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Al-hyperaccumulator Vochysiaceae from the Brazilian Cerrado store aluminum in their chloroplasts without apparent damage

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ABSTRACT

We investigated the pattern of aluminum (Al) accumulation in leaf tissues of native hyperaccumulator Vochysiaceae species *Qualea grandiflora*, *Callisthene major*, and *Vochysia pyramidalis*, from the Brazilian Cerrado. Non-accumulator *Sclerolobium paniculatum* was used as a control species. We expected a strong compartmentalization of Al in non-active leaf cell compartments such as cell walls and vacuoles in Al-accumulating species and the absence of Al in critical metabolic sites such as the chloroplasts. Plant leaves were harvested in the field and cut in small segments for histological analysis; hematoxylin dye was used for Al localization in tissues. Results of soil analysis of the three sites and the concentration of Al in leaves indicated that there is no direct relationship between Al availability in soils and Al hyperaccumulation among the Vochysiaceae species evaluated. The cross-sections of leaf tissues showed hematoxylin color in the palisade and spongy parenchyma cells (chloroplast) of *Q. grandiflora* and *C. major*. The vascular system of *Q. grandiflora* was not colored, but some cells from the xylem region of *C. major* were stained. In contrast, the adaxial and abaxial epidermal cells of *V. pyramidalis* were colored by hematoxylin, as were some cells from the vascular bundle, but color formation was not observed in the cells of palisade parenchyma. Al was not detected in leaves of *S. paniculatum*. We concluded that, although hyperaccumulation of Al is a common trait in the Vochysiaceae family, the processes of storage and detoxification in leaf tissues differ among the species. Two of the three hyperaccumulator species use chloroplasts as a sink for Al, with no apparent signs of toxicity. Therefore, the physiological role of Al in plant tissues remains to be elucidated.

1. Introduction

Most common aluminum (Al)-tolerant plants are termed Al excluders because they usually restrict Al to the symplasm of root cells and, therefore, prevent its accumulation in aboveground tissues (less than 0.1 mg Al kg^{-1} DW). The physiological mechanisms for this feature are mostly related to exudation of organic acids (malate, citrate), which present a high affinity for binding to Al in the root cell apoplasm or in the rhizosphere (Ma et al., 2001). However, the levels of Al tolerance in such species, usually crop plants, are relatively low when compared to the levels observed in wild species that originate from soils with high levels of soluble Al (Branquinho et al., 2007).

Native communities in savannas and tropical rain forests are rich in species that have developed and inherited survival strategies for coping with the restricting soil-edaphic conditions, such as high soil acidity, high Al saturation, and low nutrient availability. Among those communities, a few species (typically woody and perennial) developed features of high Al accumulation in leaves, with levels exceeding 1,000 mg Al kg^{-1} DW. These species are also called Al hyperaccumulators and they are frequent in the Euphorbiaceae, Myrtaceae, Rubiaceae, Melastomataceae and Vochysiaceae families (Cuenca et al., 1991; Jansen et al., 2002a,b). Recently, Olivares et al. (2009) reported Al-accumulators species in ferns from the Lycopodiaceae, Gleicheneaceae and Cyatheaceae families, native in acid soils in the Neotropics of Venezuela.

Hyperaccumulation of metals, predominantly heavy metals, is a trait present in over 450 species across families, orders and genera of vascular plants (Maestri et al., 2010). The trait of hyperaccumulation of metals is reported to be non-randomly distributed and

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rather common in certain plant clades or families (Broadley et al., 2001). In the case of Al hyperaccumulation, the remarkable review by Jansen et al. (2002a,b) indicated that this trait has no significance at higher taxonomic levels (due to the lack of reliable markers), but does have significance in a few taxa. In the Vochysiaceae family, the trait of Al hyperaccumulation is reported to be present in all species (Jansen et al., 2002b). The Vochysiaceae family occurs predominantly in the Neotropical regions, with five genera: *Vochysia*, *Erisma*, *Qualea*, *Callisthene* and *Salvertia*. Only one genus of this family occurs in the old world (Africa): *Erismadelphus* (Passos and França, 2003). This botanic family is an important component of the Cerrado flora, a savannah-type vegetation that has developed on acidic soils in the central region of Brazil. Several species are also found in the adjacent gallery forests, which form corridors along streams and river basins within the savannah.

