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

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

OS MECANISMOS DE TOLERÂNCIA AO ALUMÍNIO EM STYRAX CAMPORUM ENVOLVEM SÍNTESE E SECREÇÃO DE ÁCIDOS ORGÂNICOS?

BRENDA MISTRAL DE OLIVEIRA CARVALHO

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

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TÍTULO DA DISSERTAÇÃO: Os mecanismos de tolerância ao alumínio em Styrax comporum envolvem síntese e secreção de ácidos orgânicos?

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“A vida não está aqui para satisfazer as nossas projeções e exigências, ela apenas está fluindo”

Mooji

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INDÍCE

RESUMO

A vegetação do Cerrado, tipicamente conhecida como ‘Savana brasileira’, é constituída por espécies que são adaptadas a crescer em solos ácidos e ricos em alumínio (Al). Usando *Styrax camporum*, uma planta lenhosa do Cerrado moderadamente acumuladora de Al, examinamos se esta espécie exclui Al de duas raízes por meio da exsudação de ácidos orgânicos (AOs) para manter baixo conteúdo de Al na folha em relação às plantas tipicamente acumuladoras de Al dessa vegetação. Para isso, medimos os ácidos cítrico, málico e oxálico exsudado por *S. camporum* cultivada em solução nutritiva contendo 0, 740 e 1480 μM Al por 30 dias, usando GC-MS. Além disso, a concentração de Al nessas soluções foi medida aos 0 e 30 dias do estudo, usando ICP-OES para estimar a absorção de Al pelas plantas. Exceto para o ácido málico, plantas expostas ao Al exsudaram mais AOs que aquelas não expostas ao Al. Depois de 30 dias, enquanto plantas expostas a 740 μM Al absorveram 40% do Al disponível na solução, plantas expostas a 1480 μM Al absorveram 60%. Ao mesmo tempo, plantas expostas a 1480 μM Al exsudaram menor concentração de ácidos cítrico e oxálico, em relação às plantas expostas a 740 μM Al. Considerando que maior exsudação de AO causou menor absorção de Al, é possível que os ácidos cítrico e oxálico estejam detoxificando o Al nessa espécie moderadamente acumuladora. Além disso, parece que, para esta espécie, concentrações crescentes de Al em solução nutritiva podem causar diminuições proporcionais nos padrões de exsudação de AOs. Portanto, a exsudação de AO parece ser um mecanismo que contribui para a adaptação de *S. camporum* para lidar com alta disponibilidade de Al nos solos ácidos do Cerrado.

Palavras-chave: *Ácido cítrico, ácido oxálico, acumuladora de alumínio, exclusão de alumínio, vegetação do Cerrado*

ABSTRACT

The Cerrado vegetation, typically known as ‘Brazilian Savanna’, is comprised of species that are adapted to grow on soils that are acidic and rich in aluminum (Al). Using *Styrax camporum*, a moderate Al-accumulating Cerrado woody plant, we examined whether this species exclude Al from their roots through organic acids (OAs) exudation in order to maintain a low leaf Al content in relation to typical Al-accumulating plants from this vegetation. For this, we measured citric, malic and oxalic acids exuded by *S. camporum* grown in a nutrient solution containing 0, 740 and 1480 μM Al for 30 days, using a GC-MS. In addition, the Al concentration in these solutions was measured at 0 and 30 days of the study using an ICP-OES in order to estimate the Al uptake by plants. Except for malic acid, plants exposed to Al exuded more OAs than those not exposed to Al. After 30 days, while plants exposed to 740 μM Al absorbed 40% of the Al available in the solution, plants exposed to 1480 μM Al absorbed 60% of it. At the same time, plants exposed to 1480 μM Al exuded lower concentration of citric and oxalic acids in relation to plants exposed do 740 μM Al. Considering that more OA exudation caused lower Al uptake, it is possible that citric and oxalic acids are Al detoxifying mechanisms in this moderate Al-accumulating species. In addition, it seems that for this species, solutions containing increasing Al concentrations may cause decreasing OA exudation patterns. Therefore, OA exudation seems to be a mechanism contributing to adaptation of S. camporum to cope with high Al availability in the acidic soils from the Cerrado.

Keywords: *Aluminum exclusion, Al-accumulator, Cerrado vegetation, citric acid, oxalic acid*

1. INTRODUÇÃO

O Alumínio (Al) é o terceiro elemento mais abundante da crosta terrestre e é encontrado no solo principalmente na forma de aluminosilicatos. No entanto, em solos ácidos [pH (em H_2O) < 5.5], que representam 30% dos solos cultiváveis (Foy, 1988; Horst et al., 2010), o Al é solubilizado para diferentes formas iônicas, especialmente o cátion trivalente (Al^{3+}), que é tóxico para a maioria das espécies. No Brasil, o pH dos solos varia entre 3,7 e 5,5, e o Al é predominante em mais de 1/3 desses solos (Abreu Jr. et al., 2003).

O principal sintoma da toxicidade ao Al é uma rápida redução do crescimento radicular (Horst et al., 2010; Sun et al., 2010), devido a danos mecânicos causados às células das raízes (Kopittke et al., 2008). Há evidências de que a primeira lesão do Al em plantas é sua ligação às matrizes pécticas da parede celular das células das raízes (Kopittke et al., 2015), inibindo a divisão e o alongamento celular nessa região (Sivaguru & Horst, 1998; Kochian et al, 2004; Doncheva et al., 2005). Além dos efeitos negativos no sistema radicular das plantas, o Al pode causar efeitos de longa distância, como diminuição do crescimento de caules (Jiang et al., 2009) e redução das trocas gasosas e reações fotoquímicas (Konrad et al., 2005; Lidon et al, 1999; Banhos et al., 2016a).

Embora a fitotoxicidade do Al seja um fator limitante para a produtividade das culturas em locais com solos ácidos, o Brasil é uma potência agrícola. A expansão agrícola brasileira, sobretudo depois de 1960, se fez à custa da substituição da vegetação nativa do Cerrado, adaptada a solos oligotróficos, ácidos, álicos e distróficos (Pinheiro & Monteiro, 2010), pelos campos de cultivo. Assim, a agricultura depende até hoje da correção do pH do solo desse locais.

