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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(BIOLOGIA VEGETAL)

Respostas de espécies nativas do Cerrado a diferentes concentrações de alumínio (Al)

ANNA CAROLINA GRESSLER BRESSAN

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

Novembro - 2018

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TÍTULO DA TESE: Respostas de espécies nativas do Cerrado a diferentes concentrações de alumínio (Al)

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"Nesses tempos de céus de cinzas e chumbos, precisamos de árvores, desesperadamente verdes. "

Mário Quintana

SUMÁRIO

Resumo

O alumínio (Al) é um elemento tóxico para muitas espécies de plantas, porém, diferentes respostas à presença deste elemento podem ser observadas. No Brasil, espécies acumuladoras e não acumuladoras de Al ocorrem naturalmente em áreas de solo ácido, como no Cerrado brasileiro, e este fato evidencia a riqueza de mecanismos desenvolvidos pelas plantas para lidar com a alta disponibilidade de Al no solo. Nesta Tese avaliamos os efeitos provocados em espécies nativas do Cerrado quando expostas a diferentes concentrações de Al. Em casa de vegetação, plantas de *Styrax camporum* foram cultivadas em solução nutritiva contendo 0, 740 e 1480 μM Al por 90 dias. Plantas expostas a 1480 μM Al apresentaram sistema radicular menos desenvolvido, menor altura da planta e baixas taxas de troca de gasosas em relação àquelas expostas a 0 e 740 μM Al. Plantas expostas a 0 e 740 μM Al apresentaram valores semelhantes para estes parâmetros, indicando que o Al não causa efeitos benéficos para o desenvolvimento desta espécie. Análises em microscopia de luz, microscopia eletrônica de varredura e microscopia eletrônica de transmissão também foram realizadas. Em um estudo de campo comparamos as concentrações foliares de Ca e Al em duas espécies do gênero *Qualea* (Vochysiaceae), ocorrendo em um fragmento de Cerrado localizado em solo calcário, com os valores apresentados pelas mesmas espécies, ocorrendo em um fragmento de Cerrado localizado em solo ácido e rico em Al. O teor foliar de Ca foi positivamente associado à saturação no solo, enquanto o teor de Al foi o mesmo nas plantas que cresceram nos dois solos. Além disso, independentemente do tipo de solo, estes elementos são armazenados em diferentes regiões da folha, indicando não existir uma competição entre estes elementos em espécies acumuladoras de Al.

Palavras-chave: Toxicidade do Al. Styracaceae. Vochysiaceae.

Abstract

Aluminum (Al) is a toxic element for many plant species, however, different responses to the presence of this element can be observed. In Brazil, Al-accumulating and non-accumulating species occur naturally in acidic soil areas, such as in the Brazilian Cerrado, and this fact evidences the richness of mechanisms developed to deal with high availability of Al in the soil. In this Thesis, we evaluated the effects caused in native species when exposed to different Al concentrations. In a greenhouse experiment, plants of *Styrax camporum* (Styracaceae) grown in a nutrient solution containing 0,740 and 1480 μM Al for 90 days. Plants exposed to 1480 μM showed a less developed root system, lower plant height and low gas exchange rates in relation to those exposed to 0 and 740 μM Al. Plants exposed to 0 and 740 μM Al showed similar values for these parameters, indicating that Al does not cause beneficial effects to development of this species. Analyses in light microscopy, scanning electron microscopy and transmission electron microscopy were also performed. In a field study, we compared the Ca and Al leaf contents in two species of the genus *Qualea* (Vochysiaceae), occurring in a Cerrado fragment located in calcareous soil, with the values presented by the same species occurring in a Cerrado fragment located in acidic soil, rich in Al. The Ca leaf content was positively associated with the soil saturation, while the Al content was the same in plants growing in both soils In addition, regardless of soil type, these elements are stored in different regions of the leaf, indicating that there is no competition between these elements in Al accumulating species.

Key words: Al-toxicity. Styracaceae. Vochysiaceae.

INTRODUÇÃO GERAL

O alumínio (Al) é o terceiro elemento químico mais abundante da crosta terrestre, e representa 8% de sua massa total (Vitorello et al., 2005; Bojórquez-Quintal et al., 2017). Em pH neutro o Al encontra-se naturalmente presente no solo, na forma de óxidos e aluminosilicatos (Matsumoto, 2000; Vardar e Ünal, 2007). Entretanto, em solos ácidos (pH < 5) os minerais são solubilizados, e formas potencialmente fitotóxicas como $Al(OH)^{2+}$, $Al(OH)_2^{+}$ e $Al(OH)_6^{3+}$, são liberadas para a solução do solo (Matsumoto, 2000; Vitorello et al., 2005; Vardar e Ünal, 2007; Schmitt et al., 2016). Dentre as formas disponíveis do alumínio, $Al(OH)_6^{3+}$, também conhecida como Al^{3+}, é considerada a mais tóxica para as plantas (Panda e Matsumoto, 2007; Vardar e Ünal, 2007; Silva et al., 2012; Bojórquez-Quintal et al., 2017; Singh et al., 2017).

O Al é considerado um importante limitador da produtividade agrícola mundial (Ma et al., 2000; Barceló e Poschenrieder, 2002; Kochian et al., 2005; Horst et al., 2010; Silva et al., 2012), principalmente em solos ácidos, os quais correspondem a 30% das terras não cobertas por gelo (von Uexküll e Mutert, 1995; Brunner e Sperisen, 2013) e 50% das terras aráveis do planeta (Vardar e Ünal, 2007; Gupta et al., 2013).

O primeiro sintoma da toxicidade do Al evidenciado nas plantas é a inibição do crescimento radicular (Barceló e Poschenrieder, 2002; Ciamporová, 2002; Kochian et al., 2005; Yang e Horst, 2015), a qual pode ser observada em poucas horas, ou minutos, após o contato das raízes com altas concentrações deste elemento (Kochian et al., 1995; Horst et al., 2010). Os mecanismos exatos, responsáveis por desencadear os sintomas de toxicidade, ainda não foram totalmente desvendados. Entretanto, nos últimos anos o conhecimento sobre os efeitos do Al nas plantas evoluiu consideravelmente, principalmente em relação às espécies cultivadas.

De acordo com a literatura, a inibição do crescimento radicular induzida pelo Al é resultado das ligações formadas entre o Al^{3+} e sítios do apoplasto e/ou simplasto das células de raízes (Kochian, 1995; Barceló e Poschenrieder, 2002; Kochian et al., 2005; Vardar e Ünal, 2007; Kopittke et al., 2015). Classificado como um elemento extremamente reativo, o Al liga-se preferencialmente a grupos negativos contendo oxigênio, como -COOH, -OH, -CO e $-PO_3$ (Kochian et al., 1995; Matsumoto, 2000; Vitorello et al., 2005). Devido à capacidade do Al^{3+} em se ligar a diversos componentes celulares, os efeitos provocados pelo Al são muito distintos entre as espécies e podem

levar a alterações fisiológicas, morfológicas, celulares e moleculares nas plantas sensíveis (Ciamporová et al., 2002; Panda e Matsumoto, 2007; Gupta et al., 2013).

Raízes danificadas pelo Al geralmente são curtas e apresentam poucos ou nenhum pelo radicular (Kochian et al., 2005; Kopittke et al., 2008; Gupta et al., 2013). Os ápices radiculares tornam-se mais largos e apresentam fissuras em sua superfície, provocadas pela desintegração e morte de células da epiderme e córtex periférico (Barceló e Poschenrieder, 2002; Ciamporová, 2002; Horst et al., 2010). Os ápices radiculares são as primeiras estruturas a entrar em contato direto com o Al e têm papel fundamental no mecanismo de percepção deste elemento (Matsumoto, 2000; Vardar e Ünal, 2007).

Recentemente, evidenciou-se que o Al tem grande afinidade com grupos carboxílicos da rede péctica do apoplasto das células de raízes (Wehr et al., 2010; Gupta et al., 2013), e alguns estudos associam esta afinidade com a inibição do crescimento radicular. A ligação com o Al pode alterar as propriedades estruturais e mecânicas da parede celular, tornando-a mais rígida e levando à redução de sua extensibilidade mecânica, a qual é necessária para o processo de expansão celular (Ciamporová, 2002; Horst et al., 2010; Gupta et al., 2013; Kopittke et al., 2015; Poschenrieder et al., 2015).

Sintomas da toxicidade do Al também são evidenciados na parte aérea das plantas, como redução da abertura estomática, redução da atividade fotossintética, clorose e necrose foliar (Gupta et al., 2013). Entretanto, geralmente são considerados consequências dos danos provocados no sistema radicular (Vitorello et al., 2005).

A exposição prolongada a altas concentrações de Al pode levar à formação de um sistema radicular danificado e, consequentemente, prejudicar a aquisição de água e nutrientes pela planta (Reyes-Díaz et al., 2015), o que, a longo prazo, pode provocar deficiência nutricional (Vitorello et al., 2005; Gupta et al., 2013). A consequência final da toxicidade do Al é crescimento com menor incorporação de biomassa, o que justifica o papel de limitador da produtividade agrícola atribuído ao Al (Vitorello et al., 2005).

Apesar de o crescimento e desenvolvimento de muitas espécies serem afetados negativamente pela presença do Al, diferentes níveis de relação à presença deste elemento podem ser observados entre as espécies vegetais (Kochian, 1995; Ma et al., 2000; Matsumoto, 2000; Ma et al., 2014).

Algumas plantas apresentam mecanismos para impedir a entrada deste elemento no interior de suas células, e assim, evitar os danos provocados pela toxicidade do Al (Horst et al, 2010; Ryan e Delhaize, 2010; Poschenrieder et al., 2015). Um destes mecanismos, vastamente identificado e apresentado por muitas espécies, é a exsudação de ácidos orgânicos (AOs – malato, citrato, oxalato e succinato) pelas raízes, os quais formam complexos estáveis com o Al no apoplasto e/ou na rizosfera, evitando sua absorção pelas plantas (Watanabi e Osaki, 2002; Ryan et al., 2011; Brunner e Spersisen, 2013).

Muitas outras plantas absorvem e acumulam Al em seus tecidos (Brunner e Sperisen, 2013; Poschenrieder et al., 2015). Neste caso, o Al adentra o simplasto e forma complexos estáveis com os ácidos orgânicos. Estes complexos são, posteriormente, armazenados nos vacúolos, permitindo que a concentração de Al livre no citosol mantenha-se baixa (Watanabi e Osaki, 2002; Reyes-Díaz et al., 2015). Além dos ácidos orgânicos, o Al pode se ligar a outros compostos no citoplasma, como ATP, RNA e compostos fenólicos (Barceló e Poschenrieder, 2002; Vardar e Ünal, 2007).

Os sítios de deposição do Al são diversificados entre as espécies. Alguns estudos demonstram que o Al se acumula predominantemente no sistema radicular (Ciamporová, 2002; Banhos et al., 2016; Schmitt et al., 2016). Outros, no entanto, demonstram grandes concentrações de Al na parte aérea (Chenery, 1948; Jansen et al., 2002). Espécies que apresentam a partir de 1000 mg de Al por kg de massa seca de caules e folhas, como *Camellia sinensis* (L.) Kuntze e *Hidrangea macrophylla* L., são classificadas como acumuladoras de Al (Ma et al., 2001; Watanabe e Osaki, 2002; Schmitt et al., 2016).

No Brasil, espécies acumuladoras de Al podem ser encontradas ocorrendo naturalmente em áreas de solo ácido, como o Cerrado (Haridasan, 1982; Bressan et al., 2016), e a concentração de Al nas folhas destas plantas pode superar, em muito, o limite de 1000 mg de Al por kg de massa seca, estabelecido para espécies cultivadas (Ma et al., 2001; Watanabe e Osaki, 2002; Schmitt et al., 2016). Entretanto, menos atenção é direcionada a estas comunidades, e poucos são os estudos que avaliam os efeitos provocados pelo Al em espécies nativas.

A vegetação do Cerrado é constituída por um mosaico de fisionomias, compostas por espécies arbóreas e herbáceas, que se distribuem desde campos,

passando por vegetação tipo savana (cerrado *sensu strictu*) até formações florestais, como o Cerradão (Coutinho, 1978; Oliveira-Fillho e Ratter, 2002; Habermann e Bressan, 2011). Estas formações são influenciadas não apenas pelo clima (sazonalidade), mas também por fatores edáficos (Pinheiro e Monteiro, 2010). Deste modo, a vegetação se desenvolve sobre solos ácidos (pH < 4.0), com alta saturação de Al trocável (m% > 70 %) e baixa concentração de nitrogênio, fósforo, cálcio e micronutrientes (Haridasan, 2008; Habermann e Bressan, 2011; Bressan et al., 2016).

As plantas do Cerrado podem ser classificadas como espécies acumuladoras e não acumuladoras de Al. Para espécies do Cerrado, plantas acumuladoras de Al apresentam entre 4.000 e 20.000 mg Al por kg de massa seca de folhas (MSF) (Haridasan, 1982; Haridasan e Araújo, 1988; Bressan et al., 2016). A maioria das plantas que acumulam Al no Cerrado são arbustos e árvores das famílias Melastomataceae, Rubiaceae, Simplocaceae e Vochysiaceae (Haridasan, 1982; Haridasan et al., 1986). Algumas espécies acumuladoras parecem não tolerar a falta de Al no solo quando cultivadas em solo calcário, como *Miconia albicans* (Sw.) DC. (Melastomataceae), *Vochysia thyrsoidea* Pohl. (Vochysiaceae) (Haridasan, 2008) e *V. tucanorum* Mart. (Souza et al., 2017). Entretanto, a grande maioria das espécies do Cerrado é de não acumuladoras de Al (Haridasan, 1982; Haridasan et al., 1986; Souza et al., 2015), plantas que apresentam de 100 a 600 mg de Al por kg de MSF (Haridasan, 1982; Jansen et al., 2002), como *Styrax ferrugineus* Nees and Mart. (Styracaceae) (Bressan et al., 2016).

Devido à grande riqueza de espécies e alto grau de endemismo, o Cerrado é considerado um *Hotspot* de biodiversidade (Myers et al., 2000), e o fato de espécies acumuladoras e não acumuladoras de Al coexistirem no mesmo ambiente evidencia a riqueza de mecanismos desenvolvidos por estas espécies para lidar com a alta disponibilidade de Al no solo. Diante do exposto, é intrigante perceber que a maioria dos estudos relacionados ao Al seja realizada com espécies cultivadas, pouco adaptadas a condições de solo ácido e álico (Barceló e Poschenrieder, 2002; Ciamporová, 2002; Kochian et al., 2005; Kopittke et al., 2015; Ma et al., 2001; Schmitt et al., 2016; Watanabe e Osaki, 2002; Yang e Horst, 2015). Nesta Tese de Doutorado, estudamos os efeitos provocados em espécies nativas do Cerrado quando expostas a diferentes concentrações de Al. Para tanto, dois experimentos foram realizados, um em casa de

vegetação e outro no campo, os quais correspondem aos capítulos 1 e 2 desta Tese, respectivamente.

O capítulo 1 apresenta os efeitos fisiológicos e celulares provocados em plantas de *Styrax camporum* Pohl. (Styracaceae) cultivadas em solução nutritiva contendo 0, 740 e 1480 μM Al, por 90 dias. Em campo, *S. camporum* apresenta entre 1000 e 1500 mg Al por kg de massa seca de folhas (Bressan et al., 2016). Entretanto, um trabalho prévio (Banhos et al., 2016) demonstrou que, quando cultivada em 1480 μM Al em solução nutritiva, esta espécie apresenta redução de brotação na parte aérea, redução nas taxas de trocas gasosas e redução na emissão de raízes laterais, e levantou a questão de que seria possível existir uma concentração entre 0 e 1480 μM de Al que fosse benéfica para esta espécie. Assim, testamos a hipótese de que *S. camporum* não apresenta sintomas de toxicidade quando exposta à concentração de 740 μM de Al podendo, inclusive, mostrar evidências de algum benefício do Al a essas plantas.

No capítulo 2 comparamos as concentrações foliares de Ca e Al de duas espécies do gênero *Qualea* (Vochysiaceae), ocorrendo em um fragmento de Cerrado localizado em solo calcário, recentemente descrito (Alves, 2017). A concentração foliar desses elementos foram comparados nestas mesmas espécies ocorrendo em um fragmento de Cerrado localizado em solo ácido e rico em Al (m% > 70%). Testamos a hipótese de que as concentrações foliares de Ca e Al encontradas nestas espécies refletem a disponibilidade destes elementos nos dois tipos de solo.

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CAPÍTULO 1

Styrax camporum, a moderate Aluminum-accumulating species from the Cerrado, does not benefit from non-toxic Al concentrations in nutrient solution

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Abstract

Styrax camporum Pohl. (Styracaceae) is a Cerrado woody species that grows on acidic soils with high aluminum (Al) saturation (m% > 50%). However, a previous study showed that 1480 μM Al in nutrient solution causes toxicity symptoms, which raises the question whether a concentration lower than 1480 μM Al could cause beneficial effects to this species. The association between Al exposure and mitochondria occurrence in root tips was also checked, as organic acids from Krebs cycle exuded by roots of *S. camporum* exposed to Al have been recently evidenced. Five-month-old plants were grown in nutrient solution with 0, 740 and 1480 μM Al for 90 days. Plants exposed to 1480 μM Al showed a less developed root system, reduced plant height and low gas exchange rates in relation to those exposed to 0 and 740 μM Al, confirming that 1480 μM Al is toxic to *S. camporum*. However, plants exposed to 0 and 740 μM Al showed similar number of leaves, plant height, root biomass, root length, total plant biomass and gas exchange rates, indicating that no beneficial effects of 740 μM Al could be observed for this species. In plants exposed to 1480 μM Al, mitochondria were not evident due to the great vacuolation of root cells, as evidenced by transmission electron microscopy analysis at 90 days.

Key words: Al^{3+}; Anatomical analysis; SEM; TEM; Styracaceae

1 Introduction

Aluminum (Al) is the third most abundant element in the Earth's crust and it naturally occurs as Al silicate and Al oxide in soils in general (Von Uexküll and Mutert 1995). In acidic soils (pH < 5.0) these Al forms can be solubilized into $Al(OH)^{2+}$, $Al(OH)_2^{+}$ and $Al(OH)_6^{3+}$ (or Al^{3+}), being the latter the most phytotoxic and over-studied (Von Uexküll and Mutert 1995; Vitorello et al., 2005; Singh et al., 2017). Approximately 30-45% of soils from the world's ice-free land are acidic (Von Uexküll and Mutert 1995), which guarantees the solubilized forms and its availability to plants.

In plants that are sensitive to Al, it causes direct effects on the roots, where it is mostly retained (> 75% of the absorbed Al) (Ciamporová, 2002; Vitorello et al., 2005; Banhos et al., 2016). One of the most conspicuous symptoms of Al toxicity is the inhibition of root growth (Kochian, 1995; Horst et al., 2010; Sun et al., 2010). Indirect effects can also be observed in the aboveground plant organs, such as reduced plant height, low biomass of stems and leaves (Vitorello et al., 2005; Singh et al., 2017, Silva et al., 2018) and low leaf gas exchange and photochemical performances (Chen et al., 2005; Jiang et al., 2008; Silva et al., 2012; Gupta et al., 2013).

On the other hand, there are plants that accumulate Al in their leaves without showing toxicity symptoms, as observed in *Camellia sinensis* (L.) Kuntze (Theaceae) and *Hidrangea macrophylla* L. (Hydrangeaceae), (Ma et al., 2001; Watanabe and Osaki, 2002). Al-accumulators usually store more than 1000 mg Al per kg dry leaves (Chenery, 1948; Jansen et al., 2002). Indeed, in this group of plants, Al may have beneficial effects as evidenced by increased root elongation and plant biomass as noted in *Melastoma malabathricum* L. (Melastomataceae), *Quercus serrata* Murray (Fagaceae) and *C. sinensis* (Bojórquez-Quintal et al., 2017).