Aluminium accumulation and nutrient uptake have been described for some species in the Cerrado (see review of Haridasan, 2008). In the first study, Haridasan (1982) showed that Al hyperaccumulation was present in the following families: Vochysiaceae (*Qualea grandiflora*, *Q. parviflora*, *Q. multiflora*, *Vochysia thyrsoidea*, *V. elliptica*), Melastomataceae (*Miconia ferruginata*, *M. pohliana*), and Rubiaceae (*Palicourea rigida*). Due to the high concentration of Al in these species, reports have suggested beneficial effects of Al in the development of *Miconia albicans* (Melastomataceae), *Q. multiflora* and *V. thirsoidea* (Vochysiaceae). Such beneficial effects would include growth stimulation and increased uptake of some nutritional elements in the presence of Al (Haridasan, 2008). A similar response to Al was described for *Melastoma malabathricum*, an Al-hyperaccumulator species that inhabits tropical rainforests in Asia (Watanabe et al., 1998).

Although there are few reports about the physiological mechanisms related to Al hyperaccumulation in plants, the available results indicate (i) the lack of a root barrier for Al absorption (Watanabe et al., 1998), (ii) Al transport in the xylem stream, as a result of transpiratory flux (Cuenca et al., 1990), (iii) Al redistribution predominantly through phloem elements, resulting in Al accumulation in cell walls (Haridasan et al., 1986), (iv) the formation of stable complexes with organic compounds (Al-oxalate) (Watanabe et al., 1998, 2005; Watanabe and Osaki, 2001) and inorganic ligands (Al–Si) (Britez et al., 2002) in cytoplasm, or (v) compartmentalization in apoplasm or vacuoles, where Al does not interfere with the metabolic activities of the cell (Watanabe et al., 1998; Ma, 2007).

The objective of this work was to investigate the pattern of Al accumulation in the leaf tissues of three Al-hyperaccumulator species that belong to the Vochysiaceae family (i.e. *Callisthene major*, *Q. grandiflora*, *V. pyramidalis*) and are native in the acidic soils of Brazilian Cerrados. These species inhabit different plant formations of Cerrado, differing in soil-edaphic conditions and Al availability. Moreover, the Al-accumulator species of this study are the most important and dominant Al-accumulator species of those environments (Andrade et al., 2002; Oliveira and Felfili, 2005; Nascimento et al., 2004). We expected a strong compartmentalization of Al in non-active leaf cell compartments such as cell walls and vacuoles in Al-accumulating species and the absence of Al in critical metabolic sites such as the chloroplasts.

2. Materials and methods

2.1. Natural environment

The plant material was collected from four species present in delimited areas of approximately 20 m × 30 m dimension, in their native environment. These sites were chosen according to the uniformity of the phytophysiognomy and soil characteristics, e.g. Site 1: Cerrado *sensu stricto* on dark red Latossol; Site 2: gallery forest on hydromorphic soil; and Site 3: semideciduous forest on Cambisol. The sites were located in the Federal District of Brazil, in natural reserves at the Embrapa Cerrados Research Center and the Biological Station of Água Emendadas (SEMARH), both located in Planaltina, and in a private natural reserve located in Sobradinho. The species evaluated in this study and their respective sites were *V. pyramidalis* Mart., present at Site 1 (15°35′0.16″S; 47°36′27.7″W); *Sclerolobium paniculatum* Vogel and *Q. grandiflora* Mart., at Site 2 (15°36′13.5″ S; 47°42′0.20″W); and *C.* major Mart., at Site 3 (15°30′38.0″S; 47°57′46.7″W).

For chemical characterization of the soils at the study sites, soil samples were collected at close vicinity to the roots of the targeted plants, from a 0- to 20-cm soil layer. Soil samples were analyzed for organic matter, pH_W (1:2.5), potential acidity (H + Al) extracted by 0.01 M calcium acetate at pH 7, exchangeable Ca, Mg, Al extracted by 1N KCl; and exchangeable K by the Mehlich-1 (0.05 M HCl + 0.0125 M H_2SO_4) extractant, according to Embrapa procedures (1999).

