



# Aluminum (Al)-induced organic acid exudation in an Al-accumulating species from the Brazilian savanna

Brenda Mistral de Oliveira Carvalho Bittencourt<sup>1</sup> · Carolina de Marchi Santiago da Silva<sup>1</sup> · Sebastião Zanao Filho<sup>2</sup> · Gustavo Habermann<sup>3</sup>

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## Abstract

**Key message** *Styrax camporum*, an Al-accumulating species from the Cerrado, seems to rely on the exudation of citric and oxalic acids to avoid excessive Al.

**Abstract** Organic acid (OA) exudation by the roots of plants to chelate aluminum (Al) and forming non-toxic complexes is a known mechanism of Al exclusion in some plants, including some Al-accumulating species. The Cerrado vegetation in South America is composed of Al non-accumulating species and some Al-accumulating species from few families, all growing healthy on acidic soils with high Al saturation but never tested for OA exudation. We elected *Styrax camporum* (Styracaceae), a Cerrado woody species that accumulates in their leaves approximately 1500 mg Al kg<sup>-1</sup> dry mass, to examine whether plantlets of this species exude OAs in response to changes in Al concentrations in a nutrient solution, for 30 days. Citric, malic and oxalic acids exuded by the roots of this species were cumulatively measured in nutrient solutions containing 0, 740 and 1480 μM Al. Also, we measured the Al concentration of whole plantlets at 0 and 30 days. Malic acid was not exuded by the plantlets, but it was detected inside root tips of plantlets exposed to Al. Plantlets exposed to 740 μM Al released more oxalic and citric acid in the nutrient solution than those exposed to 0 and 1480 μM Al after 30 days. On the other hand, between 0 and 30 days, plantlets exposed to 740 μM Al increased the Al concentration (in whole plantlet) by three times while those exposed to 1480 μM Al, by seven times. This higher OA exudation associated with lower Al uptake at 740 μM Al suggests an Al exclusion mechanism that is impaired at higher Al concentrations. This is the first report showing that an Al-accumulating species from the Cerrado exudes OAs in response to the Al concentration in the root environment.

**Keywords** Aluminum exclusion · Cerrado vegetation · Citric acid · Oxalic acid · *Styrax camporum*

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✉ Gustavo Habermann  
gustavo.habermann@unesp.br

- <sup>1</sup> Departamento de Botânica, Instituto de Biociências, Programa de Pós-Graduação em Ciências Biológicas (Biologia Vegetal), Universidade Estadual Paulista, UNESP, Av. 24-A, 1515, Rio Claro, SP 13506-900, Brazil
- <sup>2</sup> Instituto de Biociências, Centro de Estudos de Insetos Sociais (CEIS), Universidade Estadual Paulista, UNESP, Av. 24-A, 1515, Rio Claro, SP 13506-900, Brazil
- <sup>3</sup> Departamento de Botânica, Instituto de Biociências, Universidade Estadual Paulista, UNESP, Av. 24-A, 1515, Rio Claro, SP 13506-900, Brazil

## 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<sub>2</sub>O) < 5.5], which comprise 30% of the world's ice-free land (von Uexküll and Mutert 1995), Al can be found as different ions, especially the phytotoxic trivalent cation (Al<sup>3+</sup>). Al decreases the root growth, leaf gas exchange and plant development (Kopitke 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, accumulating Al without apparent damage to their tissues. Species belonging to approximately 45 families (being Melastomataceae, Rubiaceae, Simplicaceae, Theaceae, and Vochysiaceae examples of those most studied ones) can be described

as Al-accumulating plants (Jansen et al. 2002). Plants that accumulate in their leaves more than 1000 mg Al kg<sup>-1</sup> DM are defined as Al-accumulators (Chenery 1948; Jansen et al. 2002).

Al-accumulating species seem to isolate Al at sites that are insensitive to Al (e.g., epidermal cells or vacuoles). Once Al enters the symplast, these species may chelate intracellular Al, forming an Al-ligand complex (mainly OAs) for the transport from roots to shoots and to leaf accumulation (Kochian et al. 2004; Ma et al. 2001; Brunner and Sperisen 2013). Besides this internal tolerance mechanism, Al-accumulating plants may also rely on exclusion mechanisms that detoxify the Al externally (in the apoplast of root cells and rhizosphere) (Horst et al. 2010; Ma et al. 2001). In this process, OAs, like citric, malic and oxalic acids are exuded by the roots of plants and form non-toxic stable complexes with Al (Kochian et al. 2004; Brunner and Sperisen 2013). This avoids the reaction of Al to sites negatively charged in the apoplast (Horst et al. 2010), which is the primary lesion of Al in plant roots (Kopittke et al. 2015), and limits its uptake into the cytosol (Brunner and Sperisen 2013). This double-handed behavior (Al accumulation and Al exclusion) can be seen in tea plants (*Camellia sinensis* L.) (Theaceae), which accumulate in their leaves approximately 6000 mg Al kg<sup>-1</sup> DM (Carr et al. 2003), and detoxify Al externally through OAs exudation (Morita et al. 2011). Similarly, buckwheat (*Fagopyrum esculentum* Moench) (Polygonaceae) and tora [*Colocasia esculenta* (L.) Schott] (Araceae), which show significant Al concentration in their leaves and shoots, exude OAs by their roots in response to Al (Ma et al. 1997; Ma and Miyasaka 1998).

