UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
BIOLOGIA VEGETAL

Espectros de raios-x em MEV indicam associação entre Al e Si em plantas acumuladoras de Al do Cerrado

ANNA CAROLINA GRESSLER BRESSAN

Dissertação apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Mestre em Ciências Biológicas (Biologia Vegetal).

Abril - 2014

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Poema 63 – TAO TE CHING

(Lao-Tsé)

À minha família.

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"A man who works with his hands is a laborer;

A man who works with his hands and his brain is a craftsman;

But a man who works with his hands, his brain and his heart is an artist"

(Louis Nizer)

ÍNDICE

1. RESUMO

Plantas acumuladoras de alumínio (Al) estão distribuídas ao redor do mundo, mas no Cerrado, ou savana brasileira, existem espécies hiper-acumuladoras sendo algumas até mesmo dependentes da presença do Al no solo. Descrevemos padrões anatômicos de armazenamento de Al nos tecidos foliares de espécies acumuladoras e não acumuladoras de Al, empregando diferentes corantes, específicos na detecção deste elemento em tecidos. Em adição, medimos o espectro de raios – x específico para Al em regiões diferentes dos tecidos das folhas, analisados através de microscopia eletrônica de varredura (MEV). Seções transversais da nervura central de folhas de plantas adultas acumuladoras, *Miconia albicans*, *M. rubiginosa* (Melastomataceae), *Qualea grandiflora*, *Q. parviflora* (Vochysiaceae) e não acumuladoras *Styrax ferrugineus* e *S. camporum* (Styracaceae) foram coradas com hematoxilina ou cromo azurol S e visualizadas em microscopia de luz. Análises em MEV e espectros de raios-x foram usados para identificar locais de deposição de Al. Cromo azurol S demonstrou ser um corante específico para Al mais nítido e contrastante em relação à hematoxilina. Confirmamos que os principais locais de acúmulo de Al são paredes celulares de tecidos não lignificados. Observou-se também a presença de grânulos, com alta concentração de Al, confinados em cavidades no parênquima cortical de uma espécie acumuladora. A descrição anatômica da deposição do Al nas folhas e o padrão consistente de o Al estar associado a paredes celulares sugere um papel mais estrutural do que fisiológico para o Al em folhas de plantas lenhosas do Cerrado.

Palavras-chave: Cerrado, alumínio, toxicidade, histoquímica.

ABSTRACT

Aluminum (Al) accumulator plants are distributed all over the world, but in the Brazilian savanna or Cerrado there are hyper accumulator species, and some are Al-dependent. We described anatomical patterns of Al storage in leaf tissues of Al-accumulators and non-accumulators using different Al-specific dyes. In addition, we measured Al-specific x-ray spectra from different regions in leaf tissues analyzed by scanning electron microscopy (SEM). Cross sections from leaves of adult plants from the Al-accumulators, *Miconia albicans, M. rubiginosa* (Melastomataceae), *Qualea grandiflora, Q. parviflora* (Vochysiaceae), and the non-accumulators *Styrax ferrugineus* and *S. camporum* (Styracaceae) were stained with hematoxylin and chrome azurol S for light microscopy. SEM analyses and x-ray spectra were used to identify sites of Al accumulation in leaves. Chrome azurol S was demonstrated to be a sharper and more contrasting Al-specific dye in relation to hematoxylin. We confirmed that the main sites for Al accumulation are cell walls of non-lignified leaf tissues of Al-accumulating plants. In these plants, we also observed Al-constitutive granules confined in cavities formed in the cortical parenchyma. The anatomical description of Al accumulation in leaves and the consistent pattern of Al association with cell walls strongly suggest structural rather than physiological roles of Al in leaves of Cerrado woody plants.

Key words: Aluminum, Cerrado, Hystochemical studies, Metal toxicity

1. INTRODUÇÃO

A fitotoxidez por alumínio (Al) tem sido apontada como um dos principais fatores limitantes da produtividade de culturas em solos ácidos, os quais representam cerca de 40% da terra arável do planeta (Ma et al, 2001). Nesses solos, minerais ricos em Al são solubilizados e a forma fitotóxica Al^{3+} torna-se disponível às plantas (Matsumoto, 2000; Vardar & Ünal, 2007). Um dos principais sintomas da toxicidade ao Al é a necrose de raízes. Por ser uma forma bastante reativa, o Al interage com diferentes sítios do simplasto e apoplasto das células e pode interferir em processos de alongamento e divisão celular, levando à inibição do crescimento radicular (Delhaize & Ryan, 1995; Matsumoto, 2000).

Por outro lado, plantas que se desenvolvem em solos naturalmente ácidos e com alto teor de Al podem evitar ou tolerar os efeitos tóxicos deste elemento (Ma, et al, 2001; Haridasan, 2008). Certas espécies resistentes são consideradas "excludentes" e podem barrar o transporte deste elemento para o interior de seus tecidos, muitas vezes através da secreção de ácidos orgânicos pelas raízes para formar compostos estáveis com o Al ainda na rizosfera (Ryan, 2011). Outras apresentam mecanismos de tolerância interna e são capazes de imobilizar o Al que adentra o simplasto em sítios como parede celular e vacúolos. (Brunner & Sperisen, 2013).

Existem espécies, porém, capazes de acumular elevadas concentrações de Al em seus tecidos sem que danos aparentes sejam evidenciados em suas estruturas internas ou externas (Haridasan, 1982). Plantas que estocam 1000 mg de Al por kg de massa seca de folhas são consideradas acumuladoras de Al (Jansen et al, 2002). Algumas destas espécies são nativas da vegetação do Cerrado e foram descritas pela primeira vez por Haridasan (1982). Desde então, o estudo da relação de plantas acumuladoras de Al com seu ambiente tem se intensificado.

Neste contexto, folhas de espécies consideradas acumuladoras e não acumuladoras de Al, nativas de fragmentos de cerrado *sensu stricto* no interior do Estado de São Paulo, foram coletadas para elaboração de análise nutricional e testes histoquímicos com emprego de corantes indicadores da presença do Al. Em adição, foram realizadas análises em microscopia eletrônica de varredura (MEV) para averiguação de detalhes em micromorfologia. O padrão de acúmulo do Al nos tecidos pode ajudar a compreender mecanismos associados à sua toxicidez e tolerância em plantas sensíveis (Matsumoto, 2000). Sendo assim, plantas do Cerrado necessitam ser

preservadas e estudadas, uma vez que em algumas o Al já foi sugerido fazer parte do metabolismo fotossintético (Andrade et al, 2011).

