTA concentrations (Araújo et al. 2014), it has been verified on root surfaces the deposition of high iron contents, i.e., part of the Fe available for absorption in the nutrient solution precipitated as iron oxide hydroxide, thus becoming unavailable for absorption by the two species. However, although the iron plaque may have reduced Fe absorption, *P. urvillei* and *S. parviflora* both accumulated high Fe contents within the roots.

In order to maintain cellular homeostasis, tolerant plants can sequester the excess of heavy metals in the vacuole (Pich et al. 2001) and cell wall (Yang et al. 2011). Thus, the positive histolocalization of iron in cortex cells and in protoxylem and metaxylem cell walls of *P. urvillei* and *S. parviflora* suggests a detoxification strategy in these species for the excess iron.

In roots, certain tissues, e.g., the endodermis, can act as barriers and therefore function as a protection against the toxic effects caused by heavy metals (Lux et al. 2004). Despite this, we observed damage in cells of the vascular

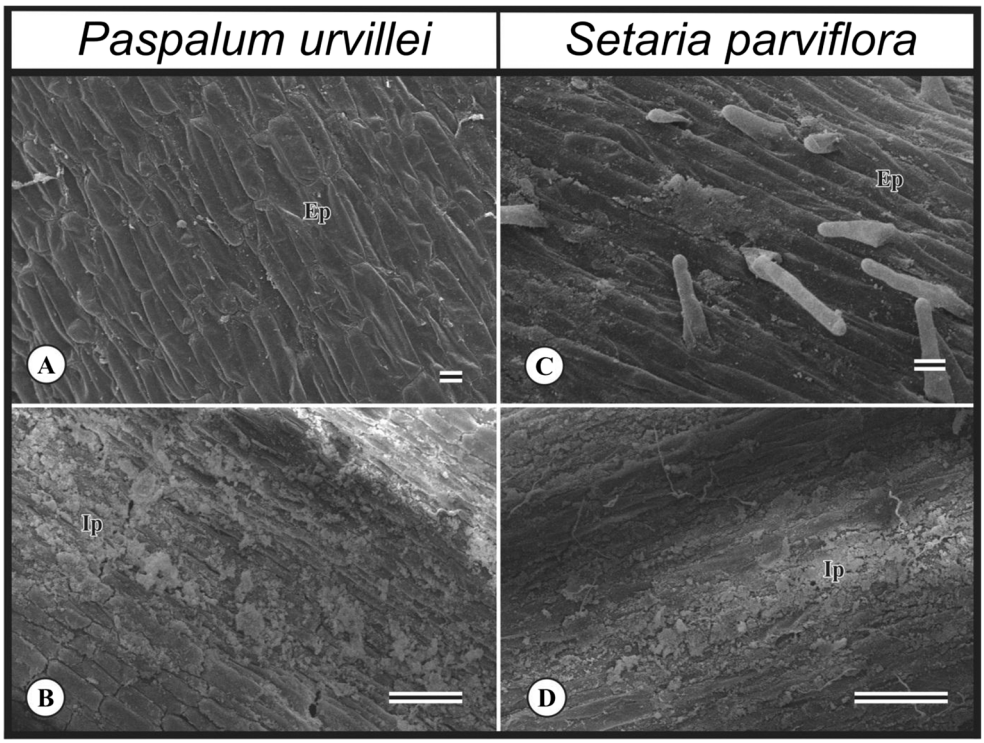

Fig. 4 Roots of *Paspalum urvillei* (**a**, **b**) and *Setaria parviflora* (**c**, **d**) from control treatment (0.009 mM Fe-EDTA in nutrient solution) (**a**, **c**) and treatment with 7 mM Fe-EDTA in nutrient solution (**b**, **d**), in scanning electron microscopy. *Ep* epidermis, *Ip* iron plaque. *Bars* **a**, **c** 20 μm; **b**, **d** 100 μm