Por outro lado, há espécies que acumulam considerável quantidade de Al sem que este cause danos aparentes aos seus tecidos. Plantas que acumulam mais de 1000 mg Al por kg de folhas secas são definidas como acumuladores de alumínio (Chenery, 1948; Jansen et al., 2002), abrangendo espécies pertencentes a cerca de 45 famílias (sendo Melastomataceae, Rubiaceae, Simplocaceae, Theaceae e Vochysiaceae exemplos das mais estudadas) (Jansen et al., 2002). Além de acumular consideráveis quantidades de Al, plantas acumuladoras podem também detoxificar o Al externamente, no apoplasto das células radiculares (Horst et al., 2010). Desta forma, ácidos orgânicos (AOs), como os ácidos cítrico, málico, oxálico e succínico são exudados pelas raízes das plantas, formando complexos estáveis com o Al e não fitotóxicos (Kochian et al., 2004; Brunner and Sperisen, 2013). Esse processo evita a reação do Al com os sítios carregados negativamente no apoplasto, onde ocorre a lesão primária do

Al nas raízes das plantas (Kopittke et al., 2015). Por exemplo, *Camellia sinensis* (Theaeae), *Fagopyrum esculentum* (Polygonaceae) e *Colocasia esculenta* (Araceae), que acumulam significativa quantidade de Al na parte aérea, exsudam AOs das raízes em resposta ao Al (Ma et al., 1997; Ma and Miyasaka, 1998; Morita et al., 2011).

Pouca atenção é dada às espécies de comunidades nativas que toleram o Al. A vegetação do Cerrado é adaptada a solos ácidos (pH < 5,0) e apresenta saturação por Al (m%) maior que 50% da capacidade da troca de cátion (Haridasan, 2008; Souza et al., 2015b; Bressan et al., 2016). Algumas espécies lenhosas do Cerrado, consideradas acumuladoras de Al, podem mostrar de 4.000 a 15.000 mg (ou mais) de Al por kg de folhas secas: *Miconia sp*. (Melastomataceae), *Palicourea rigida*, *Rudgea viburnoides* (Rubiaceae), *Qualea sp*. and *Vochysia sp*. (Vochysiaceae) (Haridasan 1982; Bressan et al., 2016; Malta et al., 2016). Por outro lado, a maiorias das espécies lenhosas desta vegetação se classificam como não acumuladoras, armazenando de 100 a 600 mg Al por kg de folhas secas (Haridasan, 1982; Souza et al., 2015a). No entanto, até onde sabemos, não há estudos sobre como as espécies acumuladoras e não acumuladoras do Cerrado lidam com a grande disponibilidade de Al no ambiente radicular. É possível que essas plantas utilizem o mecanismo de exclusão por meio da exsudação de AOs pelas raízes.

Styrax camporum (Styracaceae) é uma planta lenhosa do Cerrado considerada moderadamente acumuladora de Al (~1500 mg/kg) (Bressan et al., 2016). Assim, é esperado que essa planta exclua o Al a partir das raízes e isso poderia explicar o menor conteúdo de Al nas folhas em relação às plantas do Cerrado tipicamente acumuladoras de Al. Em um estudo anterior, *S. camporum* apresentou trocas gasosas reduzidas e diminuição da parte aérea quando cultivada em solução nutritiva contendo 1480 μM Al, comparada às plantas não expostas ao Al (Banhos et al., 2016b), indicando que 1480 μM Al pode ser tóxico para essa espécie. Assim, sugere-se que uma concentração menor de Al não cause danos a essa espécie, podendo induzir maior exclusão de Al.

No presente estudo, nós testamos a hipótese de que a exsudação de AOs está associada com a presença de Al no ambiente radicular de *S. camporum* cultivada em solução nutritiva. Além disso, esperamos que 740 μM Al cause um aumento na exsudação de AOs em relação às plantas expostas a 1480 μM Al. Para isso, quantificamos os ácidos cítrico, málico e oxálico exsudados pelas raízes de *S. camporum* cultivada em solução nutritiva por 30 dias.

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Capítulo único:

First report of organic acid exudation in a moderate aluminum-accumulating species from the Brazilian savanna*

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Abstract

Background and Aims

The Cerrado vegetation, typically known as 'Brazilian Savanna', is comprised of species that are adapted to grow on soils that are acidic and rich in aluminum (Al). Using *Styrax camporum*, a moderate Al-accumulating Cerrado woody plant, we examined whether this species excludes Al from their roots through organic acids (OAs) exudation in order to maintain a low leaf Al content in relation to typical Al-accumulating plants from this vegetation.

Methods

We measured citric, malic and oxalic acids exuded by *S. camporum* grown in nutrient solution containing 0, 740 and 1480 μM Al for 30 days, using a GC-MS. In addition, the Al concentration in these solutions was measured at 0 and 30 days of the study using an ICP-OES in order to estimate the Al uptake by plants.

Results

Except for malic acid, plants exposed to Al exuded more OAs than those not exposed to Al. After 30 days, while plants exposed to 740 μM Al absorbed 40% of the Al available in the solution, plants exposed to 1480 μM Al absorbed 60% of it. At the same time, plants exposed to 1480 μM Al exuded lower concentration of citric and oxalic acids in relation to plants exposed do 740 μM Al.

Conclusions

Considering that more OA exudation caused lower Al uptake, it is possible that citric and oxalic acids are Al detoxifying mechanisms in this moderate Al-accumulating species. In addition, it seems that, for this species, solutions containing increasing Al concentrations may cause decreasing OA exudation patterns. Therefore, OA exudation seems to be a mechanism contributing to adaptation of *S. camporum* to cope with high Al availability in the acidic soils from the Cerrado.