Less attention is paid to species from native communities that tolerate Al. The Cerrado vegetation in South America, broadly known as ‘Brazilian Savanna’, is composed of a mosaic of physiognomies that are adapted to grow on soils that are acidic (pH < 5.0) and show Al saturation (m %) higher than 50% of the cation exchange capacity (Haridasan 1982, 2008; Souza et al. 2015b; Bressan et al. 2016; Malta et al. 2016). The great majority of Cerrado woody species are non-accumulating species [plants that accumulate in their leaves less than 1000 mg Al kg<sup>-1</sup> DM (Chenery 1948)], and some of them have been reported to store between 100 and 600 mg Al kg<sup>-1</sup> DM (Haridasan 1982; Souza et al. 2015a). This large group of woody species occurs in this vegetation along with few species considered Al-accumulators [leaf Al concentration > 1000 mg kg<sup>-1</sup> DM (Chenery 1948)], such as *Miconia* spp. (Melastomataceae), *Palicourea rigida* Kunth, *Rudgea viburnoides* (Cham.) Benth (Rubiaceae), *Qualea* spp., *Vochysia* spp. (Vochysiaceae) and *Styrax camporum* Pohl. (Styracaceae) (Haridasan 1982; Bressan et al. 2016; Malta et al. 2016). Some studies have shown that these species rely on internal tolerance mechanisms to cope with high Al in the soil, in particular, Al

immobilization by compartmentalization (Haridasan et al. 1986; Andrade et al. 2011; Scalón et al. 2013). However, up to date, there are no studies describing any Al exclusion mechanism in Al-accumulating species from the Cerrado.

*S. camporum* is a Cerrado woody species that accumulates in their leaves approximately 1500 mg Al kg<sup>-1</sup> DM (Bressan et al. 2016), being considered an Al-accumulating species by the widely accepted criterion (Chenery 1948). The only study that investigates *S. camporum* response to Al in nutrient solution showed that 1480 μM Al causes lower leaf gas exchange rates and reduced shoot growth in relation to plants not exposed to Al (Banhos et al. 2016b), suggesting that this Al concentration is toxic to this species. Al toxicity effects in Al-accumulating plants have also been demonstrated in tea plants that grow and survive in nutrient solution containing up to 1000 μM Al, but show reduced shoot growth when exposed to 4000 μM Al (Morita et al. 2008). This species exudes increasing amounts of OAs when exposed from 0 to 800 μM Al (Morita et al. 2011). Another study showed that root OAs content is retained or decreased when tea plants are exposed from 800 to 4000 μM Al (Morita et al. 2008). Thus, once 1480 μM Al seems to be toxic to *S. camporum*, a lower Al concentration in nutrient solution could not harm this plant, possibly due to an increased OAs exudation and consequent Al exclusion. Similar to tea plant, tora and buckwheat that accumulate considerable amount of Al in their leaves and exude OA in response to Al (Ma et al. 1997; Ma and Miyasaka 1998; Morita et al. 2011), it is possible that this double-handed behavior (Al accumulation and Al exclusion) also occurs in *S. camporum*.

Here, we tested if *S. camporum*, an Al-accumulating plant, exudes OAs in response to changes in Al concentrations in the nutrient solution. For this, we measured citric, malic and oxalic acids exuded by roots of *S. camporum* plantlets grown in a nutrient solution containing 0, 740 and 1480 μM Al for 30 days.

## Material and methods

### Plant material and experimental conditions

Ripe fruits of five plants of *Styrax camporum* were collected in a Cerradão remnant (22°15'S and 47°00'W; 860 m of altitude) 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<sup>-2</sup> s<sup>-1</sup>) for 17 months, until reaching 8 ± 1 cm in height, as this species shows slow growth and development. The plantlets were then transferred

to 50-mL Falcon tubes (1 plant per tube) containing 45 mL of a nutrient solution.