In the Cerrado vegetation in South America, also known as 'Brazilian savanna', the woody plant community can be divided into Al-accumulating and non-accumulating species (Souza et al., 2015). Al-accumulators species from the cerrado show between 4000 and 20,000 mg Al per kg dry leaves in this vegetation (Haridasan, 1982; Haridasan and Araújo, 1988), and belong to Melastomataceae, Rubiaceae, Simplocaceae and Vochysiaceae families (Haridasan, 1982; Bressan et al., 2016; Malta et al., 2016). However, most of the woody species from the Cerrado are considered non-accumulators, and both groups grow well on soils that are dystrophic, acidic (pH < 4.5)

and with high Al saturation (m% > 50%) (Haridasan, 1982; Habermann and Bressan, 2011; Bressan et al., 2016).

Styrax camporum Pohl. (Styracaceae), a Cerrado woody species, stores approximately 1500 mg Al per kg dry leaves, as evidenced in a field study (Bressan et al., 2016), and could be considered a moderate Al-accumulating species. However, when cultivated in nutrient solution with 1480 μM Al, it retains 70% of the absorbed Al in the root system, and this concentration seems to be toxic to this species, causing reduced flushing and low gas exchange rates (Banhos et al., 2016). Being a native species widely distributed in the Cerrado physiognomies and growing well in soils that are acidic and rich in Al (Kissmann et al., 2012), we expect that a concentration lower than 1480 μM Al could cause beneficial effects to *S. camporum*.

In addition, a previous study has found organic acids (OAs) (malic, citric and oxalic acids) exudation by roots of *S. camporum* seedlings exposed to 0, 740 and 1480 μM Al in nutrient solution (Carvalho et al., 2018). Exuded OAs form non-toxic stable complexes with Al (Kochian et al., 2004; Brunner and Sperisen, 2013) and consequently avoids the phytotoxic reactions of free Al in the metabolism (Brunner and Sperisen, 2013; Singh et al., 2017). As these OAs are synthesized in Krebs cycle in the mitochondrial matrix (Taiz et al., 2017), we expect to find association between Al exposure and the occurrence of mitochondria in root apices of *S. camporum*.

Growing *S. camporum* plants in nutrient solution with 0, 740 and 1480 μM Al for 90 days, we measured biometric data, leaf gas exchange rates, photochemical parameters and leaf water potential. Al concentration in plant organs was also evaluated at 90 days. In addition, root apices were collected for light microscopy, scanning electron microscopy (SEM) and also for transmission electron microscopy (TEM).

2 Material and methods

2.1 Plant material and experimental conditions

Mature fruits of *Styrax camporum* Pohl. (Styracaceae) were collected from ten adult plants from Cerrado fragments in the municipality of Corumbataí and Itirapina, São Paulo state, Southeastern Brazil. The seeds germinated under controlled conditions (germination chamber at constant 25ºC), according to Kissmann and Habermann (2013). The seedlings were cultivated in a substrate (Plantmax, Campinas, SP, Brazil) without Al in a greenhouse under semi-controlled conditions. Seventy-five plants with

five months of age were transferred to opaque plastic boxes (50 cm x 30 cm x 15 cm; 20 L), containing nutrient solutions with 0, 740 and 1480 μM Al.

We used a nutrient solution (Furlani and Furlani, 1988) with a chemical composition based on Clark's solution (Clark 1975) that has been used to study Al toxicity in plants (Banhos et al., 2016; Silva et al., 2018). However, we diluted its macro- and micronutrient concentrations by seven in order to resemble the nutrient composition of Cerrado soils (Habermann and Bressan 2011; Souza et al. 2015), as also performed by Banhos et al. (2016) for studying Al toxicity in *S. camporum*. It consisted of 196.11 μM $Ca(NO_3)_2$ 4 H_2O, 72.43 μM NH_4NO_3, 32.06 μM KCl, 32.46 μM K_2SO_4, 31.23 μM KNO_3, 69.03 μM $Mg(NO_3)_2$ 6H_2O, 4.3 μM KH_2PO_4, 3.72 μM $FeSO_4$ 7H_2O, 3.4 μM NaEDTA, 0.5 μM $MnCl_2$ 4H_2O, 1.41 μM H_3BO_3, 0.13 μM $ZnSO_4$ 7 H_2O, 0.03 μM $CuSO_4$ 5H_2O, 0.06 μM $NaMoO_2$ 2 H_2O. This solution resulted in the following macronutrients (in mM): NO_3^- 0.137; NH_4^+ 0.058; P, 0.0019; K, 0.123; Ca, 0.204; Mg, 0.047; S, 0.031; and micronutrients (in μM): Cl, 30.58; Fe (EDTA), 3.32; B, 1.19; Mn, 0.41; Zn, 0.10; Cu, 0.04; Mo, 0.04. We observed that this solution caused no nutrient deficiency in the plants. The Al treatments were provided through $AlCl_3$ 6H_2O, and both 740 and 1480 μM Al caused no Al precipitation. The pH of the aerated solution was kept at 4.0 ± 0.1 to maintain Al as soluble as possible, and the nutrient solution was completely replaced every 15 days. Nominal 740 μM Al and 1480 μM Al supply resulted in 630 ± 32 μM Al and 1088 ± 20 μM Al, respectively.

Fifteen boxes (five per each treatment) stood on benches inside a greenhouse with semi-controlled conditions (784.7 ± 112.6 μmol photons m^{-2} s^{-1}; approximately 13h of natural photoperiod; 24.2 ± 0.7ºC). Expanded polystyrene (Isopor®) 50 × 30 cm plates (2-cm thick), with five equidistant holes (3.0 cm in diameter) each, were floated on the nutrient solution, and the plants were fixed in these holes with polyurethane foam strips that were placed around the plant collar.

2.2 Experimental design

The plants were exposed to the three treatments (0, 740 and 1480 μM Al) for 90 days. One day after planting (DAP) and at 90 DAP, biometric data (number of leaves, leaf area, plant height, root length and biomass of organs) were assessed. Leaf gas exchange rates and photochemical parameters were measured at 1, 15, 30, 60 and 90 DAP. Leaf water potential and Al concentration in plant organs were evaluated at 90

DAP. In addition, root apices were collected for light microscopy, scanning electron microscopy (SEM) (30, 60 and 90 DAP) and also for transmission electron microscopy (TEM) (90 DAP).

2.3 Biometric parameters

Using five replicates (plant) per treatment, the leaves, stems (plus petioles) and roots were separated in order to measure the biometric parameters. The number of leaves was counted and the plant height and root length were assessed with a ruler (cm). The total leaf area per plant was measured with an area meter (LI-3100C, LI-COR). Leaf, stem and root samples were oven-dried at 60°C until constant mass, and biomass (g) was measured using a scale.

2.4 Leaf gas exchange rates

The CO_2 assimilation (*A*, μmol m^{-2} s^{-1}) and transpiration (*E*, mmol m^{-2} s^{-1}) rates, stomatal conductance (*gs*, mol m^{-2} s^{-1}) and intercellular CO_2 (*Ci*) were measured in five replicates (plant) with a portable open gas exchange system (LI-6400xt, LI−COR, Lincoln, NE, USA). The CO_2 concentration entering the leaf cuvette (LCF chamber; 2 cm^2, LI-COR) averaged 400 μmol mol^{-1}, as provided by the 6400-01 CO_2 mixer (LI-COR). Measurements were performed between 9:00 and 11:30h (Banhos et al., 2016) on cloudless days. The photosynthetic photon flux density (PPFD) was provided by an artificial light source (6400-40 LCF, LI-COR), which was set to provide 1500 μmol photons m^{-2} s^{-1} in the leaf cuvette, as this value saturates *A* for *S. camporum* (Habermann et al., 2011). The vapor pressure deficit (VPD) inside the leaf cuvette was 2.05 ± 0.18 kPa, which means that the relative humidity in the (sample) chamber was 64.7 ± 2.3 %.

2.5 Photochemical parameters

Chlorophyll *a* fluorescence was measured in five replicates (plant) with a portable modulated fluorometer (6400-40 LCF, LI-COR), which was integrated into the LI-6400xt gas exchange system. The saturating light pulse was approximately 7000 μmol m^{-2} s^{-1} during 0.7 s. For calculating maximum quantum yield of photosystem II (PSII) (Fv/Fm), leaves were dark-adapted for 30 min (Bolhàr-Nordenkampf and Öquist, 1993) with aluminum foils, before measuring the fluorescence. Fm and Fv are maximum and variable fluorescence in dark-adapted leaves, respectively. The effective

quantum yield of PSII (ΦPSII) was calculated as (Fm' - Fs)/Fm', where Fm' and Fs indicate the maximum and the steady state fluorescence in light-adapted leaves, respectively. Apparent electron transport rate (ETR = ΦPSII PPFD 0.5 0.85) was calculated, using 0.5 as the fraction of excitation energy distributed to PSII, and 0.85 as the fractional light absorbance. The proportion of open PSII reactions centers (qP) was measured as (Fm' - Fs)/(Fm' - Fo'), where Fo' is the minimal fluorescence from light-adapted leaves (Bolhàr-Nordenkampf and Öquist, 1993).

2.6 Leaf water potential

Leaf water potential was measured in three replicates (plant) at predawn (Ψpd) and midday (Ψmd; under maximum VPD), and values in MPa were obtained by the pressure chamber method (Turner, 1981), using a DIK-7000 (Daiki Rika Kogyo, Tokyo, Japan) chamber.

2.7 Aluminum concentration in plant organs

This parameter was measured using five replicates. The root samples were washed thrice with deionized water in order to remove residual Al from the nutrient solution. Dried samples of leaves, stems (plus petioles) and roots were sent to a routine plant nutrition laboratory at Instituto Agronômico de Campinas (IAC, Campinas, SP, Brasil) where these were ground and digested in a solution of sulfuric:nitric:percloric acids (1:10:2, v/v/v). After digestion, the Al concentration was determined by the atomic absorption spectrophotometer method (Sarruge and Haag, 1974) and expressed as mg Al per kg dry mass.

2.8 Light microscopy

Root tips (~ 0.5 cm in length and 1mm in diameter) were collected and immediately fixed in Karnovsky solution (with 0.1 M phosphate buffer, at pH 7.3; overnight at 4°C) (Karnovsky, 1965). The samples were dehydrated in increasing ethanol series [30, 50, 70, 90 (one hour each), and 100% (three times, one hour each)], then infiltrated with resin (Historesin, Leica instruments, Germany) and ethanol 100%, at a ratio of 1:1, overnight. After 24h, samples were infiltrated with pure resin, reserved overnight and then polymerized in blocks. Longitudinal sections were obtained with a rotary microtome and mounted on glass slides that were immersed (5 min) in a toluidine

blue solution (pH 4.5) for staining (at room temperature) (O'brien et al., 1964). Then, the glass slides were washed under tap water to remove excess of dye and mounted with Entellan® (Merck, Darmstadt, Germany). All sections were observed under light microscope (DMLB, Leica Microsystems, Wetzlar, Germany) and the images were captured with a digital camera (DFC-290, Leica Microsystems, Germany) functionally attached to the DMLB.

2.9 Scanning electron microscopy (SEM)

The root longitudinal segments were fixed in a Karnovsky solution as described previously (Karnovsky, 1965), and dehydrated in an increasing acetone series of 50, 70, 90, 95, and 2 x 100%, kept for 15 min in each step. The samples were then transferred to the drying apparatus to the critical point (CPD 030, Bal-tec, Balzers, Liechtenstein), where they were passed through CO_2 baths until the material was completely dry. The samples were mounted on stubs made of brass using carbon tape (double sided carbon tape, 8 mm in width; Electron Microscopy Science, EMS, USA). The images were obtained from a scanning electron microscope (TM 3000, Hitachi, Japan) operated at 15 kV.

2.10 Transmission electron microscopy (TEM)

Samples previously fixed in Karnowsky solution were washed with PBS buffer (0.1M and pH 7.2) twice (15 min each) and post-fixed with osmium tetroxide solution (1% in 0.1% sodium cacodylate buffer, 1 M) for 2 hours in the dark. The samples were then washed with PBS buffer twice (15 min each), washed with 10% ethanol (15 min) and contrasted with uranyl acetate solution (0.2 g uranyl acetate + 10 mL of 10% ethanol) for 2 hours. The samples were then dehydrated, first in increasing series of ethanol (30%, 90% and 95% for 15 min in each of these solutions and three times of 15 min in 100% ethanol), then in 100% ethanol solution + 100% acetone (1:1 v/v) for 15 min and finally in 100% acetone for 15 min. Subsequently the samples were infiltrated with acetone solution + Epon - Araldite resin (29.3g Araldite 6005 + 30.8g Epon 812 + 49.9g DDSA) (1:1:1, v/v/v) overnight, and then in pure resin + 4 drops of catalyst (4 GT) overnight. The samples were polymerized in pure resin with catalyst in silicone molds. The resin blocks were sectioned in ultramicrotome (Reichert Supernova, Leica Microsystems, Germany) with disposable glass razors.

Transverse sections of the root apices (150 to 240 nm thick) were stained with

toluidine blue and preserved on permanent slides mounted with Entellan® (Merck), and analyzed under light microscopy. Subsequently, five sections with 90 to 100 nm thickness were accommodated on 200 mesh copper screens. The screens were then contrasted in uranyl acetate (2%) for 45 min and lead citrate (0.4%) for 10 min. The contrast was done on a petri dish coated with aluminum foil (to avoid light incidence) with NaOH pellets inside in order to avoid precipitation of lead acetate. Subsequently, they were washed in the following sequence: deionized water, NaOH solution (0.02N) and three more times in deionized water. The samples were then observed under transmission eletron microscope JEM1011 (JEOL, USA).

2.11 Data analysis

A one-way analysis of variance (ANOVA) was conducted between plants exposed to 0, 740 and 1480 μM Al to test differences in the number of leaves, leaf area, plant height, root length, biomass of leaf, stem, root and total plant biomass at 0 and 90 DAP, as well as in *A*, *E*, *gs*, *Ci*, Fv/Fm, ΦPSII, ETR and qP, separately, at 1, 15, 30, 60 and 90 DAP. This same procedure was conducted to test differences in Ψpd, Ψmd and Al concentration in the plant organs at 90 DAP. The Tukey test ($\alpha = 0.05$) was used to conduct post hoc comparisons to determine the least significant difference between mean results. In addition, a Student t-test ($\propto = 0.05$) was used to test differences in the biometric parameters for each treatment (0, 740 and 1480 μM Al) between 0 and 90 DAP.

3 Results

Plants exposed to 1480 μM Al were visually affected by Al, showing a less developed root system and reduced plant height in relation to those exposed to 0 and 740 μM Al, at 90 DAP (Fig. 1). In addition, plants exposed to 0 and 740 μM Al showed more and larger leaves in relation to those of plants exposed to 1480 μM Al (Fig. 1).

Plants exposed to 1480 μM Al showed lower values of biometric parameters in relation to those exposed to 0 and 740 μM Al, at 90 DAP (Fig. 2). There was no increase in plant height (Fig. 2E), root length (Fig. 2G) and stem and root biomass (Fig. 2D, 2F) between 0 and 90 DAP. In this same period, plants exposed to 1480 μM Al shed part of their leaves and, consequently, reduced the number of leaves (Fig. 2A), leaf biomass (Fig. 2B), leaf area (Fig. 2C) and total plant biomass (Fig. 2H). On the other

hand, between 0 and 90 DAP, plants exposed to 0 and 740 μM Al showed increased number of leaves (Fig. 2A), plant height (Fig. 2E), root biomass (Fig. 2F), root length (Fig. 2G) and total plant biomass (Fig. 2H).

In relation to plants exposed to 0 and 740 μM Al, the plants exposed to 1480 μM Al showed lower values of *A* (Fig. 3A), *gs* (Fig. 3B) and *E* (Fig. 3C), mainly at 60 and 90 DAP. The intercellular CO_2 (*Ci*) was lower in plants exposed to 1480 μM Al only at 15 DAP (Fig. 3D). Plants exposed to 0 and 740 μM Al showed the same leaf gas exchange rates throughout the study (Fig. 3).

The maximum quantum efficiency of PSII (Fv/Fm) was, in general, similar between treatments, and at 30 DAP plants not exposed to Al showed lower values than those exposed to Al (Fig. 4A). The effective quantum efficiency of PSII (ΦPSII) (Fig. 4B), ETR (Fig. 4C) and qP (Fig. 4D) were variable until 30 DAP, and at 60 DAP plants exposed to 0 μM Al and 1480 μM Al showed the highest and the lowest values, respectively, while those exposed to 740 μM Al exhibited intermediate values. However, at 90 DAP, these responses were different: 740 μM Al > 0 μM Al > 1480 μM Al (Fig. 4).

Predawn leaf water potential (Ψpd) was similar between treatments (Fig. 5). Plants exposed to 1480 μM Al showed 60% lower midday leaf water potential (Ψmd) in relation to those exposed to 0 and 740 μM Al (Fig. 5).

Plants exposed to 1480 μM Al accumulated more Al than those exposed to 0 and 740 μM Al (Fig. 6). Regardless of the Al concentration in the nutrient solution, most of this metal was retained in the root system, being 75% in the roots of plants exposed to 1480 μM Al, 95% in the roots of those exposed to 740 μM Al and, even in the plants cultivated in the solution with nominal 0 μM Al, 80% of the Al available was retained in their roots (Fig. 6).

Plants exposed to 740 and 1480 μM Al showed wider root tips (Fig. 7E, 7F and 7H and 7I) when compared to sharp arrow-like root tips of plants not exposed to Al (Fig. 7D and 7G), mainly from 60 DAP. The hypodermis from the distal region in the root tips of plants exposed to 1480 μM Al showed larger and round-shape cells evidenced since 30 DAP (Fig. 7C, 7F and 7I) when compared to the regular shape of cells from this tissue in the roots of plants exposed to 0 and 740 μM Al at 30 DAP (Fig. 7A and 7B), 60 DAP (Fig. 7D and 7E) and 90 DAP (Fig. 7G and 7H). The cortex in the

root tips of plants exposed to 1480 μM Al exhibited rectangular-shape and vacuolated cells evidenced from 30 DAP (Fig. 7C, 7F and 7I), while cortical cells in root tips of plants exposed to 0 and 740 μM Al were smaller and round-shape (Fig. 7A, 7B, 7D, 7E, 7G and 7H). At 90 DAP, the vascular cylinder in root tips of plants exposed to 1480 μM Al showed more lignin deposition, as evidenced by stronger blue-green stains (Fig. 7I), and differentiation of this tissue is noted closer to the root apex in relation to that of plants exposed to 0 and 740 μM Al (Fig. 7G and 7H).

Plants exposed to 1480 μM Al showed root apices with more epidermal discard (Fig. 8C, 8F and 8I) than those from plants exposed to 0 (Fig. 8A, 8D and 8G) and 740 μM Al (Fig. 8B, 8E and 8H) at every DAP. At 90 DAP, cracks and fissures were conspicuously observed in root apices of plants exposed to 1480 μM Al (Fig. 8I).

