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REGULAR ARTICLE

X-ray spectra in SEM and staining with chrome azurol S show Al deposits in leaf tissues of Al-accumulating and non-accumulating plants from the cerrado

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Abstract
Aims Aluminum (Al) accumulating plants are distributed throughout the world. In the Cerrado, Al-accumulating and non-accumulating species coexist. Therefore, we anatomically/histochemically explore the sites of Al deposits in Al-accumulating species, and we also ask whether Al can be observed in non-accumulating species.
Methods The anatomical patterns of Al storage in leaf tissues of Al-accumulating [*Miconia albicans*, *M. rubiginosa* (Melastomataceae), *Qualea grandiflora*, and *Q. parviflora* (Vochysiaceae)] and non-accumulating species [(*Styrax ferrugineus* and *S. camporum* (Styracaceae)] were described using different Al indicator dyes: hematoxylin and chrome azurol S (CAS). In addition, Al-specific x-ray spectra from different regions of leaf tissues were measured and analyzed by scanning electron microscopy (SEM).
Results When compared to hematoxylin, it was confirmed by x-ray spectra in SEM that CAS was a more contrasting indicator of Al presence. Silica granules associated to Al were observed on cell walls of non-lignified leaf tissues of Al-accumulating species. However, granules were also found in leaf midribs of *S. camporum*.
Conclusions The anatomical description of Al accumulation in leaves and the consistent pattern of Al association with cell walls strongly suggest that Al has structural rather than physiological roles in leaves of Cerrado woody plants, and that Al is perhaps isolated from metabolism.

Keywords Al^{3+} · Brazilian savanna · Histochemical studies · Melastomataceae · Styracaceae · Vochysiaceae

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Introduction

Acidic soils (pH < 5.0) occupy approximately 30 % of the world's ice-free land areas, where 67 % support woodlands and forests, and 18 % savannas (vonUexküll and Mutert 1995). Acidic soils usually present low base (K, Ca and Mg) saturation (BS) and, consequently, high values of potential acidity ($H^+ + Al^{3+}$) and exchangeable aluminum (Al) (Robson 1989). Poor soils and edaphic Al are known to limit the growth of crop plants (Yang et al. 2013), as Al causes ruptures in roots, binding itself to cell walls of rhizodermis and increasing its rigidity while reducing the ability of outer cells to elongate (Kopittke et al. 2008). Some plants form complexes of malate and citrate with Al in the root tip, and specific transporters are

responsible for excluding these organic acid complexes, explaining the resistance of some genotypes, which are called Al excluders (Ryan et al. 2011).

However, Al-accumulating plants are able to grow on acidic and Al-rich soils. Examples come from plants from the Rubiaceae family found in swamp forests in Brazil (Britez et al. 2002), Melastomataceae growing in highly disturbed landscapes in Mexico (González-Santana et al. 2012), 127 Melastomataceae species from different parts of the world (Jansen et al. 2002), 11 from Rubiaceae species distributed worldwide (Jansen et al. 2003), and even from tea plants – Theaceae (Carr et al. 2003; Tolrà et al. 2011).

In the Cerrado, which comprises grasslands, savannas and forests physiognomies, vegetation grows on more acidic (pH < 4.0) soils with Al saturation > 70 % (Haridasan 2008; Habermann and Bressan 2011). In the Cerrado, Al-accumulating plants were first described in 1982, and because of edaphic conditions, Al-accumulating and non-accumulating species were identified; while the former accumulates between 1000 up to 15,000 (or more) mg Al per kg dry leaves, the latter presents between 600 and 1000 mg/kg (Haridasan 1982). According to this author, most Al-accumulating plants in the Cerrado are shrubs and trees from Melastomataceae, Rubiaceae and Vochysiaceae.

Using Al indicators (dyes) in hystochemical tests, Al deposition was observed on cell walls of the phloem of the midrib and secondary veins of Al-accumulating plants from the Cerrado (Haridasan et al. 1986). These authors also showed that xylem fibers and xylem vessels do not get stained, while cell walls of the collenchyma, spongy parenchyma and guard cells do. This pattern was reported for other Al-accumulating plants that are not from the Cerrado (Britez et al. 2002; Carr et al. 2003; Tolrà et al. 2011; González-Santana et al. 2012), although in *Melastoma malabathricum*, *Tibouchina urvilleana* (Melastomataceae) and *Symplocos chinensis* (Symplocaceae), all Al-accumulators, Al was evidenced only in trichomes and epidermis and none in the mesophyll (Maejima et al. 2014).

Inferring physiological roles for the Al in the metabolism of these plants, however, is quite challenging. For example, *Vochysia thyrsoidea* (Vochysiaceae) and *Miconia albicans* (Melastomataceae) from the Cerrado exhibit leaf chlorosis and poor growth when cultivated in alkaline calcareous soils (Haridasan 2008). Recently, Al has been suggested to have some unknown role in chloroplasts, since it was observed in these organelles (Andrade et al. 2011).

Another dissension among researchers derives from the use of different dyes as Al-indicators. Most studies use hematoxylin (González-Santana et al. 2012; Andrade et al. 2011) and aluminon (Chenery 1948; Haridasan et al. 1986; Jansen et al. 2002), but it has been demonstrated that these dyes also react with Fe, Cu and Zn rather than reacting specifically with Al deposited in plant tissues. Here, we compared hematoxylin with chrome azurol S (CAS) and demonstrate that the latter is more efficient.

In addition, leaf Al concentration is not always concomitantly measured when investigating Al-accumulating plants, and micromorphology has not yet been used to study Al-accumulating plants from the Cerrado. Thus, besides confirming anatomical patterns of Al deposition in leaf tissues from these plants, in this paper we analytically quantified Al in leaves from some Al-accumulating and non-accumulating species growing in the field. As a novelty, we analyzed the micromorphology (Al-specific x-ray coupled to scanning electron microscope) to identify where Al-accumulating plants store Al in their leaves, and we hypothesized that Al cannot be evidenced in non-accumulating species.