We used a nutrient solution with 1/7 ionic strength with a chemical composition based on Clark's solution (Clark 1975) that was already used to study Al toxicity in *S. camporum* (Banhos et al. 2016b). This ionic strength was used to resemble the nutrient composition of Cerrado soils (Souza et al. 2015b; Bressan et al. 2016). For instance, Kopittke et al. (2010) observed that the soil solution from an Australian acidic soil exhibits nutrient concentrations that are approximately seven-fold lower than those in Hoagland & Arnon's nutrient solution. Although nutrient concentrations in solutions (mass per liquid volume) cannot be compared with nutrient saturation assessed in soils (ionic charges per volume of a solid matrix), Banhos et al. (2016b) demonstrated that this nutrient solution causes no nutrient deficiency in *S. camporum* saplings (30 cm in height). It consisted of 196.11  $\mu\text{M}$   $\text{Ca}(\text{NO}_3)_2 \cdot 4 \text{H}_2\text{O}$ , 72.43  $\mu\text{M}$   $\text{NH}_4\text{NO}_3$ , 32.06  $\mu\text{M}$   $\text{KCl}$ , 32.46  $\mu\text{M}$   $\text{K}_2\text{SO}_4$ , 31.23  $\mu\text{M}$   $\text{KNO}_3$ , 69.03  $\mu\text{M}$   $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 4.3  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$ , 3.72  $\mu\text{M}$   $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 3.4  $\mu\text{M}$   $\text{NaEDTA}$ , 0.5  $\mu\text{M}$   $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 1.41  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 0.13  $\mu\text{M}$   $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ , 0.03  $\mu\text{M}$   $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.06  $\mu\text{M}$   $\text{NaMoO}_2 \cdot 2 \text{H}_2\text{O}$ . This solution resulted in the following macronutrients (in mM): 0.137  $\text{NO}_3^-$ ; 0.058  $\text{NH}_4^+$ ; 0.0019 P; 0.123 K; 0.204 Ca; 0.047 Mg; 0.031 S; and micronutrients (in  $\mu\text{M}$ ): 30.58 Cl; 3.32 Fe (EDTA); 1.19 B; 0.41 Mn; 0.10 Zn; 0.04 Cu and 0.04 Mo.

In a previous study using this same nutrient solution (Banhos et al. 2016b), we observed that *S. camporum* exposed to 1480  $\mu\text{M}$  Al showed reduced shoot growth and leaf gas exchange, suggesting that this concentration may result in a higher Al availability in relation to Cerrado soils, where this Al-accumulating species grows well without any apparent damage or toxicity symptoms. Thus, considering that 1480  $\mu\text{M}$  Al may be toxic to *S. camporum*, a lower Al concentration could be also tested. Therefore, besides macro and micronutrients, the solution contained 0, 740 and 1480  $\mu\text{M}$  Al provided through  $\text{AlCl}_3 \cdot 6 \text{H}_2\text{O}$ . The nutrient solution with nominal 0  $\mu\text{M}$  Al showed Al concentration below 0.04  $\mu\text{M}$  Al during the whole experiment (Table S1; Supplementary material). The nutrient solution with nominal 740  $\mu\text{M}$  Al resulted in  $582.5 \pm 4.9 \mu\text{M}$  Al, while the nutrient solution with nominal 1480  $\mu\text{M}$  Al resulted in  $1198.7 \pm 6.9 \mu\text{M}$  Al (Table S1; Supplementary material). The pH of the solution in each tube was monitored daily with a pH meter (Tec-2; Tecnal, Piracicaba, Brazil) and maintained at  $4.0 \pm 0.1$  to keep Al as soluble as possible. Aeration of the solution in each tube was performed using aquarium pumps.

The tubes were wrapped with aluminum foil to avoid light to the roots and nutrient solution and were kept in racks, on benches in the laboratory, under controlled conditions ( $25 \pm 1^\circ\text{C}$ ;  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ; 12 h of photoperiod). The plantlets were fixed at the mouth of the tubes using

polyurethane foam strips that were placed around the plantlet collar.