In transversal sections, cortical cells in root tips of plants exposed to 0 μM Al showed regular size (Fig. 9A), while those in plants exposed to 740 μM Al (Fig. 10A) and 1480 μM Al (Fig. 11A) showed distinct size between the peripheral and internal cortex. Cortical cells in root tips of plants exposed to 0 μM Al showed vacuoles with low volume, integral aspects of nucleus and nuclear envelope, abundant starch granules, endoplasmic reticulum and profuse mitochondria with integral aspects (Fig. 9B-G). In plants exposed to 740 μM Al, the peripheral cortex cells showed vacuoles with different sizes and volumes, integral aspects of nucleus and nuclear envelope (Fig. 10B, 10C and 10D) and abundant mitochondria (Fig. 10B and 10C). However, internal cortex cells showed larger vacuoles (Fig. 10E, 10F and 10G) and deposits of electron-dense material (Fig. 10E). Starch granules were also observed in the peripheral (Fig. 10B and 10C) and internal (Fig. 10F) cortical cells. In plants exposed to 1480 μM Al, xylem and phloem cells are irregularly larger and show thick cell walls (Fig. 11A). In these plants, the nucleolus became extremely electron-dense (Fig. 11B-F) and deposits of electron-dense material were also observed (Fig. 11B, 11C, 11E and 11F). Peripheral and internal cortical cells of plants exposed to 1480 μM Al showed numerous vacuoles with great volumes, but organelles were not evident in this treatment (Fig. 11B-G).

4 Discussion

In general, plants that store more than 1000 mg Al per kg dry leaves are considered Al-accumulators (Chenery, 1948; Jansen et al., 2002). Cerrado woody plants that accumulate up to 600 mg Al per kg dry leaves are considered Al non-accumulating

species, while Al-accumulating species from this vegetation store between 4,000 and 20,000 mg Al per kg dry leaves (Haridasan, 1982; Haridasan and Araújo, 1988). In the field, *S. camporum* accumulates approximately 1500 mg Al per kg dry leaves (Bressan et al., 2016). *S. camporum* could, then, be considered a moderate Al-accumulating species. Thus, one would not expect this species to exhibit Al toxicity symptoms when growing in the presence of Al, because *S. camporum* is widely distributed in the physiognomies of the Cerrado (Kissmann et al., 2012). However, when cultivated in nutrient solution with 1480 μM Al this species showed leaf shedding, reduced root length, low biomass of roots and stems (Fig. 1C and Fig. 2), as well as reduced leaf gas exchange rates (Fig. 3) and low ΦPSII and ETR at 30, 60 and 90 DAP (Fig. 4B and 4C). Similar results were observed for this same species growing in a nutrient solution with 1480 μM Al (Banhos et al., 2016). Taken together, these observations suggest that 1480 μM Al is toxic to this moderate Al-accumulating species.

The number of leaves, leaf area, leaf biomass, plant height, root length, stem, root and total plant biomass were similar between plants cultivated in nutrient solution with 0 μM Al and 740 μM Al (Fig. 2). In addition, plants exposed to 0 and 740 μM Al showed the same leaf gas exchange rates throughout the study (Fig. 3). These results indicate that 740 μM Al is not toxic for this species, although no beneficial effects of this Al concentration could be observed either, consequently denying our hypothesis. Beneficial effects of Al seem to be dose-responsive and species-dependent; however, there is evidence that in Al-accumulating species not from the Cerrado, such as *Camellia sinensis* (L.) Kuntze (Theaceae), *Melastoma malabathricum* L. (Melastomataceae) and *Quercus serrata* Murray (Fagaceae), the Al promotes root elongation and biomass enhancement (Bojórquez-Quintal et al., 2017). For *C. sinensis*, the absence of Al may also cause slow root elongation and plant growth (Tsuji et al., 1994; Fung et al., 2008). In the case of Al-accumulating species from the Cerrado, *Vochysia thyrsoidea* Pohl. growing in a medium without Al showed leaf chlorosis, and *Miconia albicans* (Sw.) Triana growing on an alkaline soil was less developed (Haridasan, 2008). In addition, *Vochysia tucanorum* (Vochysiaceae) growing on a calcareous soil without Al available showed chlorotic and necrotic leaves (Souza et al., 2017). On the other hand, our results show that *S. camporum* growing in nutrient solution with 0 μM Al did not exhibit physiological or morphological damages (Fig. 1A

and Fig. 2), suggesting that this species may not depend on this metal for growth and development.

Compared to plants exposed to 0 μM Al and 740 μM Al, the less developed root system in plants exposed to 1480 μM Al (Fig. 1) could suggest less roots contributing to water uptake, and this could explain the low Ψmd (Fig. 5) and reduced leaf gas exchange rates (Fig. 3), possibly due to a lack of water supply to the mesophyll. For instance, the low *gs* values observed in plants exposed to 1480 μM Al, especially after 30 DAP, reflects the low *A* and *E* values during the same period. Therefore, even for this moderate Al-accumulating species from the Cerrado, the Al may cause inhibition of root growth (Fig. 2F and 2G), leading to a possible low water uptake and supply to the mesophyll (Fig. 5), with negative consequences for gas exchange rates, especially *gs* (Fig. 3B), *A* (Fig. 3A) and *E* (Fig. 3C). Thus, our results reiterate similar results that were observed by Banhos et al. (2016), suggesting that Al concentration higher than or close to 1480 μM Al in the nutrient solution may have systemic negative impacts for this species.

Although field studies show that Al-accumulating Cerrado woody species store Al in the leaves (Haridasan, 1982; Andrade et al., 2011; Malta et al., 2016; Bressan et al., 2016), and not investigating the Al concentration in their roots probably due to the difficulty to study their roots in the field, studies using nutrient solution (Banhos et al., 2016) or soils with contrasting Al availability (Souza et al., 2017) have demonstrated that the Al is retained mainly in the root system of Al-accumulating species from this vegetation. In the present study, *S. camporum* exposed to 1480 μM Al retained 75% of the absorbed Al in their roots, while plants exposed to 740 μM Al retained 95% of it in the root system (Fig. 6), corroborating the few studies using Al-accumulating Cerrado woody species growing in nutrient solution or in contrasting soils. *S. camporum* is a moderate Al-accumulating species, as demonstrated in a field study (Bressan et al., 2016), and here we demonstrate that the more Al available in the nutrient solution the more Al uptake by *S. camporum* and accumulated in its leaves and shoots, but mainly in its roots (Fig. 6).

Although *S. camporum* is a moderate Al-accumulating species, our results demonstrate that 1480 μM Al in nutrient solution is toxic to this species, and this is associated with anatomical characteristics observed in the root apices. The longer the exposure and the higher the Al concentration in the nutrient solution more damage was

observed in the epidermis and hypodermis in the root apices (Fig. 7 and Fig. 8). At 90 DAP, plants exposed to 1480 μM Al showed cracks and ruptures in the apex surface (Fig 8I). In Al non-accumulating species that are sensitive to this metal, such as soybean [*Glicine max* (L.) Merr.] and wheat (*Triticum sp*. L.), the Al caused cracks in the root surface, which was attributed to disintegration and death of epidermis, hypodermis and next cortical cells (Ciamporová, 2002). In another study, root epidermal cells of *Zea mays* L. underwent to a complete disintegration when exposed to toxic Al concentration in nutrient solution (Ciamporová, 2000). In the case of these studies (Ciamporová, 2000; 2002), an irregular expansion of the cells within the root tissues was suggested to cause mechanical stress leading to disintegration of peripheral root tissues. Similarly, in the present study, 1480 μM Al caused progressive disorganization in the epidermis and hypodermis, which may have mechanically contributed to the intense discard of epidermal cells (Fig. 7 and Fig. 8).

The longer the exposure and the higher the concentration of Al in the nutrient solution, the more vacuolation in the cortical cells, mainly in internal ones (Fig 9, Fig 10 and Fig 11). In studies performed with barley (*Hordeum vulgare* L.) (Ikeda and Tadano, 1993) and oat (*Avena sativa* L.) (Marienfeld et al., 1995), cells in the root apex showed a progressive enhanced vacuolation when exposed to toxic Al concentration in nutrient solution. Internal Al tolerance in plants suggests that the Al may be confined into vacuoles (Brunner and Sperisen, 2013; Poschenrieder et al., 2015; Reyes-Díaz et al., 2015). For instance, Al protein transporters investigated in mutants of *Arabidopsis thaliana* (Larsen et al., 2007) and *Oryza sativa* L. (Huang et al., 2012) are assumed to exist in the tonoplast, in order to transfer Al from the cytosol to the vacuole. However, the mechanisms involved in tonoplast transport and sequestration of Al are not fully understood (Huang et al., 2012). Therefore, the intense vacuolation of cortical cells observed in the root apices of plants exposed to 1480 μM Al (Fig. 11) could have dislocated the cortical cells towards the peripheral direction, partially explaining the wider root tips (Fig. 7E, 7F and 7H and 7I) when compared to sharp arrow-like root tips of plants not exposed to Al (Fig. 7D and 7G).

In the present study, plants exposed to 1480 μM Al showed cortical cells with extremely electron-dense nucleolus (Fig. 11B-F) and spots of electron-dense material in the cytoplasm (Fig. 11B, 11C, 11E and 11F). Plants of *Theobroma cacao* L. exposed to 2220 μM Al in nutrient solution showed deposits of electron-dense materials in the xylem parenchyma and endodermis of roots and increased concentration of reactive

oxygen species (ROS) (Almeida et al., 2015), suggesting an association between these micromorphological injures and oxidative stress caused by the Al. Indeed, many studies have indicated oxidative stress in plant Al toxicity (Yamamoto et al., 2002; Yamamoto et al., 2003; Singh et al., 2017). We did not measure enzymatic or molecular responses to check ROS production between the Al treatments, but the electron-dense nucleolus and material found in *S. camporum* exposed to Al could indicate the occurrence of oxidative stress, and this topic deserves further investigations in Al-accumulating Cerrado woody species.

Finally, we expected to find a great number of mitochondria in plants exposed to Al in relation to those grown without Al, as a previous study evidenced organic acid exudation in *S. camporum* seedlings exposed to 0, 740 and 1480 μM Al in nutrient solution (Carvalho et al., 2018), and organic acids are synthesized in Krebs cycle, in the mitochondrial matrix. In plants exposed to 740 μM Al mitochondria were observed in cells of the peripheral cortex (Fig. 10B, 10C). However, in plants exposed to 1480 μM Al, organelles, including mitochondria, were not evident due to the great vacuolation of cells (Fig. 11B-G). Therefore, we could not confirm any association between Al exposure and conspicuous occurrence of mitochondria.

It has been demonstrated that 1480 μM Al in nutrient solution is toxic to *S. camporum* (Banhos et al., 2016), but here we demonstrate that an intermediate Al concentration (740 μM Al), although not toxic, cannot cause beneficial effects either for this moderate Al-accumulating species from the Cerrado. In addition, we also point out that the longer the exposure and the higher the concentration of Al in the nutrient solution, the more vacuolation in the cortical cells, and that a progressive disorganization in the epidermis and hypodermis may have mechanically contributed to the intense discard of epidermal cells.

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Figures:

Fig. 1 General view of *S. camporum* plants after growing in a nutrient solution containing 0 (A), 740 (B) and 1480 µM Al (C), for 90 days. Scale bar = 15 cm.

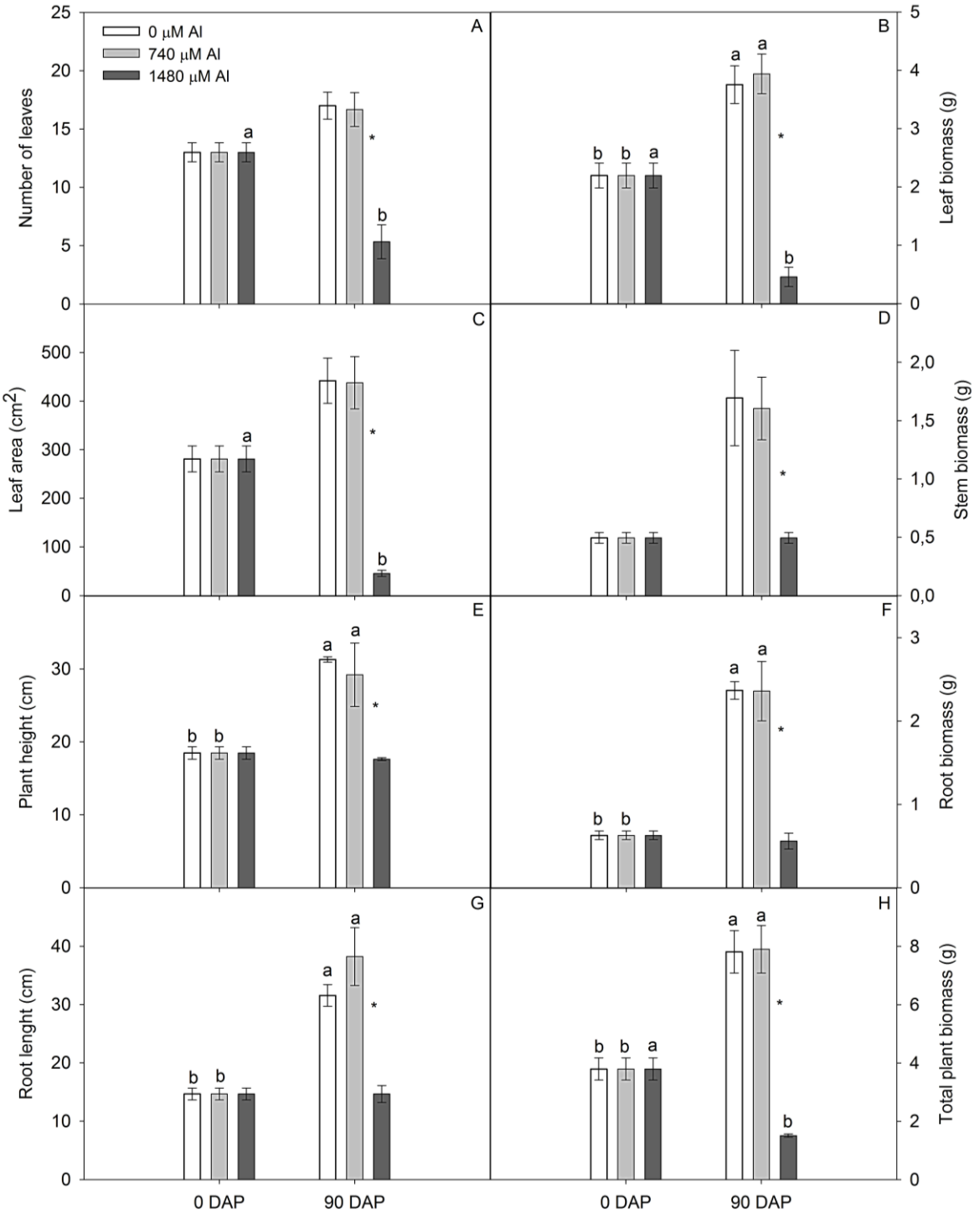

Fig. 2 Mean values (n = 5 plants) of biometric parameters (A, C, E and G) and biomass of organs (B, D, F and H) of *S. camporum* plants at 0 and 90 days after planting (DAP) in nutrient solution containing 0, 740 and 1480 µM Al. For each treatment, distinct letters indicate significant differences ($P < 0.05$) between 0 and 90 DAP. Asterisks indicate significant difference ($P < 0.05$) between treatments at 90 DAP. Vertical bars = s.d.

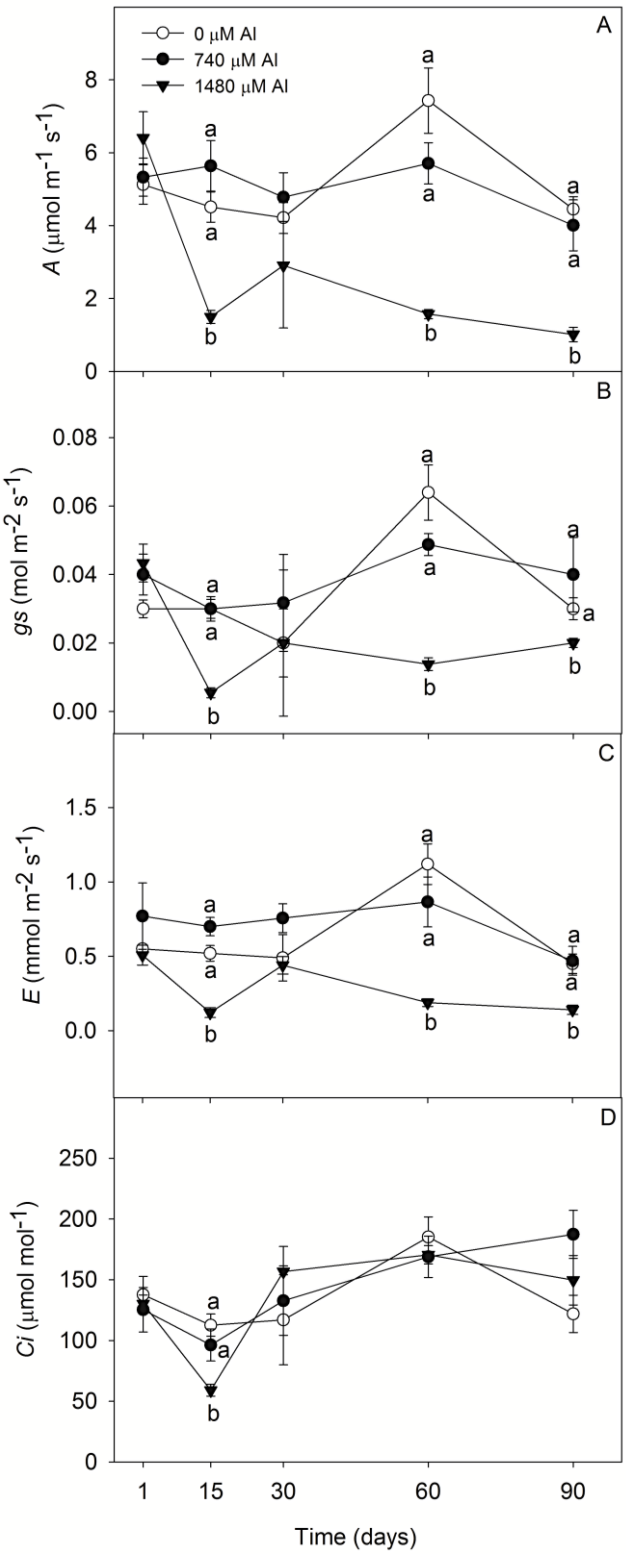

Fig. 3 Mean values (n = 5 plants) of gas exchange rates (A, B, C) and intercellular CO_2 (D) of *S. camporum* plants grown for 90 days in nutrient solution containing 0, 740 and 1480 μM Al. For each evaluation date, distinct letters indicate significant differences ($P < 0.05$) between treatments. Bars = s.e.