Keywords: Aluminum exclusion, Cerrado vegetation, citric acid, oxalic acid, *Styrax camporum*

Introduction

Aluminum (Al) is the third most abundant element in the Earth's crust and, in the soil it is present as aluminosilicate and other precipitated forms, which are harmless to plants (Brunner and Sperisen, 2013). In acidic soils [pH (in H_2O) < 5.5], which comprise 30% of world's ice-free land (von Uexküll and Mutert, 1995), Al can be found as different ions, especially the phytotoxic trivalent cation (Al^{3+}). Al decreases root growth, leaf gas exchange and plant development (Kopittke et al., 2008; Horst et al., 2010; Banhos et al., 2016a). On the other hand, some plants may present mechanisms to cope with Al toxicity. For instance, there are plants that accumulate Al without apparent damage to their tissues. Species belonging to approximately 45 families (being Melastomataceae, Rubiaceae, Simplocaceae, Theaceae, and Vochysiaceae examples of those most studied ones) can be described as Al-accumulating plants (Jansen et al., 2002). Plants that accumulate more than 1000 mg Al per kg dry leaves are defined as Al-accumulators (Chenery, 1948; Jansen et al., 2002).

Despite accumulating considerable amounts of Al, Al-accumulating plants may also rely on mechanisms that detoxify Al externally (in the apoplast of root cells) (Horst et al., 2010). In this process, organic acids (OA) like citric, malic, oxalic and succinic are exuded by the roots of plants and form non-toxic stable complexes (Kochian et al., 2004; Brunner and Sperisen, 2013). This avoids the reaction of Al to sites negatively charged in the apoplast, which is the primary lesion of Al in plant roots (Kopittke et al., 2015). For instance, tea plants (*Camellia sinensis*) (Theaceae), buckwheat (*Fagopyrum esculentum*) (Polygonaceae) and tora (*Colocasia esculenta*) (Araceae), which accumulate significant Al content in their leaves and shoots exude OAs from roots in response to Al (Ma et al., 1997; Ma and Miyasaka, 1998; Morita et al., 2011).

Less attention is paid to species from native communities that tolerate Al. The Cerrado vegetation, broadly known as 'Brazilian Savanna', is comprised of a mosaic of physiognomies that are adapted to grow on soils that are acidic (pH < 5.0) and shows Al saturation (m%) higher than 50% of the cation exchange capacity (Haridasan, 1982; Haridasan, 2008; Souza et al., 2015b; Bressan et al., 2016; Malta et al., 2016). This condition has contributed to the adaptation of Al-accumulating and non-accumulating species, being few species considered Al-accumulating (4000–15000 mg/kg): *Miconia sp.* (Melastomataceae), *Palicourea rigida*, *Rudgea viburnoides* (Rubiaceae), *Qualea sp.* and *Vochysia sp.* (Vochysiaceae) (Haridasan 1982; Malta et al., 2016). On the other hand, the great majority of woody species from this vegetation is comprised of non-accumulating

species (100–600 mg/kg) (Haridasan, 1982; Souza et al., 2015a). However, up to date, as far as we are aware, there are no studies about how Al-accumulating and non-accumulating species from the Cerrado cope with Al in the soils of these areas. Thus, it is possible that plants from these groups show Al exclusion through OA exudation by their roots.

Styrax camporum (Styracaceae) is considered a moderate Al-accumulating Cerrado woody plant (~1500 mg/kg) (Bressan et al., 2016). Thus, it is expected that this plant exclude Al from their roots and this could explain its low leaf Al content in relation to those of typical Al-accumulating Cerrado plants. In a previous study, *S. camporum* grown in nutrient solution with 1480 μM Al showed decreased leaf gas exchange rates and reduced shoot growth in relation to plants not exposed to Al (Banhos et al., 2016b). This indicates that 1480 μM Al might be toxic to this species, and a lower Al concentration would not harm this plant, and could induce a higher Al exclusion.

Here we tested the hypothesis that OA exudation is associated with the presence of Al in the root environment of *S. camporum* grown in nutrient solution. In addition, we also expected that 740 μM Al causes an increased OA exudation in relation to plants exposed to 1480 μM Al. For this, we measured citric, malic and oxalic acids exuded by roots of *S. camporum* grown in a nutrient solution for 30 days.

Material and Methods

Plant material and experimental conditions

Ripe fruits of five plants of *Styrax camporum* Pohl. were collected in a Cerradão remnant (22°15’ S and 47°00’ W) in the municipality of Corumbataí, São Paulo State, Brazil. The seeds were germinated under controlled conditions (25 °C), as suggested by Kissmann and Habermann (2013), and seedlings were grown in vermiculite in a screen-house (200 μmol photons m^{-2} s^{-1}) for 17 months. The plants (8 ± 1 cm in height) were then transferred to 50-mL Falcon tubes (1 plant per tube) containing 45 mL of a nutrient solution.

The nutrient solution was adapted from Banhos et al. (2016b) and shows a chemical composition based on the solution proposed by Clark (1975). It consisted of 1372.8 μM $Ca(NO_3)_2$ 4 H_2O, 507 μM NH_4NO_3, 224.4 μM KCl, 227.2 μM K_2SO_4, 218.6 μM KNO_3, 483.2 μM $Mg(NO_3)_2$ 6H_2O, 12.9 μM KH_2PO_4, 26.01 μM $FeSO_4$ 7H_2O, 23.8 μM NaEDTA, 3.5 μM $MnCl_2$ 4H_2O, 9.9 μM H_3BO_3, 0.9 μM $ZnSO_4$ 7 H_2O, 0.2 μM $CuSO_4$ 5H_2O, 0.4 μM $NaMoO_2$ 2 H_2O. In a previous study (Banhos et al., 2016b), we observed that *S. camporum* exposed to 1480 μM Al showed reduced shoot growth and leaf gas exchange, suggesting that

this concentration may be toxic to this moderate Al-accumulating species, and that a lower Al concentration should be tested. Therefore, besides macro and micronutrients, the solution contained 0, 740 and 1480 µM Al provided through $AlCl_3$ 6 H_2O. The nutrient solution was not replaced in the tubes, but deionized water was added in each tube to complete the 45 mL every day. The pH of the solution in each tube was monitored daily and maintained at 4.0 ± 0.1 to keep Al as soluble as possible. Aeration of the solution in each tube was performed using aquarium pumps. The Al concentration in this solution was measured in the beginning and at the end of the study using an inductively coupled plasma optical emission spectroscopy (ICP-OES) (Varian, Vista-MPX/Australia). This procedure was performed to estimate the Al uptake by plants because measuring the Al concentration directly from plant tissues would be limited by the minimum dry mass sample required for analytical procedures.