## Experimental design

We measured organic acids (citric, malic and oxalic) exuded by the plantlets roots, directly in the nutrient solution. For this, we used 45 tubes containing the nutrient solution (15 tubes containing 0  $\mu\text{M}$  Al, 15 tubes with 740  $\mu\text{M}$  Al and 15 tubes with 1480  $\mu\text{M}$  Al) with one plantlet per tube. The total solution volume from three tubes (replicate) of each of the three treatments was collected (destructive procedure) at 1, 5, 10, 15 and 30 days after transfer (DAT). Organic acids (citric, malic and oxalic) exuded by the roots of plantlets into the collected nutrient solution were measured using a gas chromatograph coupled to a mass spectrometer (GC–MS) (see details below). The nutrient solution was not replaced in the tubes so we could measure the accumulated OAs from the moment the plantlet was transferred to the tube until the moment that total volume of nutrient solution was collected from each tube on each date. Therefore, to replace the transpired water (vapor) through leaves of plantlets, everyday, deionized water was added in each tube to complete the 45 mL.

As malic acid was not detected in the nutrient solution of any of the Al treatments at any DAT, we performed a second experiment to investigate the malic acid concentration inside root tips ( $0.5 \pm 0.1$  cm in length). For this, we used 27 tubes containing the nutrient solution (nine tubes containing 0  $\mu\text{M}$  Al, nine tubes with 740  $\mu\text{M}$  Al and nine tubes with 1480  $\mu\text{M}$  Al) and plantlets of the same age/size under the same experimental conditions. The root tips were collected (destructive analysis) at 1, 15 and 30 DAT and internal malic acid was quantified also using GC–MS. In addition to the root tips, at 1 and 30 DAT, we measured the Al concentration in whole plantlets. On the same dates, nutrient solution was also collected to measure the Al, macro and micronutrients concentrations.

## Detection and quantification of organic acids in the solution and malic acid in root tips

Exuded OAs were concentrated after drying the 45 mL solution in a forced-air oven at  $80^\circ\text{C}$ . Samples were esterified according to Fischer's method (methylation) (Fischer and Speier 1895). Then, we added 700  $\mu\text{L}$  of methanol (HPLC/Spectro) and 300  $\mu\text{L}$  of sulfuric acid (3.5 M). The samples were shaken and kept for 1 h at  $70^\circ\text{C}$  for catalyzing the reaction. After adding 1 mL of hexane (HPLC/Spectro), 100  $\mu\text{L}$  of the apolar phase was collected and analyzed using a GC–MS (GC-2010/GCMSQP2010 Plus, Shimadzu, Japan), with an automatic sample injector (AOC-20i).

To establish a relationship between GC–MS peak areas and the OAs concentrations found in the nutrient solution with 0, 740 and 1480  $\mu\text{M}$  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  $\mu\text{g mL}^{-1}$ ). For each concentration, all the three OAs were added in the same nutrient solution. Three replicates were used for each concentration and values were plotted to generate the standard curves. This method was already used to detect and quantify OAs exuded by *Citrus limonia* L. (Rutaceae) in nutrient solution with 0 and 1480  $\mu\text{M}$  Al (Silva et al. 2019).

Malic acid content in the root tips ( $0.5 \pm 0.1$  cm in length) was extracted by osmosis and alkaline gradient, immersing the root tips in 1.5 mL 40 mM  $\text{Na}_2\text{CO}_3$  for 24 h. 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  $\mu\text{L}$  of methanol (HPLC/Spectro) and 100  $\mu\text{L}$  of sulfuric acid (3.5 M). After shaking, the samples were kept for 1 h at 70 °C for catalyzing the reaction. Then, we added 1 mL of hexane (HPLC/Spectro), collected 100  $\mu\text{L}$  of the apolar phase, and analyzed it using GC–MS. This method was already used to detect and quantify OAs in root tips of *C. limonia* in nutrient solution with 0 and 1480  $\mu\text{M}$  Al (Silva et al. 2019).

In the GC–MS, we used a 30 m-length and 250  $\mu\text{m}$ -diameter fused-silica microcolumn (RTX-5MS, Restek), and analytical ultra-pure helium (99.9999%, White Martins®) was used as a carrier gas. The injector temperature was 250 °C (Splitless mode) and the injection volume was 1  $\mu\text{L}$ . Column gas flow was maintained at 41  $\text{cm s}^{-1}$ . The initial column temperature was 50 °C with a 4 min step. After that, at a 10 °C  $\text{min}^{-1}$  rate, it achieved 70 °C. Then, it was increased to 250 °C at a 25 °C  $\text{min}^{-1}$  rate, and maintained for 0.8 min, completing 14 min running. Mass detector was a simple quadrupole type with 70 eV electronic impact ionization. The GC–MS interface temperature was 250 °C and 230 °C to the ionizer. The detector potential was relative to tuning, with a 40–450  $m/z$  detection range (scanner mode).