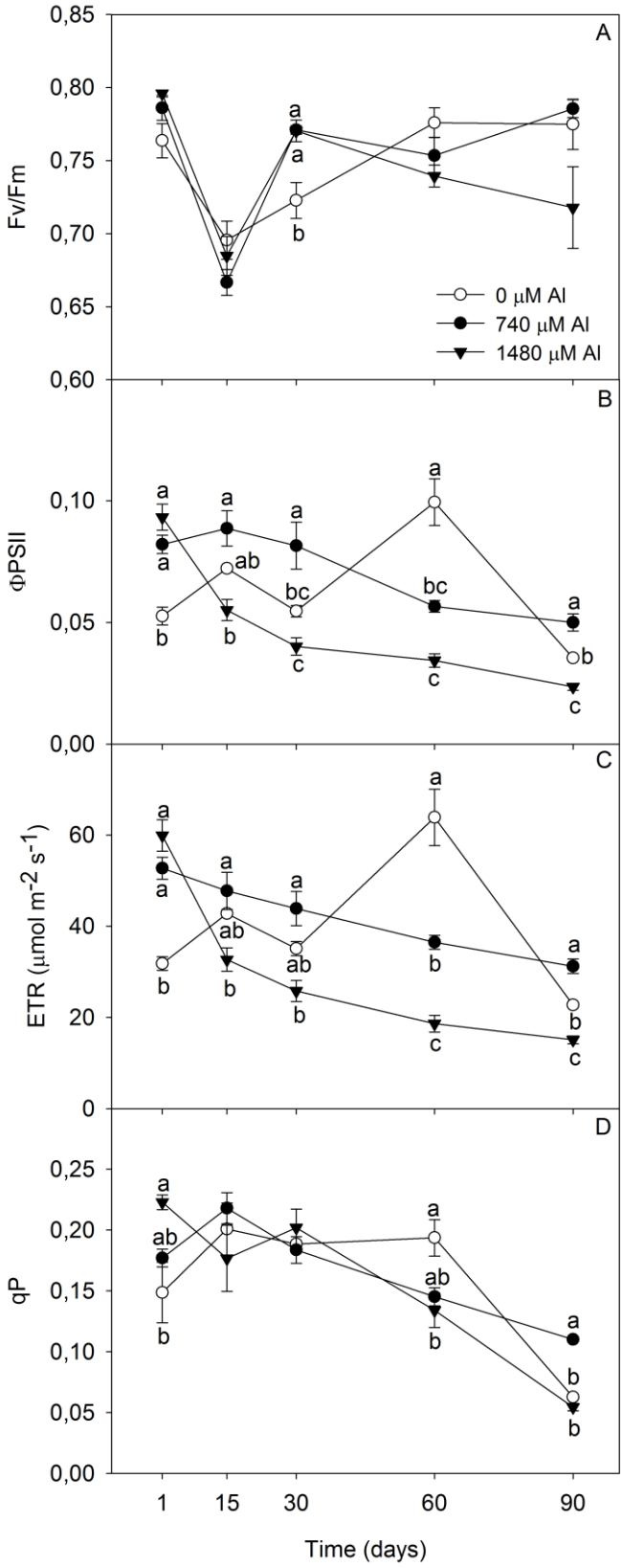

Fig. 4 Mean values (n = 5 plants) of photochemical parameters of *S. camporum* plants grown for 90 days in nutrient solution containing 0, 740 and 1480 µM Al. For each evaluation date, distinct letters indicate significant differences ($P < 0.05$) between treatments. Bars = s.e.

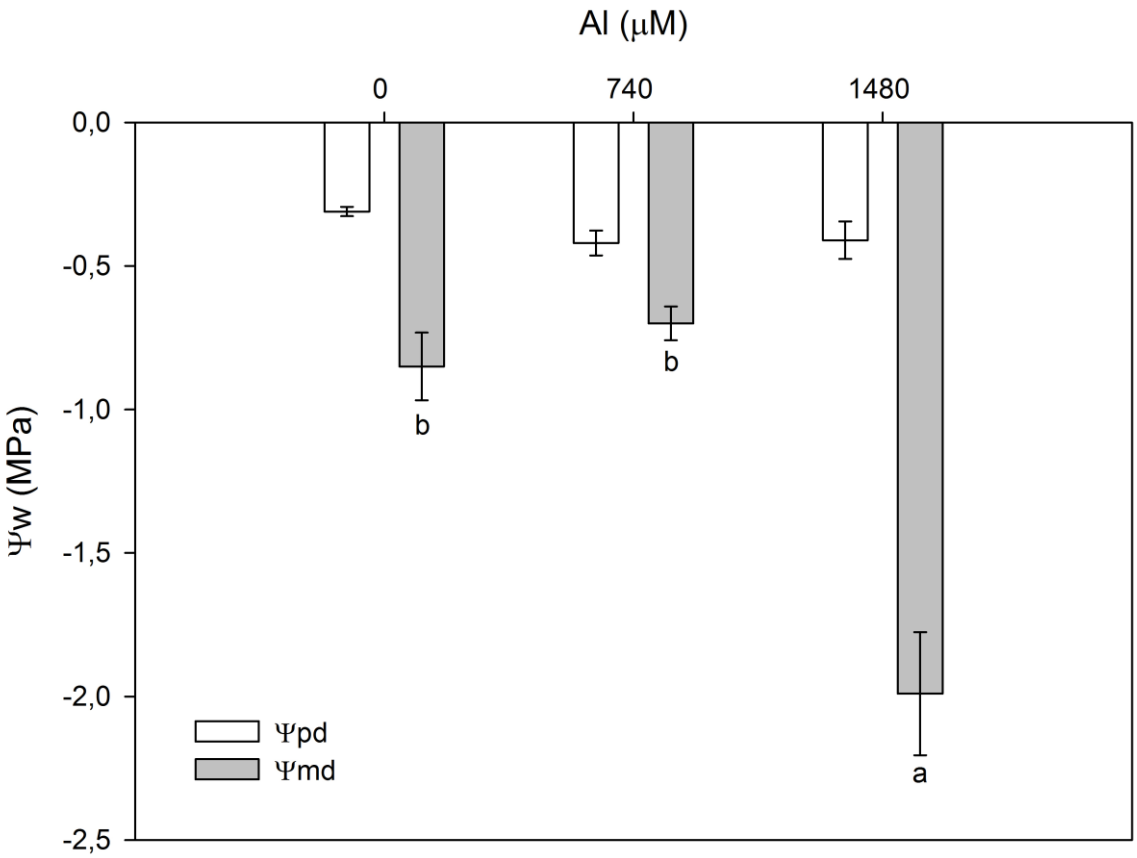

Fig. 5 Leaf water potential at predawn (Ψpd) and midday (Ψmd) of *S. camporum* plants grown in nutrient solution containing 0, 740 and 1480 μM Al, at 90 DAP. Distinct letters indicate significant differences ($P < 0.05$) between treatments. Bars = s. e.

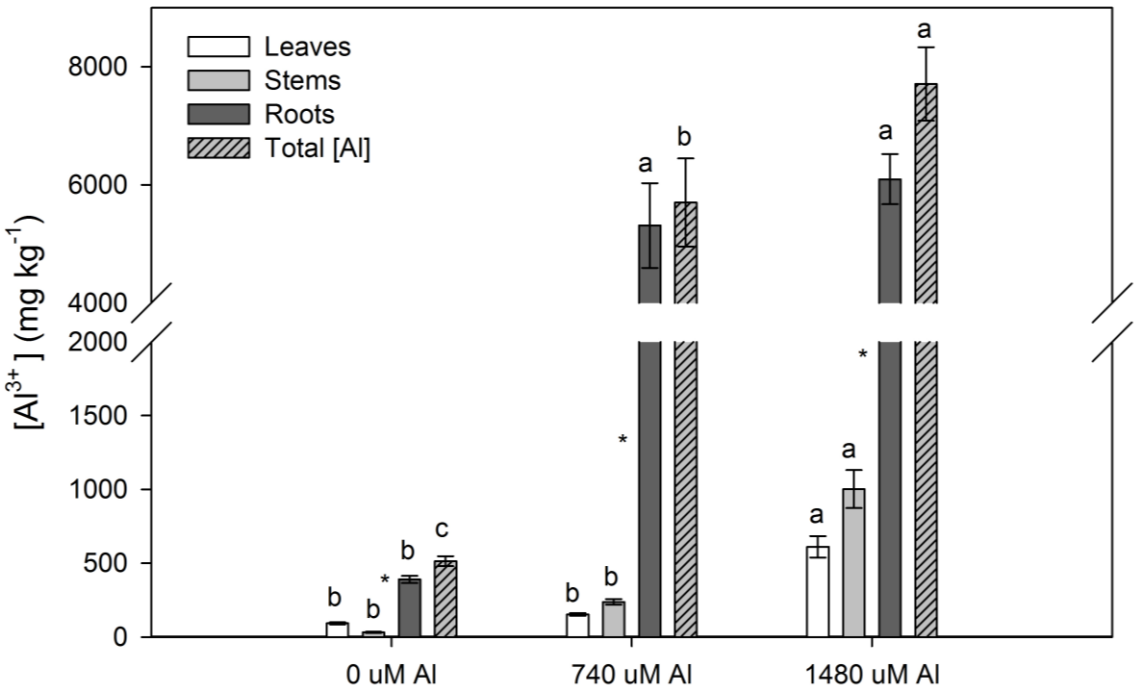

Fig. 6 Mean values (n = 5 plants) of aluminum (Al) concentration in the leaves, stems, roots and in the whole plant of *S. camporum* cultivated for 90 days in nutrient solution containing 0, 740 and 1480 μM Al. For each plant organ (or the whole plant), distinct letters indicate significant differences ($P < 0.05$) between the three treatments. For each Al treatment, asterisks indicate significant difference ($P < 0.05$) between plant organs. Bars = s. e.

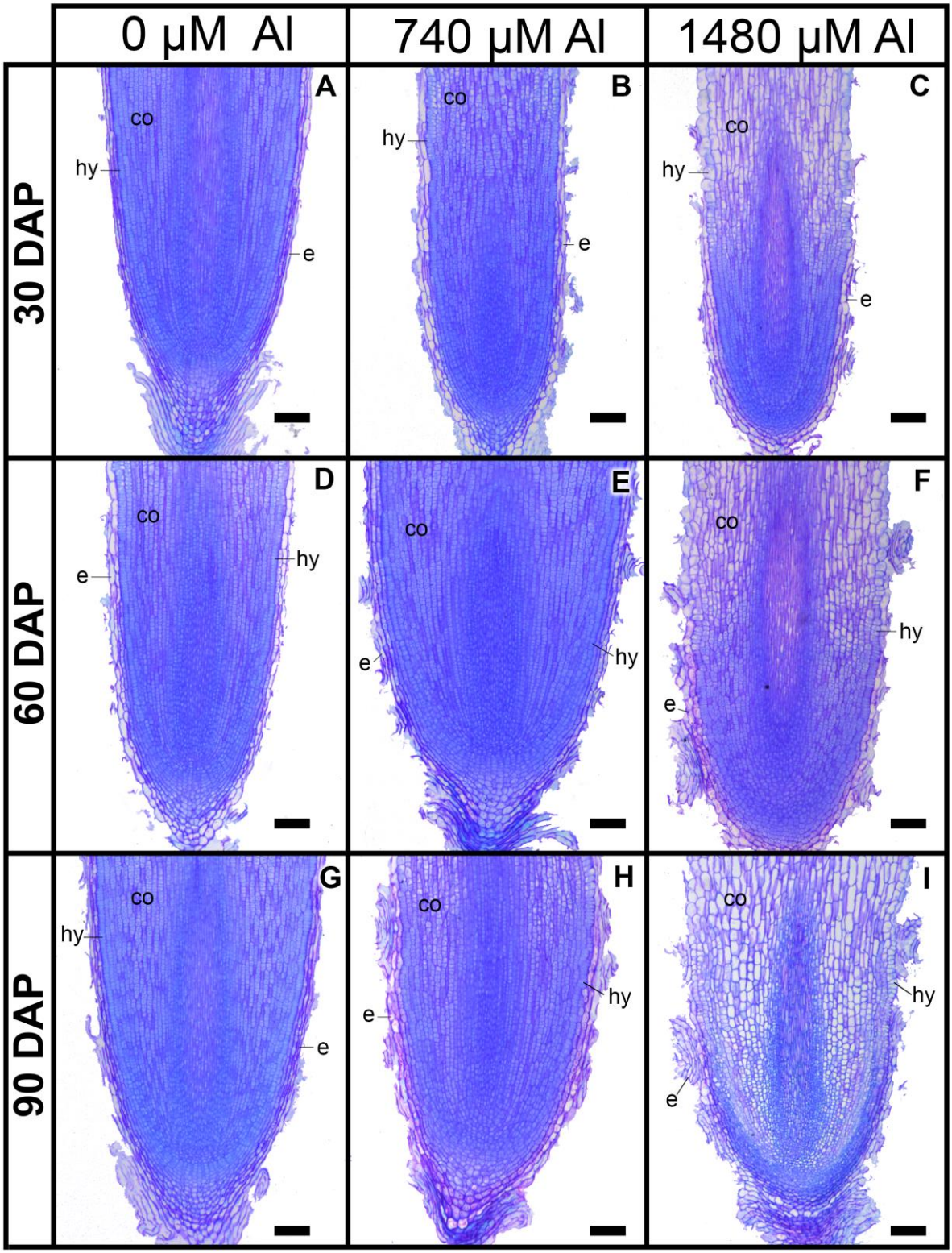

Fig. 7 Light microscopy: Longitudinal sections of root apices of *S. camporum* grown in nutrient solution containing 0, 740 and 1480 µM Al at 30 days after planting (DAP) (A, B, C), 60 DAP (D, E, F) and 90 DAP (G, H, I). e = epidermis; hy = hypodermis; co = cortex. Scale bar = 100 µm.

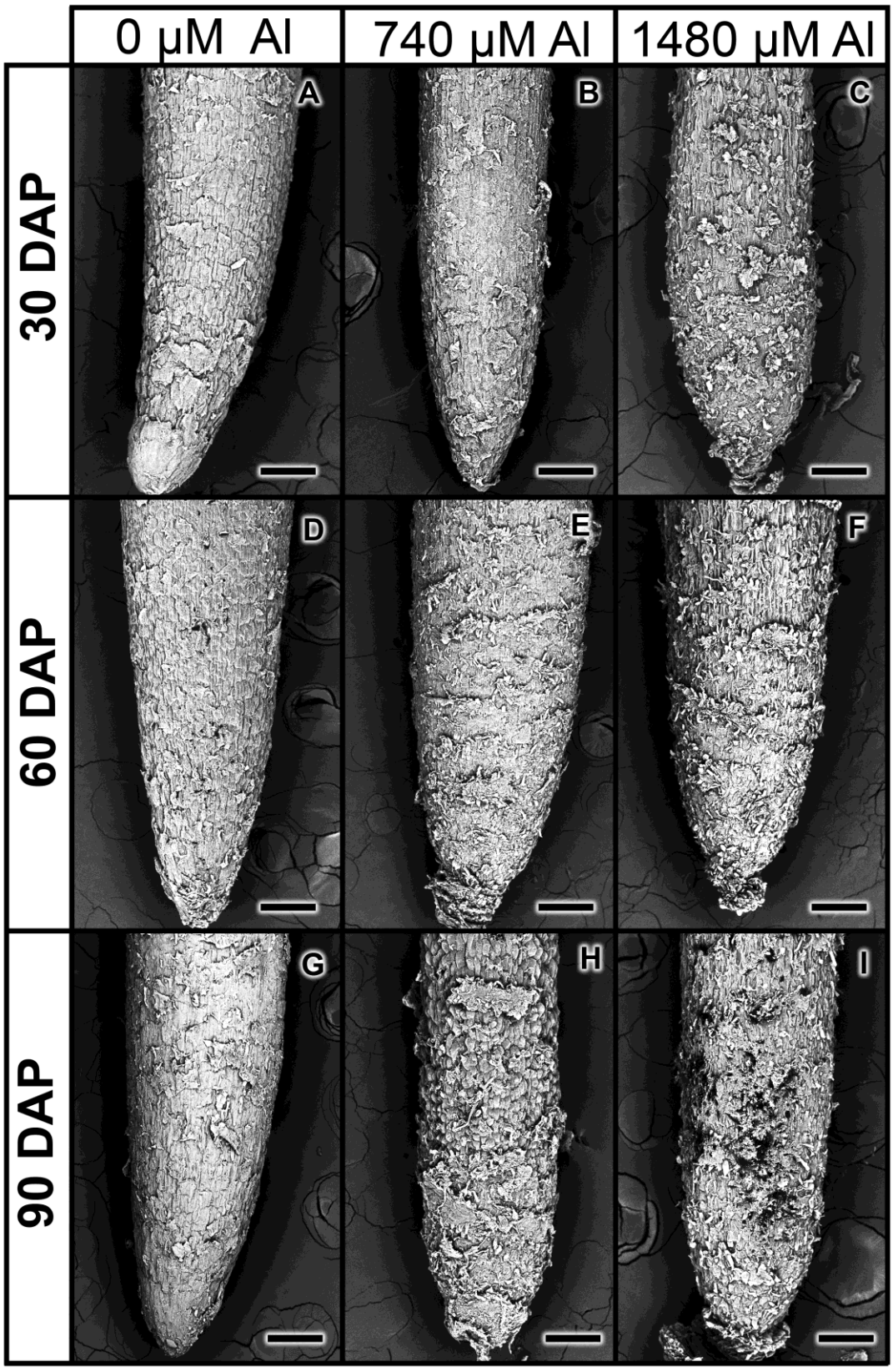

Fig. 8 Scanning electron microscopy (SEM): Root apices of *S. camporum* grown in nutrient solution containing 0, 740 and 1480 µM Al at 30 days after planting (DAP) (A, B, C), 60 DAP (D, E, F) and 90 DAP (G, H, I). Scale bar = 200 µm.

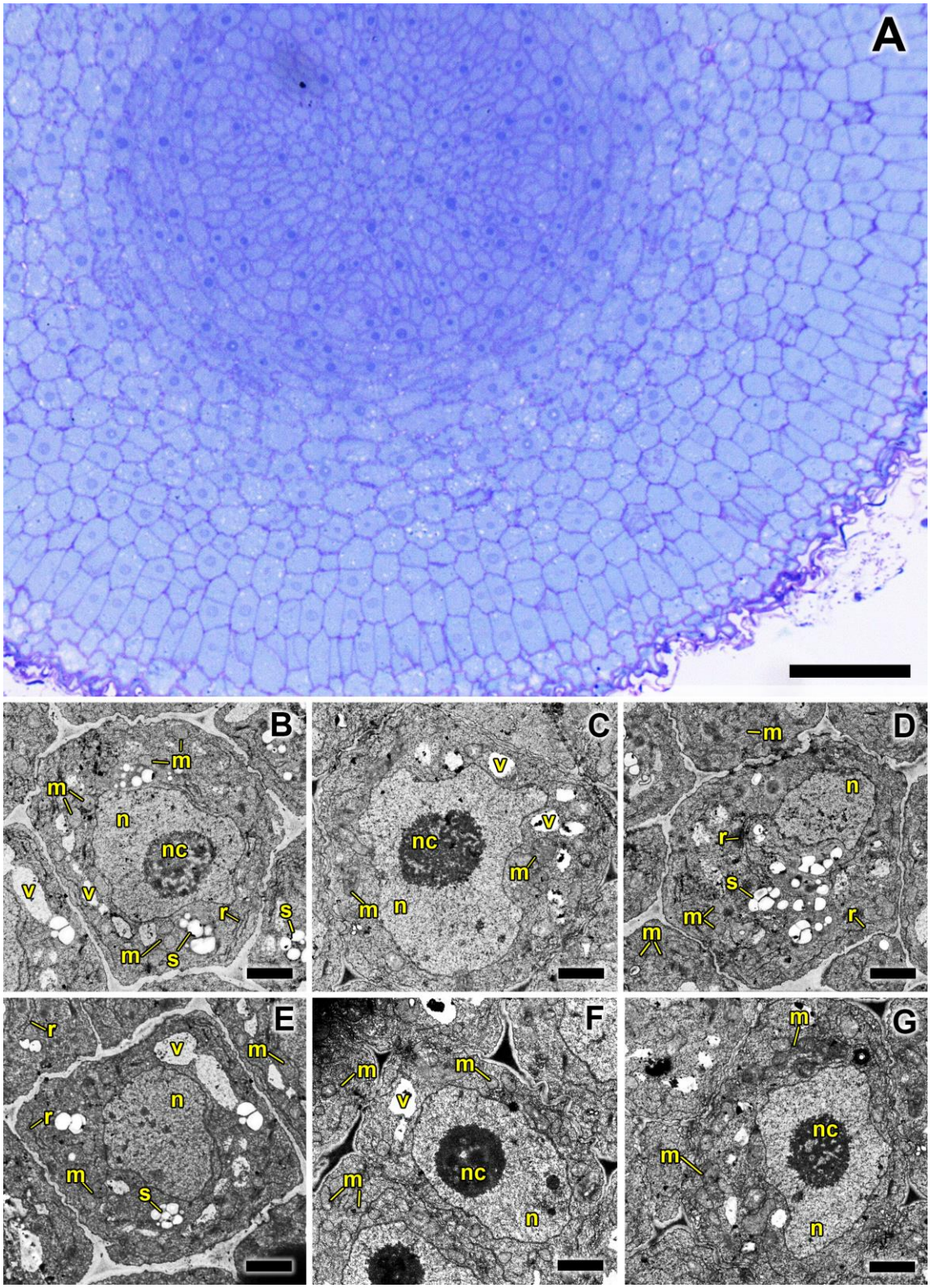

Fig. 9 Transversal section of root apices of *S. camporum*, grown for 90 days in nutrient solution containing 0 μM Al, observed in light microscopy (A) and transmission electron microscopy (B - G). m = mitochondria; n = nucleus; nc = nucleolus; v = vacuole; r = endoplasmic reticulum; s = starch granules. Scale bar: A = 25 μm; B - G = 2 μm.