The tubes were wrapped with aluminum foil to avoid light to the nutrient solution, and were kept in racks, on benches in the lab, under controlled conditions (25 ± 1 °C; 200 µmol photons m^{-2} s^{-1}; 12h of photoperiod). The plants were fixed at the mouth of the tubes using polyurethane foam strips that were placed around the plant collar.

Experimental design

Using three plants per treatment (0, 740 and 1480 µM Al), we collected the nutrient solution from the tubes at 1, 5, 10, 15 and 30 days after transfer (DAT), totaling 45 tubes. Eighteen extra tubes (3 tubes per treatment) were used to measure the Al concentration at 0 and 30 DAT. Organic acids (citric, malic and oxalic) exuded by the plants were measured in the nutrient solution using a gas chromatograph coupled to a mass spectrometry (GC-MS). In addition, a previous gene expression analysis performed using plants grown for 60 days at 0 and 740 µM Al showed that *ALMT* (aluminum activated malate transporter) and *MATE* (multidrug and toxic compound extrusion) had been differentially expressed (data not published). Thus, we measured the expression of these genes at 1, 15 and 30 DAT using quantitative real-time PCR (q-PCR) in plants exposed to 0, 740 and 1480 µM Al. As malic acid was not detected in any treatment at any DAT, and ALMT was expressed at 15 DAT, we performed an extra experiment to investigate the malic acid content inside root tips collected at 1, 15 and 30 DAT also using GC-MS.

Standard curves for detection of organic acids

To check the method and the GC-MS parameter ability to detect, separate and quantify the OAs, we set up a standard curve for each OA by adding six concentrations of monohydrated citric (Fluka – Thermo Fisher Scientific, USA), malic (Fluka – Thermo Fisher Scientific, USA) and oxalic acids standards (Merck, Germany) (5, 10, 30, 50, 70 and 100 µg/mL) in methanol.

In order to establish a relationship between GC-MS peak areas and the OAs concentrations found in the nutrient solution with 0, 740 and 1480 µM Al, we set up three standard curves for each OA by adding six concentrations of citric, malic and oxalic acids standards (25, 50, 100, 200, 300 and 400 µg/mL). Three replicates were used for each concentration and values were plotted to generate the standard curves, and the equations with their respective R^2 are shown on Table 1.

Exuded organic acids analysis

Exuded OAs were concentrated after drying the 45 mL solution in a forced-air oven at 80°C. Samples were esterified according to Fischer method (methylation) (Fischer and Speier, 1895). Then, we added 700 µL of methanol (HPLC/Spectro) and 300 µL of sulphuric acid (7N). The samples were shaken and kept for 1h at 70°C for catalysing the reaction. After adding 1 mL of hexane (HPLC/Spectro), 100 µL of apolar phase were collected and analysed using a GC-MS system (GC-2010/GCMSQP2010 Plus, Shimadzu, Japan), with an automatic sample injector (AOC-20i).

Malic acid content in the root tips

Malic acid content in the root tips were extracted by osmosis and alkaline gradient, immersing the root tips (0.5 ± 0.1 cm in length) in 1.5 mL 40 mM Na_2CO_3 for 24h. Then, 1 mL of the extracting solution was collected and dried completely at 80°C. We esterified the samples by methylation (Fischer and Speier, 1895), and added 400 µL of methanol (HPLC/Spectro) and 100 µL of sulfuric acid (7N). After shaking, the samples were kept for 1h at 70°C for catalysing the reaction. Then, we added 1 mL of hexane (HPLC/Spectro), collected 100 µL of apolar phase, and analysed using GC-MS.

GC-MS parameters

In the GC-MS, we used a 30 m-length and 250 µm-diameter fused-silica microcolumn (RTX-5MS, Restek), and analytical ultra-pure helium (99.9999%, White Martins) was used as

carrier gas. The injector temperature was 250°C (Splitless mode) and the injection volume was 1 µL. Column gas flow was maintained at 41 cm/s. The initial column temperature was 50°C with a 4 min step. After that, at a 10°C/min rate, it achieved 70°C. Then, it was increased to 250°C at a 25°C/min rate, and maintained for 0.8 min, completing 14 min running. Mass detector was a simple quadrupole type with 70eV electronic impact ionization. The GC-MS interface temperature was 250°C and 230°C to the ionizer. The detector potential was relative to tunning, with a 40 to 450 m/z detection range (scanner mode).

Gene expression analysis

We used quantitative PCR to analyze the expression of *ALMT* and MATE genes in roots. As reference genes we used actin and elongation factor alpha (EFα), designed by Silva et al. (2017) for *Styrax* genus. Primers for actin were: AGCTGGAGACTGCAAAGAGC and TTCCATTCCAATCAATGACG, for elongation factor: GCAACCACGCCAAAGTATTC and TGTTGTCACCCTCAAAACCA, for ALMT: AATTTCCACATCGCAACCTC and TGAGCAATGAAGCCACTGTC, and for MATE: GCTTGGAAGGCTGGTCATAC and CTTGTGTCAAGGCTGATTGC. Three replicates were used to quantify gene expression. Biological replicate consisted of one *S. camporum* individual under control (0 µM Al) or Al conditions (740 and 1480 µM Al). Each biological sample was run (qPCR) in three technical replicates.