2.2. Sample collection for Al content determination and Al localization in plant tissues

We monitored leaf phenology for the four studied species along the year of 2005. Plants started to flush during the final days of dry season (August–September). The samples for determination of Al and nutrients content in tissues and for histological analysis were collected in December, in the middle of wet season, when about 60% of the tree leaves of the four species were in early maturity stages. We also sampled leaves from the subsequent dry season in July 2006, when the majority of leaves were in senescent stages. We sampled leaves from the edge of the tree crown, on four individuals of each species. For the histological analysis, the leaves selected were the first pair of fully expanded leaves (between third and fourth node). All sampled leaves came from adult individuals (tall enough to present reproductive phases).

Al and nutrients content in dry matter were determined in leaves dried at 70 °C, grind and digested using with a mixture of concentrated $HClO_4$ and H_2O_2 and analyzed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Thermo Jarrel Ash IRIS/AP), according to Embrapa procedures (1999).

2.3. Hematoxylin staining of leaf tissues

Fully expanded leaves were collected for light microscopy. The leaf blade and central vein were cut into 0.2 cm × 0.5 cm pieces and immediately fixed with FAA (5% formaldehyde [37%], 5% glacial acetic acid, 60% ethanol, 30% H_2O (v/v), for 24 h. Next, the samples were dehydrated in graded ethanol series and embedded in paraplast (Paraplast X-TRA, Oxford Labware) resin. Sections of 10-μm thickness were cut using a Leica rotary microtome and placed on 2% 3-thethoxysilylpropylamine (TESPA) slides. The paraplast was removed from the sections by xylene and rehydrated in solutions of decreasing ethanol concentration. Finally, the slides with cross-sections were totally immersed in hematoxylin solution (2.0 g of hematoxylin + 0.2 g of IO_3K/l) at room temperature, for 40 min and washed several times in distilled water. After staining, the slides were air-dried and mounted in Permount, examined with a Zeiss Axiophot light microscope and then photographed. Al localization in the tissues was represented by a purple staining that is typical of Al reaction with hematoxylin. Hematoxylin reacts with Al (III) species and the resulting Al–hematoxylin complex forms a purple color (Baker, 1962).

The microscope slides for histological analysis were prepared at Microscope Laboratory in Embrapa Recursos Genéticos e Biotecnologia, in Brasilia (DF, Brazil).

Table 1
Al content in soils and in leaves of native species from the Cerrado.

Site	Species	Al in leaves[a]		Soil characteristics[b]										
		December/2005	June/2006	pH	O.M.	Al	Ca	Mg	K	P	CECeff.	CEC pH7	V	m
		g kg^{-1} DW			g dm^{-3}		cmol$_c$ dm^{-3}			mg dm^{-3}	cmol$_c$ dm^{-3}			%
(1) Cerrado *sensu stricto*	*S. paniculatum*	0.1 (0.0)	0.2 (0.0)	4.8 (0.0)	41.7 (6)	0.74 (0.2)	0.05 (0.0)	0.06 (0.0)	0.09 (0.0)	0.3 –	0.94 (0.3)	8.3 (1.1)	2.3 (0.8)	78.7 (3.7)
	Q. grandiflora	3.9 (0.7)	5.0 (1.1)	4.9 (0.1)	39.9 (4)	0.75 (0.1)	0.08 (0.0)	0.05 (0.0)	0.08 (0.0)	<0.1 –	0.96 (0.1)	7.8 (0.2)	2.6 (0.3)	78.2 (1.7)
(2) Gallery forest	*V. pyramidalis*	5.8 (0.7)	7.3 (0.7)	5.3 (0.1)	31.5 (13)	1.06 (0.4)	0.11 (0.0)	0.35 (0.1)	0.33 (0.2)	2.6 (1.2)	1.85 (0.6)	7.3 (2.3)	10.9 (3.1)	57.8 (9.4)
(3) Semideciduous forest	*C. major*	6.5 (0.3)	8.8 (0.4)	4.9 (0.2)	29.0 (9)	1.40 (0.5)	0.59 (0.3)	1.11 (0.3)	0.91 (0.7)	1.6 (1.4)	5.38 0.3	10.3 (1.3)	39.0 (2.0)	25.1 (8.6)

[a] Samples of mature leaves collected in December/2005 (middle of wet season) and June/2006 (beginning of dry season.
[b] Soil samples (0- to 20-cm depth); Al, Ca, Mg extracted by 1 *N* KCl; P and K, by Mehlich-1 extractant; V = base saturation; m = Al saturation at effective CEC (CECeff.); ( ) = standard deviation, with $n = 3–4$ replicates.