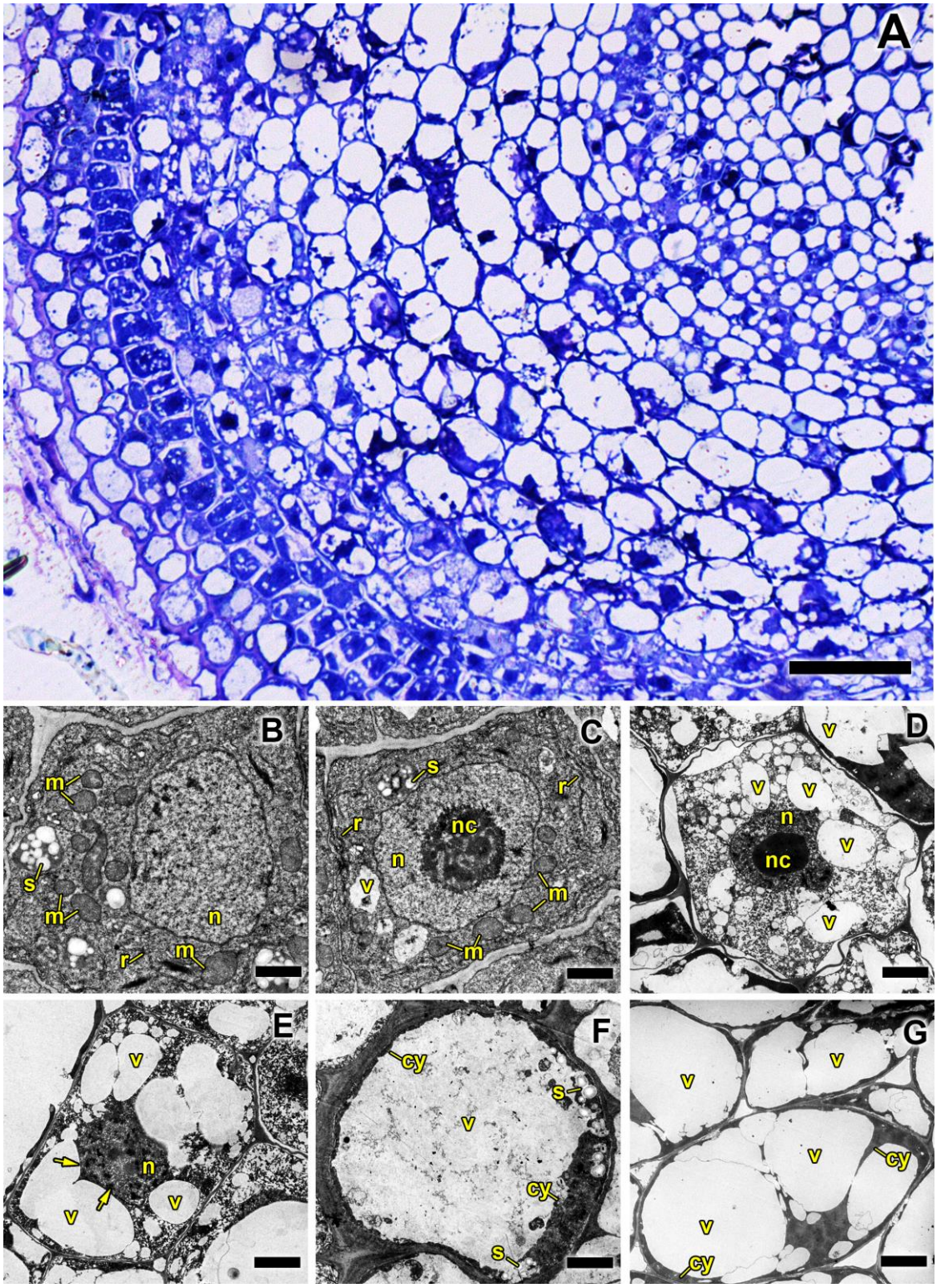

Fig. 10 Transversal section of root apices of *S. camporum*, grown for 90 days in nutrient solution containing 740 μM Al, observed in light microscopy (A) and transmission electron microscopy (B-G). Peripheral cortical cells = B, C and D. Internal cortical cells = E, F and G. m = mitochondria; n = nucleus; nc = nucleolus; v = vacuole; cy = cytoplasm; r = endoplasmic reticulum; s = starch granules. Arrows indicate deposits of electron-dense material. Scale bar: A = 25 μm; B - G = 2 μm.

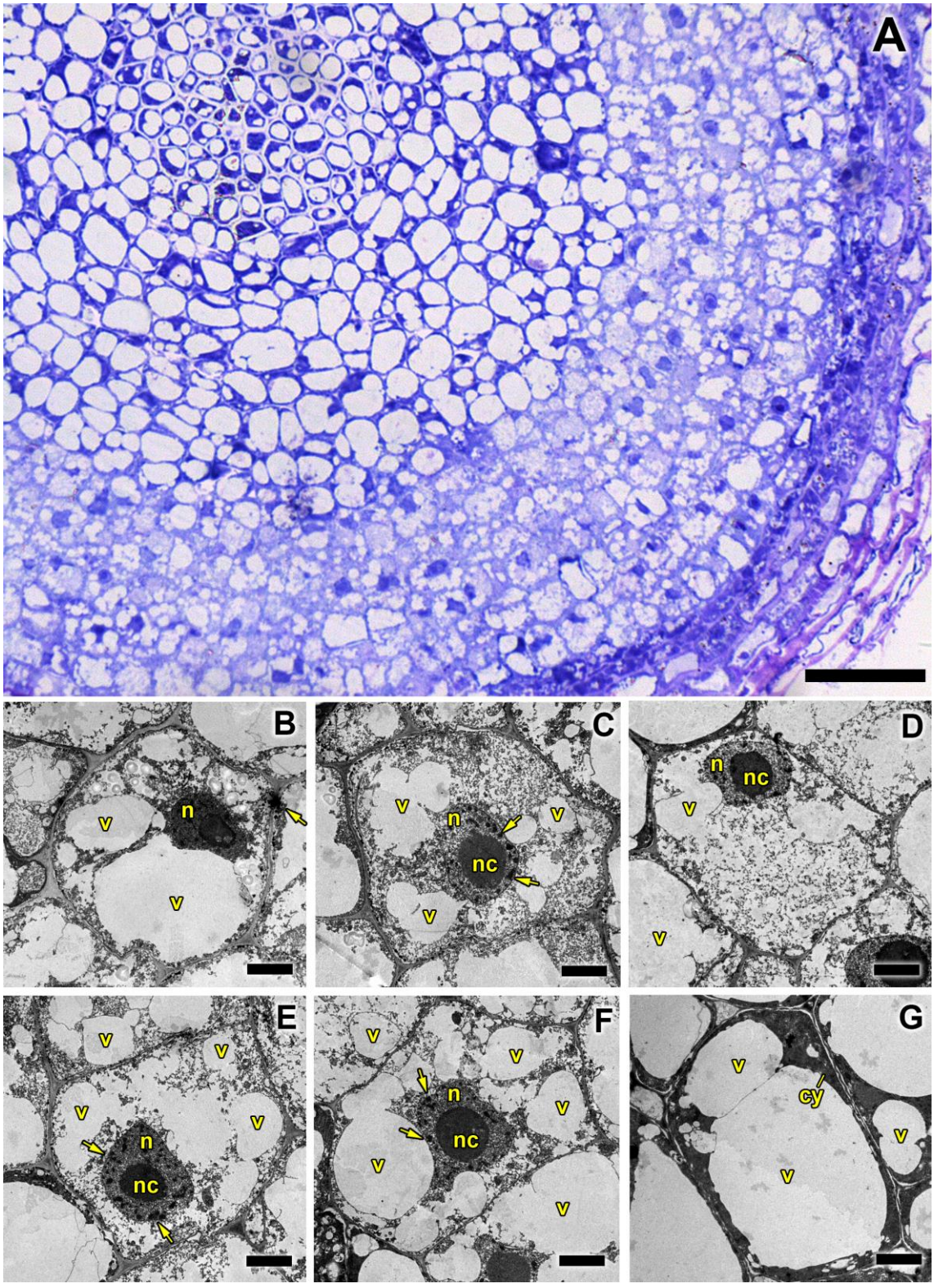

Fig. 11 Transversal section of root apices of *S. camporum*, grown for 90 days in nutrient solution containing 1480 μM Al, observed in light microscopy (A) and transmission electron microscopy (B - G). Peripheral cortical cells = B, C, D and E. Internal cortical cells = F and G. n = nucleus; nc = nucleolus; v = vacuole; cy = cytoplasm. Arrows indicate deposits of electron-dense material. Scale bar: A = 25 μm; B-G = 2 μm.

CAPÍTULO 2

Aluminum-accumulating Vochysiaceae species growing on a calcareous soil in Brazil

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Abstract

Cerrado woody species are divided into a small group of aluminum (Al)-accumulating species and the rest of the woody species. Both groups grow well on acidic and Al-rich soils. We found a Cerrado remnant growing on a calcareous soil with high calcium (Ca) and low Al saturations (m%). We checked whether Al deposition differs between leaf veins and leaf blade, and predicted that plants grown on the acidic soil store more Al than those grown on the calcareous soil. Adult plants of *Qualea grandiflora* and *Q. parviflora*, two Al-accumulators, were found in this area, and we compared leaf Ca and Al concentrations with those of the same species growing on a dystrophic Cerrado soil. Leaf Ca concentration reflected differences between the soil types, and Ca was more accumulated in leaf veins. However, Al accumulation was independent of m%, and it was more deposited in the leaf blade of both species, which was confirmed by hystochemical reactions and X-ray spectra in SEM analysis (EDS). The leaf tissue to which Al is preferentially allocated in the leaf blade could not be distinguished. Granules in epidermal cells exhibiting high Al EDS peaks suggest an important allocation for this metal.

Key words: Al; Calcium; Leaf blade; *Qualea sp*; SEM analysis; X-ray spectra

1 Introduction

Plants usually reflect the availability of nutrients and the characteristics of the soils on which they grow (Haridasan, 2001; Haridasan and Araújo 2005). The past expansion of agriculture into areas primarily occupied by native vegetation has involved, in most cases, soil correction (lime application) and the use of fertilizers (Ratter et al., 1997; Habermann and Bressan-Smith, 2013). For native plants, however, concepts of nutrient deficiencies, toxicities and soil pH, well established for crop species, are not appropriate (Haridasan, 2008). The Cerrado vegetation in South America, broadly known as 'Brazilian savanna', shows between 1000 and 2000 species per ha (Ratter et al., 2003), and this flora grows well on soils that are acidic [soil pH (in $CaCl_2$) < 5], poor in phosphorus (P) and base saturation [BS = Calcium (Ca) + Potassium (K) + Magnesium (Mg)] and with high aluminum (Al) saturation (m% > 70%) (Haridasan, 2008; Andrade et al., 2011; Habermann and Bressan, 2011; Souza et al., 2015; Bressan et al., 2016).

The Cerrado is a mosaic of vegetation physiognomies including woodlands, scrublands and grasslands, which together are referred to as Cerrado *sensu lato* (Oliveira Fillho and Ratter, 2002). Woody species are distributed in forest physiognomies, such as the 'Cerradão' (augmentative of 'Cerrado' in Portuguese) and in the cerrado *sensu stricto* (*s. str.*), which is a savanna-type physiognomy with an herbaceous understory, scattered shrubs and trees with high irradiance at the soil level (Ribeiro and Walter, 2008; Kissmann et al., 2012). These species grow naturally on Al-rich dystrophic soils of the Cerrado with no apparent damage to their metabolism (Andrade et al., 2011; Souza et al., 2015), and can be divided into Al-accumulating and non-accumulating species (Haridasan, 1982; Souza et al., 2015).

Aluminum-accumulating species from the Cerrado belong mainly to Melastomataceae, Rubiaceae, Simplocaceae and Vochysiaceae families (Haridasan, 1982; Andrade et al., 2011; Souza et al., 2015; Malta et al., 2016). In general, plants showing more than 1000 mg Al per kg dry leaves are classified as Al-accumulators (Chenery, 1948; Jansen et al., 2002). However, in the Cerrado, Al-accumulating species exhibit between 4000 and 20,000 mg Al per kg dry leaves (Haridasan, 1982; Haridasan and Araújo, 1988). Therefore, of the 1000-2000 species per ha in the Cerrado (Ratter et al., 2003), just few species from few families are Al-accumulators, although all of them grow on dystrophic soils from the Cerrado.

Up to date, no association or interference of Al with any metabolic activity has been observed in these plants. Previous studies have showed Al deposition in non-lignified tissues of the mesophyll of Al-accumulating species from the Cerrado, such as phloem, collenchyma and chlorophyll parenchyma (Haridasan et al., 1986; Andrade et al., 2011; Malta et al., 2016). Aluminum presence has been evidenced in the chloroplasts of Al-accumulating Cerrado woody species (Andrade et al., 2011; Malta et al., 2016). However, besides the limitations related to the techniques used in these studies to demonstrate Al presence in this organelle (qualitative data), Al has also been found in association with granules in leaf midribs (Bressan et al., 2016) and with leaf hypodermal and epidermal cells (Pereira et al., 2018), both leaf tissues with low metabolic activity. This raises the possibility of compartmentalization of excessive Al or structural allocation of Al in these plants. Thus, Al could be either associated with metabolically active tissues as spongy and palisade parenchyma (leaf blade), which could be less likely, or with low metabolic components, *i.e*, the leaf structure (veins).

Here we found a unique cerrado *s. str*. remnant growing on a calcareous soil with pH (in $CaCl_2$) = 5.0, increased Ca (16.4 mmolc/dm^3) and low Al (m% = 3.6%)

availabilities in relation to average Cerrado soils. In this area, we found adult plants of *Qualea grandiflora* Mart. and *Q. parviflora* Mart. (Vochysiaceae), two Al-accumulating woody species from the Cerrado. As far as we are aware, Al-accumulating species have rarely been found growing on calcareous soil with low Al saturation. We measured specific leaf area (SLA), leaf Ca and Al concentration as well as Al- hystochemical reactions and X-ray spectra in scanning electron microscopy (SEM) in leaves of these two species and compared with plants of these same species growing on a typically dystrophic Cerrado soil. This soil was acidic with pH (in $CaCl_2$) = 4.0, and showed low Ca (3.4 mmolc/dm^3) and high Al (m% = 63%) saturation. We used whole leaves for measuring Al and Ca concentration, but we also separated them into leaf blade and leaf veins in order to check whether Al deposition differs between structural (veins) and metabolically active tissues (blade). In addition, we test the hypothesis that leaf Al accumulation in the plants grown on the acidic soil is higher than that of those grown on the calcareous soil. The present research is important as to test Al accumulation patterns in adult plants naturally growing on contrasting soils.

2 Material and methods

2.1 Studied areas and plant material

The field study was conducted in two experimental sites within two fragments of cerrado *s. str.* showing soils with contrasting characteristics, where two Al-accumulating species (*Qualea grandiflora* and *Q. parviflora*) grow naturally. *Qualea grandiflora* accumulates between 4000 and 6000 mg Al per kg dry leaf, while *Q. parviflora*, around 10,000 mg Al per kg dry leaf (Haridasan, 1982; Bressan et al., 2016).

One of the areas was on the São Vicente farm (19º 10’ 39” S and 49º 42’ 60” W; 680 m of altitude) in the municipality of Ituiutaba, Minas Gerais (MG) state, southeast

Brazil (Fig. S1; Supplementary material). We identified the mineral calcite in calcareous rocky outcrop, sparsely present in the area as evidenced by the rock coloration (whitish) and effervescent reaction with dilute hydrochloric acid as also observed by Alves et al (2018) in a study conducted in a similar Cerrado remnant close to the experimental area. The soil profile was shallow (~ 50-60 cm in depth) and it was classified as calcareous neosol, herein called 'calcareous soil' (Fig. 1C). The other area was on the Campininha farm (22° 15' 19" S and 47° 09' 30" W; 680 m of altitude), within the Reserva Biológica de Mogi Guaçu, in the municipality of Mogi Guaçu, São Paulo state (SP), southeast Brazil (Fig. S1; Supplementary material). The soil profile was deeper in this area and it was classified as latosol, herein called 'acidic soil' (Fig. 1D).

Five adult trees of *Qualea grandiflora* Mart. and *Q. parviflora* Mart. (Vochysiaceae) were assessed in March 2017 in both experimental areas. In the area with the calcareous soil (Ituiutaba, MG) the plants from these species were 2-3 m in height and showed canopies of 2-3 m in diameter (Fig. 1E, F), exhibiting an open vegetation profile (Fig. 1A), while in the cerrado area with the acidic soil (Mogi Guaçu, SP) these plants showed more than 7 m in height and canopies of 2-3 m in diameter (Fig. 1G, H), exhibiting a denser vegetation profile (Fig. 1B).

2.2 Experimental design

In each of these two sites, soil samples were collected at 20–40 cm of depth. Physical (Table 1) and fertility parameters (Table 2), such as soil Al saturation (m%) were measured to confirm the contrasting characteristics of the calcareous and acidic soils. We divided the canopy into four quadrants (N, S, E, W), and healthy, sun-exposed fully expanded (mature) leaves of both species occurring in both areas were collected to

measure the specific leaf area (SLA), calcium (Ca) and Al contents in leaf samples that were separated into whole leaves, leaf blades and leaf veins to identify the region where Ca and Al are most stored in these organs. These elements were elected because they were the most contrasting elements between the studied areas (Table 2). We sought Al presence in leaf tissues using histochemical tests with chrome azurol S, an Al presence indicator (Kukachka and Miller, 1980; Bressan et al., 2016). In addition, Al-specific x-ray spectra from different regions of leaf tissues were measured and analyzed by scanning electron microscopy (SEM).

2.3 Soil characteristics

Five soil samples were collected 1-2 m close to each of the five plants in each experimental site and taken to the Soil Science Lab at University of São Paulo (Esalq, USP, Piracicaba, SP) for routine soil physical (according to Embrapa, 1997) and chemical (fertility) analyses, including soil pH (in $CaCl_2$). The procedures for the chemical analyses were performed according to van Raij et al. (2001) and described in English by Dantas and Batalha (2011).

2.4 Specific leaf area (SLA) and leaf Ca and Al concentration

Five leaves per plant of each species from both areas were used for obtaining one leaf disc of pre-determined area (2 cm in diameter) per each leaf. Leaf discs were oven-dried at 60°C until constant mass. SLA was calculated as the ratio between leaf area (cm^2) and leaf dry mass (g) (Habermann and Bressan, 2011).