Five serial dilutions of genomic DNA from *Styrax camporum* were amplified using the internal primers in order to access the amplification efficiency during qPCR analysis. GoTaq® qPCR Master Mix with SYBR® fluorescence (Promega, Madison, USA) was used with the following cycle specifications: 95°C for 5 min (1 cycle), 95°C for 10 s, 60°C for 30 s, 72°C for 30 s (40 cycles) followed by a melting curve analysis (1% slope temperature; 60–95°C), performed in a 7500 fast real time PCR system (Applied Biosystems, Foster City, USA). Amplification efficiencies, respectively for actin, elongation factor, *ALMT* and *MATE* were 95%, 89.4%, 99.6% and 95.7%. Total RNA was extracted from root samples using the RNeasy plant mini kit (Qiagen, Hilden, Germany). Two µg of total RNA were treated with TURBO DNA-freeTM (Ambion, Carlsbad, USA) and reverse transcribed using an oligo-dT primer and Super ScriptTM IV (Life Technologies, Carlsbad, USA), according to the manufacturer's protocol. cDNAs were used to qRT-PCR analyses, where amplification conditions were the same of amplification efficiency tests. qPCR data was analyzed based on

the procedure of Pfaffl (2001). Expression was normalized using the geometric mean of the RQ values of actin and EFα.

Data analysis

For each date of analysis, a one-way analysis of variance (Anova) was performed to test for differences in OAs and *ALMT* gene expression between plants exposed to 0, 740 and 1480 μM Al. The Tukey test ($\alpha = 0.05$) was used to conduct post-hoc comparisons to estimate the least significant differences between mean results of the three treatments.

Results

The GC-MS method was adequate to evaluate the targeted OAs, and a comparison between their retention times showed that citric, malic and oxalic acids could be differentiated in the chromatogram (Fig. 1). The standard curves used for each OAs were also representative ($R^2 > 0.99$; Table 1).

Exudation of OAs by *S. camporum* was observed in all treatments. However, plants exposed to Al exuded more OAs than those not exposed to Al, mainly after 10 days (Fig. 2). Al-induced citric acid exudation was not significantly higher before 10 DAT when plants exposed to 1480 μM Al peaked exudation, while plants exposed to 740 μM Al peaked citric acid exudation at 15 DAT.

Al-induced oxalic acid exudation occurred before 24h of Al exposure because, at 1 DAT, plants exposed to 740 and 1480 μM Al exhibited higher oxalic acid exudation than plants not exposed to Al (Fig. 2B). Plants exposed to 1480 μM Al exuded more oxalic acid than those exposed to 740 and 0 μM Al, at 5, 10 and 15 DAT (Fig. 2B). At 30 DAT, plants exposed to 740 exuded more oxalic acid in relation to those exposed to 1480 and 0 μM Al (Fig. 2B).

Malic acid was not detected in the nutrient solution. However, at 15 DAT *ALMT* was 25 times more expressed in plants exposed to 740 μM Al when compared to those not exposed to Al, and this difference was only 10.6 times for plants exposed to 1480 μM Al (Fig. 3A). Similarly, at 15 DAT, malic acid content in the root tips of plants exposed to 740 μM Al was twice as high as those exposed to 1480 μM Al (Fig. 3B). The expression of *MATE* was not significantly different between plants exposed to 0, 740 and 1480 μM Al (data not shown).

The nutrient solution with nominal 0 μM Al showed Al concentration below 0.04 μM Al during the whole experiment. The nutrient solution with nominal 740 μM Al showed a 40% decrease in Al concentration between 0 and 30 DAT, while this reduction was of 60% in the nutrient solution with nominal 1480 μM Al (Fig. 4).

Discussion

As far as we are aware, this is the first evidence of the OA exclusion mechanism occurring in a Cerrado woody species. *S. camporum* is considered a moderate Al-accumulating species, and it stores approximately 1500 mg Al per kg dry leaves (Breassan et al., 2016). On the other hand, Al-accumulating woody species from this vegetation store between 3000 and 15000 mg Al per kg dry leaves (Haridasan, 1982; Bressan et al., 2016; Malta et al., 2016).

Although Al-accumulating species store considerable Al content in their shoots and leaves, these plants also possess mechanisms for detoxifying Al externally (in the apoplast). For instance, the Al-accumulating plants *Camellia sinensis* (Morita et al., 2011), *Fagopyrum esculentum* (buckwheat) (Ma et al., 1997) and *Colocasia esculenta* (tora) (Ma and Miyasaka, 1998) were reported to exude OA from roots in response to Al. This could be especially important because there are almost 2000 woody species in the Cerrado vegetation (Ratter et al., 2003) that are adapted to grow on soils that are acidic and rich in Al (m% > 50%) (Haridasan, 2008), and only some species from few families (Melastomataceae, Rubiaceae and Vochysiaceae) have been identified as Al-accumulating plants (Haridasan, 1982; Bressan et al., 2016; Malta et al., 2016).

In the present 30-day study, while plants exposed to 740 μM Al absorbed 40% of the available Al in the solution, plants exposed to 1480 μM Al absorbed 60% of it. At the same time, plants exposed to 1480 μM Al exuded lower concentration of citric acid between 15 and 30 DAT, and oxalic acid at 30 DAT. This suggests that citric and oxalic acids are Al detoxifying mechanisms in this species considering that more OA exudation were associated with lower Al uptake. Also, these data infer that plants exposed to 740 μM Al exclude more Al through OA exudation when compared to those exposed to 1480 μM Al. This may explain why *S. camporum* plants exposed to 1480 μM Al for 91 days exhibited reduced leaf gas exchange and shoot growth in relation to plants not exposed to Al (Banhos et al., 2016b). Thus, as occurs in *Camellia sinensis* - that accumulates approximately 6000 mg Al per kg dry leaves (Carr et al., 2003) and also exude OA (Morita et al., 2011) - *S. camporum* accumulates

considerable concentration of Al in their leaves (Bressan et al., 2016) but also uses the exclusion mechanism to cope with Al in their roots.