### Aluminum, macro and micronutrients in the nutrient solution

The Al, K, Ca, Mg, P, S, B, Cu, Fe, Mn, and Zn concentration in the nutrient solution was measured using an inductively coupled plasma optical emission spectroscope (ICP-OES) (Varian, Vista-MPX/Australia). This method allows measuring all the nutrient species in the solution, i.e., free and complexed forms. The amoniacal- and nitrate-N were measured by the distillation method, according to Bremner and Keeney (1966).

### Aluminum concentration in plantlets

Dried samples of whole plantlets were sent to a routine plant nutrition laboratory at Instituto Agronômico de Campinas (IAC, Campinas, SP, Brazil) where they were ground and digested in a solution of sulfuric:nitric:perchloric acids (1:10:2, v/v/v). After digestion, Al concentration was determined by the atomic absorption spectrophotometer method (Sarruge and Haag 1974) and expressed as mg Al  $\text{kg}^{-1}$  DM.

### Data analysis

The experiment was carried out in a completely randomized design with three treatments and (destructive) collections distributed over time (1, 5, 10, 15 and 30 DAT). For each date, a one-way analysis of variance (Anova) was performed (after checking for normal data distribution and homogeneous variance of data) to test for differences in OAs and Al concentration between plantlets exposed to 0, 740 and 1480  $\mu\text{M}$  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. In addition, a Student's *t* test was used to test for differences in plantlet Al concentration between 0 and 30 DAT.

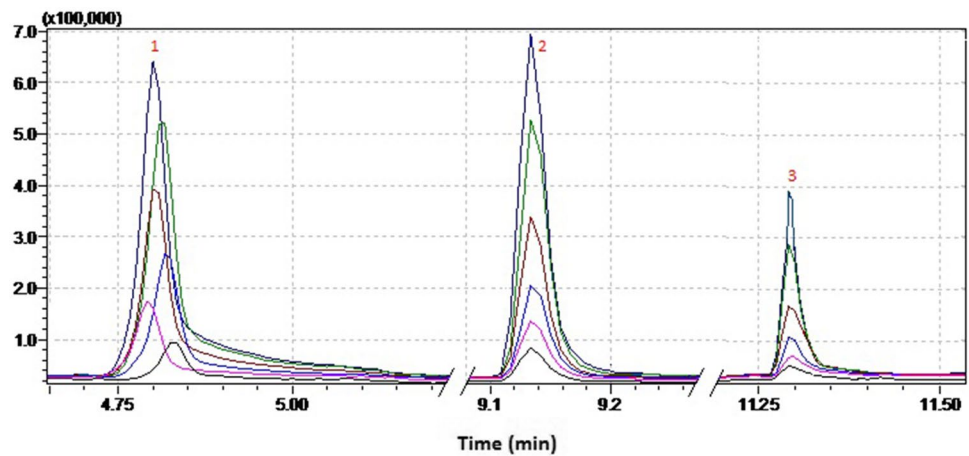
To compare OAs concentrations over time, a one-way repeated measures analysis of variance (RM-Anova) was performed (also after checking for normal data distribution and homogeneous variance of data) for each OA and Al concentration to test for differences between evaluation dates. The Holm–Sidak method ( $\alpha=0.05$ ) was used to conduct post hoc pairwise multiple comparisons. The statistical package available in SigmaPlot 12.0 software was used, and standard deviation is given in all figures and tables.

### Results

The method using GC–MS 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 of the OAs were also representative ( $R^2 > 0.99$ ; Table 1).

The exudation of OAs by *S. camporum* was observed in all treatments. At 1 DAT, plantlets exposed to 740 and 1480  $\mu\text{M}$  Al showed higher oxalic acid concentration in the nutrient solution than those not exposed to Al (Fig. 2a), indicating that Al-induced oxalic acid exudation occurred before 24 h of Al exposure. Plantlets exposed to 1480  $\mu\text{M}$  Al exuded oxalic acid between 1 and 5 DAT and, after this date, oxalate exudation stopped, while those exposed to 740  $\mu\text{M}$  Al continued exudation of oxalate between 10 and 30 DAT (Fig. 2a; Table 2). Thus, plantlets exposed to 1480  $\mu\text{M}$  Al

**Fig. 1** GC–MS chromatogram for the standard solutions of six concentrations of the OAs in methanol. Different line colors represent different concentrations ( $\mu\text{g mL}^{-1}$ ): 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



**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  $\mu\text{M}$  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 $\mu\text{M}$ 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 $\mu\text{M}$ 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 $\mu\text{M}$ Al	$y = 8.8615x + 10.447$ $R^2 = 0.9901$	$y = 3.0731x + 9.7316$ $R^2 = 0.9976$	$y = 0.7873x + 5.375$ $R^2 = 0.9952$

showed higher oxalic acid concentration in the nutrient solution than those exposed to 0 and 740  $\mu\text{M}$  Al at 5, 10 and 15 DAT (Fig. 2a), while plantlets exposed to 740  $\mu\text{M}$  Al showed higher concentration of this OA than those exposed to 0 and 1480  $\mu\text{M}$  Al at 30 DAT (Fig. 2a).