Grande parte da vegetação original do Cerrado foi substituída pela agricultura e até hoje, a pesquisa agronômica apenas desprezou os genes das plantas nativas. A destruição do Cerrado e sua riqueza biológica demonstram a prioridade para sua conservação em nível global (Myers *et al*., 2000). Diante do exposto, comparamos a eficiência da hematoxilina e do cromo azurol S em testes histoquímicos de identificação de Al em tecidos foliares de espécies acumuladoras e não acumuladoras nativas do Cerrado. Em adição, foram feitas análise em microestrutura com MEV e análises nutricionais das folhas.

2. REVISÃO DE LITERATURA

O alumínio é um dos elementos mais abundantes do planeta (Delhaize & Ryan, 1995. Uma vez disponível pode causar fitotoxidez em espécies cultivadas, sobretudo em solos ácidos (pH < 5) (von Uexküll & Mutert, 1995). Por conta disto, o Al tem sido apontado como um dos principais fatores limitantes à produtividade agrícola em solos ácidos, os quais representam cerca de 40% da terra arável do mundo (Ma et al, 2001).

A maior parte do Al no solo encontra-se na forma de óxidos e aluminosilicatos, os quais são inofensivos às plantas. Porém, em condições de acidificação do solo ou em solos naturalmente ácidos, os minerais são solubilizados e a forma fitotóxica, Al^{3+}, é liberada para a solução do solo (Matsumoto, 2000; Vardar & Ünal, 2007).

Um dos principais sintomas da toxicidade do Al são a necrose do meristema radicular e raízes curtas (Delhaize & Ryan, 1995; Matsumoto, 2000). A forte ligação estabelecida entre o Al e componentes doadores de oxigênio torna-o capaz de interagir com diferentes sítios do apoplasto e simplasto e interferir nos processos de alongamento e divisão celular, o que leva à inibição do crescimento radicular (Ma et al, 2001). Vazquez et al (1999) observaram severos distúrbios na homeostase de cátions no apoplasto de raízes e aumento na espessura da parede de células corticais em espécies consideradas sensíveis. Rupturas celulares e redução da capacidade de alongamento celular também foram constatadas por Kopittke et al (2009).

No entanto, plantas que se desenvolvem em solos naturalmente ácidos e com alto teor de Al podem evitar ou tolerar os efeitos tóxicos deste elemento (Ma, et al, 2001; Haridasan, 2008). O termo “resistência” refere-se à propriedade de uma planta em

apresentar pouco ou nenhum dano em seu crescimento ou desenvolvimento quando submetida a altas concentrações de Al (Brunner & Sperisen, 2013).

Certas espécies resistentes são consideradas "excludentes" e podem barrar o transporte deste elemento para o interior de seus tecidos (Ryan et al., 2011). Ácidos orgânicos sob a forma de ânions (citrato, succinato, oxalato e malato) são secretados para fora das células e formam complexos altamente estáveis com o Al na rizosfera, impedindo-o de adentrar ao simplasto (Brunner & Sperisen, 2013). Em algumas espécies a formação destes complexos ocorre no ápice da raiz e posteriormente são eliminados através de transportadores específicos (Ryan et al. 2011).

Outras espécies, porém, apresentam mecanismos de tolerância interna e podem quelar o Al simplástico no citosol em complexos não tóxicos e imobilizá-lo em outros sítios, como apoplasto e vacúolos (Haridasan, 1986; Brunner & Sperisen, 2013). Mecanismos de tolerância parecem ser comuns em espécies endêmicas de regiões com solos ácidos, como nos trópicos, onde a capacidade de lidar com o estresse por Al é fundamental para a sobrevivência (Ryan & Delhaize, 2010).

Existem ainda espécies capazes de acumular grandes concentrações deste elemento em seus tecidos sem que qualquer dano seja evidenciado em suas estruturas internas ou externas. Plantas que estocam a partir de 1000 mg de Al por kg de massa seca de folhas são consideradas acumuladoras de Al (Jansen et al, 2002). Exemplos de acumuladoras incluem espécies de Rubiaceae de florestas alagadas (Britez et al., 2002), espécies de Melastomataceae de diferentes partes do mundo (Jansen et al., 2002) e espécies de considerável importância econômica, como *Camellia sinensis* L. (Carr et al, 2003; Tolrà et al., 2011).

O Cerrado é um complexo de biomas ocorrentes no Brasil internacionalmente reconhecido e abrange formações savânicas, campestres e florestais ecologicamente relacionadas (Batalha & Mantovani, 2001). A vegetação se desenvolve em solos antigos, ácidos (pH < 4), ricos em Al e com baixos teores de matéria orgânica (Haridasan, 2000; Habermann & Bressan, 2011). Tais características fazem deste um ambiente hostil para a maior parte das plantas cultivadas. Ainda assim, são muitas as espécies que compõe a flora nativa do Cerrado (Castro et al., 1999).

Espécies acumuladoras de Al foram descritas para o Cerrado pela primeira vez por Haridasan (1982). Este autor descreveu a maior parte das espécies acumuladoras de Al como árvores e arbustos das famílias Melastomataceae, Rubiaceae e Vochysiaceae.

O padrão da localização do Al nos tecidos é de grande importância para a compreensão de mecanismos associados à toxidez e tolerância ao Al (Matsumoto, 2000). Sítios de deposição podem ser verificados através de testes histoquímicos que empregam corantes indicadores da presença deste elemento (Haridasan et al, 1986; Andrade et al, 2011). A hematoxilina é um corante amplamente utilizado na detecção de Al em tecidos vegetais (González-Santana et al., 2012; Andrade et al, 2011), mas outros como aluminon (Jansen et al., 2002; Haridasan, 1986) e cromo azurol (Kukachka & Miller, 1985) também podem ser aplicados com este propósito.