Intriguingly, we could not find malic acid in the nutrient solution, although it was detected when analytical standard was added in nutrient solutions containing 0, 740 and 1480 μM Al (R^2 > 0.99; Table 1). This suggests that nutrient solution did not interfere with the detection of malic acid and was not exuded by plants in any of the treatments. Therefore, we presume that *S. camporum* exudes only citric and oxalic acids in response to Al, like *Acacia auriculiformis*, *Eucaliptus camaldulensis* and *Melaleuca cajuputi* (Nguyen et al., 2003; Tahara et al., 2008).

On the other hand, we found significant contents of malic acid in the root tips of plants exposed to 740 μM Al in relation to those exposed to 0 and 1480 μM Al, mainly at 15 DAT, when *ALMT* was more expressed in plants exposed to 740 μM Al in relation to those exposed to 0 and 1480 μM Al. This indicates a corresponding pattern between the expression of *ALMT* gene and the content of this OA in the roots, although it was not exuded. This could be explained by the fact that *ALMT* family may not be involved with organic acid exudation. For instance, the maize genes Zm*ALMT1* and Zm*ALMT2* are involved in ion homeostasis (Sharma et al. 2016), and a similar role is played at the vacuolar level by At*ALMT0* in *Arabidopsis thaliana*, which mediates malic acid uptake on the tonoplast (Kovermann et al. 2007; Meyer et al. 2010; Sasaki et al. 2010; De Angeli et al. 2013b). Moreover, the presence of citric and oxalic acids and the lack of malic acid in the nutrient solution might be due to the fact that citric acid can detoxify 2–3 times more Al (Ma et al. 1997) than oxalate or malate can (Ryan et al. 1995; Ma 2000). Indeed, the three OA anions form complexes with Al with the following order of strength: citrate > oxalate > malate (Brunner and Sperisen, 2013).

This is the first report of Al exclusion through OA exudation in *S. camporum*, a Cerrado woody species that grows on soils that are acidic and rich in Al. This species relies on exudation of mainly citric and oxalic acids that may form complexes with Al in the apoplast in order to cope with Al in the soil. Considering that this species is a moderate Al-accumulating plant, this mechanism could explain the low Al accumulation in their leaves when compared to Al-accumulating plants from this vegetation, and this OA exclusion mechanism merits further investigation in the few species of Al-accumulating plants from the Cerrado.

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Table:

Table 1. Equations of calibration curves and correlation coefficients for each OA after adding six concentrations of citric, malic and oxalic acids in methanol (MeOH) and nutritive solution containing 0, 740 and 1480 μM Al

Standard addition	Citric acid	Malic acid	Oxalic acid
MeOH	y = 0.5588x + 4.8989 R^2 = 0.9968	y = 0.638x + 1.1854 R^2 = 0.9983	y = 0.2992x + 6.614 R^2 = 0.9942
0 μM Al	y = 1.9885x + 64.046 R^2 = 0.9901	y = 1.1511x + 13.861 R^2 = 0.9972	y = 0.7827x + 5.0585 R^2 = 0.9911
740 μM Al	y = 4.661x + 20.359 R^2 = 0.9949	y = 1.5978x + 6.413 R^2 = 0.998	y = 0.7878x + 2.8177 R^2 = 0.9934
1480 μM Al	y = 8.8615x + 10.447 R^2 = 0.9901	y = 3.0731x + 9.7316 R^2 = 0.9976	y = 0.7873x + 5.375 R^2 = 0.9952

Figures:

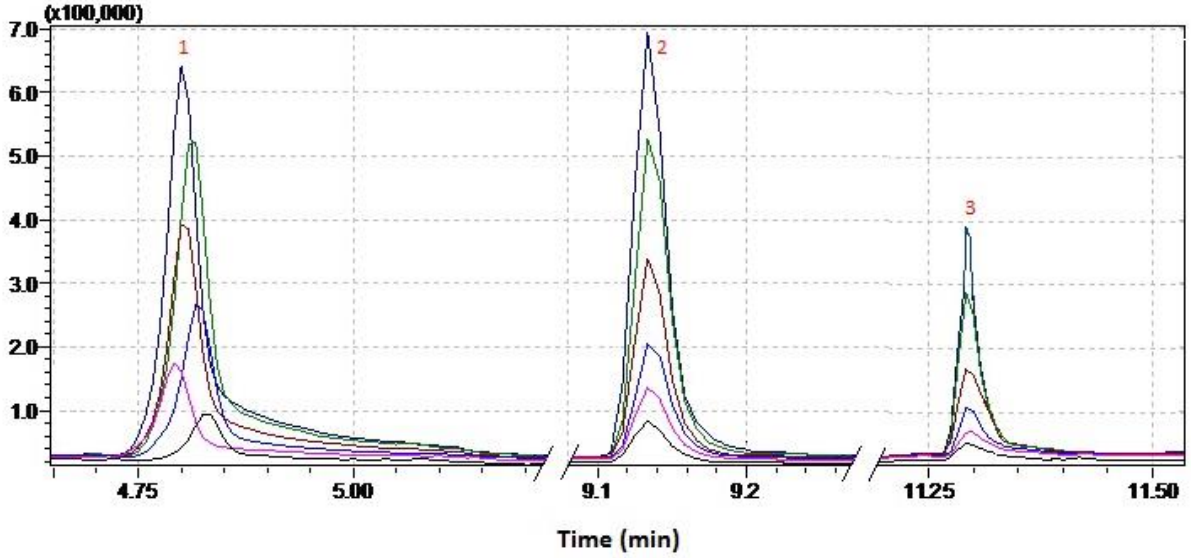

Fig. 1. GC-MS chromatogram for the standard solutions of six concentrations of the OAs in methanol. Different line colors represent different concentrations (μg/mL): black, 5; pink, 10; blue, 30; brown, 50; green, 70; dark blue, 100. Peaks in time retention order: 1, oxalic acid; 2, malic acid; 3, citric acid.