Material and methods

Plant material

Leaves of Al-accumulating and non-accumulating plants were collected in the field. Al-accumulating plants were *Qualea grandiflora* Mart., *Q. parviflora* Mart. (Vochysiaceae), *Miconia albicans* (SW) Triana and *M. rubiginosa* (Bonpl.) DC (Melastomataceae), and the non-accumulating species were *Styrax ferrugineus* Nees & Mart. and *S. camporum* Pohl. (Styracaceae). These were adult trees (5–10 m tall) and shrubs (3–5 m tall) naturally occurring in cerrado *sensu stricto* (savanna-type physiognomy) remnants and in a forest-type physiognomy called ‘*Cerradão*’ (a more densely treed savanna, whose name is the augmentative of ‘Cerrado’ in Portuguese).

These species from Vochysiaceae and Melastomataceae have been described as Al-accumulating plants since 1982 (Haridasan 1982). The two Al non-accumulating species

from the Styracaceae family were chosen not only because they do not appear on the list of Al-accumulating plants (Haridasan 1982), but also because we knew previously (unpublished data) that they accumulate low amounts of Al in their leaves, which makes them good contrasting plants growing on the same soils as Al-accumulating plants do in the Cerrado.

Study sites

The field sites were located in the municipalities of Mogi-Guaçu, Itirapina and Corumbataí in São Paulo state, Brazil, in the southern part of the Cerrado vegetation in South America. In Mogi-Guaçu, plants were found in a cerrado *sensu stricto* remnant and also in a Cerradão area, on '*Campininha*' farm at the 'Reserva Biológica de Mogi-Guaçu' (22° 15′ 19″ S 47° 09′ 30″ W; 680 m of altitude; 340 ha), a Cerrado area that has been preserved in its natural condition since 1950.

In Itirapina, plants were found in a cerrado *sensu stricto* fragment (22°13′ S 47° 53′ W; 610 m of altitude; 260 ha) on 'São José da Conquista' farm; and in Corumbataí, plants were growing in a Cerradão fragment (22° 15′ S, 47° 00′ W; 860 m of altitude; 38.7 ha).

Experimental strategy

In each of these three sites, soil samples were collected at 20–30 cm of depth, where the uptake of nutrients takes place for most native plants (Wigley et al. 2013). Fertility parameters, but most importantly, soil Al saturation (Al%) was measured to support our results of leaf Al accumulation. We sought Al presence in leaf tissues using histochemical tests with different dyes. For this, leaves from the Al-accumulating and non-accumulating species were cut and stained with hematoxylin and CAS, and were compared to non-dye treated cuts. In addition, Al-specific x-ray spectra from different regions of leaf tissues were measured and analyzed by scanning electron microscopy (SEM).

Soil fertility and leaf Al concentration

Five soil samples were randomly collected in each site and taken to the Soil Science Lab at the University of São Paulo (Esalq, USP, Piracicaba, SP) for routine soil chemical (fertility) analysis (pH in $CaCl_2$), which was performed according to van Raij et al. (2001), and the procedures are described in English by Dantas and Batalha (2011).

In each of the three sites, at least five plants of each species available in the area were identified and had their leaves (leaf lamina with its petiole) collected. For this, each plant had its canopy subdivided into the four geographical quadrants (N, S, E and W) where mature fully expanded leaves were collected. The leaf samples (± 40 leaves per tree) were, then, taken to the Plant Nutrition Lab at the University of São Paulo (Esalq, USP, Piracicaba, SP) for analysis of Al concentration. The leaves were oven-dried at 60 °C to constant dry mass, ground and digested in a solution of sulfuric:nitric:percloric acids (1:10:2, v/v/v). After digestion, Al concentrations were determined by the atomic absorption spectrophotometer method (Sarruge and Haag 1974) and expressed as mg Al per kg dry leaves.

Anatomical studies

Some shoots collected from each plant were separated and taken freshly (moistened and put in a plastic bag) to the Plant Anatomy Lab at the São Paulo State University (Unesp) for the anatomical analyses of their leaves.

The leaves were fixed in FAA 50 (37 % formaldehyde, glacial acetic acid, 50 % ethanol; 1:1:18 v:v:v) and preserved in 70 % alcohol (Johansen 1940), according to Andrade et al. (2011). We also stained fresh tissues with both dyes, and the staining patterns were the same as those obtained when the plant material was fixed (FAA 50) and preserved (70 % alcohol). The anatomical study was based on consecutively-sliced cross sections from the same leaf segments (1 cm^2) from leaf midribs containing part of the leaf lamina for each species. These sections were cut manually with a razor blade. Non-dye treated cuts were immersed in distilled water for 15 min before mounting semi-permanent glass slides. Dye-treated cuts were stained for 45 min (at room temperature) as no staining difference was observed after 20, 30, 40 or 60 min in the staining solutions. These were washed thrice (15 min each) in distilled water and mounted in semi-permanent glass slides. All cuts were, then, observed under light microscope (DMLB, Leica Microsystems, Wetzlar, Germany). The images were captured with a digital camera (DFC-290, Leica Microsystems, Germany) functionally attached to the DMLB.

Chrome azurol S (CAS), or Mordant blue 29 (3″-sulpho-2″,6″-dichloro-3,3′-dimethyl-4-hydroxyfuchson-5,5′-dicarboxylic acid), 50 % purity (Sigma-Aldrich, St. Louis, MO, USA) solution was prepared dissolving 20 mL of a 41.3 mM CAS solution (25 g/L) into 80 mL of a 760 mM and 4.82 pH sodium acetate solution (Kukachka and Miller 1980). Therefore, CAS final concentration was 8.3 mM (5 g/L) (pH = 4.76 ± 0.01).

Hematoxylin (7,11b-dihydroindeno[2,1-*c*]chromene-3,4,6a,9,10(6*H*)-pentol), P.A. (Impex, Diadema, SP, Brazil) solution was prepared dissolving 80 mL of a 8.3 mM hematoxylin solution (2.5 g/L) into 20 mL of a 4.7 mM and 5.2 pH KIO_3 solution (Andrade et al. 2011). Therefore, hematoxylin final concentration was 6.6 mM (2 g/L) (pH = 5.8 ± 0.05).

Micromorphological studies

For the scanning electron microscopy (SEM), the leaf segments were fixed in a 2.5 % (v/v) Karnovsky solution (with 0.1 M phosphate buffer, at pH 7.3; overnight at 4 °C) and dehydrated in an increasing acetone series of 50, 70, 90, 95, and 2 × 100 %, kept for 15 min in each step, before mounting these leaf segments on stubs.