Citric acid concentration in the nutrient solution of plantlets exposed to 740 and 1480  $\mu\text{M}$  Al was not significantly higher than that exposed to 0  $\mu\text{M}$  Al until 10 DAT, indicating that this OA exudation was not Al induced before this date (Fig. 2b). Plantlets exposed to 740  $\mu\text{M}$  Al exuded citric acid between 10 and 15 DAT and, after this date, citrate exudation stopped (Fig. 2b; Table 2), although this OA concentration was kept higher than those in the solutions containing 0 and 1480  $\mu\text{M}$  Al at 15 and 30 DAT (Fig. 2b).

Malic acid was not detected in the nutrient solution, as described above (see the experimental design). At 15 DAT, malic acid content in the root tips of plantlets exposed to 740  $\mu\text{M}$  Al was three times higher than those exposed to 1480  $\mu\text{M}$  Al (Fig. 3). No malic acid was detected in root tips of plantlets exposed to 0  $\mu\text{M}$  Al at 15 and 30 DAT or exposed to 740  $\mu\text{M}$  Al at 30 DAT because it was below the method detection limit (Fig. 3).

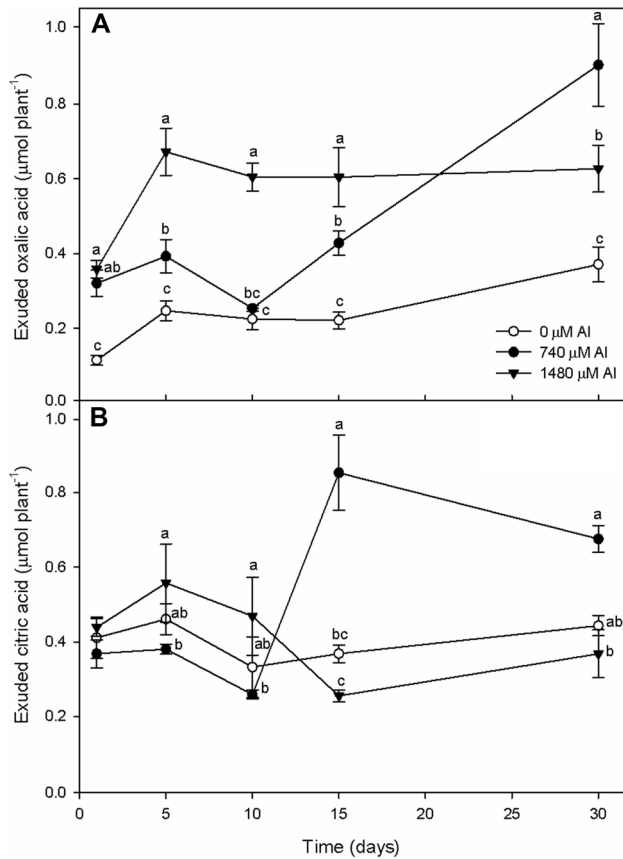
The Al concentration in plantlets exposed to 0  $\mu\text{M}$  Al was the same at 0 and 30 DAT. Plantlets exposed to 740  $\mu\text{M}$  Al tripled the Al concentration between 0 and 30 DAT,

while this increase was of seven times in plants exposed to 1480  $\mu\text{M}$  Al (Fig. 4).

## Discussion

As far as we are aware, this is the first report evidencing that *S. camporum*, considered an Al-accumulating species, exudes citric and oxalic acids by its roots in response to the Al concentration in the root environment. In the field, this species accumulates in their leaves approximately 1500 mg Al  $\text{kg}^{-1}$  DM (Bressan et al. 2016), while Al-accumulating woody species from Melastomataceae, Rubiaceae and Vochysiaceae from this vegetation store in their leaves between 3000 and 15,000 mg Al  $\text{kg}^{-1}$  DM (Haridasan 1982; Bressan et al. 2016; Malta et al. 2016).