3. Results

The average values for the soil characteristics of the studied sites are described in Table 1. There were relatively minor differences in the soil exchangeable Al among the three sites. However, there was a marked difference in Al saturation ("m" values) among these sites, with values ranging from 25 to 80% in the semideciduous forest and in Cerrado *sensu stricto* sites, respectively. The Al content in leaves of three Vochysiaceae species ranged from 3.9 to 6.5 g Al kg^{-1} leaf DW in *Q. grandiflora* and *V. pyramidalis*, respectively. Despite the fact that Site 2 had about 80% of soil effective cation exchangeable capacity (CEC) occupied by Al, *S. paniculatum* accumulated less than 0.1 g Al kg^{-1} leaf DW (Table 1), indicating that this plant probably possesses effective mechanisms for Al exclusion from its leaves. The Al concentration in leaves were significantly different between the two seasons, and Al concentration increased with time, for all investigated species, except for *Q. grandiflora*, which concentrations hardly changed (Table 1).

Figs. 1–4 show photographs of tissue cross-sections from central vein or blade stained with hematoxylin, for the three Al-hyperaccumulator species and for the Al non-accumulator species from leaves collected in the wet season. The sites of Al accumulation were the same for cross-sections from leaf samples collected in dry season (slides not shown). Certain aspects of leaf sections differed among these cross-sections, as follows. In the cross-sections of both leaf tissues from *S. paniculatum*, the purple color was not observed, indicating the absence of Al in the vascular system and leaf blades of this species (Fig. 1).

As for the *Q. grandiflora* species, we observed an absence of stain in the vascular system (Fig. 2, A and A1). In the blade, the presence of Al was shown by a slightly positive reaction of the dye in the cell wall, in the epidermis cells of both abaxial and adaxial faces (Fig. 2, B and B1). In contrast, the palisade and spongy parenchyma tissues were deeply stained, with a strong staining density among the chloroplasts.

In the central vein of *C. major* tissues (Fig. 3), some cells of the xylem region and the pith parenchyma were stained. In blade tissues, there was an absence of staining of epidermis cells, on both adaxial and abaxial sides. However, the cuticle was clearly stained, indicating the presence of Al–hematoxylin complexes on the blade surface. Similarly to *Q. grandiflora*, a deep staining of some palisade and spongy parenchyma was observed.

Cross-section of central veins of *V. pyramidalis* indicated the presence of Al in the vascular tissues, in the xylem region (Fig. 4). The cuticle and cell walls of adaxial and abaxial epidermis were strongly stained. In contrast to the other two Al-hyperaccumulator species evaluated in this study, the cells of palisade parenchyma were not stained in this species. Only the cell walls of spongy parenchyma cells were stained in this morphological region, while their respective chloroplasts were not stained. This aspect suggested that the Al ions were prevented from entering into the cells in this species.

4. Discussion

The Al-hyperaccumulator species from the Vochysiaceae family can grow in soils with marked differences in edaphic conditions, such as water and nutrient availability, which, in some way, seem to affect the plants' spatial distribution in the natural environment. Although *Q. grandiflora* can be found in other environments (Ratter et al., 2003), it is common in the Cerrado *sensu stricto* phytophysiognomy, with well-drained Ferralsols that present low cation availability (Ca^{2+}, Mg^{2+}) and very high Al^{3+} soil saturation. The *V.*

