BIOLOGIA PLANTARUM 60 (3): 572-578, 2016 DOI: 10.1007/s10535-016-0607-5

Citric acid secretion induced by aluminum in two *Stylosanthes* species

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Abstract

Secretion of organic acids (OAs) by roots has been suggested to be an important mechanism of Al resistance in many species. In *Stylosanthes*, the participation of OAs in the mechanism of Al resistance is poorly understood. We aimed to study the production and secretion of OAs by two Brazilian *Stylosanthes* species with different Al resistance. *Stylosanthes capitata* and *S. guianensis* were treated with Al at different concentrations in 0.5 mM $CaCl_2$ (pH 4.0), and then root elongation, Al and OA content, OA secretion into the external solution, and the activity of citrate synthase (CS) were measured. Al-induced secretion of citric acid was also evaluated in the presence of protein synthesis and anion channel inhibitors. *S. guianensis* accumulated lower amounts of Al in its roots and displayed less inhibition of root elongation compared to *S. capitata*. Citric and malic acids were the most abundant OAs in the roots, and their content decreased with the Al treatment, except for citric acid in *S. guianensis*. Citric acid was the only OA secreted into the nutrient solution by the Al-treated plants of both species, but more by *S. guianensis*. Citrase synthase activity decreased in *S. capitata* but increased in *S. guianensis* with the Al treatment, and it may have a crucial role in the maintenance of citric acid content in the roots of *S. guianensis*. The use of anion channel and protein synthesis inhibitors reveal that anion channels were likely involved in the secretion of citric acid, and channel protein transcription was up-regulated by exposure to Al in *Stylosanthes*.

Additional key words: aluminum resistance, citrate synthase, malic acid, organic acids, *Stylosanthes capitata, S. guianensis*.

Introduction

Aluminum toxicity is an important limiting factor of crop productivity in acidic soil. In tropical and subtropical regions, approximately 40 - 50 % of the potentially arable soil is acidic, with pH values of less than 5.5, and approximately 60 % of this soil is located in the tropics and subtropics (Kochian *et al.* 2004).

The primary symptom of Al toxicity in plants is the inhibition of root elongation, which occurs a few minutes after exposure to this element (Barceló and Poschenrieder 2002, Kochian *et al.* 2004, Doncheva *et al.* 2005). However, despite several studies recently conducted on this subject, the mechanisms by which Al inhibits root elongation and plants tolerate excess Al remain essentially unknown (Sun *et al.* 2014).

Organic acid (OA) secretion to the external environment, where these acids would form complexes with the most toxic form of Al (Al^{3+}) and consequently decrease its phytotoxicity, is currently one of the more accepted proposed mechanisms of Al resistance (Ma *et al.* 2001, Kochian *et al.* 2004). These OAs could form stable complexes with Al in the external solution preventing its binding to negatively charged sites on the cell wall or plasma membrane, metabolites and/or major subcellular structures within the symplasm (Ryan and Delhaize 2010, Singh and Chauhan 2011). Several OAs, such as citric, malic, oxalic, and malonic acids, have been detected in the nutrient solution of several plant species after Al exposure (Martins *et al.* 2013, Yang *et al.* 2013), and often combinations of two or more OAs (Du *et al.* 2009, Martins *et al.* 2013). An Al-induced secretion of OAs into the nutrient solution follows two patterns: *1*) plants display an almost immediate response to Al treatment by OA secretion, or *2*) there is a lag of several hours between Al exposure and OA secretion (Ma *et al.* 2001). In plants fitting the first pattern, a rapid secretion of these acids appears to involve the activation of only

Submitted 12 August 2015, *last revision* 3 December 2015, *accepted* 18 January 2016.

Abbreviations: 9AC - anthracene-9-carboxylic acid; CHM - cycloheximide; CS - citrate synthase; NIF - niflumic acid; OA - organic acid.