Stubs are also made of Al, which could interfere with the Al spectra emited from the plant material. Therefore, before mounting the plant material directly on stubs, we compared x-ray spectra obtained from stubs (with no plant material) when covered and not covered with two layers of a carbon tape (Double sided carbon tape, 8 mm in width; Electrom Microscopy Science, EMS, USA). We noted that the Al from stubs has no influence on spectra obtained from the plant material.

The images were obtained from a scanning electron microscope (TM 3000, Hitachi, Japan) operated at 15 kV, and the Al (1.48–1.55 keV), as well as silicon (Si; 1.73–1.83 keV) and calcium (Ca; 3.69–4.02 keV) detection was performed using an x-ray energy dispersive detector (Swift ED 3000, Hitachi, Japan). The counts were done over a 60-s period, spectra were recorded and qualitative data were expressed as counts to the second ratio (relative intensity).

The same plant material was compared under light microscopy and SEM to check the correspondence between images and their respective spectrum of emitted Al.

Results

Soils from the three experimental sites were acidic (pH < 4.0) with low concentrations of P, K, Ca and Mg, resulting in low BS. These soils also showed relatively low cation exchange capacity (CEC), while Al% was between 77 and 86 % (Table 1).

Nutritional analysis of leaves showed that the most Al-accumulating species were those from Vochysiaceae and Melastomataceae (*Q. parviflora* > *M. albicans* > *M. rubiginosa* > *Q. grandiflora*), whereas *S. ferrugineus* showed the lowest Al concentration in its leaves (Table 2). Surprisingly, *S. camporum*, which is not considered an Al-accumulating species, showed approximately 1000 mg Al per kg dry leaves.

Hematoxylin and CAS reacted positively with all Al-accumulating species (Figs. 1, 2, 3 and 4), but negatively with those considered non-accumulating ones (Figs. 5 and 6). For *M. rubiginosa*, hematoxylin stained in red (Fig. 1c, d), and CAS, in purple (Fig. 1e, f), exhibiting a clear contrast with non-dye treated cuts (Fig. 1a, b). The same colors were observed for *M. albicans* when stained with hematoxylin (Fig. 2c, d) and CAS (Fig. 2e, f) when compared with its non-dye treated cuts (Fig 2a, b). However, CAS contrasted the positive and negative reactions in the midrib and leaf lamina more intensely when compared to hematoxylin (Figs. 1 and 2). When hematoxylin was used, intercellular spaces and cell walls of both the parenchyma and collenchyma of the midrib (Fig. 2d) did not react as clear as when CAS was used (Fig. 2f).

We observed positive reactions on cell walls of epidermal surfaces, phloem, parenchyma, and collenchyma of the midrib (Table 3). Positive reactions were evident in vacuoles of the parenchyma of the midrib (Fig. 1f, 3d and 4b), and these reactions also showed Al as content of phloem cells (Fig. 1d and f). Cell walls of both the xylem and sclerified cells reacted negatively (Table 3) with hematoxylin (Fig. 1d and 2d) and CAS (Fig. 1f, 2f, and 3d).

Chrome azurol S also stained phloem cell walls in the vascular bundle of *M. rubiginosa* (Fig. 3b). The palisade parenchyma stained in red when treated with CAS, contrasting with its purple positive reaction noticed for the spongy parenchyma (Fig. 3b). We observed no reactions with the internal contents of chloroplasts.

Table 1 Soil fertility indexes in the soil (20–30 cm in depth) of Cerrado areas where the leaves were sampled

Site	pH	P	S	K^+	Ca^{2+}	Mg^{2+}	Al^{3+}	H^++Al^{3+}	CEC	BS	BS%	Al^{3+}%	BO_3^-	Cu^{2+}	Fe^{2+}	Mn^{2+}	Zn^{2+}
		mg dm^{-3}		mMol$_{charges}$ dm^{-3}							%						
Itirapina	3.8 ± 0.1	1.3 ± 0.58	*	< 0.7	< 3.0	< 1.0	7.7 ± 0.57	33.3 ± 4.0	34.2 ± 4.2	1.2 ± 0.1	3.7 ± 0.57	86.3 ± 0.57	<0.12	0.53 ± 0.23	47.3 ± 8.02	1.17 ± 0.40	< 0.4
Corumbatai	3.8 ± 0.1	2.0 ± 1.0	*	< 0.7	< 3.0	< 1.0	9.67 ± 2.5	46.3 ± 12.0	49.0 ± 11.6	2.7 ± 0.6	6.0 ± 1.73	77.3 ± 6.65	0.18 ± 0.00	0.93 ± 0.15	87.3 ± 37.5	1.13 ± 0.20	0.47 ± 0.06
Mogi-Guaçu	3.7 ± 0.1	3.3 ± 0.58	*	< 0.7	< 3.0	< 1.0	13.3 ± 2.3	54.0 ± 3.4	57.1 ± 3.1	3.1 ± 1.6	5.7 ± 2.88	81.7 ± 8.38	0.21 ± 0.00	1.60 ± 0.79	69.0 ± 35.5	16.2 ± 13.75	< 0.4

CEC Cation exchange capacity, *BS* Base saturation

* Sulfur not determined

The Al and Si x-ray spectra obtained from phloem cell walls of *M. rubiginosa* (spectrum 1 in Fig. 3e), which reacted positively with CAS (Fig. 3d), were more prominent (Fig 3f) than those obtained from thicker cell walls of the parenchyma of the midrib (spectrum 2 in Fig. 3e), reinforcing the phloem as an important Al-accumulating site. This points out CAS as an efficient indicator of Al in leaf tissues. In addition, positive reaction with CAS was noted in the epithelium of a secretory cavity in *M. rubinosa* (Fig. 3c).

Remarkable structures were noted for Al-accumulating and non-accumulating plants. For instance, we observed cavities of 50–100 μm in diameter in the parenchyma of leaf midribs of *Q. parviflora* (Fig. 4a-b), which did not stain with CAS. When thicker cuts of the same plant material were analyzed by SEM, these cavities seemed to be filled with solid granules (Fig. 4c). In addition, when Al and Si x-ray spectra obtained from these granules (spectrum 2 in Fig. 4c) were compared to those from a xylem cell wall (spectrum 4 in Fig. 4c), these granules revealed conspicuous x-ray emission intensities (Fig. 3d).