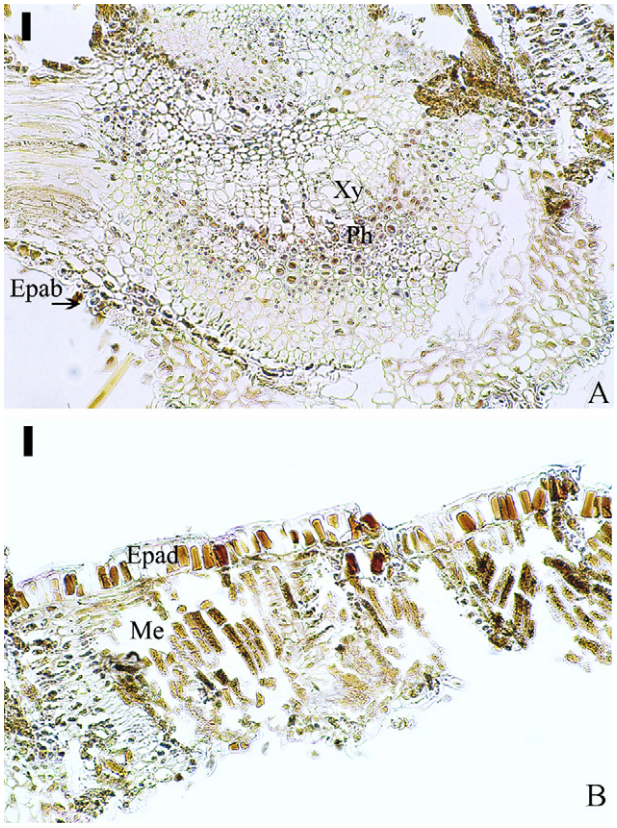

Fig. 1. Cross-section of (A) central vein and (B) leaf blade of *S. paniculatum* after staining with hematoxylin. Xy, xylem; Ph, phloem; Epad, epidermis adaxial; Epab, epidermis abaxial (scale bar: A and B = 35 μm).

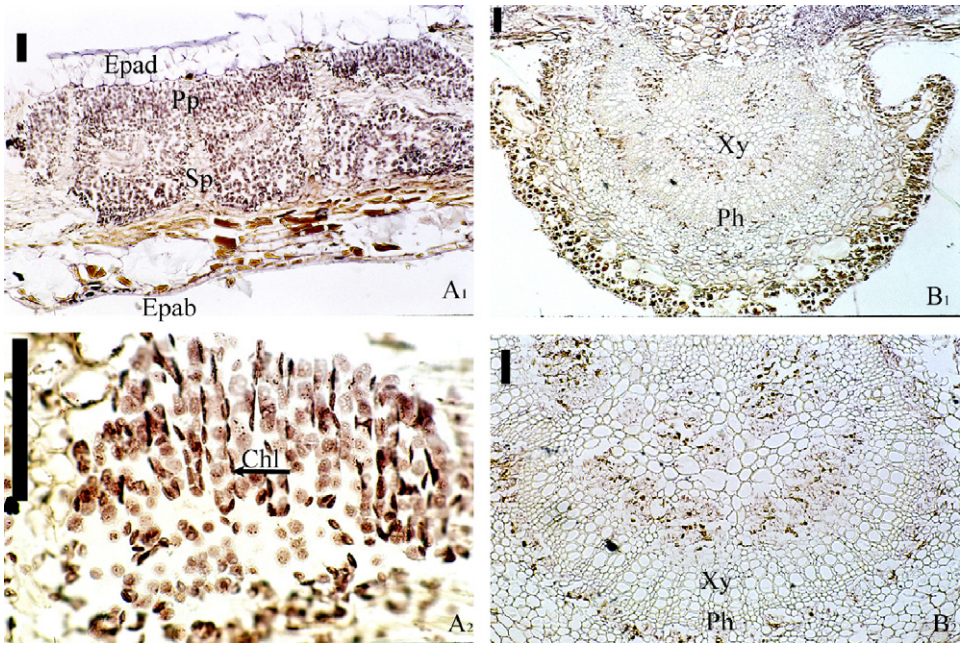

Fig. 2. Cross-section of (A1 and A2) central vein and of (B_1 and B_2) leaf blade from *Q. grandiflora* after staining with hematoxylin. The presence of Al in the tissues is indicated by a purple color. Xy, xylem; Ph, phloem; Epad, epidermis adaxial; Epab, epidermis abaxial; Pp, palisade parenchyma; Sp, spongy parenchyma; Chl, chloroplast (scale bar: A_1 = 45 μm; A_2 = 60 μm; B_1 = 150 μm; B_2 = 100 μm).

pyramidalis trees studied in this work were grown in a gallery forest, which is characterized by more superficial water table, soil pH around 5 and low levels of exchangeable Al and exchangeable nutrients. The *C. major* species was present in areas of higher soil fertility and higher soil CEC, as well as low Al saturation levels. These soil characteristics are observed in areas of semideciduous forest phytophysiognomies. In spite of the differences in Al availability in soils of the three sites evaluated in the present study, all Vochysiaceae species studied were Al hyperaccumulators, confirming the