Através da análise anatômica de folhas de espécies nativas do Cerrado Haridasan et al, (1986) salienta que células de fibras e elementos de vaso em xilema não coram durante os testes com aluminon, o que sugere serem estes sítios negativos para a deposição do Al. Por outro lado, paredes de células de colênquima, parênquima esponjoso e células-guarda mostraram-se sítios positivos para a presença (Haridasan et al., 1986). Este padrão também foi observado em outras espécies acumuladoras, mas não nativas do Cerrado (Brítez et al., 2002; Tolrà et al., 2011; González-Santana et al., 2012).

Possíveis vantagens adaptativas ou promoção de benefícios em reações metabólicas estimuladas pelo Al em espécies tolerantes e acumuladoras não são bem elucidadas, mas desde o trabalho de Haridasan (1982) o estudo da relação destas espécies com seu ambiente tem se intensificado.

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Capítulo Único:

X-ray spectra in SEM show Al and Si association in Al-accumulating plants from the Cerrado[1]

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Abstract

- ***Background and Aims*** Aluminum (Al) accumulator plants are distributed all over the world, but in the Cerrado there are hyper accumulator species, and some are Al-dependent. We described anatomical patterns of Al storage in leaf tissues of Al-accumulators and non-accumulators using different Al-specific dyes. In addition, we measured Al-specific x-ray spectra from different regions in leaf tissues analyzed by scanning electron microscopy (SEM).
- ***Methods*** Cross sections from leaves of adult plants from the Al-accumulators, *Miconia albicans*, *M. rubiginosa* (Melastomataceae), *Qualea grandiflora*, *Q. parviflora* (Vochysiaceae), and the non-accumulators *Styrax ferrugineus* and *S. camporum* (Styracaceae) were stained with hematoxylin and chrome azurol S for light microscopy. SEM analyses and x-ray spectra were used to identify sites of Al accumulation in leaves.
- ***Key results*** Chrome azurol S was demonstrated to be a more contrasting indicator of Al presence in relation to hematoxylin, as evidenced by x-ray spectra in SEM. We confirmed that the main sites for Al accumulation are pectin-rich cell walls of non-lignified leaf tissues of Al-accumulating plants. In these plants, we also observed silica grains associated to Al confined in cavities formed in the cortical parenchyma.
- ***Conclusions*** The anatomical description of Al accumulation in leaves and the consistent pattern of Al association with cell walls strongly suggest structural rather than physiological roles of Al in leaves of Cerrado woody plants.

Key words: Aluminum, Cerrado, Hystochemical studies, Metal toxicity

Introduction

Acidic soils (pH < 5.0) occupy approximately 30% of the world's ice-free land areas and, from these, 67% support woodlands and forests, whereas savannas grow on 18% acidic soils (von Uxküll and Mutert, 1995). Acidic soils usually present low base (K, Ca and Mg) saturation (BS) and, consequently, high potential acidity (H^+ + Al^{3+}) and exchangeable aluminum (Al) (Robson, 1989). Poor soils and Al are known to limit growth of crop plants (Yang et al., 2013), as Al causes ruptures in roots, as it binds to cell wall of rhizodermis increasing its rigidity and reducing the ability of the outer cells to elongate (Kopittke et al. 2009). Some plants form complexes between malate and citrate with Al in the root tip, and specific transporters are responsible for excluding these organic acid complexes, therefore explaining 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, inhabiting swamp forests in Brazil (Britez et al., 2002), Melastomataceae growing in highly disturbed landscapes in Mexico (Gonzáles-Santana et al., 2012), from 127 species from Melastomataceae coming from different parts of the world (Jansen et al., 2002), from 11 species from Rubiaceae distributed worldwide (Jansen et al., 2003), and even from tea plants – Theaceae (Carr et al., 2003; Tolrà et al., 2011).

In the Cerrado, which is comprised of grasslands, savannas and forests physiognomies, the vegetation grows on more acidic (pH < 4.0) soils, rich in Al (Haridasan, 2008; Habermann and Bressan, 2011). In the Cerrado, Al-accumulating plants were first described in 1982, and because of edaphic conditions, Al-accumulators and non-accumulators were identified, in such a manner that the former accumulates between 1000 up to 15,000 mg Al per kg dry leaves, whereas the later, between 600 and

1000 mg/kg (Haridasan, 1982). According to this author, the most Al-accumulating plants in the Cerrado are shrubs and trees from Melastomataceae, Rubiaceae and Vochysiaceae. Since then, some aspects of Al within these plants have been described.

Using indicators (dyes) of Al 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 fiber cells and xylem do not stain, while cell walls of the collenchyma, spongy parenchyma and guard cells do. This pattern was reported for other Al-accumulating non-Cerrado plants (Britez et al., 2002; Carr et al., 2003; Tolrà et al., 2011; González-Santana et al., 2012). Inferring physiological roles for Al in the metabolism of these plants, however, is quite challenging, since *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 Al was observed in these organelles (Andrade et al., 2011).

Another dissention among papers comes 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 these dyes have been demonstrated to also react with Fe, Cu and Zn rather than reacting specifically with Al deposited on plant tissues. In the present paper, we compared hematoxylin with chrome-azurol S and demonstrate that the later 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.

Therefore, besides confirming anatomical patterns of Al deposition in leaf tissues from both groups of Cerrado plants, in the present paper we analytically quantified Al in their leaves. As a novelty, we analyzed the micromorphology, to identify where these plants may be storing Al in their leaves, evidencing the Al presence with Al-specific x-ray emission detected by a dispersive detector mounted on a scanning electron microscope.

Material and methods

Plant materials

We collected leaves of Al-accumulating and non-accumulating plants 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 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* (augmentative of Cerrado in Portuguese).

These species from Vochysiaceae and Melastomataceae are described as Al-accumulating plants since 1982 (Haridasan, 1982). For Al non-accumulating species, we chose these two species from Styracaceae not only because they do not appear on the list of Al-accumulating plants (Haridasan, 1982), but also because we knew previously that they accumulate low amounts of Al in their leaves, therefore, being good contrasting plants growing on the same soils where Al-accumulating plants grow in the Cerrado.

Study sites

The field sites were located in the municipalities of Mogi-Guaçu, Itirapina and Corumbataí, in the São Paulo state, Brazil, on 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, both vegetation forms occurring on the '*Campininha*' farm within the Reserva Biológica de Mogi-Guaçu (22º 15' 19"S 47º 09' 30"W; 680 m of altitude; 340 ha), which is a Cerrado area long preserved (since 1950) in its natural condition.