In contrast to our prediction, granules were also embedded in the collenchyma of leaf midribs of *S. camporum*, although these were smaller than those from Al-accumulating species and only observed by SEM (Fig. 5c, d). Therefore, these structures could not react with CAS (Fig. 5b). However, the Al and Si x-ray spectra emission intensities obtained from these granules were not as conspicuous (spectrum 1 in Fig. 5e) as those from *Q. parviflora* (spectrum 2 in Fig. 4d). We also observed some storage structures in *S. ferrugineus*'s leaf midribs (Fig. 6c-d), which did not react with CAS either (Fig. 6b). These structures showed negligible Al-specific x-ray emission intensity (spectrum 1 in Fig. 6e) when compared to those found in granules from *Q. parviflora* (spectrum 2 in Fig. 4d). However, conspicuous Ca-specific emission intensity was observed from these structures in *S. ferrugineus* (spectrum 1 in Fig. 6e), which characterize them as druses (calcium crystals).

Discussion

Our results show that CAS gives sharper contrasting results compared to hematoxylin when reacting with Al in plant tissues. Aluminon has long been used to identify Al-accumulating plants by the intensity of colors (Chenery 1948; Jansen et al. 2002) produced by

Table 2 Aluminum concentrations in leaves of Al-accumulating and non-accumulating species growing in the studied Cerrado areas

Family	Species	Site	Al concentration mg kg^{-1}
Vochysiaceae	*Q. grandiflora*	Itirapina	3833.4 ± 691.1
		Mogi-Guaçu	4035.4 ± 2439.7
		Corumbataí	5442.1 ± 804.3
	Q. parviflora	Mogi-Guaçu	9876.3 ± 1004.9
Melastomataceae	*M. albicans*	Itirapina	3116.7 ± 1463.3
		Mogi-Guaçu	8628.8 ± 1185.8
	M. rubiginosa	Itirapina	2284.2 ± 242.6
		Mogi-Guaçu	5457.8 ± 499.5
Styracaceae	*S. ferrugineus*	Itirapina	300.9 ± 48.6
		Mogi-Guaçu	218.2 ± 16.5
	S. camporum	Corumbataí	1366.9 ± 338.8

the reaction between this dye and Al-rich plant material. For *Q. grandiflora*, hematoxylin has already been used to identify Al in the mesophyll (Andrade et al. 2011). In fact, different dyes (ferron, morin, pyrocatechol violet, aluminon, CAS and hematoxylin) can detect monomeric Al (Al^{3+}) (Wehr et al. 2010). Pyrocatechol violet,

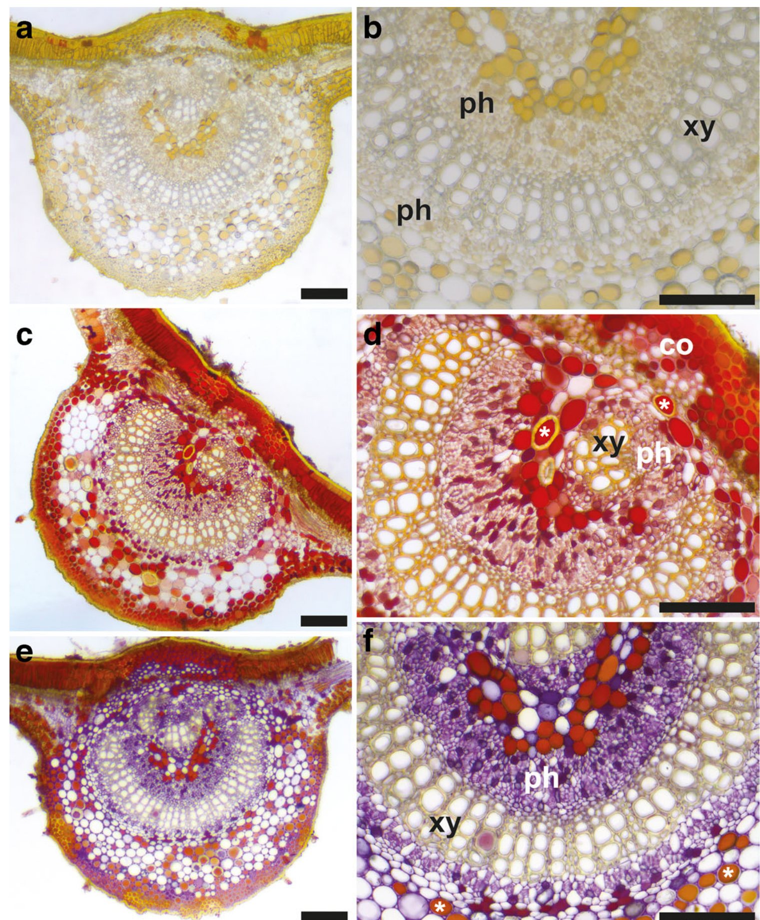

Fig. 1 General aspects (**a**, **c** and **e**) and respective details (**b**, **d** and **f**) from serial cross-sections of leaf midribs of *Miconia rubiginosa*. **a** and **b**: non-stained; **c** and **d**: stained with hematoxylin; **e** and **f**: stained with chrome azurol S. co = collenchyma; ph = phloem; xy = xylem; * = sclerified cells. Scale bars: **a**, **c** and **e** = 200 μm; **b**, **d** and **f** = 100 μm