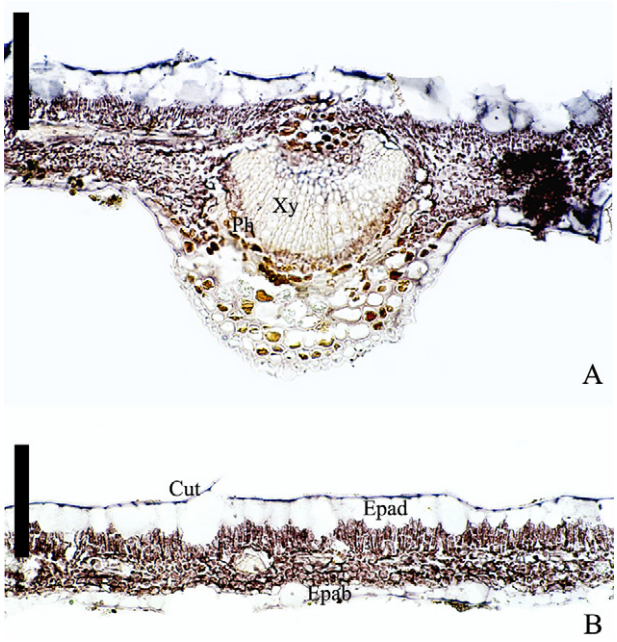

Fig. 3. Cross-section of (A) central vein and (B) leaf blade of *C. major* species after staining with hematoxylin. The presence of Al in the tissues is indicated by a purple color. Xy, xylem; Ph, phloem; Epad, epidermis adaxial; Epab, epidermis abaxial; Cut, cuticle; Pp, palisade parenchyma; Sp, spongy parenchyma (scale bar: A = 90 μm; B = 95 μm).

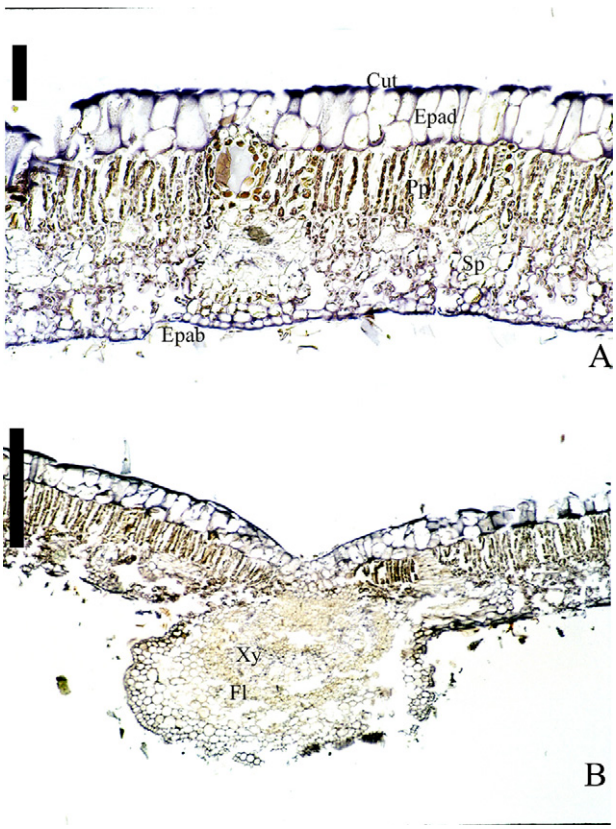

Fig. 4. Cross-section of (A) central vein and (B) leaf blade of *V. pyramidalis* species after staining with hematoxylin. The presence of Al in the tissues is indicated by a purple color. Xy, xylem; Ph, phloem; Epad, epidermis adaxial; Epab, epidermis abaxial; Cut, cuticle; Pp, palisade parenchyma; Sp, spongy parenchyma (scale bar: A = 50 μm; B = 270 μm).

observations of Haridasan (1982). Recent reports on the evolutionary control of elemental composition in leaves of different plant species indicated that, with regard to Al, about 49.5% of variation in the presence of this element in leaves was associated with plant family or key clade. Even though phylogeny represents the sum of all pastselection pressures, such as soil chemistry and climate (Watanabe et al., 2007), results of soil analysis of the three sites in the present study indicated that, at least in present-day situations, there is no direct relationship between Al availability in soils and Al hyperaccumulation among the Vochysiaceae species evaluated.