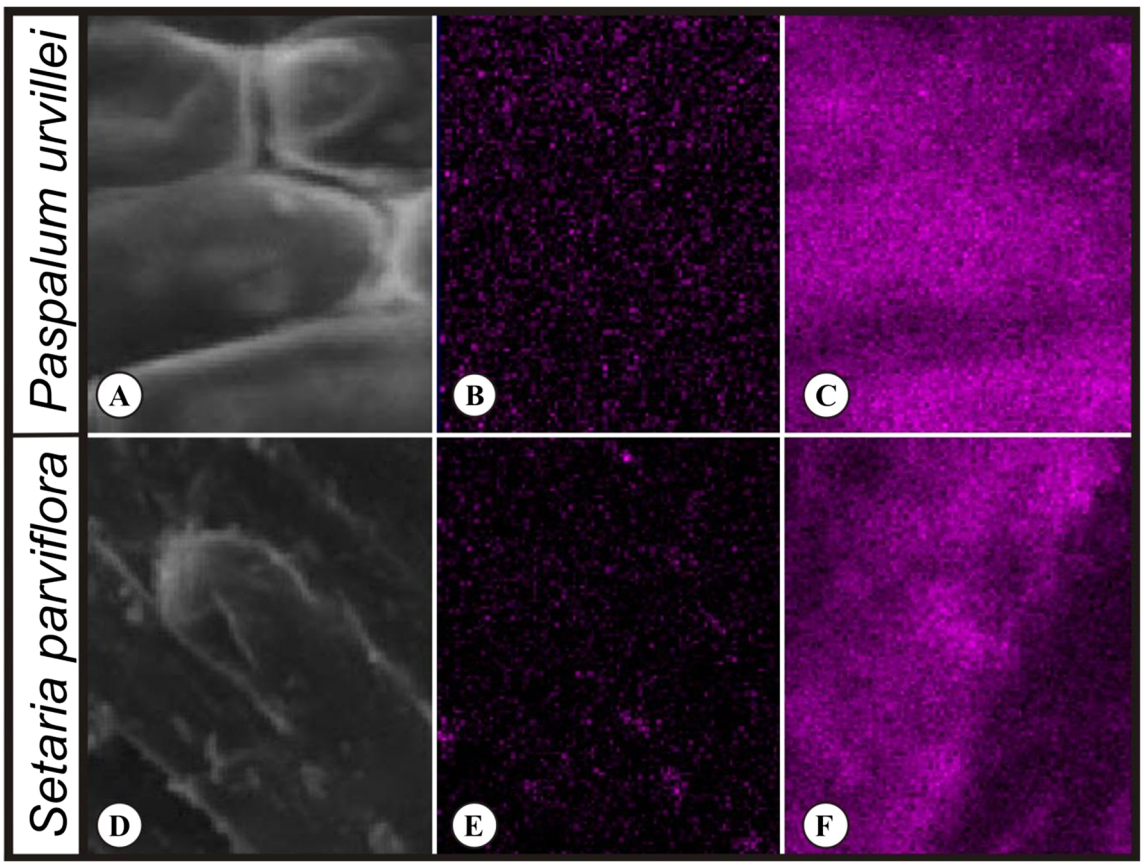

Fig. 5 Roots of *Paspalum urvillei* (**a**–**c**) and *Setaria parviflora* (**d**–**f**) from control treatment (0.009 mM Fe-EDTA in nutrient solution) (**b**, **e**) and treatment with 7 mM Fe-EDTA in nutrient solution (**c**, **f**), in energy dispersive X-ray microanalysis. *Pink dots* mark the presence of iron on root surface

cylinder in the two studied species, which therefore suggests that such barriers were not effective against iron toxicity. The cicatrization tissue in *P. urvillei* also acts as a barrier that would prevent damage progress toward healthy tissues (Siqueira-Silva et al. 2012), as observed in some plant species exposed to different pollutants (Silva et al. 2005; Sant'Anna-Santos et al. 2006). In our study, this tissue was not effective on barring the entry of Fe, as demonstrated by the presence of structural damage.