In Itirapina, plants were found in a cerrado *sensu stricto* fragment (22º13'S 47º53'W; 610 m of altitude; 260 ha) on the São José da Conquista farm; and in Corumbataí, plants were growing in a Cerradão fragment (22º15'S, 47º00'W; 860 m of altitude; 38.7 ha).

Experimental Description

In each of these three sites, soil samples were collected at 20-30 cm of depth, where most roots are able to uptake nutrients in the soil of native areas (Wigley et al., 2013). 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, which was performed according to Embrapa (1997).

In each of the three sites, at least five plants of each species available in the area were identified and had its leaves collected. Mature fully expanded leaves were collected from healthy shoots on each of the four geographical quadrants (N, S, E and W) of the plant canopy. The four leaf subsamples composed the leaf (leaf lamina with its petiole) sample (≈ 40 leaves), which was taken to the Plant Nutrition Lab at the University of São Paulo (Esalq, USP, Piracicaba, SP) for analysis of Al concentration in

the tissue. For this, leaves were oven-dried at 60°C to constant dry mass, ground and digested in a sulfuric:nitric:percloric acids (1:10:2, v/v/v) solution. After digestion, concentrations of Al were determined by the atomic absorption spectrophotometer method and expressed as mg Al per kg dry leaves.

From shoots collected from each plant, some were separated and taken freshly (moistened and put in a plastic bag) to the lab for anatomical analyses.

Anatomical studies

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). The anatomical study was based on cross sections from leaf segments (1 cm^2) form the leaf midrib containing part of the lamina. Sections were hand-made with a razor blade, stained with hematoxylin or chrome azurol S and observed under light microscope (DMLB, Leica Microsystems, Wetzlar, Germany).

Chrome azurol S (3’’-sulpho-2’’,6’’-dichloro-3,3’-dimethyl-4-hydroxyfuchson-5,5’-dicarboxylic acid) dissolving 0.5 g of chrome azurol S (Sigma Aldrich) and 5 g of sodium acetate (buffer) in 80 mL of distilled water, and completing it to 100 mL (Kukachka and Miller, 1980).

Hematoxylin (7,11b-dihydroindeno[2,1-*c*]chromene-3,4,6a,9,10(6*H*)-pentol) as a 2g/L solution was prepared with 0.2 g of hematoxylin and 0.02 g of $NaIO_3$ dissolved in 100 mL distilled water.

Micromorphological studies

For scanning electron microscopy (SEM), 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^{o}C) and

dehydrated in an increasing acetone series of 50, 70, 90, 95, and 2 x 100%, with 15 min each step. To avoid interference in the Al detection, we compared spectra obtained from the aluminum stubs only with that from aluminum stubs covered with one or two layers of a carbon tape (Double sided carbon tape, 8 mm in width; Electrom Microscopy Science, EMS, USA), and observed that the Al from stubs had no influence on the spectra obtained. The images were acquired in a scanning electron microscope (TM 3000, Hitachi, Japan) operated at 15 kV, and the Al detection was performed using an x-ray energy dispersive detector (Swift ED 3000, Hitachi, Japan). The counts were made over a 60-s period, spetra were recorded and data were expressed as the counts to second ratio.

We tried to compare the same plant material under light microscopy and SEM to check the correspondence between images, and their respective spectrum of Al emitted.

Results

Soils from the three experimental sites were acidic (pH < 4.0) with low concentration of P, K, Ca and Mg, resulting in low BS too. These soils also showed relatively low CEC, while Al^{3+}% 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, exhibited approximately 1000 mg Al per kg dry leaves. No direct relationship between Al availability in the soil and Al accumulation by plants was observed because Al concentrations in the soil of the three sites were

extremely high (Table 1), while leaf Al concentrations differed more than 36 times between species (Table 2).

Hematoxylin and chrome azurol S reacted positively with all Al-accumulating species (Fig. 1, 2 and 3), but negatively with those considered non-accumulators (Fig. 4 and 5). Positive reactions with hematoxylin stained in red, and in purple when chrome azurol S was used, exhibiting a clear contrast with non-dye-treated cuts (Fig. 1a, f), for *M. rubiginosa* (Fig. 1b-c) and *M. albicans* (Fig. 1g-h). However, chrome azurol S contrasted the positive and negative reactions in midrib and leaf lamina more intensely (Fig. 1). When hematoxylin was used, intercellular spaces and cell walls in the cortex (Fig. 1i) did not react as clear as when chrome azurol S was used (Fig. 1j).

In the midrib we observed positive reactions in the cell wall of both epidermal surfaces, phloem, cortical parenchyma and collenchyma (Table 3). Positive reactions were also evident in vacuoles of the cortical parenchyma (Fig. 1e, 2c e 3b). Xylem and fiber cells reacted negatively with both dyes, regardless of the species (Table 3; Fig. 1, 2 and 3).

Chrome azurol S also stained phloem cell walls in the vascular bundle of *M. rubiginosa*. Cellular contents of the palisade parenchyma stained in red, differing from the purple positive reaction between chrome azurol S and the spongy parenchyma (Fig. 2b). No positive reaction with internal contents of chloroplasts was observed.

The x-ray spectrum obtained from thicker cell walls in the cortical parenchyma of *M. rubiginosa*, which reacted negatively with chrome azurol S (Fig. 2d), was lower than that obtained from phloem cell walls (Fig. 2e-f), reinforcing the phloem as an important Al-accumulating site. This points out chrome azurol S as an efficient indicator of Al in leaf tissues. In addition, a positive reaction with chrome azurol S was noted in the epithelium of a secretory cavity in *M. rubinosa* (Fig. 2c).

Remarkable structures were noted for Al-accumulating and non-accumulating plants. For instance, we observed cavities of 50-100 μm in diameter in the cortical parenchyma of leaf midribs of *Q. parviflora* (Fig. 3a-b). When thicker cuts of the same plant material were analyzed in SEM, these cavities seemed to be fulfilled by solid granules (Fig. 3c). In addition, when the x-ray spectrum obtained from these granules was compared to that from a xylem cell wall (Fig. 3c), these granules revealed conspicuous x-ray emission intensities, and specific not only for Al, but also for silicon (Fig. 3d).