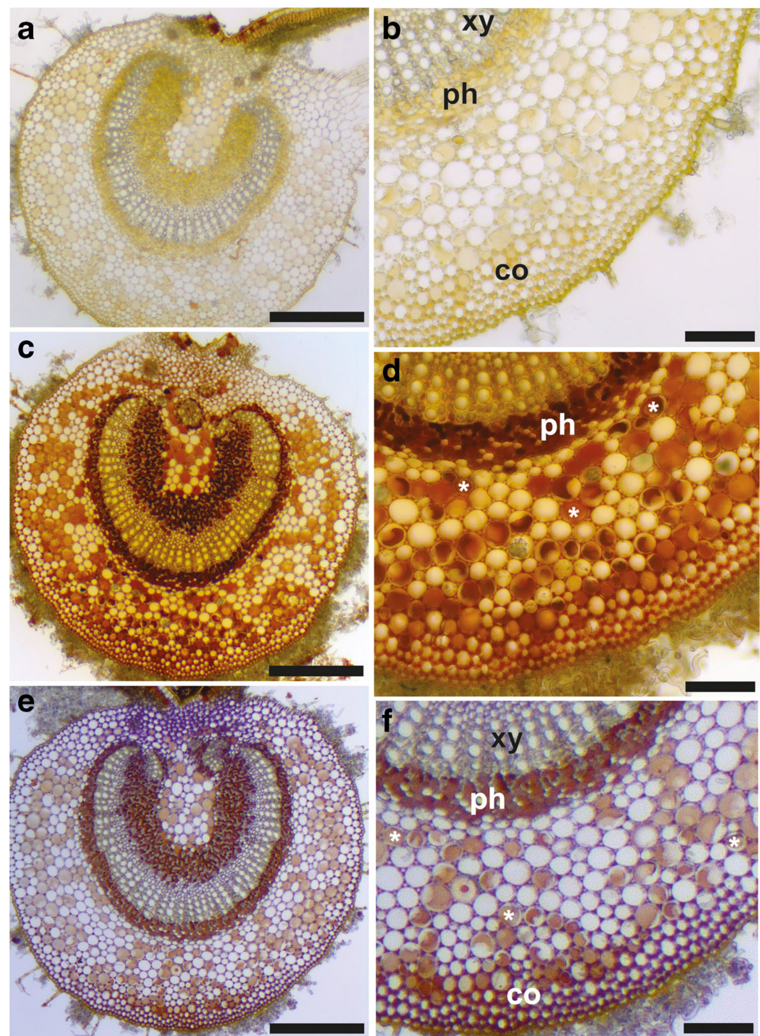

Fig. 2 General aspects (**a**, **c** and **e**) and respective cortex details (**b**, **d** and **f**) from serial cross-sections of leaf midribs of *Miconia albicans*. **a** and **b**: non-stained; **c** and **d**: stained with hematoxylin; **e** and **f**: stained with chrome azurol S. co = collenchyma; ph = phloem; xy = xylem; * = sclerified cells. Scale bars: **a**, **c** and **e** = 200 μm; **b**, **d** and **f** = 100 μm

however, also react with Fe, Cu and Zn (González-Santana et al. 2012), and hematoxylin is also known to react with Fe salts. Chrome azurol S can also react with Fe, Cu and Zn. However, in acidic solutions (pH < 5.0) these reactions are minimized. The interference of Fe in Al-CAS complexes is much larger at pH 5.1 than at pH 4.6 (Pakalns 1965). For this same reason, a Na-acetate buffer solution is recommended when using CAS as an Al indicator (Wehr et al. 2010), and the CAS solution we used showed 4.76 ± 0.01 pH.

On plant cell walls, small Al-hydroxy species, which are present on pectins and other cell wall compounds (Wehr et al. 2010), can be indicatively stained by CAS (Kennedy and Powell 1986). The color given by the reaction between an Al indicator and the tissue is also important and dependent on the pH of the solution, but the highest absorbance of Al-CAS complexes is observed within 470–550 nm, regardless of the pH in the 3.0–5.0 pH range, where the indicative color of Al presence is purple (Wehr et al. 2010), the same color we observed in Al-accumulating plants. Chrome azurol S has been used even to estimate Al concentration in plant material (Jansen et al. 2000). The use of CAS is considerably more reproducible and selective than aluminon (Pakalns 1965). Therefore, we suggest CAS and not hematoxylin to anatomically observe Al accumulation in plant tissues.

Our results also show that Al is anatomically associated with tissues composed mainly by primary cell walls (phloem, collenchyma, epidermis and parenchyma) and hematoxylin, but most importantly, CAS does not stain tissues containing lignin,

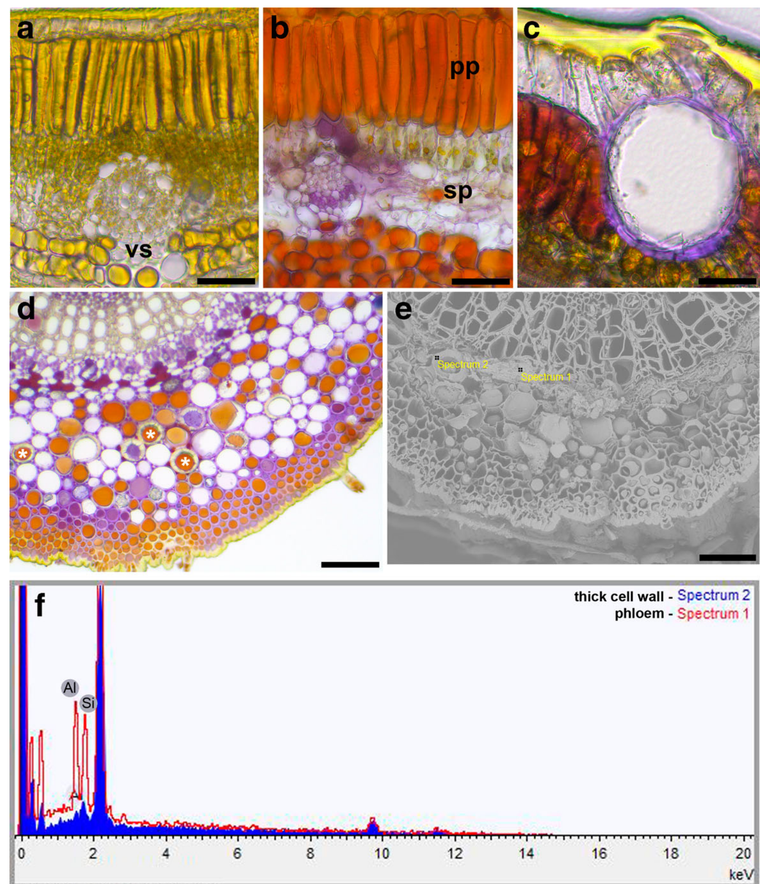

Fig. 3 Anatomical analyses from cross-sections of leaf lamina (**a**, **b** and **c**) and abaxial midrib (**d**) of *M. rubiginosa* performed under light microcopy (**a**, **b**, **c** and **d**) and SEM (**e**). **a**: non-stained; **b**, **c** and **d**: stained with chrome azurol S. **vs** = vascular sheath; **pp.** = palisade parenchyma; **sp.** = spongy parenchyma; * = sclerified cells; **e**: Al- and Si-specific energy-dispersive x-ray spectra from the phloem and a thick cell wall in the cortical parenchyma. Scale bars: **a**, **b** and **c** = 50 μm; **d** = 100 μm; **e** = 30 μm