Acknowledgments: The authors would like to thank the Fundação de Amparo à Pesquisa do Estado de Minas Gerais for the financial support for this study and the Conselho Nacional de Desenvolvimento Científico e Tecnológico for the fellowship award. The authors also thank Dr. Celso Dornelas Fernandes (Embrapa Beef Cattle) and Dr. Marcelo Ayres Carvalho (Embrapa Cerrados) for providing the *Stylosanthes* seeds.

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pre-existing proteins (Delhaize and Ryan 1995), whereas in those fitting the second pattern, the induction of a gene that encodes carrier proteins is required (Ma 2000, Ma *et al.* 2001).

The genus *Stylosanthes* belongs to the family *Fabaceae* and consists of approximately 50 species and subspecies, which are predominantly herbaceous and native to tropical and subtropical regions of Asia, Africa, and America, especially South America. In Brazil, there are 25 native *Stylosanthes* species found primarily in areas of savannah vegetation (Costa *et al.* 2008). Among these species, *S. guianensis, S. capitata*, and *S. macrocephala* are mostly used in the formation of mixed pastures in Brazil due to their adaptation to low-fertility acidic soils and tolerance of drought, pests, and diseases (Botrel *et al.* 1985). A study of six genotypes of *S. guianensis* and *S. scabra* demonstrated that Al resistance is associated with secretion of citric acid to the external environment (Li *et al.* 2009). In contrast, Sun *et al.* (2014) studied other genotypes of *S. guianensis* and suggested that Al resistance is associated with secretion of malic acid. Other studies, such as the one conducted by Du *et al.* (2009), indicate that yet other OAs may be involved in Al resistance. In this context, the present study aimed to determine the effect of Al on production and secretion of OAs by roots of two *Stylosanthes* species and to determine the mechanism by which Al regulates biosynthesis and secretion of OAs.

Materials and methods

Plants and cultivation: The seeds of *Stylosanthes capitata* Vogel and *Stylosanthes guianensis* (Aubl.) Sw. cv. Mineirão (species with different Al resistance) were selected by size and shape, scarified to break dormancy and then surface sterilized with 2.5 % (m/v) sodium hypochlorite solution for 1 min. The seeds were germinated in trays containing *Tropstrato® HT* substrate with daily watering in a growth room with a 16-h photoperiod, an irradiance of 230 μmol m^{-2} s^{-1}, a temperature of 25 ± 1 ºC, and a relative humidity of 65 %. Fifteen days after sowing, the seedlings were transferred to containers filled with 1/5 strength Clark's nutrient solution (Clark 1975), pH 4.5, under continuous aeration. The nutrient solution was changed every two days, and its ionic strength progressively increased until a half of the total ionic strength was reached. The pH of the nutrient solution was adjusted daily by adding 1 M HCl or 1 M NaOH. The seedlings were maintained under these conditions for 15 d and then transferred to 0.5 mM $CaCl_2$ solution, pH 4.0, for 24 h before application of the treatments.

Effects of aluminum on root elongation and aluminum content: Six seedlings of each *Stylosanthes* species were exposed to different concentrations of Al (0, 75, 150, 300, 600, and 900 μM) applied in the form of $AlCl_3$ in a 0.5 mM $CaCl_2$ solution, pH 4.0. In a parallel experiment, six seedlings of each *Stylosanthes* species were exposed to 0 or 200 μM Al in a 0.5 mM $CaCl_2$ solution, pH 4.0, for 12, 24, and 48 h. Primary root length was measured before the beginning of the experiment and after the Al exposure. The effect of Al was expressed as the percentage inhibition of root elongation: $RE\ [\%] = [(RE_{-Al} - RE_{+Al}) / RE_{+Al}] \times 100$; where RE means root elongation. Roots were washed with running tap water, 0.1 mM HCl, and rinsed with deionized water. Collected root samples were placed in 2 M HCl for 48 h (Ma *et al.* 2004), and Al content was determined in the extract by inductively coupled plasma optical emission spectrometry (*Optima 8300 ICP-OES*, *Perkins Elmer*, Watham, MA, USA) at a wavelength of 394.4 nm.