The leaf samples (± 50 leaves with petioles per tree) were separated into whole leaves, leaf blades and leaf veins (primary and secondary veins) using a razor blade, and then taken to the Plant Nutrition Lab at Instituto Agronômico de Campinas (IAC) for

routine analysis of Ca and Al concentration. The plant material were oven-dried at 60°C to constant mass, ground and digested in a solution of sulfuric:nitric:percloric acids (1:10:2, v/v/v). After digestion, Ca and Al concentration were determined by the atomic absorption spectrophotometric method (Sarruge and Haag 1974), and Ca was expressed as g per kg dry mass while Al, as mg/kg dry mass.

2.5 Anatomical studies

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 Bressan et al. (2016). We also stained fresh tissues with chrome azurol S in order to compare the staining pattern, which were the same as those obtained when the plant material was fixed and preserved. The anatomical study was based on consecutively-sliced cross sections from the same leaf segments (1 cm^2) from leaf midribs and leaf blade for both species. These sections were cut manually with a razor blade. Cuts were stained for 45 min (at room temperature) according to Bressan et al. (2016). The cuts were washed thrice (15 min each) in distilled water and mounted in semi-permanent glass slides. The cuts were observed under light microscope (DMLB, Leica Microsystems, Wetzlar, Germany), and the images were captured with a digital camera (DFC-290, Leica Microsystems, Germany) attached to the DMLB.

Chrome azurol S (CAS), or Mordant blue 29 (3-sulpho- 2", 6"- dichloro-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 pH 4.82 sodium acetate solution (Kukachka and Miller 1980). CAS final concentration was 8.3 mM (5 g/L) (pH = 4.76 ± 0.01).

2.6 Micromorphological analyses

The leaf segments were fixed in a 2.5 % (v/v) Karnovsky solution (1965) (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. Then, they were mounted on stubs made of brass using carbon tape (double sided carbon tape, 8 mm in width; Electrom Microscopy Science, EMS, USA). 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) 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 contrasted under light microscopy and SEM in order to check the correlation between hystochemical results and their respective Al emission spectrum.

2.7 Data Analysis

A Student t-test ($\propto = 0.05$) was used to test differences in physical and chemical parameters between calcareous and acidic soils. A two-way analysis of variance (two-way ANOVA) was conducted using the soil type factor (calcareous and acidic soil) and the species factor (*Qualea grandiflora* and *Q. parviflora*) to test the SLA. In order to test Ca and Al leaf concentrations we used a three-way ANOVA also considering the leaf region factor (whole leaf, blade and veins). The Tukey test ($P < 0.05$) was used to conduct post hoc comparisons to determine the least significant difference between mean results of SLA, Ca and Al leaf concentrations.

3 Results

Although physical analysis of soils from both areas showed similar characteristics (Table 1), the fertility between these soils was significantly different. The calcareous soil showed pH 5.0, m% of 3.6% (< 2 mmolc dm^{-3}) and 53.3 mmolc dm^{-3} of CEC, while the acidic soil exhibited pH 4.0, m% of 63% (7.8 ± 0.5 mmolc dm^{-3}) and 33.7 mmolc dm^{-3} of CEC. Although P and sulphur (S) concentrations were the same between the soils from both areas, the calcareous soil showed BS 85% higher than the acidic soil, and K, Ca and Mg availabilities were 60%, 80% and 93% higher in the calcareous soil in relation to the acidic soil, respectively (Table 2).

The specific leaf area (SLA) was similar for plants of the same species, regardless of the soil on which they grew (Fig. 2; Table S1, Supplementary material). *Qualea grandiflora* and *Q. parviflora* showed higher leaf Ca concentration in the plants growing on the calcareous soil in relation to those growing on the acidic soil (Fig. 3; Table S2, Supplementary material). Leaf Ca concentration was higher in the veins in relation to the blade and whole leaf for both species growing on the calcareous soil (Fig. 3; Table S2, Supplementary material). For plants growing on the acidic soil, the leaf vein showed higher leaf Ca concentration in relation to the leaf blade, and the whole leaf showed intermediate values (Fig. 3).

Qualea grandiflora and *Q. parviflora* showed the same leaf Al concentration between the soil types (Table S3; Supplementary material). The Al concentration in whole leaves and leaf blades was higher than that in leaf veins (Fig. 4), regardless of the soil type (Table S3; Supplementary material).

The pattern of Al accumulation was similar for both species, at both sites (Table 3). Hystochemical tests with cromo azurol S showed the presence of Al in non-lignified

tissues in the central vein (Fig. 5A, 5B, 5E and 5F) and the chlorophyll parenchyma (Fig. 5C, 5D, 5G and 5H) of both species when grown on both soil types.

The X-ray analysis confirmed the results obtained with histochemical tests and revealed, in the mid vein, prominent Al peaks in the phloem region when contrasted with the xylem ones, both for *Q. grandiflora* between the soil types (Fig. 6B and 6H) and similarly for *Q. parviflora* (Fig. 7B and 7H).

In addition, the collenchyma region showed a conspicuous Al peak in relation to sclerified cells, both for *Q. grandiflora* between the soil types (Fig. 6C and 6I) and similarly for *Q. parviflora* (Fig. 7C and 7I). The palisade parenchyma showed pronounced Al peaks in relation to the cuticle for *Q. grandiflora* grown in the acidic soil (Fig. 6K) and for *Q. parviflora* grown on the calcareous (Fig. 7E) and acidic soil (Fig. 7K). However, the palisade and spongy parenchyma of *Q. parviflora* grown on the calcareous soil showed similar relative intensity of Al peaks (Fig. 7F).

In addition, we observed Al accumulation in granules in epidermal cells of the leaf blade of both species (Fig. 6D, 6J and 7J). The Al peaks in these granules were conspicuous when contrasted with the palisade parenchyma (Fig. 6E, 6L e 7L) and cuticle (Fig. 6F).

4 Discussion

In the present study, we observed that *Qualea grandiflora* and *Q. parviflora* showed a positive association between leaf Ca concentration and Ca saturation in the soil. Both species showed higher Ca concentrations in the leaves of plants grown on the calcareous soil when compared to those grown on the acidic soil (Fig. 3; Table S2, Supplementary material). Plants may reflect the availability of nutrients and the characteristics of the soils on which they grow (Haridasan, 2001; Haridasan and Araújo

2005). In a previous study, leaf Ca concentration of semi-deciduous woody plants grown on a calcareous soil was higher than that of plants grown on an arenitic soil (Haridasan and Araújo, 2005). In another study, the application of up to 8.7 t of lime per ha on a dystrophic dark red latosol caused an increase of 87% in the concentration of Ca in the leaves of a Cerrado vegetation (Vilela and Haridasan, 1994). More recently, leaves of *Vochysia thyrsoidea*, another Al-accumulating species (Haridasan, 1982), collected from plants growing on a calcareous soil (18.3 mmol Ca dm^{-3} soil) in a cerrado area (Serra do Cipó, Brazil) exhibited increased leaf Ca concentration (in upper epidermis) when compared to plants grown on an acidic soil (1 mmol Ca dm^{-3} soil) (Pereira et al., 2018). Therefore, our results showing direct correspondence between leaf Ca concentration and soil Ca saturation are in agreement with most studies. On the other hand, in the case of the Al, this association between leaf concentration and its saturation in the soil did not occur in the present study. *Qualea grandiflora* and *Q. parviflora* accumulated ~6000 and ~18.000 mg Al per kg dry leaves, respectively, regardless of the soil type (Fig. 4). These values are close to those already reported for these species in similar studies (Haridasan, 1982; Bressan et al., 2016). These results do not confirm our hypothesis that plants grown on the calcareous soil with low Al saturation show lower leaf Al concentration in relation to plants growing on the acidic soil.

Previous studies have showed that Al-accumulating species store Al in their leaves when growing on eutrophic soils with low Al saturation (Andrade et al., 2011; Malta et al, 2016). These studies have demonstrated Al accumulation in plants of Vochysiaceae (Andrade et al., 2011) and Rubiaceae families (Malta et al., 2016) when the contrast in Al saturation between dystrophic soils [m% = 78.2 (Andrade et al., 2011); m% = 96.7 (Malta et al., 2016)] and eutrophic soils [m% = 25.1 (Andrade et al., 2011); m% = 50.9 (Malta et al., 2016)] were of 53.1% and 45.8%, respectively. In the

present study, the contrast in Al saturation between the calcareous and acidic soil was more pronounced, and m% in the calcareous soil was close to zero. The calcareous soil showed m% = 3.6 ± 0.87% and the acidic soil, m% = 63 ± 2.64%, resulting in a contrast of 59.4%. This means that of the total 53.3 mmolc dm^{-3} (CEC) in the calcareous soil, less than 2 mmolc dm^{-3} were occupied by Al, while in the acidic soil, of the total 33.7 mmolc dm^{-3} (CEC), 7.8 ± 0.5 mmolc dm^{-3} were occupied by Al (Table 2). Thus, although not confirming our hypothesis, we add further evidence that there is no direct relationship between Al availability in the soil and Al accumulation by Al-accumulating species. In a study of natural distribution of woody species (Haridasan and Araújo, 2005), of nine species showing more than 1000 mg Al per kg dry leaves, five occurred on a calcareous soil [pH (in KCl) ~ 6.0] rich in Ca and with low Al saturation, while three of them were common between the calcareous and an arenitic acidic [pH (in KCl) ~ 4.0] soil rich in Al. This suggests that the distribution of Al-accumulating Cerrado woody species, widely known to occur on acidic dystrophic soils from the Cerrado (Haridasan 2008), may also occur on calcareous eutrophic soils, as evidenced by Haridasan and Araujo (2005) and Pereira et al. (2018). In another study of Cerrado plant communities grown on soils with contrasting properties, Haridasan and Araujo (1988) show that Al-accumulating plants may not be restricted to strongly acidic and dystrophic soils. Therefore, it is intriguing that these species are able to accumulate Al when growing either on acidic dystrophic soils and calcareous soils extremely poor in Al, such as the one we found in Ituiutaba, MG, Brazil. Assessing the Al deposition pattern in leaf tissues could provide evidence in this regard.

Our results also showed that Ca concentration is higher in the leaf veins in relation to the blade, regardless of the species and the soil on which these plants grew (Fig. 3; Table S2, Supplementary material). The structural relation of Ca in plants is

relatively well known. Calcium is important in the formation of new cell walls in the meristem, and it is required for normal functioning of plant cell membranes, being relatively immovable under a situation of Ca deficiency (Epstein and Bloom, 2005; Taiz et al., 2015). Thus, the Ca presence in higher concentration in leaf veins in relation to the leaf blade reinforces its structural role for these Vochysiaceae species too. On the other hand, Al concentration was higher in the leaf blade in relation to the veins (Fig. 4). In *Q. grandiflora* and *Callisthene major* (Vochysiaceae), cross sections of leaf tissues treated with hematoxylin, an Al indicator, showed positive reaction in the palisade and spongy parenchyma cells (Andrade et al., 2011). In *Rudgea viburnoides* (Rubiaceae), another Al-accumulating species from the Cerrado, increased fluorescence obtained with confocal microscopy (tissues treated with lumogallion) was observed in the palisade parenchyma (Malta et al., 2016), although these results should be taken with care because of the auto-fluorescence from the chlorophyll parenchyma. In *Miconia rubiginosa* (Melastomataceae), also an Al-accumulating woody species from the Cerrado, hystochemical tests with chrome azurol S (CAS) showed positive reactions in the spongy parenchyma (Bressan et al., 2016). These studies, however, rely exclusively in staining and fluorescence techniques that, apart from being only qualitative, are subjective. Recently, the use of quantitative cryo-SEM and EDS (that quantifies tissue-specific element concentration) evidenced that *Q. grandiflora* and *Q. parviflora* accumulate Al in the upper and lower epidermis and also hypodermal cells (Pereira et al. 2018), leaf tissues with low metabolic activity. The fact that Al concentration was higher in the leaf blade in relation to the veins (Fig. 4) could be because the "leaf blade" included the epidermis, hypodermis and also the mesophyll, and we could not tell exactly where the Al could be allocated. In the present study, we also observed Al presence (CAS positive reaction) in the palisade and spongy parenchyma of both

species grown on both soil types (Fig 5C, 5D, 5G and 5H), but this qualitative analysis should be taken with care too, and this merits further investigation. On the other hand, as observed in previous studies (Haridasan et al., 1986; Andrade et al., 2011; Bressan et al., 2016; Malta et al., 2016), while reactions between Al indicators and non-lignified cells are positive, in xylem vessels and sclerified cells they are negative, a pattern that was also confirmed in the present study (with CAS) (Table 3). This pattern of Al presence in non-lignified tissues was also confirmed by the X-ray spectra in SEM analysis (Fig. 6 and 7). In Al-accumulating species not from the Cerrado, like tea plants (*Camellia sinensis* L.) (Theaceae), the Al in the xylem sap form complexes with citrate and is translocated from roots to shoots (Morita et al. 2004). Therefore, upon reaching the mesophyll of Al-accumulating plants, the pattern of Al deposition may be simply a matter of chemical affinity, as Al seems to react with non-lignified tissues, including the epidermis, hypodermis, and also the mesophyll. Indeed, there is evidence that Al is bound tightly to pectate and primary cell wall material (Wehr et al., 2010).

SEM analysis also showed granules located in epidermal cells of both species, which showed conspicuous Al peaks when contrasted with the palisade parenchyma (Fig 6D, 6E, 6J, 6L, 7J and 7L). The association between Al and these granules has also been observed by Bressan et al. (2016). As mentioned above, Al was found to accumulate more in upper and lower epidermis than the spongy and palisade mesophyll of *Qualea grandiflora* and *Q. parviflora* (Pereira et al., 2018). These results may indicate an alternative form of deposition for this metal in Al-accumulating plants from the Cerrado. Therefore, a likely role for Al in these plants remains to be evidenced, and Pereira et al. (2018) propose that a specific pattern of Al allocation (in leaves) would be important so as to not interfere with P availability (precipitation in metabolically active cells) in the leaf metabolism. In addition, the evidence that leaf Al accumulation and its

availability in the soil are independent, while leaf Ca concentration was higher in leaves of plants grown on the calcareous soil suggests that the uptake of Ca is unlikely to compete with that of Al in the species studied here. Accordingly, Al uptake by Al-accumulating plants seems to be independent of the soil fertility and saturation of Ca, Mg and K in the soil (Haridasan and Araújo, 1988). Furthermore, high Al concentration in leaves of Al-accumulating species does not seem to interfere with the uptake and storage of nitrogen (N), P, K, Ca and S that occur between the juvenile and mature leaf phases (Souza et al., 2015). Identifying competition between nutrients uptake is difficult, especially in field studies, but our results suggest lack of competition between Al and Ca uptake by Al-accumulating species.

The specific leaf area (SLA) was similar for plants of the same species, regardless of the area in which they grew (Fig. 2; Table S1, Supplementary material). This parameter is usually responsive to the light environment (Gvinish, 1988; Liu et al., 2016). Accordingly, congeneric pairs from the Cerrado vegetation show increased SLA when grown in the understory of a riparian forest in relation to the edge of a forest physiognomy of the Cerrado called 'Cerradão' and the open vegetation of a cerrado *s. str*. (Habermann and Bressan, 2011). In relation to Al-accumulating plants, there is evidence showing that SLA increases from the juvenile to the mature leaf phase, and that high SLA is important in these plants for the warm and wet season, when sunlight capture is critical for growth of new branches (Souza et al., 2015). Thus, based on the studies cited above, in the present study, as we collected only fully-expanded mature leaves, SLA could not be expected to differ between plants of the same species growing on both soil types, even though the plant community grown on the acidic soil showed a denser profile than that grown on the calcareous soil (Fig. 1A and 1B). Taken together, it seems that leaf Ca concentration, that was different between plants grown on both soil

types, was not able to influence SLA of mature leaves of the Al-accumulating plants studied here.

The Cerrado vegetation usually grows on acidic soils with high Al saturation, but here we found a Cerrado plant community, including Al-accumulating species, growing on a calcareous soil with low Al saturation. Leaf Ca concentration reflected differences between the soil types, while leaf Al concentration was independent of m%. As a novelty, we showed that Al was more accumulated in the leaf blade, but we could not distinguish quantitatively to which leaf tissue in the blade (mesophyll, lower or upper epidermis) the Al was significantly allocated. There is quantitative evidence from the literature showing that Al is preferentially allocated to the epidermis (Pereira et al., 2018), which corroborates our data as granules in epidermal cells of both species were found exhibiting conspicuous Al peaks when contrasted with those from the palisade parenchyma. This challenges the possibility that the Al might have a physiological function in these plants, and further evidence in this regard remains to be found.

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Tables:

Table 1. Mean values (n = 5 ± s.e.) of physical parameters of a calcareous and an acidic soil from cerrado *sensu stricto* remnants, from Ituiutaba, MG (Calcareous) and Mogi Guaçu, SP (Acidic), in southeastern Brazil

Soil type	Total Sand	Clay (in H_2O)	Clay (in NaOH)	Silt	Flocculation
	---------- g kg^{-1} ----------				%
Calcareous	780.6 ± 9.52 **a**	25 ± 0 **a**	135 ± 12.74 **b**	84.2 ± 6.99 **a**	80.8 ± 1.82 **a**
Acidic	692.6 ± 13.48 **b**	30 ± 5 **a**	224.8 ± 13.69 **a**	82.4 ± 4.90 **a**	86.6 ± 2.27 **a**

Different letters represent significant difference between soil types by Student *t*-test

Table 2. Fertility parameters, macro and micronutrient contents of a calcareous and an acidic soil from cerrado *sensu stricto* remnants, from Ituiutaba, MG (Calcareous) and Mogi Guaçu, SP (Acidic), in southeastern Brazil

Soil type	pH (in $CaCl_2$)	P	S	K	Ca	Mg	Al	BS	CEC
		----------mg dm^{-3}--------		---------- mmolc.dm^{-3} ----------					
Calcareous	5 ± 0.05 **a**	4 ± 0 **a**	8.6 ± 0.67 **a**	2.16 ± 0.13 **a**	16.4 ± 1.02 **a**	15.4 ± 1.07 **a**	< 2 **b**	33.96 ± 1.93 **a**	53.36 ± 1.42 **a**
Acidic	4.08 ± 0.03 **b**	4 ± 0 **a**	10.6 ± 0.87 **a**	0.9 ± 0 **b**	3.4 ± 0.24 **b**	1 ± 0 **b**	7.8 ± 0.5 **a**	4.56 ± 0.26 **b**	33.76 ± 3.18 **b**

Soil type	Cu	Fe	Mn	Zn	Al (m%)
	----------mg dm^{-3}----------				%
Calcareous	0.96 ± 0.07 **a**	44 ± 1.73 **a**	5.32 ± 0.20 **a**	0.2 ± 0 **a**	3.6 ± 0.87 **b**
Acidic	0.48 ± 0.04 **b**	54.2 ± 8.44 **a**	1.6 ± 0.14 **b**	0.2 ± 0 **a**	63 ± 2.64 **a**

BS = Base saturation = K+Ca+Mg; CEC = Cation exchange capacity;

Different letters represent significant difference between soil types by Student *t*-test

Table 3. Patterns of hystochemical reactions with chrome azurol-S (Al indicator) in the central midrib and mesophyll of leaves of *Qualea grandiflora* and *Q. parviflora* from cerrado *sensu stricto* remnants growing on a calcareous (Ituiutaba, MG) and an acidic soil (Mogi Guaçu, SP), in southeastern Brazil.