such as xylem and sclerified cells. Moreover, Al is anatomically associated with the middle lamella, as CAS x Al positive reaction was evidenced in the parenchyma of the midrib of *M. albicans* (Fig. 2f). This indicates that the lack of pectin may be a possible impairment of Al biding to the cell wall, corroborating Wehr et al. (2010). The cell wall of both epidermal surfaces seems to be the primary site for Al accumulation in leaves. This pattern was already reported for other Al-accumulating plants not from the Cerrado, such as *Faramea marginata* [Rubiaceae (Britez et al. 2002)], *Camellia sinensis* [Theaceae (Carr et al. 2003; Tolrà et al. 2011)] and *Conostegia xalapensis* [Melastomataceae (González-Santana et al. 2012)]. Pyrocatechol violet stains the cell wall of the spongy parenchyma, but not the sclerenchyma or the xylem of *F. marginata* (Britez et al. 2002). In *Melastoma malabathricum*, *Tibouchina urvilleana* (Melastomataceae) and *Symplocos chinensis* (Symplocaceae) grown under 500 μM Al, pyrocatechol violet stained trichomes and leaf epidermis (Maejima et al. 2014) whose cells do not contain lignin.

Although Haridasan et al. (1986) already reported the above-mentioned patterns of Al-tissue reaction with aluminon in Al-accumulating plants from the Cerrado, evidence for physiological function(s) of Al in the metabolism of these plants is sought. As these authors showed the positive reaction of Al in the phloem of leaf midribs of these plants, and later found significant amounts of Al in seeds of Al-accumulating plants (Haridasan 2008), a phloem transport mechanism for Al has been proposed. Using cryo-fixation of plant material, which avoids retranslocation of elements during sample preparation for observation with low energy x-ray fluorescence spectro-microscopy, Tolrà et al. (2011) were able to confirm Al storage in the leaf phloem of *C. sinensis*. These findings strongly suggest that Al-citrate, Al-malate or Al-oxalate (Brunner and Sperisen 2013) flowing through the xylem sap (Watanabe and

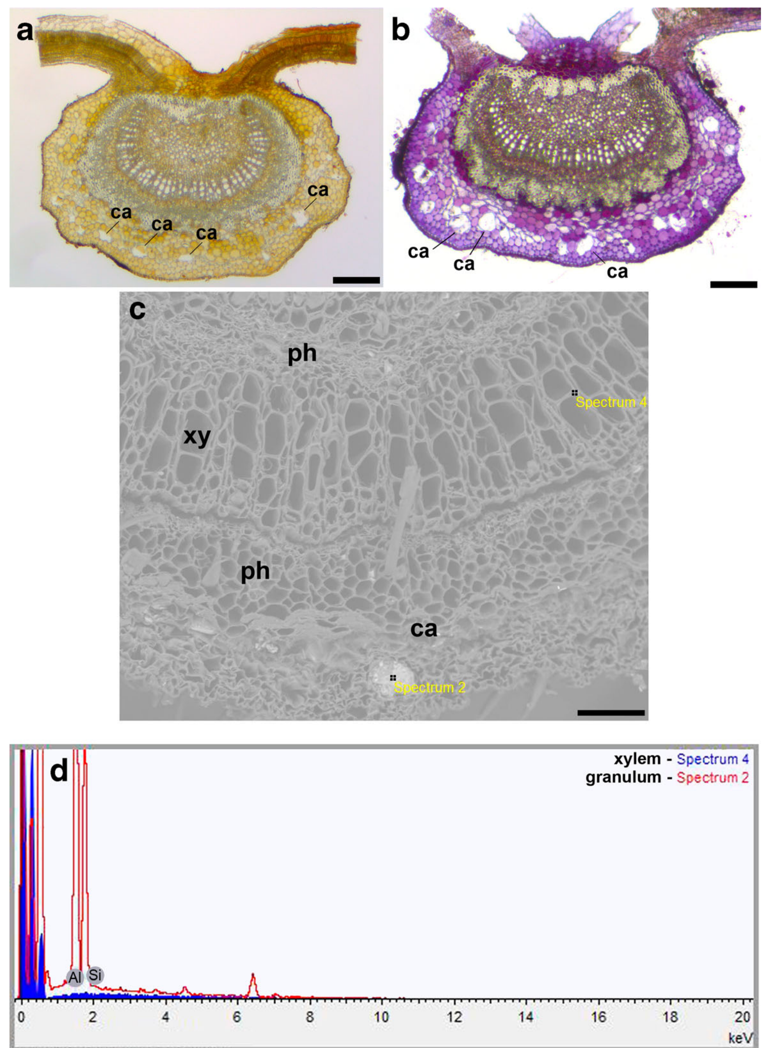

Fig. 4 Anatomical analyses from cross-sections of leaf midribs (**a**, **b** and **c**) of *Q. parviflora* performed under light microscopy (**a** and **b**) and SEM (**c**). **a**: non-stained; **b**: stained with chrome azurol S; ca = cavities; **d**: Al- and Si-specific energy-dispersive x-ray spectra from the xylem and a granule in the cortical parenchyma. Scale bars: **a** and **b** = 200 μm; **c** = 50 μm

Osaki 2001) reaches the leaves through the transpiration stream (Shen and Ma 2001) and moves symplastically, eventually reacting with pectins (Wehr et al. 2010) on the phloem cell wall. However, such similar mechanism was not yet evidenced for Al-accumulating species from the Cerrado due to a lack of semi-controlled studies with these plants growing on non-Al contaminant soils and Al-rich soils.

In contrast to Andrade et al. (2011), who observed hematoxylin staining of the palisade leaf parenchyma with staining density among the chloroplasts of *Q. grandiflora*, our results show no hematoxylin or CAS (positive) reactions with this tissue in leaves of this species, which is also in accordance with Haridasan et al. (1986). This unusual observation made by Andrade et al. (2011) for Al-accumulating plants is unique in the recent literature (Brunner and Sperisen 2013). Furthermore, we found no positive reaction of any dye with the internal contents of chloroplasts.