Effects of aluminum on organic acid content in roots and on secretion of organic acids: Ten seedlings of each *Stylosanthes* species were transferred to containers filled with 300 cm^3 of a 0.5 mM $CaCl_2$ solution, pH 4.0, and then treated with either 0 μM or 200 μM Al applied in the form of $AlCl_3$. After 1, 2, 4, 8, 16, 24, and 48 h, the seedlings were removed from the containers and OAs of the root systems were extracted in 80 % (v/v) ethanol according to the method described by Cambraia *et al.* (1983). The nutrient solution was lyophilized and then solubilized in 3 cm^3 of deionized water. Both the root extract and the nutrient solution were further purified by cation-exchange chromatography (*Dowex AG50-WX8*, *Bio-Rad Laboratories*, Hercules, CA, USA, 200 - 400 mesh, H^+ form) followed by anion-exchange chromatography (*Dowex AG1-X8*, 200 - 400 mesh, formate form). The OA fraction was then eluted from the last column with 20 cm^3 of 2 M formic acid (Cambraia *et al.* 1983). The samples of the eluates and a known quantity of tartaric acid, used as internal standard, were evaporated to dryness and then converted into trimethylsilyl derivatives by reaction with pyridine and bis (trimethylsilyl)-trifluoroacetamide (BSTFA) (Jham *et al.* 2002). The OAs were separated and quantified by a gas chromatograph (*GC2014*, Shimadzu, Tokyo, Japan) equipped with a flame ionization detector. A *Rtx-1* capillary column (30 m × 0.25 mm; 100 % dimethyl-polysiloxane) was used in the chromatographic separation. An initial column temperature of 100 ºC (2 min) was gradually raised at a rate of 10 ºC min^{-1} until reaching a final temperature of 250 ºC. Injector and detector temperatures were 290 and 300 ºC, respectively. Ultrapure nitrogen was used as carrier gas at a flow rate of 1.2 cm^3 min^{-1} with a 1:10 split ratio. Concentrations of OAs in the samples were estimated by comparison with authentic standard acids.

Effect of aluminum on citrate synthase (EC 2.3.3.1) activity: Six seedlings of each *Stylosanthes* species were exposed to 0 or 200 μM Al in a 0.5 mM $CaCl_2$ solution, pH 4.0, for 24 h. After this time, root samples (0.4 g)

were collected and macerated in liquid nitrogen. Next, the plant material was homogenized in 2.0 cm^3 of a 100 mM Tris-HCl buffer (pH 8.0), 5.0 mM Na_2EDTA, 5.0 mM $MgCl_2$, 10 % (v/v) glycerol, 0.1 % (v/v) *Triton X-100*, and 0.5 mM phenylmethylsulphonyl fluoride (PMSF) (Hayes and Ma 2003). The homogenate was filtered through glass wool, centrifuged at 20 000 *g* and 4 ºC for 10 min, and the resulting supernatant was used as enzyme source. Citrate synthase (CS) activity was determined by adding 0.1 cm^3 of the crude enzyme extract to 0.8 cm^3 of a reaction medium containing a 50 mM Tris-HCl buffer (pH 8.0), 5 mM $MgCl_2$, 100 μM 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), 0.2 mM acetyl CoA, and 0.5 mM oxaloacetate. The reaction was initiated by adding oxaloacetate, and a rate of absorbance change was monitored at 412 nm for 1 min at 25 ºC (Hayes and Ma 2003). Specific enzyme activity was calculated using a coefficient of absorbance of 13.6 M^{-1} cm^{-1}). Total protein content was determined by method of Lowry *et al*. (1951).

Effects of inhibitors of anion channels and protein synthesis: Ten seedlings of each *Stylosanthes* species were treated with 0 or 200 μM Al, 200 μM Al *plus* 50 μM niflumic acid (NIF), 10 μM anthracene-9-carboxylic acid (9AC), or 30 μM cycloheximide (CHM) in a 0.5 mM $CaCl_2$ solution, pH 4.0. After 24 h, the nutrient solutions were collected, lyophilized, and content of OAs was measured by gas chromatography as described before. Root samples were removed, washed, and used to determine Al content as described above.