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 in SEM (Fig. 4c, d). But the x-ray emission intensities obtained from these granules were not as high (Fig. 4d-e) as those from *Q. parviflora* (Fig. 3d). In *S. ferrugineus* we also observed some storage structures in their leaf midribs. These structures showed very low Al-specific x-ray emission intensity, but high Ca-specific emission, which characterize them as druses (Fig. 5c-e).

Discussion

Our results show that chrome azurol S gives sharper contrasting images in relation 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 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, chrome azurol S and hematoxylin) can detect monomeric Al (Al^{3+}) (Wehr et al., 2010). Pyrocatechol violet, however, also react with Fe, Cu and Zn

(Gonzalez-Santana et al., 2012), and hematoxylin is also known to react with Fe salts. On the plant cell wall, small Al-hydroxy species, which are present on pectins and other cell wall compounds (Wehr et al., 2010), can be indicatively stained by chrome azurol S (Kennedy and Powell, 1986). The color given by the reaction between an Al indicator and the tissue is also important and dependent on the solution pH, but the highest absorbance of Al-chrome azurol S complexes are observed within 470-550 nm, which is independent of the pH in the range of pH 3.0-5.0, being purple the indicative color of Al presence (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). Therefore, we suggest chrome azurol S and not hematoxylin to anatomically observe Al accumulation in plant tissues.

Our results also show that the Al is anatomically associated with cell walls containing pectins (phloem, collenchyma, epidermis and cortical parenchyma), and hematoxylin but most importantly, chrome azurol S do not stain tissues containing lignin, such as xylem and fibers. This indicates that the lack of pectin may be a possible impediment for the grip of Al to the cell wall, which corroborates 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, such as *Faramea marginata* [Rubiaceae (Britez et al., 2002)], *Camellia sinensis* [Theaceae (Carr et al., 2003; Tolrà et al., 2011)] and *Conostegia xalapensis* [Melastomataceae (Gonzalez-Santana et al., 2012)]. Pyrocatechol violet stains the cell wall of the spongy parenchyma, but not the sclerenchyma or xylem of *F. marginata* (Britez et al., 2002). Therefore, our results reinforce primary cell walls, rich in pectins, rather than secondary cell walls, which are poor in pectins, as the main site for Al accumulation.

Although Haridasan et al. (1986) also reported the above-mentioned patterns of Al-tissue reaction with aluminon in Al-accumulating plants, we pursue evidence for physiological function(s) of Al in the metabolism of these plants. As these authors also showed 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 Osaki, 2001) reaches the leaves through the transpiration stream (Shen and Ma, 2001) and moves symplastically, eventually binding pectins (Wehr et al., 2010) on the phloem cell wall.

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 chrome azurol S 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 internal contents of chloroplasts. The Al-constitutive granules observed into cavities of the cortical parenchyma in the midrib of the Al-accumulating plant, *Q. parviflora*, and non-accumulating species, such as *S. camporum*, suggest a structural, rather than functional role of Al in leaves of Cerrado plants. On the other hand, granules associated with Al and Si, as evidenced by specific x-ray emission intensities in Al-accumulating plants may also suggest that these plants attempt,

somehow, to isolate the Al from the metabolism. In fact, Si-associated grains, usually secreted into cavities, have already been described for Vochysiaceae and Styracaceae (Metcalfe and Chalk, 1989). But these authors did not risk assigning any physiological role for these grains. Silicon and Al seem to be associated (Britez et al, 2002), but, once more, no functional role are described for these salts.

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), what 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 this descriptive work, we demonstrate a high efficiency of chrome azurol S as an Al-specific dye when compared to hematoxylin. In addition, we confirm the cell wall of leaf tissues as the main site for Al accumulation and, as a novelty we show that Al-constitutive granules are embedded on the cell wall of non-lignified leaf tissues. These endorsements and new findings diminish substantially a possible physiological role of Al in leaves of Cerrado woody plants.

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Tables:

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+}%
		----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
Corumbataí	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
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

Site	BO_3^-	Cu^{2+}	Fe^{2+}	Mn^{2+}	Zn^{2+}
	----------mg dm^{-3}----------				
Itirapina	<0.12	0.53 ± 0.23	47.3 ± 8.02	1.17 ± 0.40	< 0.4
Corumbataí	0.18 ± 0.00	0.93 ± 0.15	87.3 ± 37.5	1.13 ± 0.20	0.47 ± 0.06
Mogi-Guaçu	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

Table 2. Aluminum concentration in leaves of Al-accumulator and non-accumulator plants 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

Table 3. Patterns of hystochemical reactions in the mesophyll of Al-accumulator and non-accumulator plants growing in the Cerrado areas

Dye	Leaf structure	Species	Cuticle	Epidermal wall		Xylem	Phloem	Cortical Parenchyma	Collenchyma	Fibers	Mesophyll	
				Adaxial	Abaxial						Palisade	Spongy
Chrome-Azurol	Central midrib	*M. albicans*	-	+	+	-	+	+	+	-	0	0
		M. rubiginosa	-	+	+	-	+	+	+	-	0	0
		Q. grandiflora	-	+	-	-	+	-	+	-	0	0
		Q. parviflora	-	+	+	-	+	+	+	-	0	0
		S. camporum	-	-	-	-	-	-	-	-	0	0
		S. ferrugineus	-	-	-	-	-	-	-	-	0	0
	Leaf lamina	*M. albicans*	-	+	+	-	+	0	0	0	-	+
		M. rubiginosa	-	+	+	-	+	0	0	0	-	+
		Q. grandiflora	-	+	+	-	+	0	0	0	-	+
		Q. parviflora	-	+	+	-	+	0	0	0	-	+
		S. camporum	-	-	-	-	-	0	0	0	-	-
		S. ferrugineus	-	-	-	-	-	0	0	0	-	-
Hematoxylin	Central mid rib	*M. albicans*	-	+	+	-	+	+	+	-	0	0
		M. rubiginosa	-	+	+	-	+	+	+	-	0	0
		Q. grandiflora	-	+	-	-	+	-	+	-	0	0
		Q. parviflora	-	+	+	-	+	+	+	-	0	0
		S. camporum	-	-	-	-	-	-	-	-	0	0
		S. ferrugineus	-	-	-	-	-	-	-	-	0	0
	Leaf lamina	*M. albicans*	-	+	+	-	+	0	0	0	-	+
		M. rubiginosa	-	+	+	-	+	0	0	0	-	+
		Q. grandiflora	-	+	+	-	+	0	0	0	-	+
		Q. parviflora	-	+	+	-	+	0	0	0	-	+
		S. camporum	-	-	-	-	-	0	0	0	-	-
		S. ferrugineus	-	-	-	-	-	0	0	0	-	-