Haridasan et al. (1986) used a cellular dye, aluminon, to identify the sites of Al accumulation in leaves of native species of the Cerrado. Some Vochysiaceae species were included among the Al hyperaccumulators evaluated in that study. Even when using techniques for Al detection in leaf tissues with similar principles (the aluminon and the hematoxylin dyes), there were differences between the present study and the study by Haridasan et al. (1986) with regard to the pattern of color formation in the leaf tissues of Cerrado native species. In Haridasan's study, aluminon was unable to react with Al when the latter was present in the palisade parenchyma of any of the Al-hyperaccumulator species evaluated, including *Q. grandiflora*. In the present study, hematoxylin indeed formed colored complexes with Al in mesophyll tissues (palisade and spongy cells) of leaves from *Q. grandiflora* and *C. major*, where Al penetrated inside the cells and was located in the chloroplasts (Figs. 2 and 3). Cotta et al. (2008) tested the differential ability of the two dyes to detect Al in foliar tissue of the same species. Their results indicated that the discrepancies observed might be, in part, due to permeability of the membranes to different dyes.

Memon et al. (1981), Cuenca et al. (1991), and Turnau et al. (2007) used X-ray microanalysis technique to identify Al accumulation sites in the leaves of tea plants (*Camelia sinensis*), *Richeria grandis* (a native species of the tropical forests of Venezuela), and of *Erica endevalensis* growing in a pyrite mine tailing substract heavily contaminated by Al, respectively. In *E. endevalensis*, Al was found, as well as Fe, in higher concentrations in the upper epidermis and the top part of leaf glandular hairs. The mesophyll cells showed only trace amounts of those elements. The authors suggested that the cell wall of epidermal tissues acted as a barrier to the entrance of Al into the cell and subsequent movement towards critical metabolic sites, as a mechanism of Al toxicity avoidance in those species. However, Cuenca et al. (1991) observed that this mechanism only occurred in the younger leaves of *R. grandis*. In the older leaves (presenescent), Al was observed at higher concentrations in the interior of mesophyll cells, where it was stored in the vacuoles and in the chloroplasts. Based on these results, the authors speculated that the ionic form of Al is as toxic to Al-accumulating species as it is for Al-sensitive species.

To the best of our knowledge, we showed for the first time, using hematoxylin as Al detector, that these two Vochysiaceae species also accumulate Al in palisade and spongy parenchyma of mature leaf, with penetration of Al into the interior of chloroplasts. Most interesting, these tissues did not show apparent signs of destruction due to toxic concentration of Al, as was observed by Cuenca et al. (1991) in the presenescent leaves of *R. grandis*. Although our results show a pattern of Al accumulation in chloroplasts of two Al-hyperaccumulator species, the chloroplasts do not play a role as storage organelles for Al detoxification of cells. The literature about effects of metals on photosynthetic apparatus is abundant (see review of Bertrand and Poirier, 2005) and in general reports toxic effects of Al. Thus, the effects of Al on photosynthetic reactions in the mesophyll cells of leaves from those species deserve to be studied in more detail, in natural and controlled conditions.

The light (or even absence of) coloration in the cell walls of epidermis and mesophyll cells of *Q. grandiflora* and *C. major* suggests that this compartment is not a preferential site for Al accumulation, as observed in leaves of tea and of *E. endevalensis* plants (Memon et al., 1981; Turnau et al., 2007). The prevention of Al toxicity by Al accumulation in the cell wall of epidermal and mesophyll cells may play a role for *V. pyramidalis*. Also, a positive color formation of cloroplasts in mesophyll cells was not observed.

5. Conclusion

Based on our results regarding soil characterization and Al accumulation in the native Vochysiaceae species investigated, we conclude that the process of hyperaccumulation of Al in these species is independent of the availability of this metal in the soil. The three Al-hyperaccumulator species evaluated in the present study showed contrasting mechanisms of Al detoxification in leaves. In *C. major* and *Q. grandiflora*, Al is not only taken up into the mesophyll cells, but it also enters the chloroplasts without apparent damage to these organelles. In contrast, it binds to the cell walls in the epidermal and mesophyll cells in *V. pyramidalis*. However, the nature of such mechanisms remains to be clarified and raises the following questions: (a) is Al found under complexed forms with organic ligands in different cellular compartments?, (b) is Al found sequestered in vacuoles?, (c) what is the role of Al in the chloroplast?, and (d) can Al exert a positive physiological effect in the development of these plants? The role of Al in chloroplasts is probably a key issue in understanding hyperaccumulation by Vochysiaceae, as little is known about the possible interaction of Al with the physiology of photosynthesis.

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