Experimental design and statistical analysis: The experiments followed factorial schemes in a randomized block design. Analysis of variance (*ANOVA*) was used to analyze results, and Tukey's test at 5 % probability was used to compare means. Quantitative data were analyzed by regression analysis using the statistical software *SAS v.9.0* (*SAS Institute*, Cary, NC, USA).

Results

Root elongation inhibition increased with the increasing Al concentration in the nutrient solution in both *Stylosanthes* species (Fig. 1*A*). Inhibition of root elongation was always more pronounced in *S. capitata* than in *S. guianensis* for all concentrations of Al used in this study. A difference in sensitivity to Al between the two species increased with the increasing concentrations of Al up to approximately 300 μM, and after this concentration, differences between the species began to decrease up to the highest tested concentration of Al. Root elongation inhibition of approximately 67 and 50 % was observed in *S. capitata* and *S. guianensis*, respectively, at an Al concentration of 200 μM, and this concentration was chosen for the subsequent experiments.

Plants of both species exposed to 200 μM Al displayed a marked inhibition of root elongation already after 12 h of the treatment (Table 1). However, this inhibition was not significantly intensified with a longer exposure up to 48 h in either species.

Aluminum content in roots of the two species of *Stylosanthes* also increased with the increasing Al concentration in the nutrient solution (Fig. 1*B*). No tendency toward stabilization of Al content in roots was observed over the Al concentrations used in this study, and the differences between the genotypes were accentuated with the increasing concentrations of Al in the nutrient solution. At the 200 μM concentration, Al content in roots was 25 % higher in *S. capitata* than in *S. guianensis*.

Six OAs were detected in roots of the two *Stylosanthes* species: oxalic, malonic, succinic, malic, trans-aconitic, and citric acids (Table 2). Malic and citric acids were the two most abundant OAs regardless of the Al treatment. In general, content of these OAs differed significantly between the species and Al treatments.

Table 1. Effects of aluminum on root elongation inhibition [%] in *Stylosanthes* species after treatment with 200 μM Al for 12, 24, and 48 h. Means ± SDs, $n = 3$. The means followed by the same capital letter do not differ between species, and followed by the same small letter between different periods of time at $P > 0.05$ according to Tukey's test.

Species	12 h	24 h	48 h
S. capitata	71.9 ± 2.8Aa	73.4 ± 5.3Aa	76.2 ± 4.0Aa
S. guianensis	48.3 ± 4.4Ba	49.1 ± 5.8Ba	52.4 ± 3.7Ba

Table 2. Content of organic acids [nmol g^{-1}(f.m.)] in roots of *Stylosanthes* species after 200 μM Al treatment for 48 h. Means ± SDs, $n = 3$. The means followed by the same capital letter do not differ between species and followed by the same small letter between different treatments at $P > 0.05$ according toTukey's test.

Species		Oxalic acid	Malonic acid	Succinic acid	Malic acid	*Trans*-aconitic acid	Citric acid
S. capitata	-Al	21.6 ± 3.6Aa	39.9 ± 0.3Aa	58.0 ± 8.3Aa	728.0 ± 9.8Aa	2.5 ± 0.9Aa	272.8 ± 12.0Aa
	+Al	14.2 ± 0.3Bb	27.2 ± 2.5Ab	34.3 ± 4.8Ab	564.2 ± 8.6Ab	3.2 ± 0.8Aa	152.0 ± 6.7Ab
S. guianensis	-Al	13.2 ± 1.8Bb	45.1 ± 0.8Aa	48.9 ± 6.8Aa	612.6 ± 7.4Ba	3.6 ± 0.7Aa	147.1 ± 1.9Ba
	+Al	26.2 ± 4,5Aa	25.1 ± 2.7Ab	37.1 ± 4.4Ab	250.0 ± 9.2Bb	5.0 ± 1.5Aa	138.9 ± 8.9Aa

Oxalic acid content declined in *S. capitata* but increased in *S. guianensis* after Al exposure. Content of malonic and succinic acids decreased with the Al treatment in both the species, but there were no differences between the two species regardless of the Al treatment.