Figures:

Fig. 1

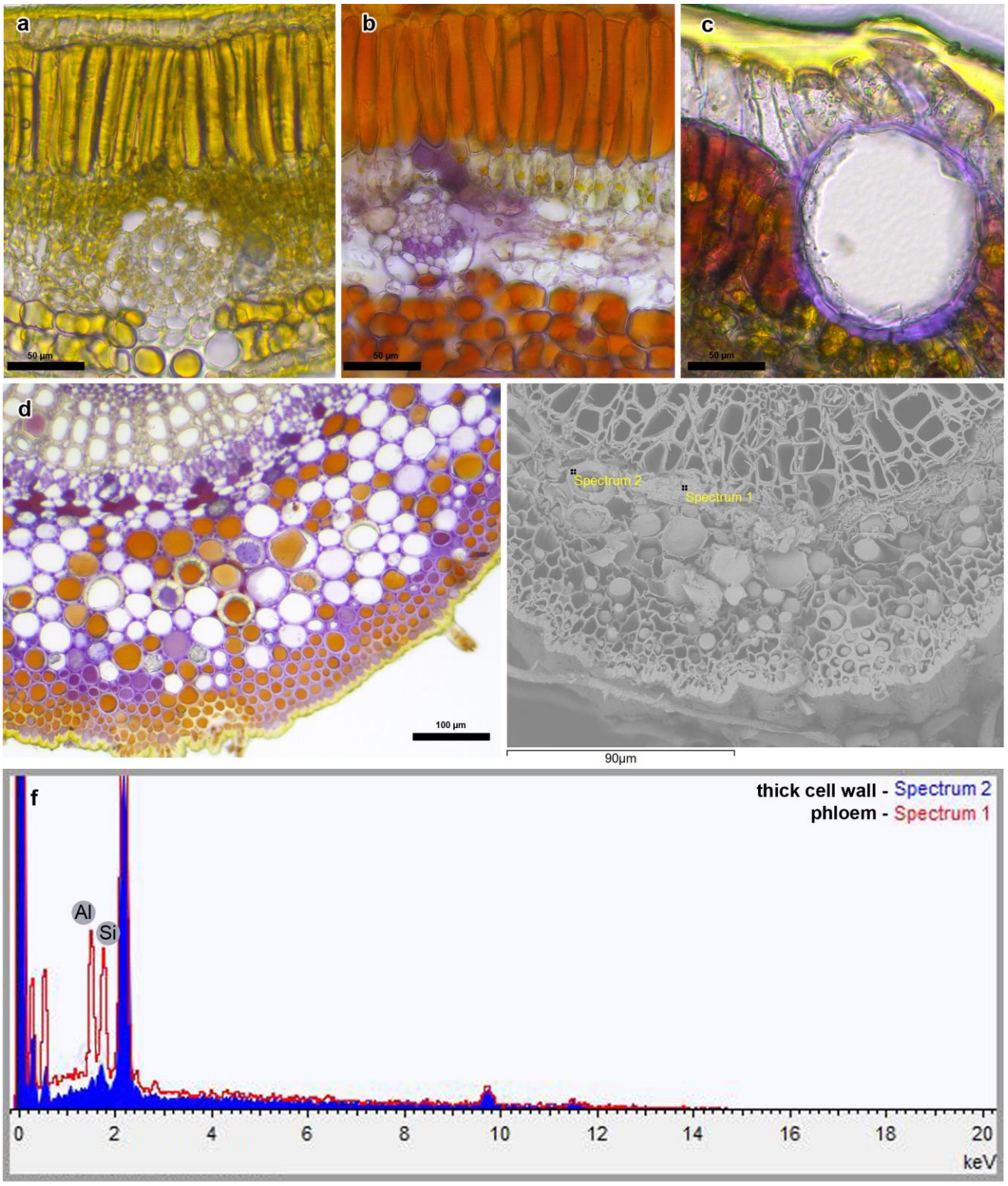
a
50 µm
b
50 µm
c
50 µm
d
100 µm
Spectrum 2
Spectrum 1
90µm
f
thick cell wall - Spectrum 2
phloem - Spectrum 1
Al
Si
0
2
4
6
8
10
12
14
16
18
20
keV