Soil type	**Species**	**Central midrib**							**Mesophyll**	
		Cuticle	Xylem	Sclerified cells	Epidermis	Phloem	Parenchyma	Collenchyma	Palisade	Spongy
Calcareous	*Q. grandiflora*	-	-	-	+	+	+	+	+	+
	Q. parviflora	-	-	-	+	+	+	+	+	+
Acidic	*Q. grandiflora*	-	-	-	+	+	+	+	+	+
	Q. parviflora	-	-	-	+	+	+	+	+	+

(-) = Negative reaction

(+) = Positive reaction

Figures:

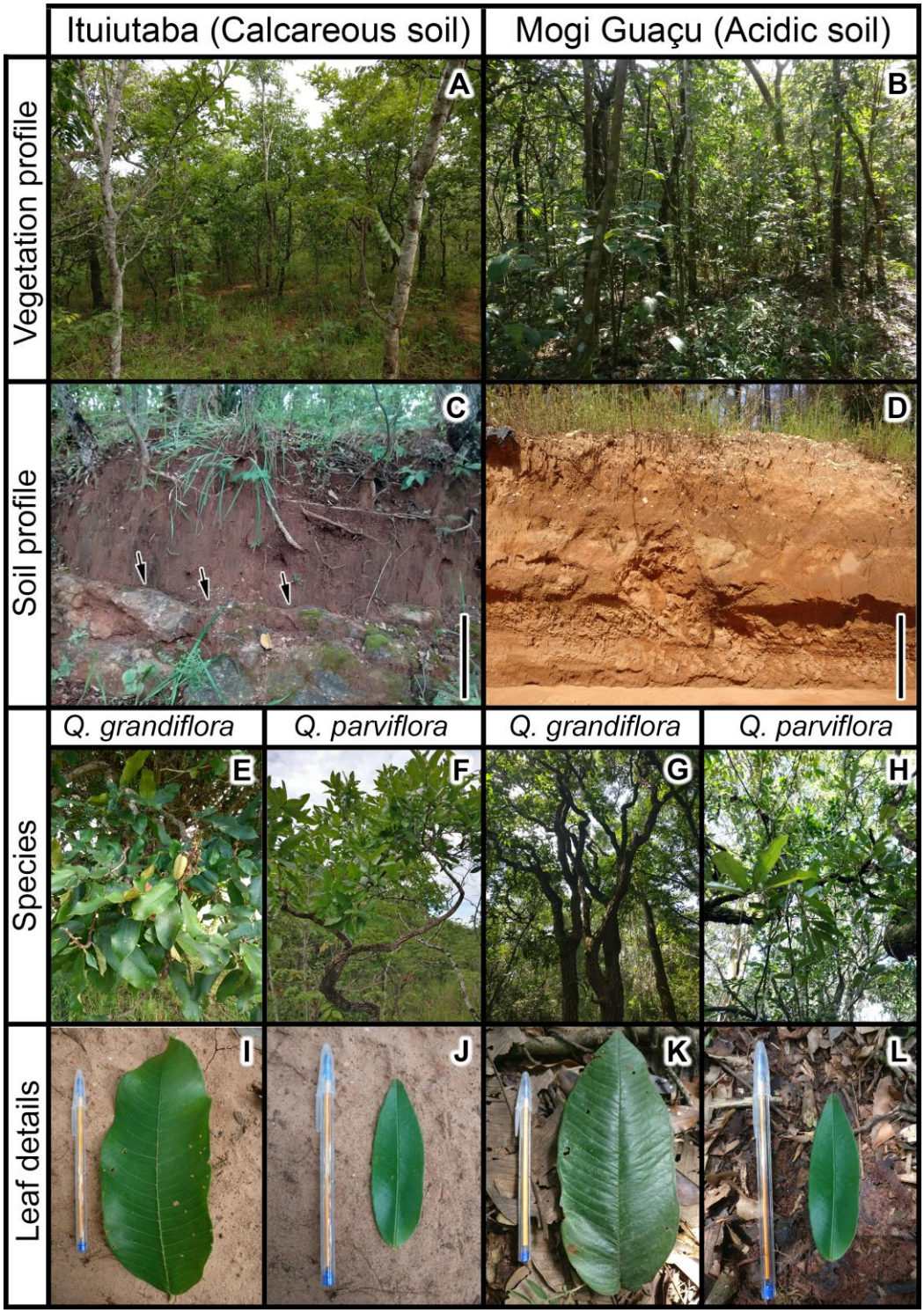

Fig. 1 Vegetation structure of cerrado *sensu stricto* remnants (**A**, **B**) growing on a calcareous soil (**C**; soil profile details) and an acidic soil (**D**; soil profile details). Plant (**E**-**H**) and leaf details (**I**-**L**) of *Qualea grandiflora* (**E**, **G**, **I**, **K**) and *Q. parviflora* (**F**, **H**, **J**, **L**). Arrows indicate the rock surface on the calcareous soil profile (**C**). Bars: 0.5 m (**C**), 1 m (**D**)

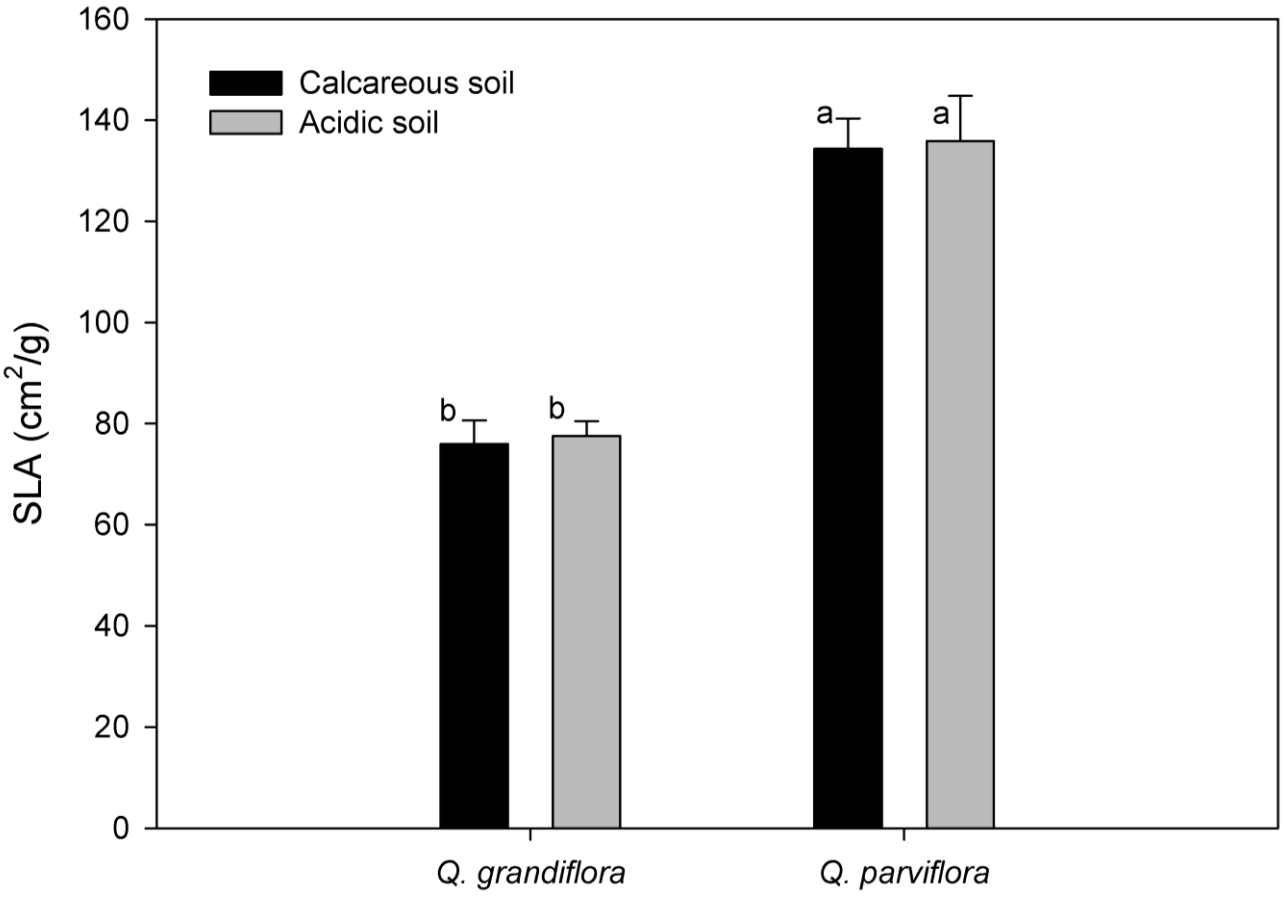

Fig. 2 Specific leaf area (SLA) of adult plants of *Qualea grandiflora* and *Q. parviflora* growing on calcareous and acidic soils in cerrado *sensu stricto* remnants in Ituiutaba (MG) and Mogi Guaçu (SP), southeastern Brazil. For each species, same letters indicate lack of significant difference between soil types by Student *t*-test. Bars = s.e.

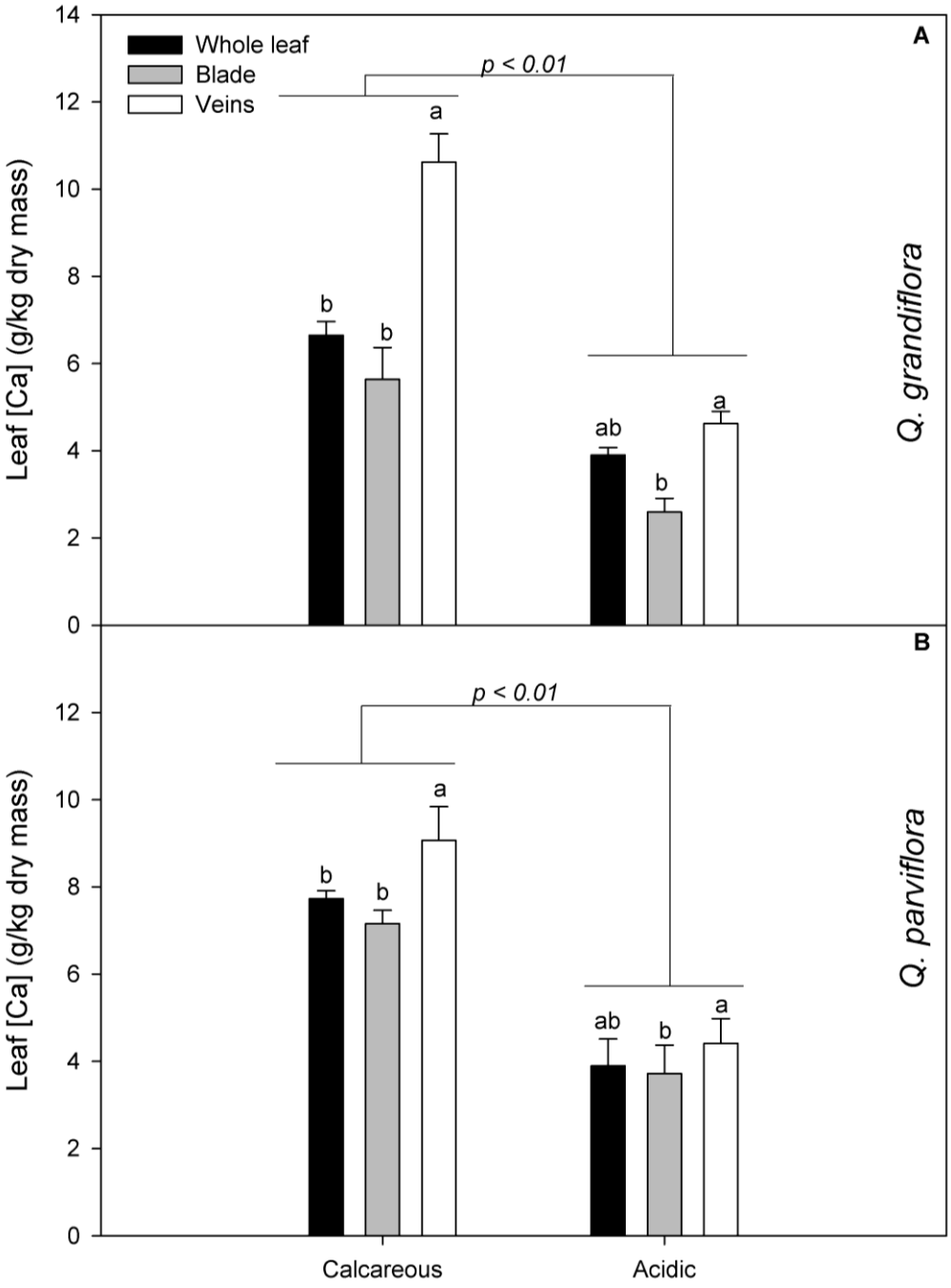

Fig. 3 Calcium (Ca) concentration in the blade, vein and in the whole leaf of adult plants of *Qualea grandiflora* (A) and *Q. parviflora* (B) growing on calcareous and acidic soils in cerrado sensu stricto remnants in Ituiutaba (MG) and Mogi Guaçu (SP), southeastern Brazil. In each species, for each soil type, different letters indicate significant differences between blade, vein and whole leaf by Tukey test ($P < 0.05$). P values indicate comparisons between soil types within each leaf region. Bars = s.e.

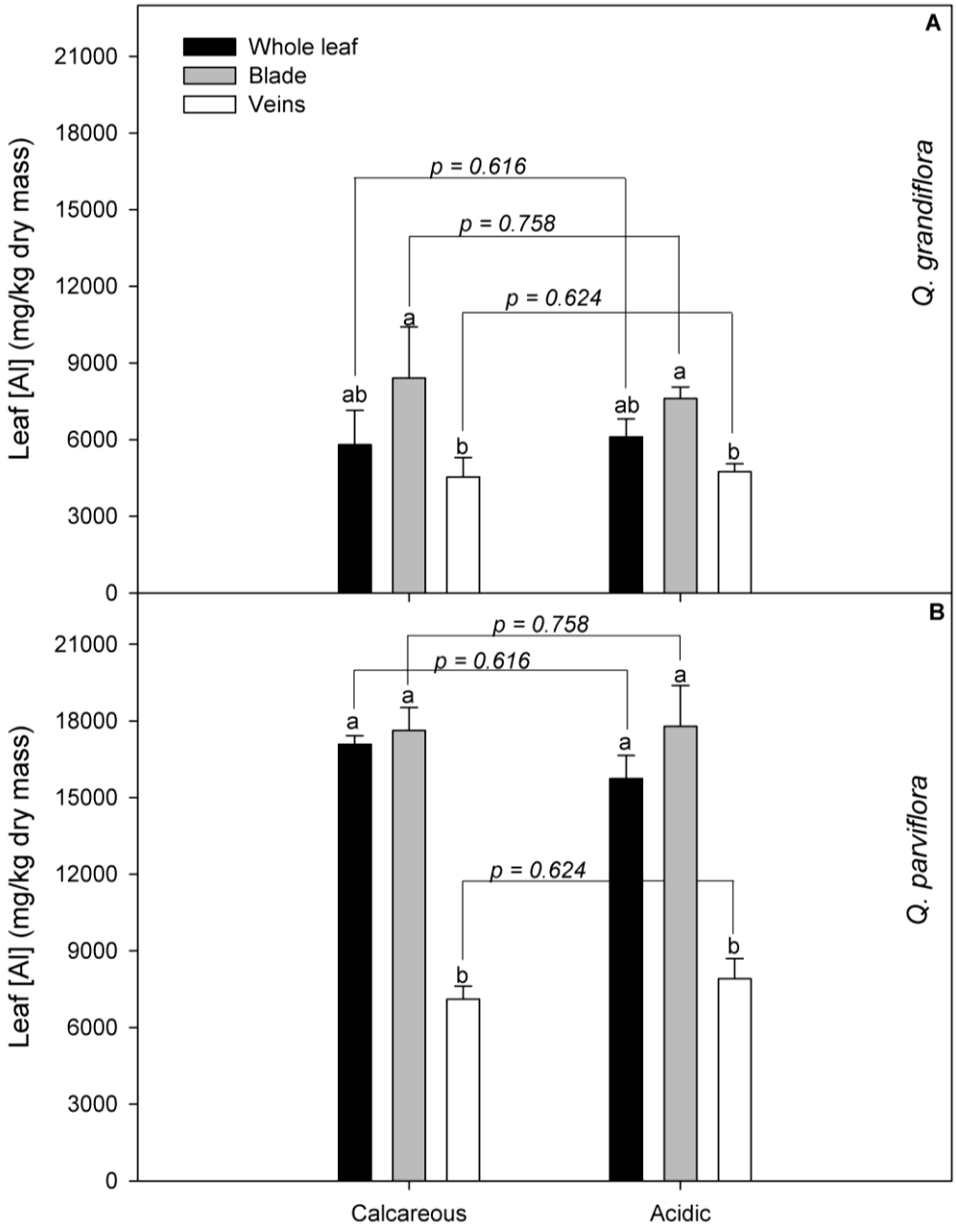

Fig. 4 Aluminum (Al) concentration in the blade, vein and in the whole leaf of adult plants of *Qualea grandiflora* (A) and *Q. parviflora* (B) growing on calcareous and acidic soils in cerrado sensu stricto remnants in Ituiutaba (MG) and Mogi Guaçu (SP), southeastern Brazil. In each species, for each soil type, different letters indicate significant differences between blade, vein and whole leaf by Tukey test ($P < 0.05$). P values indicate comparisons between soil types within each leaf region. Bars = s.e.

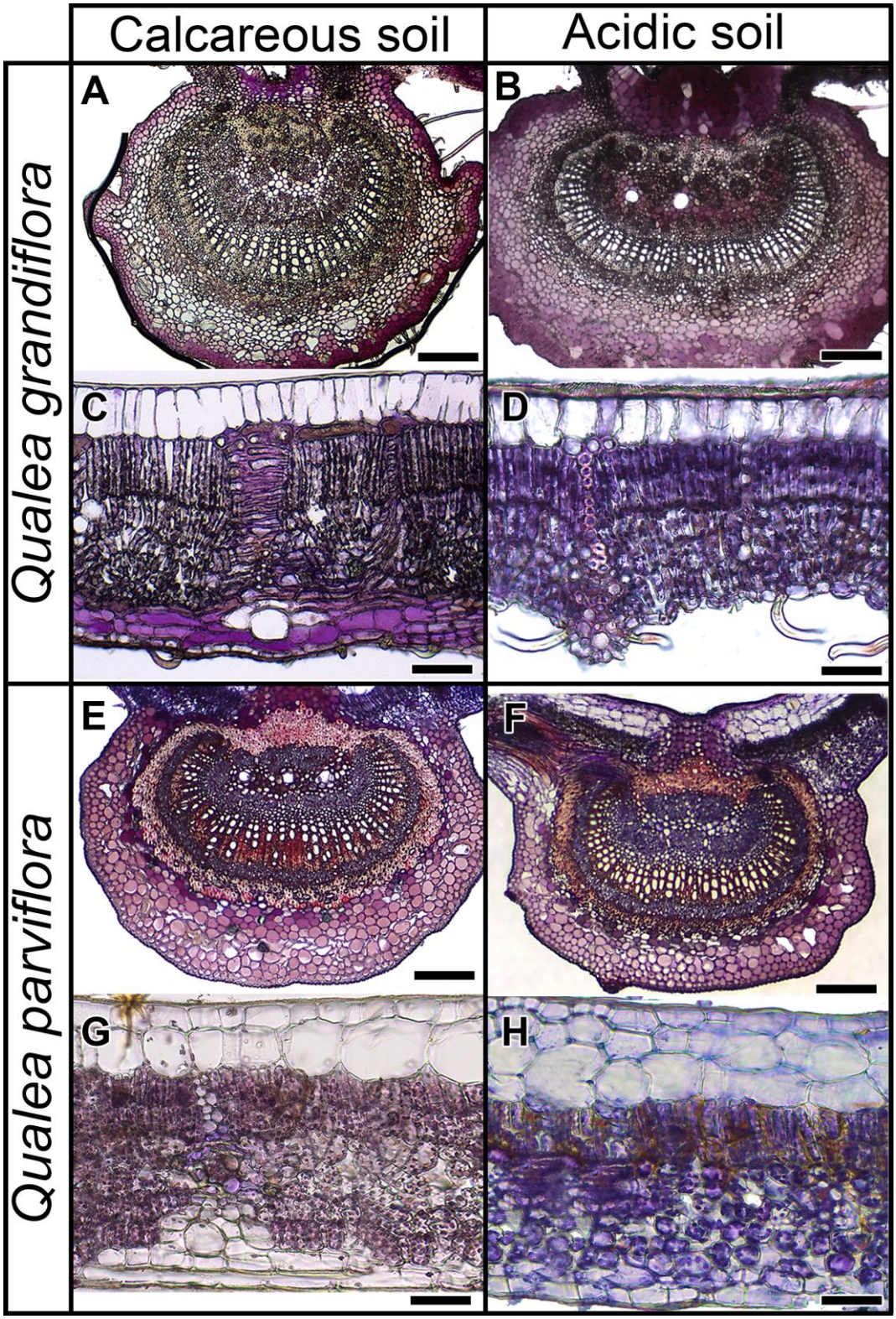

Fig. 5. Light microscopy: Histochemical reactions with chrome azurol-S (Al - indicator) in central vein and leaf blade of *Q. grandiflora* and *Q. parviflora* grown on a calcareous (Ituiutaba, MG, Brazil) (A, B, C and D) and on an acidic soil (Mogi Guaçu, SP, Brazil) (E, F, G and H). Bars: 100 µm (A, B, E and F); 50 µm (C and D); 30 µm (G and H).