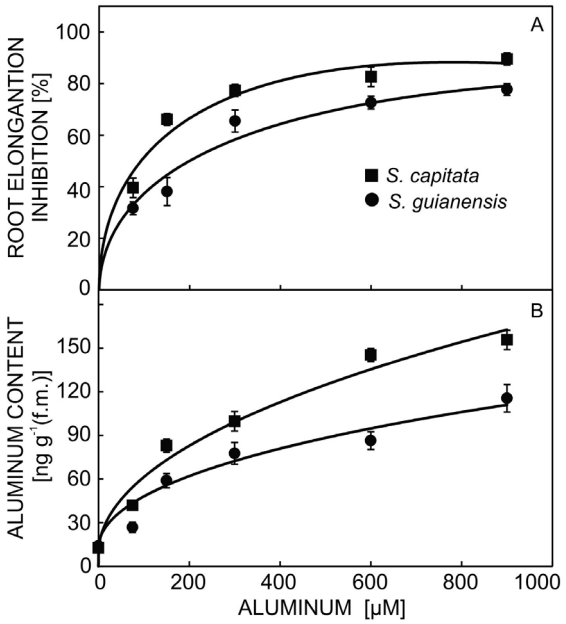

Fig. 1. Effects of different Al concentrations on root elongation inhibition RE (*A*) and Al content in roots (*B*) of two *Stylosanthes* species after 24 h. Means ± SDs, $n = 3$. Equations of the curves in *A*: $RE_{S.c.} = -0861 + 6.376\sqrt{x} - 0.114x$, $R^2 = 0.98$; $RE_{S.g.} = -1.559 + 4.500\sqrt{x} - 0.060x$; $R^2 = 0.97$; and in *B:* $Al_{S.c.} = 8.20 + 5.389\sqrt{x} - 0.008x$, $R^2 = 0.96$; $Al_{S.g.} = 12.22 + 3.717\sqrt{x} - 0.014x$, $R^2 = 0.92$.

Content of *trans*-aconitic acid was not affected by the Al treatment, and there was no difference between the species. Malic acid content was always higher in *S. capitata* than in *S. guianensis*, and after the Al treatment, it decreased by 23 and 59 % in

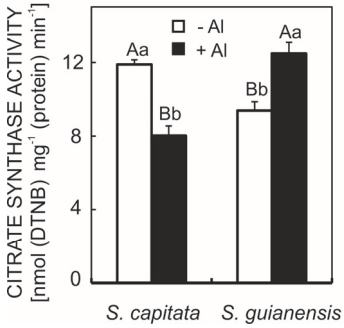

Fig. 2. Aluminum effect on citrate synthase activity in roots of two *Stylosanthes* species after treatment with 200 μM Al for 24 h. Means ± SDs, $n = 3$. The means followed by the same capital letter do not differ between species, and followed by the same small letter between Al treatments at $P > 0.05$ according to Tukey's test.

S. capitata and *S. guianensis*, respectively. Content of citric acid decreased by 45 % in *S. capitata* but did not change in *S. guianensis* after the Al treatment. The significant differences between the control plants of these two species disappeared after the Al treatment.

Activity of CS in roots of the control plants was 33 % higher in *S. capitata* than in *S. guianensis* (Fig. 2). However, in the presence of Al, CS activity decreased approximately 32 % in *S. capitata*, whereas it increased by 34 % in *S. guianensis*. Under this conditions, *S. guianensis* exhibited enzyme activity 56 % higher than *S. capitata*.

Citric acid was the only OA secreted in significant quantities to the external solution. In the absence of Al, roots of the two species secreted virtually no citric acid into the nutrient solution even after 48 h of the treatment (Fig. 3). After 8 h of exposure to Al, *S. capitata* and *S. guianensis* secreted only small quantities of citric acid to the external nutrient solution. After this time, secretion of this OA increased rapidly with exposure time to Al in both the species although it was always more pronounced in *S. guianensis*. After 48 h of exposure to Al, *S. guianensis* secreted 2.2 times more citric acid than *S. capitata* [638 and 287 nmol g^{-1} (f.m.), respectively]. During the experimental period, there was no trend toward stabilization of secretion of citric acid to the external solution, especially in *S. guianensis*. The average rate of citric acid secretion to the nutrient solution was 13 and 6 nmol g^{-1} (f.m.) h^{-1} for *S. guianensis* and *S. capitate*, respectively.