Fig. 2

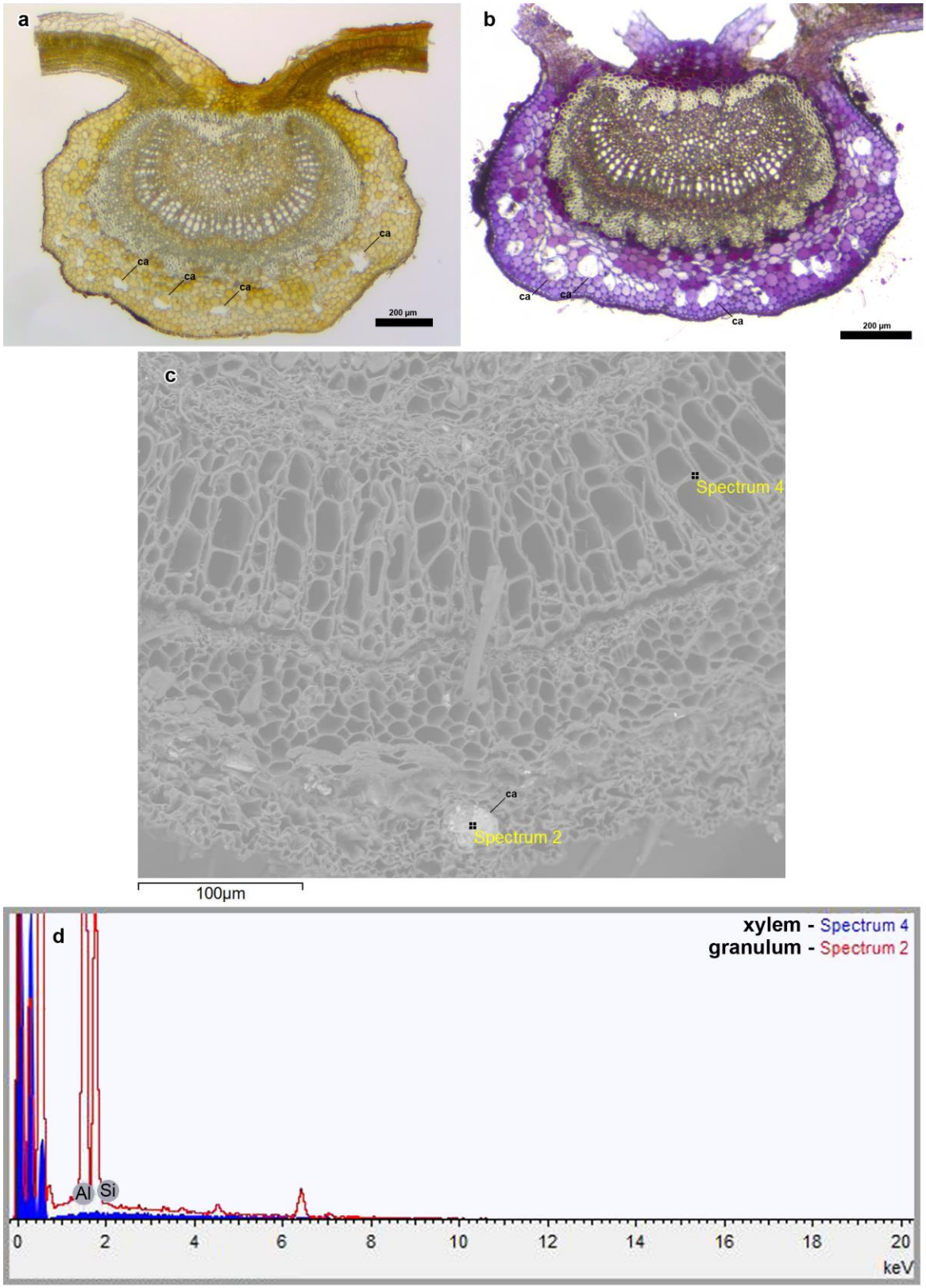
a
ca
ca
ca
ca
200 µm
b
ca
ca
ca
200 µm
c
Spectrum 4
ca
Spectrum 2
100µm
d
xylem - Spectrum 4
granulum - Spectrum 2
Al
Si
0
2
4
6
8
10
12
14
16
18
20
keV

Fig. 3

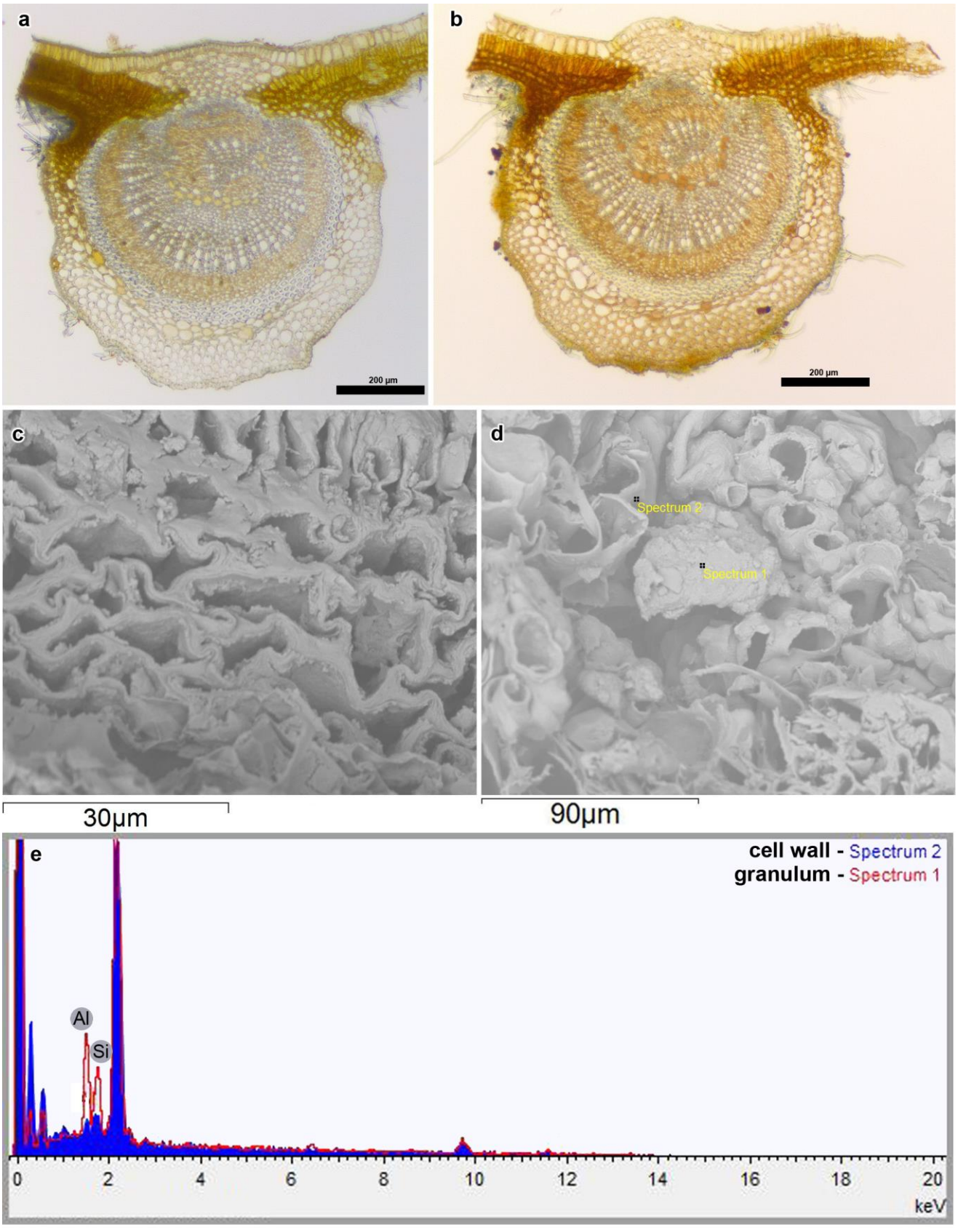
a
200 µm
b
200 µm
c
d
Spectrum 2
Spectrum 1
30µm
90µm
e
cell wall - Spectrum 2
granulum - Spectrum 1
Al
Si
0
2
4
6
8
10
12
14
16
18
20
keV