Decreasing levels of both copper and potassium in root dry matter (Araújo et al. 2014) of *P. urvillei* and *S. parviflora* have been found at treatments with high Fe-EDTA concentrations in hydroponic solution. Thus, the anatomical alterations observed in roots of *P. urvillei* and *S. parviflora* are likely to have occurred due to accumulation of high iron levels in tissues and/or to nutritional disorder resulting from the deposition of iron plaque on the roots. Reduction in the amounts of copper and potassium can compromise xylem lignification. According to Marschner (1995), copper and/or potassium deficiency in higher plants causes insufficiency in lignification of xylem vessels and structural weakening of the cell wall. Thus, the shape deformation in xylem cells detected in roots of *P. urvillei* and *S. parviflora* may have been caused by problems in the synthesis of lignin, as a consequence of nutritional disorder.

Certain results observed in the two species, such as protoplast retraction and cell collapse, may have resulted from plasma membrane deterioration. This phenomenon is likely to occur at high iron content conditions, in which there is a predisposition to an increased production of ROS (Briat et al. 2010), which in turn can induce peroxidation of lipids that compose plasma membrane (Sinha et al. 1997). Plasma membrane deterioration may also have influenced the absorption of some nutrients, since both species presented nutritional disorder when subjected to excess iron (Araújo et al. 2014).

The impending action of oxidative stress in cells can be mitigated by strategies like chelation of heavy metals by organic molecules such as phenolic compounds, which can directly neutralize ROS (Sytar et al. 2013). However, in the studied species, such compounds were not detected by histochemical analysis in tissues with high iron contents. *P. urvillei* and *S. parviflora* may possibly possess some other effective kind of antioxidant mechanism, like enzyme activity, since complete development of their life cycle occurs in a site with excess iron. We suggest that further studies that would elucidate the antioxidant system of the two species should be performed. We also suggest that additional experiments should be conducted with a longer period of exposure to Fe than the one presented in this paper, which would lead to a better understanding of the behavior of *P. urvillei* and *S. parviflora* in iron-rich environments.

Conclusions

P. urvillei and *S. parviflora* showed morphological and anatomical alterations caused by high iron amounts in root tissues. Both species presented typical visual symptoms of stress by excess iron. However, considering the high Fe contents accumulated, these symptoms were not severe enough as to cause, for example, such a cell collapse that would damage the entire organ

The positive histolocalization of iron in cortex cells and in protoxylem and metaxylem cell walls in *P. urvillei* and *S. parviflora* suggests a detoxification strategy in these species for the excess iron. Furthermore, the two species may possess strategies that allow iron compartmentalization, and consequently their survival in environments with high iron levels, even presenting visual symptoms and anatomical damage.

Anatomical damage was observed in both species. Microscopic analyses were therefore effective in evaluating the real damage caused by excess iron. Since both species develop naturally in an iron-rich environment and are able to complete their life cycle, anatomical damage was not so severe as to compromise the development of *P. urvillei* and *S. parviflora*.

Acknowledgments The authors thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the approval of a PNADB Project (Programa Nacional de Apoio e Desenvolvimento da Botânica); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the approval of a SISBIOTA-BRASIL (proc. CNPq 563335/2010-6; proc. FAPESP 2010/52319-6) Project and for the Research Productivity Scholarships granted to L.C. Silva (309170/2012-5) and A.A. Azevedo (307538/2010-9); Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) for the approval of projects CRA-APQ-01361-12 and RDP–00195-10; Secretaria de Estado de Ciência, Tecnologia e Ensino Superior (SECTES) and Projeto Floresta Escola, for financial support; Hilda Maria Longhi Wagner, for species identification; and Núcleo de Microscopia e Microanálise of Universidade Federal de Viçosa (UFV).

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