The Al-constitutive granules observed into cavities of the parenchyma in the midrib of the Al-accumulating plant, *Q. parviflora* (Fig. 4b, c), *and of the non-accumulating species*, *S. camporum* (Fig. 5d), suggest that Al can be evidenced in both groups of plants from the Cerrado, and this invalidates our hypothesis that Al can be observed only in Al-accumulating species. Although detectable with SEM/EDS in *Q. parviflora* (Fig. 4d), Al was hardly stained by CAS in the cavities (Fig. 4b). This occurs probably because CAS does not stain Al not bound to the cell wall, such as in

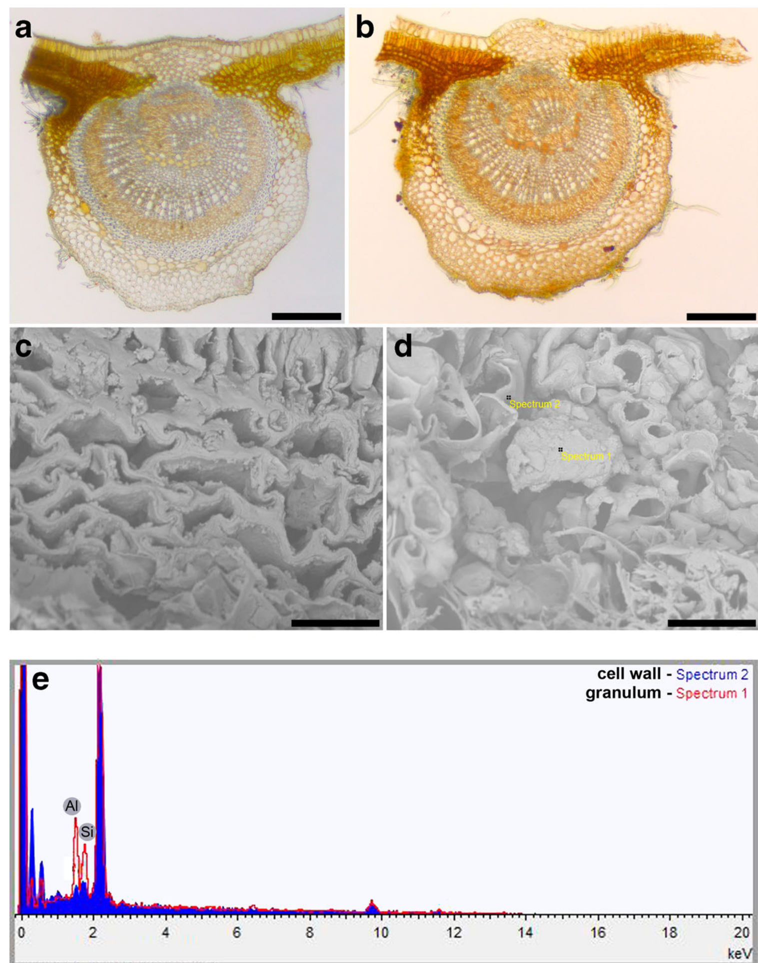

Fig. 5 Anatomical analyses from cross-sections of leaf midribs of *S. camporum* performed under light microscopy (**a** and **b**) and SEM (**c** and **d**). **a**: non-stained; **b**: stained with chrome azurol S. **c**: general view of the collenchyma region, evidencing small granules; **d**: details from the same region of the collenchyma showing a granule in an amplified view; **e**: Al- and Si-specific energy-dispersive x-ray spectra from a granule and from a collenchyma cell wall. Scale bars: **a** and **b** = 200 μm; **c** = 15 μm; **d** = 45 μm

granules, and CAS positive reaction is dependent on pectin-Al complexes in order to stain in purple at the correct pH (Wehr et al. 2010). In the case of *S. camporum*, granules were not observed under light microscopy, and CAS did not stain the plant material (Fig. 5b), although Al was detected by SEM/EDS in granules (Fig. 5d, e). Nevertheless, *S. camporum* showed more than 1300 mg Al per kg dry leaves (Table 2), and it could be reclassified as an Al accumulator. According to Chenery (1948), plant species showing at least 1000 mg Al per kg dry leaves or shoots can be considered Al accumulators.

These Al-constitutive granules observed in *Q. parviflora* and *S. camporum* may suggest a structural rather than functional role of Al in leaves of Cerrado plants. The Al concentration in mature leaves of *M. albicans*, *M. rubiginosa*, *M. fallax* and *Q. grandiflora* increases by more than 320 % in relation to their young leaves (Souza et al. 2015), suggesting that the granules we observed may be an effect of Al accumulation during leaf expansion, rather than existing structures in these species. On the other hand, we cannot tell whether plants of the same species not exposed to Al also show these cavities. Studies using these native species cultivated hydroponically in order to access their responses to Al are extremely rare.

Granules associated with Al and Si, as evidenced by specific x-ray emission intensities in the Al-accumulating plants may also suggest that these plants attempt, somehow, to isolate the Al from metabolism. Not only granules associated with Al and Si, as found in *Q. parviflora* (Fig. 4d) and *S. camporum* (Fig. 5e), but non-granular Al and Si association was also found in the phloem of *M. rubiginosa* (Fig. 3e, f).