Fig. 4

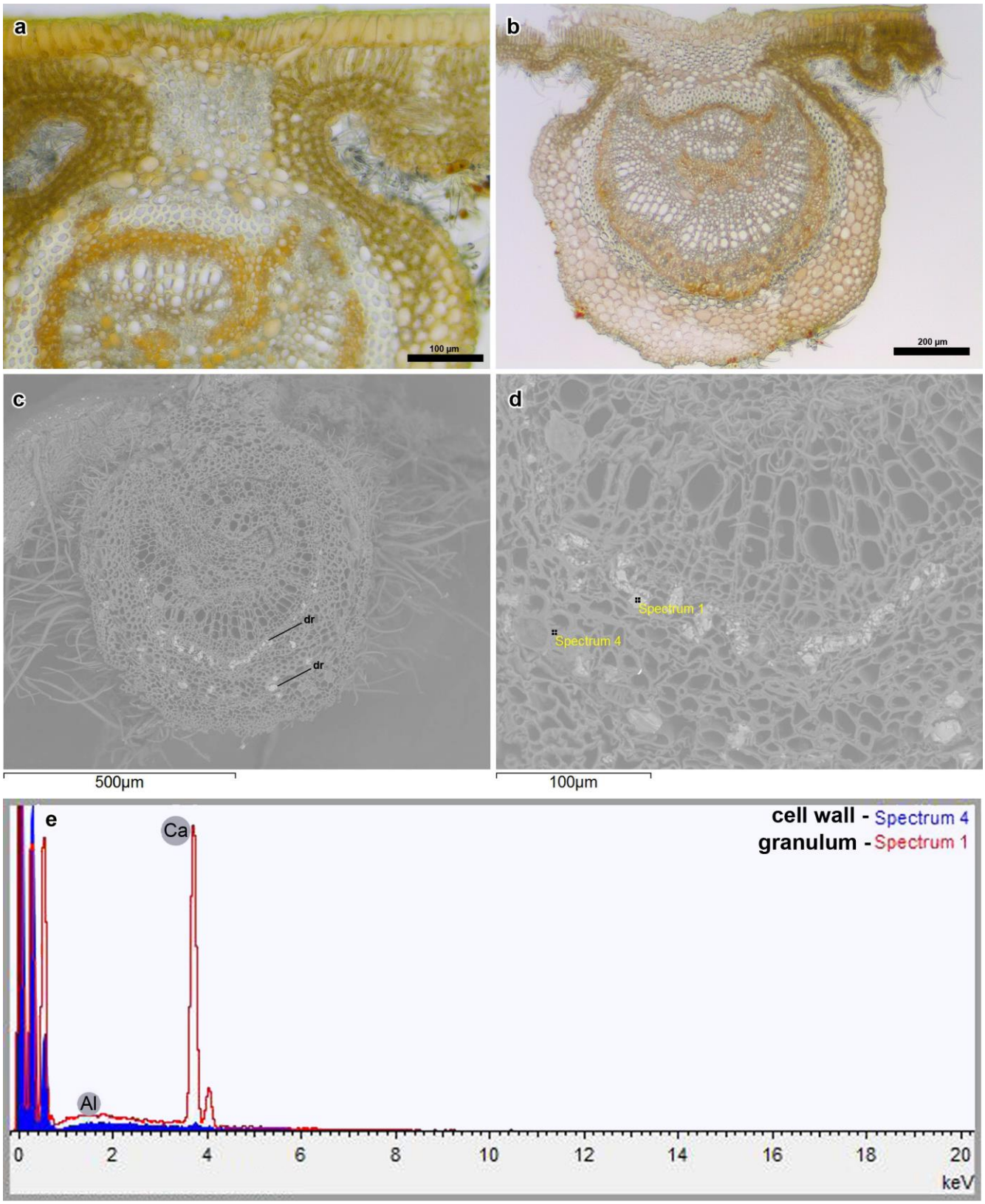
a
100 µm
b
200 µm
c
dr
dr
500µm
d
Spectrum 1
Spectrum 4
100µm
e
Ca
Al
cell wall - Spectrum 4
granulum - Spectrum 1
0
2
4
6
8
10
12
14
16
18
20
keV

Fig. 5

Figure captions

FIG. 1. Photomicrographs of cross-sections of leaf midribs of *Miconia rubiginosa* (a-e) and *M. albicans* (f-J). The presence of Al in the tissue is indicated by red (hematoxylin; b, d, g and i) and purple (chrome azurol S; c, e, h and j) colors. Non-treated sections (a and f) were used as the control. The presence of Al in the tissue is indicated by purple color with Cromo azurol and red with hematoxylin.

FIG. 2. Light microscopy (a, b, c and d) and SEM (e and f) of cross-sections of leaf laminas (a, b and c) and midrib (d) of *M. rubiginosa*. The presence of Al in the tissue is indicated by purple color (chrome azurol S; b, c, d). Al-specific energy-dispersive x-ray spectrum from phloem and a thick cell wall cell in the cortical parenchyma from the midrib (e, f).

FIG. 3. Light microscopy (a and b) and SEM (c) of cross-sections of leaf midrib of *Q. parviflora*. The presence of Al in the tissue is indicated by purple color (chrome azurol S; b). Al-specific energy-dispersive x-ray spectrum was measure from the xylem and granules in the interior of cavities (d).

FIG. 4. Light microscopy (a and b) and SEM (c and d) photographs of cross-sections of leaf midrib of *S. camporum*. The absence of purple color indicates that chrome azurol S reacted negatively with the tissue (b). General view of the collenchyma region in SEM (c), evidencing small granules, and details (d) from the same region showing a granule in an amplified view. Al-specific energy-dispersive x-ray spectrum from granules and collenchyma cell wall (e).

FIG. 5 Light microscopy (a and b) and SEM (c and d) photographs of cross-sections of leaf midrib of *S. ferrugineus*. The absence of purple color indicates that chrome azurol S reacted negatively with the tissue (b). General view of the central midrib (c) and details from the same region showing Ca oxalate crystals - druses (d). Al- and Ca- specific energy-dispersive x-ray spectrum from druses and parenchyma cell wall (e).