In the present study, after 30 days, while plantlets exposed to 740  $\mu\text{M}$  Al showed in the whole plantlet  $1556.6 \pm 102.9$  mg Al  $\text{kg}^{-1}$  DM, plantlets exposed to 1480  $\mu\text{M}$  Al showed  $3533.4 \pm 211.3$  mg Al  $\text{kg}^{-1}$  DM (Fig. 4). This indicates that the former absorbed 40% of the available Al in the solution, and the latter 60% of it (Table S1; supplementary material). *S. camporum* saplings cultivated under 1480  $\mu\text{M}$  Al using the same nutrient solution that was used in the present study accumulated approximately 3500 mg Al  $\text{kg}^{-1}$

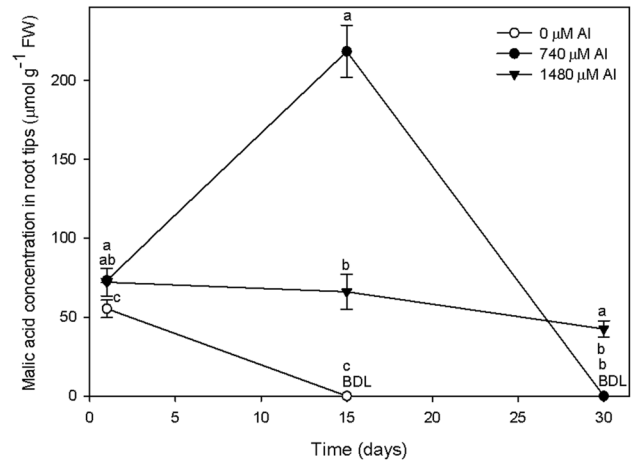


**Fig. 2** Oxalic (a) and citric (b) acids exuded by the roots of *Styrax camporum* plantlets grown in nutrient solutions containing 0, 740, and 1480 μM Al, and accumulated for 1, 5, 10, 15 and 30 days. For each evaluation date, different letters indicate significant difference by Tukey test ( $P < 0.05$ ) between 0, 740, and 1480 μM Al. Bars represent standard deviation

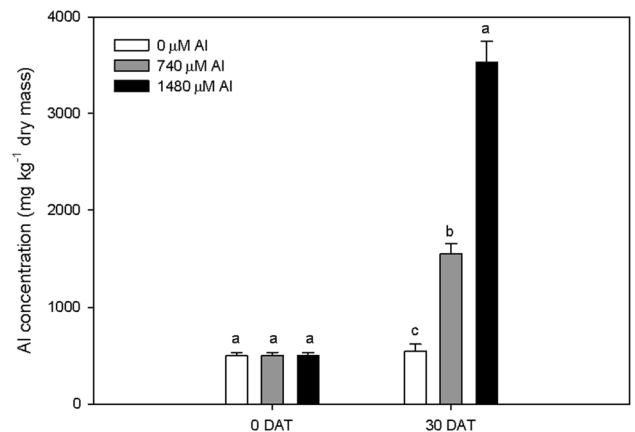
**Table 2** Statistical comparison (RM-Anova) between evaluation dates for each Al concentration and organic acid (OA)

OA	DAT	0 μM Al	740 μM Al	1480 μM Al
Oxalic	1–5	*		*
	5–10			
	10–15		*	
	15–30	*	*	
Citric	1–5			
	5–10		*	*
	10–15		*	*
	15–30		*	
Malic	1–15	*	*	
	15–30		*	

Asterisks represent significant difference by Holm–Sidak method ( $\alpha = 0.05$ ) between 1 and 5, 5 and 10, 10 and 15, and 15 and 30 days after transference (DAT)



**Fig. 3** Malic acid concentration 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, different letters represent significant difference by Tukey test ( $P < 0.05$ ) between 0, 740 and 1480 μM Al. BDL means values below the method detection limit. Bars represent standard deviation



**Fig. 4** Aluminum concentration in whole plantlets at 0 and 30 DAT. For each evaluation date, different letters represent significant differences by Tukey test ( $P < 0.05$ ) between 0, 740 and 1480 μM Al. BDL means values below the method detection limit. Bars represent standard deviation

dry plant material (Banhos et al. 2016b). Therefore, even using plantlets in the present study, these showed similar Al concentration as exhibited by larger plants in other studies.

One nutrient that usually interferes with the Al availability in nutrient solutions is phosphorus (P), resulting in precipitated forms of  $AlPO_4$  (Yang et al. 2011), but in the present study P was maintained under low concentration even in the solution containing 0 μM Al (Table S1; supplementary material). This P concentration does not seem to be limiting to the growth and development of *S. camporum*, as evidenced in a study (Banhos et al. 2016b) testing the same

species and using the same solution, which shows considerably lower P concentration in relation to Hoagland and Arnon solution, for example. Taken together, it is unlikely that Al might have reacted with any other anion in the solution, reinforcing the Al uptake observed during the 30 days (Fig. 4).