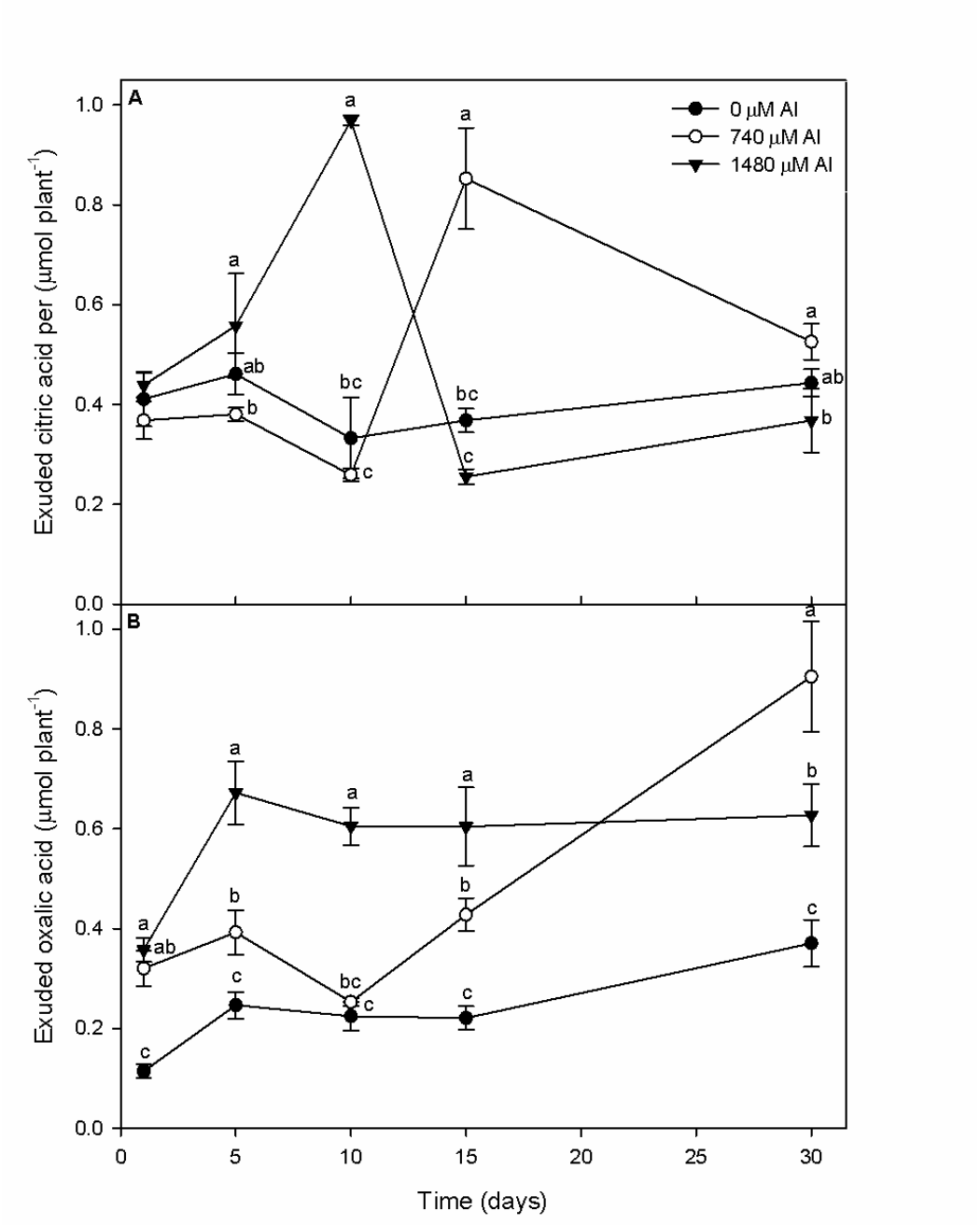

Fig. 2. Citric (A) and oxalic (B) acids exuded by the roots of *Styrax camporum* plants grown in nutrient solutions containing 0, 740, and 1480 μM Al, for 1, 5, 10, 15 and 30 days. For each evaluation date, different letters indicate significant difference by Tukey test ($P < 0.05$) between 0, 740, and 1480 μM Al.