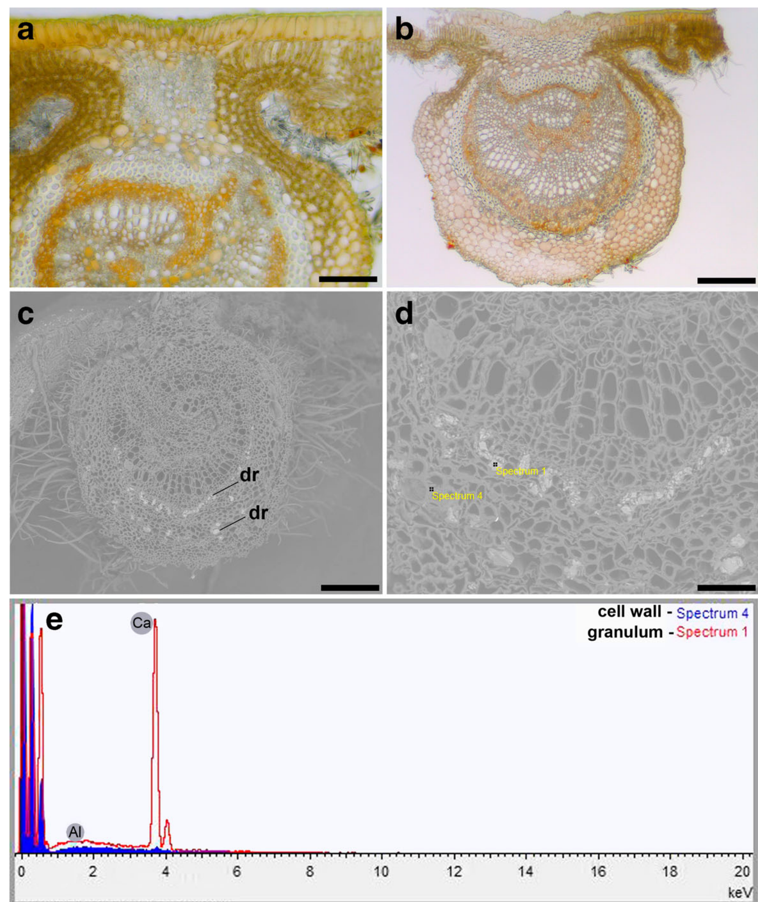

Fig. 6 Anatomical analyses from cross-sections of leaf midribs of *S. ferrugineus* performed under light microscopy (**a** and **b**) and SEM (**c** and **d**). **a**: non-stained; **b**: stained with chrome azurol S; **c**: general view of the central midrib; **d**: details from the same region of the central midrib showing calcium oxalate crystals - druses. **e**: Al- and Ca-specific energy-dispersive x-ray spectra from calcium crystals and a cell wall in the cortical parenchyma. **dr** = druses. Scale bars: **a** = 100 μm; **b** = 200 μm; **c** = 165 μm; **d** = 50 μm

Precipitating Al with Si in the apoplast or symplast would constitute a detoxification mechanism since the aluminosilicate and solid Al-Si compounds are considered to be less toxic than monomeric Al (Al^{3+}) (Britez et al. 2002), which can react with pectins on the cell wall (Wehr et al. 2010) and impair cell development. Si-associated grains, usually secreted into cavities, were already observed in Vochysiaceae and Styracaceae (Metcalf and Chalk 1989). But these authors did not risk assigning any physiological role to these grains. In the case of non-granular Si and Al association in the phloem (of *M. rubiginosa*), we cannot tell whether the binding to Si is involved in Al abundance in this tissue because we did not analytically measure Si in the plants and could not calculate Si:Al ratios. Moreover, there is no information about *M. rubiginosa* or Melastomataceae being Si-accumulator, and this should also merit further studies. Silicon and Al seem to be associated in *Faramea marginata* (Rubiaceae) (Britez et al. 2002), but no functional roles are described for these salts either.

One could still argue that the granules we observed filling the cavities in *Q. parviflora* (Figs. 4b, c) could be possible contamination or solidified artifact due to its fixation process or sectioning during the preparation of SEM. However, the Al and Si x-ray spectra emission from these granules were considerably prominent (Fig. 4d), not suggesting them as artifacts, contamination or methodological errors. Andrade et al. (2011) also showed these cavities in *Q. grandiflora* and *Callisthene major* (Vochysiaceae), although these authors did not explore the cavities' contents. These same cavities were also described for *Q. grandiflora* (unpublished data - MSc. Dissertation, Renata Cristina Costa e Silva, University of Brasilia, UnB, Brazil). Our study is the first report suggesting that these cavities contain Al- and Si-rich granules. Therefore, further investigation is necessary.

Table 3 Patterns of hystochemical reactions in the mesophyll of Al-accumulating and non-accumulating species growing in the studied Cerrado areas

Dye	Leaf structure	Species	Cuticle	Epidermal wall		Xylem	Phloem	Parenchyma	Collenchyma	Sclerified cells	Mesophyll	
				Adaxial	Abaxial						Palisade	Spongy
Chrome-Azurol	Central midrib	*M. albicans*	−	+	+	−	+	+	+	−		
		M. rubiginosa	−	+	+	−	+	+	+	−		
		Q. grandiflora	−	+	−	−	+	−	+	−		
		Q. parviflora	−	+	+	−	+	+	+	−		
		S. camporum	−	−	−	−	−	−	−	−		
		S. ferrugineus	−	−	−	−	−	−	−	−		
	Leaf lamina	*M. albicans*	−	+	+	−	+				−	+
		M. rubiginosa	−	+	+	−	+				−	+
		Q. grandiflora	−	+	+	−	+				−	+
		Q. parviflora	−	+	+	−	+				−	+
		S. camporum	−	−	−	−	−				−	−
		S. ferrugineus	−	−	−	−	−				−	−
Hematoxylin	Central midrib	*M. albicans*	−	+	+	−	+	+	+	−		
		M. rubiginosa	−	+	+	−	+	+	+	−		
		Q. grandiflora	−	+	−	−	+	−	+	−		
		Q. parviflora	−	+	+	−	+	+	+	−		
		S. camporum	−	−	−	−	−	−	−	−		
		S. ferrugineus	−	−	−	−	−	−	−	−		
	Leaf lamina	*M. albicans*	−	+	+	−	+				−	+
		M. rubiginosa	−	+	+	−	+				−	+
		Q. grandiflora	−	+	+	−	+				−	+
		Q. parviflora	−	+	+	−	+				−	+
		S. camporum	−	−	−	−	−				−	−
		S. ferrugineus	−	−	−	−	−				−	−

(+) = positive reactions

(−) = negative reactions

In angiosperms, crystals are formed by calcium oxalate and carbonate, but when Ca is low or not available in the soil as to form crystals, it may be replaced with other element (Metcalf and Chalk 1989), which might occur for Al-accumulating and non-accumulating plants. Notwithstanding, these mechanisms (Si and Ca grains associated with Al) still do not explain the Al-dependence of some species in the Cerrado (Haridasan 2008).

In conclusion, we emphasize the high efficiency of CAS as an Al dye when compared to hematoxylin. In addition, our results reinforce that the primary cell wall is the main site for Al accumulation in these plants. We show that Al- and Si-constitutive granules are embedded on the cell wall of non-lignified leaf tissues of both Al-accumulating and non-accumulating species. Therefore, our results obtained from the few Al-accumulating species available in the Cerrado challenge the possibility that the Al might have a physiological function in these plants. Being found on the (primary) cell wall and as granules decreases a possible physiological role for Al in leaves of Cerrado woody plants. These findings also suggest that the Al is accumulated in these sites as a possible mechanism to detoxify Al in leaves of these plants.

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