At the same time, plantlets exposed to 1480  $\mu\text{M}$  Al showed a lower concentration of citric acid in the nutrient solution between 15 and 30 DAT, and oxalic acid at 30 DAT, compared to plantlets exposed to 740  $\mu\text{M}$  Al. This suggests that citric and oxalic acids are Al detoxifying mechanisms in this species, as more OA was accumulated in the nutrient solution of plantlets exposed to 740  $\mu\text{M}$  Al (Fig. 2) and it was associated with lower Al uptake within the 30 days (Table S1, supplementary material; Fig. 4). One could argue that Al uptake would be proportional to the Al availability. However, for Al-accumulating species from the Cerrado there is no direct relationship between Al availability and Al accumulation by the plant (Andrade et al. 2011; Nogueira et al. 2019). Plantlets exposed to 740  $\mu\text{M}$  Al tripled the Al concentration in 30 days, while those exposed to 1480  $\mu\text{M}$  Al (two times more Al available) absorbed *seven times more* Al within the same period (Fig. 4) and showed lower accumulated OAs concentration in the nutrient solution (Fig. 2). This reinforces that, for *S. camporum*, Al uptake is not proportional to the Al available in the solution, and also suggests that the OAs exudation is an Al detoxifying mechanism in this species.

Accordingly, the root elongation of *Zea mays* is 60% higher when exposed to purified cell sap of buckwheat leaves containing 20  $\mu\text{M}$  Al complexed with oxalic acid in comparison to when exposed to 20  $\mu\text{M}$  Al provided with  $\text{AlCl}_3$  (Ma et al. 1998), indicating that OA-Al complex is not phytotoxic. External Al detoxification through OAs exudation is a widely known mechanism, which avoids Al uptake (Kochian et al. 2004; Horst et al. 2010; Brunner and Sperisen, 2013). In this regard, our data suggest that plantlets exposed to 740  $\mu\text{M}$  Al exclude more Al through OA exudation when compared to those exposed to 1480  $\mu\text{M}$  Al. This may explain why, in a previous study, *S. camporum* saplings exposed to 1480  $\mu\text{M}$  Al in nutrient solution for 91 days showed reduced leaf gas exchange and shoot growth in relation to saplings not exposed to Al (Banhos et al. 2016b). In the field, adult plants of *S. camporum* accumulate in their leaves  $\sim 1500$  mg Al  $\text{kg}^{-1}$  DM (Bressan et al. 2016) and here we found that this species seems to also use the exclusion mechanism to cope with Al in the root environment in nutrient solution (Fig. 2; Fig. 4; Table S1, supplementary material). This double-handed behavior (Al accumulation and Al exclusion) also occurs in *Camellia sinensis* that accumulates in their leaves approximately 6000 mg Al  $\text{kg}^{-1}$  DM (Carr et al. 2003)

and also exude OA (Morita et al. 2011). Furthermore, *S. camporum* accumulates less Al in their leaves when compared to Al-accumulating species from Melastomataceae, Rubiaceae and Vochysiaceae from the Cerrado vegetation. This leads us to suspect that these (hyper) accumulators from these families, possibly, do not exhibit considerable root Al exclusion through OA exudation.

Intriguingly, we could not find malic acid in the nutrient solution, although it was detected when the analytical standard was added in the nutrient solutions containing 0, 740 and 1480  $\mu\text{M}$  Al ( $R^2 > 0.99$ ; Table 1). This suggests that the nutrient solution did not interfere with the detection of malic acid, so plantlets did not exude it in any of the treatments. Therefore, our data show that *S. camporum* exudes only citric and oxalic acids in response to Al, like *Acacia auriculiformis*, *Eucalyptus camaldulensis* and *Melaleuca cajuputi* (Nguyen et al. 2003; Tahara et al. 2008).

On the other hand, we found significant content of malic acid in the root tips of plantlets exposed to 740  $\mu\text{M}$  Al in relation to those exposed to 0 and 1480  $\mu\text{M}$  Al, mainly at 15 DAT (Fig. 3). 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 (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 evidencing that an Al-accumulating species native from Cerrado, *S. camporum*, exudes citric and oxalic acids by its roots in response to the Al concentration in the root environment. Our results suggest that this species relies on the exudation of citric and oxalic acids to deal with Al in the root environment.

**Author contribution statement** BMOCB and GH conceived and designed the experiments; BMOCB, SZF and CMSS performed the experiments; BMOCB, CMSS and GH analyzed the data; BMOCB and GH wrote the manuscript.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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