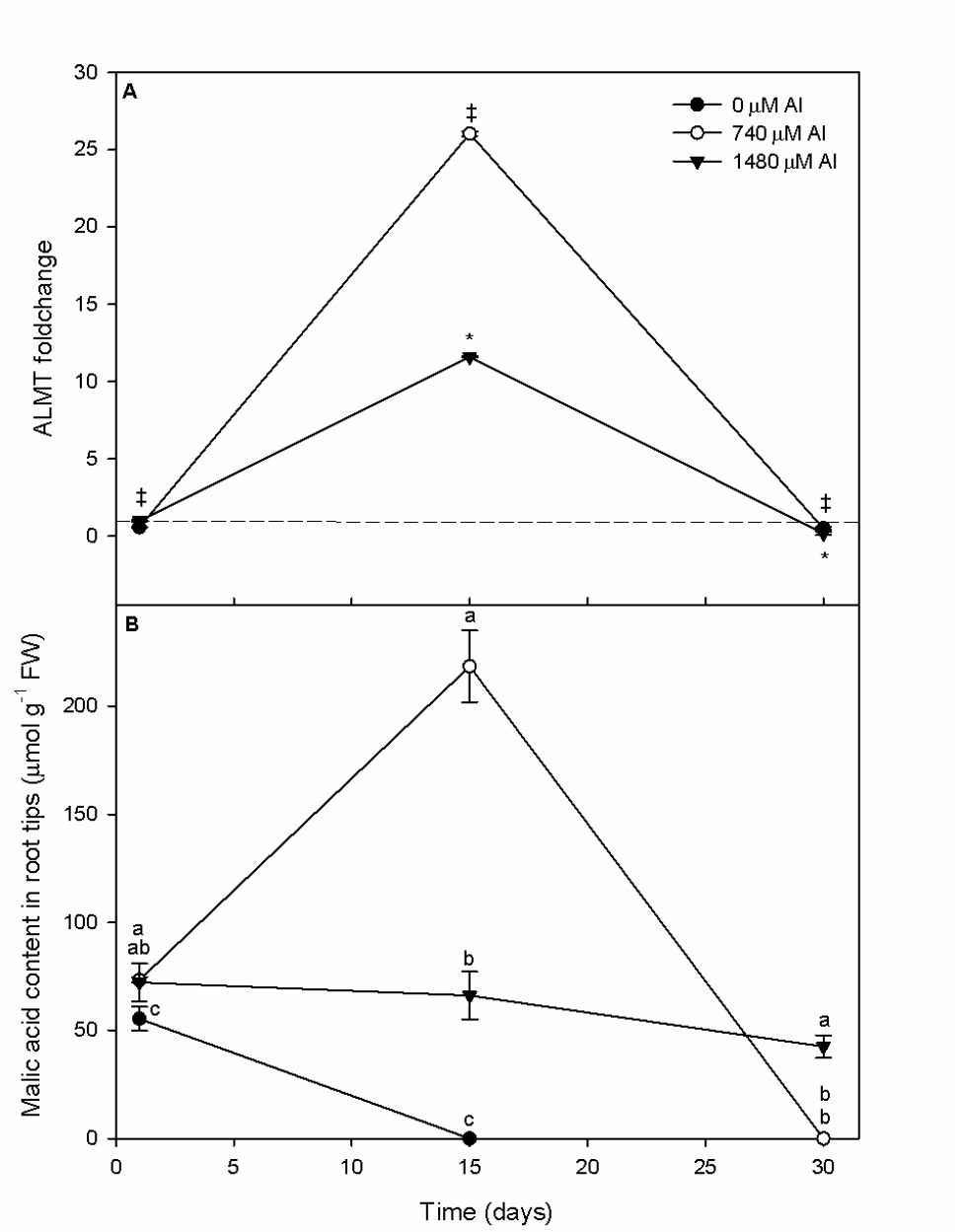

Fig. 3. Gene expression of *ALMT* channel (A) and malic acid content (B) in root tips of *Styrax camporum* grown in nutrient solutions containing 0, 740, and 1480 μM Al, for 1, 15, and 30 days. For each evaluation date, asterisks, double cross (‡) (A), and different letters (B) represent significant difference by Tukey test ($P < 0.05$) between 0, 740 and 1480 μM Al. Dashed line (A) is a reference (one fold change in gene expression of plants not exposed to Al).

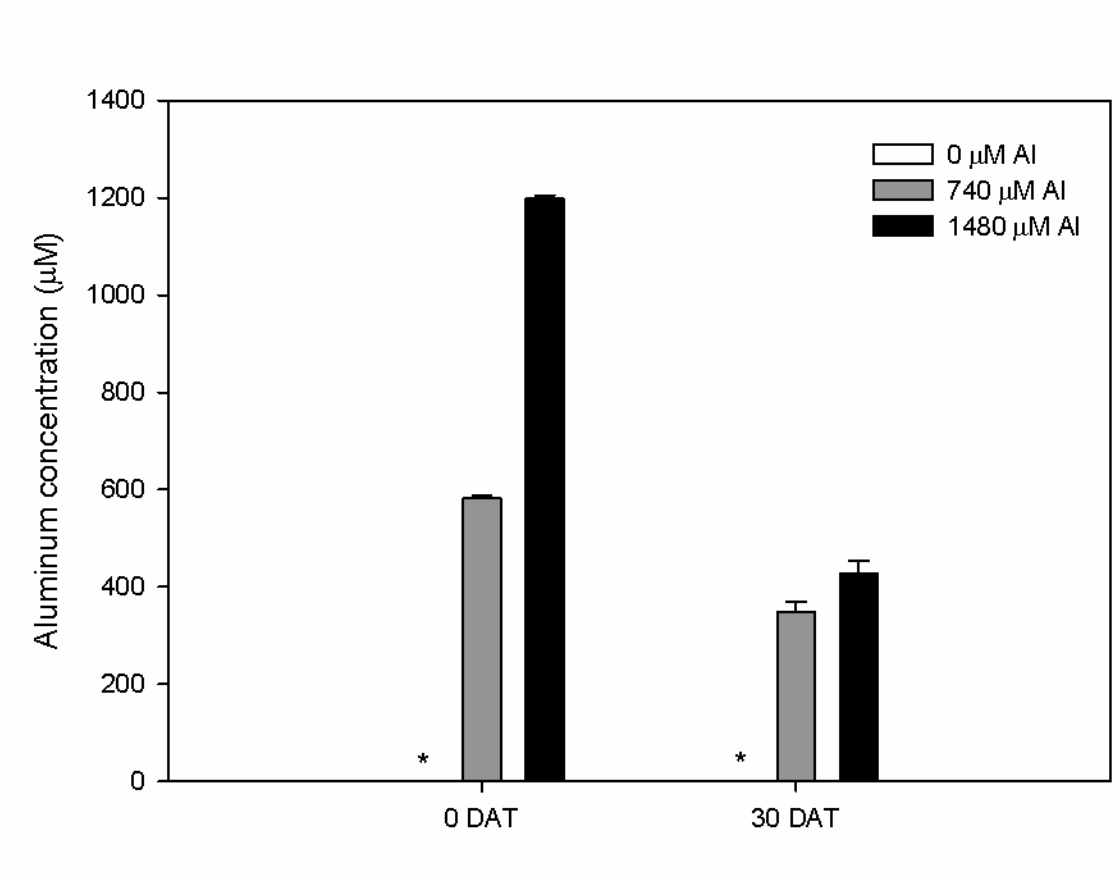

Fig. 4. Aluminum concentration in the nutrient solutions containing nominal concentration of 0, 740 and 1480 μM Al at 0 and 30 days of the study. Asterisks indicate values < 0.04 μM Al.

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