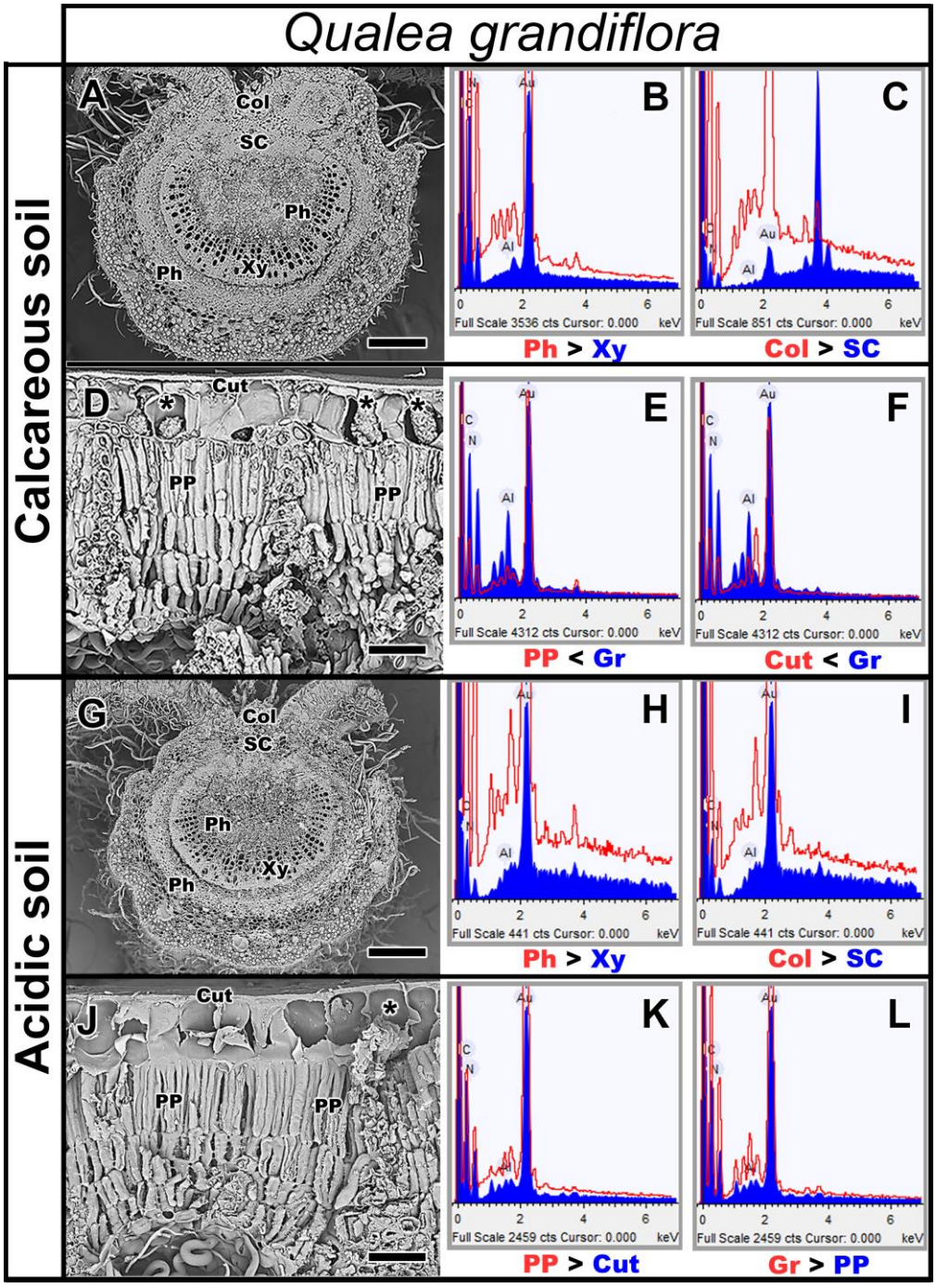

Fig. 6. Scanning electron microscopy (SEM): Central vein and leaf blade of *Q. grandiflora* grown on a calcareous (Ituiutaba, MG, Brazil) (A and D) and on an acidic soil (Mogi Guaçu, SP, Brazil) (G and J). X-ray analysis qualitatively comparing the intensity of the Al presence in different tissues, in the same sample: in the central vein (B, C, H, and I) and in the leaf blade (E, F, K and L). Ph = phloem; Xy = xylem; Col = collenchyma; SC = sclerified cells; PP = palisade parenchyma; Gr = granulum; Cut = cuticle; Asterisk (*) indicates granules containing Al. Bars: 100 µm (A and G); 50 µm (D and J).

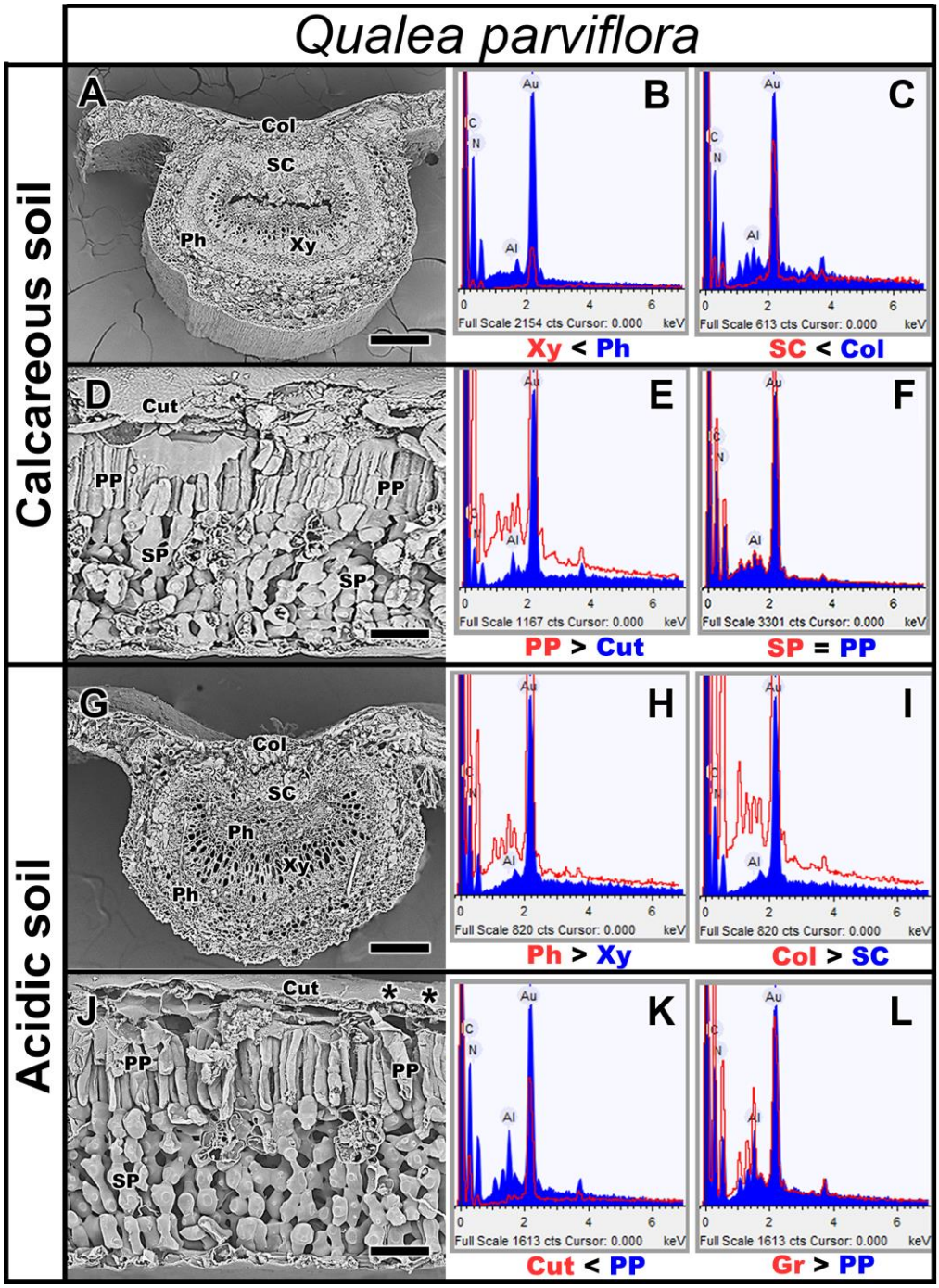

Fig. 7. Scanning electron microscopy (SEM): Central vein and leaf blade of *Q. parviflora* grown on a calcareous (Ituiutaba, MG, Brazil) (A and D) and on an acidic soil (Mogi Guaçu, SP, Brazil) (G and J). X-ray analysis qualitatively comparing the intensity of the Al presence in different tissues, in the same sample: in the central vein (B, C, H, and I) and in the leaf blade (E, F, K and L). Ph = phloem; Xy = xylem; Col = collenchyma; SC = sclerified cells; PP = palisade parenchyma; SP = spongy parenchyma; Gr = granulum; Cut = cuticle; Asterisk (*) indicates granules containing Al. Bars: 100 µm (A and G); 30 µm (D and J).

Supplementary material:

Tables:

Table S1. Results of two-way analysis of variance (ANOVA) between soil type and species for the specific leaf area in *Qualea grandiflora* and *Q. parviflora*.

Factor	***P* values**
Soil type	0.846
Species	< 0.001
Soil type x Species	0.999

Table S2. Results of three-way analysis of variance (ANOVA) between soil type, species and leaf region for leaf Ca concentration in *Qualea grandiflora* and *Q. parviflora*.

Factor	***P* values**
Soil type	< 0.001
Species	0.279
Leaf region	< 0.001
Soil type x Species	0.936
Soil type x Leaf region	0.011
Species x Leaf region	0.017
Soil type x Species x Leaf region	0.241

Table S3. Results of three-way analysis of variance (ANOVA) between soil type, species and leaf region for leaf Al concentration in *Qualea grandiflora* and *Q. parviflora*.

Factor	***P* values**
Soil type	0.853
Species	< 0.001
Leaf region	< 0.001
Soil type x Species	0.980
Soil type x Leaf region	0.756
Species x Leaf region	< 0.001
Soil type x Species x Leaf region	0.617

Figure:

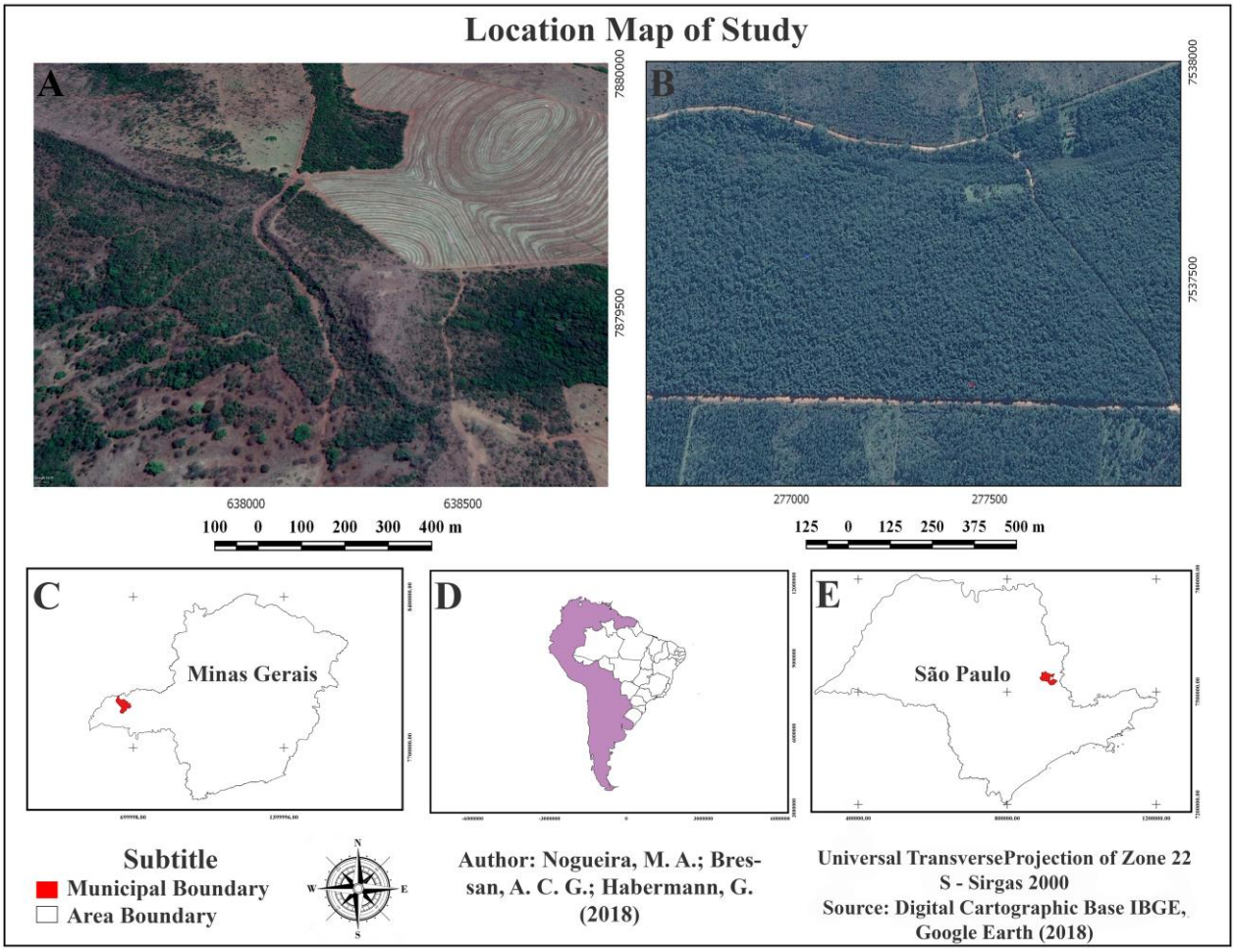

Fig. S1 Location map of the study area of cerrado *sensu stricto* remnants growing on a calcareous soil in the municipality of Ituiutaba, Minas Gerais state (**A, C**) and on an acidic soil in the municipality of Mogi Guaçu, São Paulo state (**B, E**), southeastern Brazil, in South America (**D**).

CONSIDERAÇÕES FINAIS

O alumínio (Al) é o terceiro elemento químico mais abundante da crosta terrestre, e encontra-se naturalmente presente no solo de várias partes do mundo. Em solos ácidos, o Al torna-se disponível para as plantas, e pode provocar sintomas de toxicidade nas espécies sensíveis. Entretanto, nem todas as espécies são prejudicadas pela presença do Al. No Cerrado espécies acumuladoras e não acumuladoras de Al coexistem no mesmo ambiente, evidenciando a riqueza de mecanismos desenvolvidos para lidar com a alta disponibilidade de Al no solo. Nesta Tese de Doutorado tivemos como objetivo apresentar as repostas de três espécies nativas do Cerrado quando expostas a diferentes concentrações de Al.

No capítulo 1 descrevemos um experimento realizado em casa de vegetação com *Styrax camporum*. Demonstramos que esta espécie apresenta sintomas de toxicidade quando cultivada em solução nutritiva contendo 1480 μM de Al, mas estes sintomas não são observados quando as plantas são exposta a 0 e 740 μM de Al. Plantas expostas à solução contendo 1480 μM de Al apresentaram sistema radicular menos desenvolvido, menor altura da planta e baixas taxas de troca de gasosas em relação àquelas expostas a 0 e 740 μM Al. Além disso, quanto maior o tempo de exposição e mais elevada a concentração de Al, mais intenso o descarte epidérmico, a desorganização dos tecidos internos (principalmente a hipoderme) e a vacuolização das células corticais dos ápices radiculares. Desta forma, pudemos perceber que *Styrax camporum* é uma espécie capaz de suportar altas concentrações de Al (740 μM). Entretanto, existe um limite para sua tolerância a este elemento, uma vez que, quando exposta a 1480 μM de Al, esta espécie apresenta sintomas de toxicidade.

No capítulo 2 comparamos a SLA e as concentrações foliares de Ca e Al em duas espécies do gênero *Qualea* ocorrendo em um fragmento de Cerrado localizado em solo calcário, com os valores apresentados pelas mesmas espécies, ocorrendo em um fragmento de Cerrado localizado em solo ácido e rico em Al. Os valores de SLA foram iguais nas duas áreas de estudo. O teor foliar de Ca nas duas espécies foi positivamente associado à saturação no solo, ou seja, quanto mais elevada a concentração de Ca no solo, mais elevado o teor de Ca observado nas folhas. Por outro lado, surpreendentemente o teor de Al foi o mesmo nas plantas que cresceram nos dois solos. Assim, nossos dados reforçam a ideia já apresentada por Haridasan e Araújo (1988) de

que a ocorrência de espécies acumuladoras de Al nativas do Cerrado não é restrita a ambientes de solos ácidos. Outro ponto interessante levantado neste trabalho foi o fato de que o Ca e o Al aparentemente se concentram em regiões distintas da folha. Para as duas espécies estudadas, independentemente do tipo de solo onde as plantas cresceram, a maior concentração de Ca foi encontrada nas nervuras, enquanto que a maior concentração de Al foi encontrada no limbo foliar. Estudos prévios realizados por Andrade et al. (2011) e Malta et al. (2016) também demonstraram o acúmulo de Al na lâmina foliar de plantas nativas do Cerrado, e sugeriram a existência de uma associação deste elemento com processos fotossintéticos. Entretanto, este padrão de deposição do Al pode ser apenas uma questão de afinidade química entre este metal e os tecidos foliares não lignificados. Desta forma, outras evidências, além do padrão de acúmulo de Al nos tecidos foliares, devem ser encontradas para que seja possível atribuir uma função a este elemento nos processos fisiológicos de plantas acumuladoras de Al.