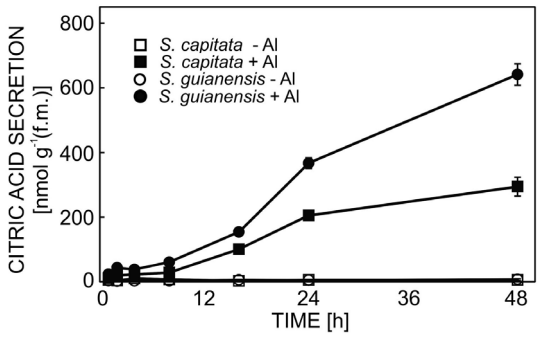

Fig. 3. Time-course of Al-induced citric acid secretion to a nutrient solution by intact roots of two *Stylosanthes* species after plant exposure to 0 and 200 μM Al. Means ± SDs, $n = 3$.

Aluminium-induced secretion of citric acid to the nutrient solution decreased by about 72 and 71 % after the treatment of *S. capitata* and *S. guianensis*, respectively, with the anion channel inhibitors NIF or 9AC (Fig. 4*A*). Reduction in Al-induced secretion of citric acid after the treatment with the protein inhibitor CHM was even greater, reaching approximately 94 and 74 % in *S. capitata* and *S. guianensis*, respectively. It is worth noting that in the presence of Al, secretion of citric acid was always higher in *S. guianensis* irrespective of the inhibitor treatment. Aluminium content in roots of the plants treated with Al *plus* the inhibitors was higher than

in the plants treated only with Al. In *S. capitata*, Al content increased approximately 11 % relative to *S. guianensis* after addition of the inhibitors to the Al-treated plants. The amount of secreted citric acid maintained an approximately inverse relationship with Al content in roots of both the *Stylosanthes* species (Fig. 4*B*).

Discussion

The two *Stylosanthes* species used in this experiment were screened from a group of six species commonly used in the formation of mixed pastures intercropped with grasses in Brazil (data not published). When treated with

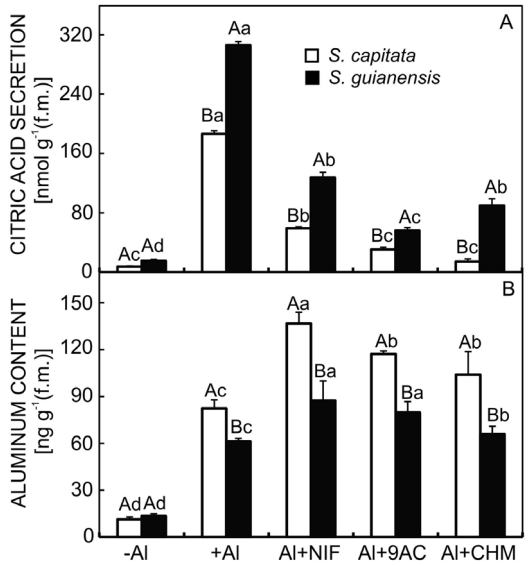

Fig. 4. Citric acid secretion to the nutrient medium (*A*) and Al content in roots (*B*) of two *Stylosanthes* species after treatment with 0 µM Al (-Al), 200 µM Al (+Al), 200 µM Al + 50 µM niflumic acid (Al+NIF), 200 µM Al + 50 µM anthracene-9-carboxylic acid (Al+9AC), and 200 µM Al + 50 µM cycloheximide (Al+CHM). Means ± SDs, n = 3. The means followed by the same capital letter do not differ between species and followed by the same small letter between Al treatments at $P > 0.05$ according to Tukey's test.

Al at concentrations up to 900 µM, they exhibited decreases in root elongation that ranged from 39 to 88 % in *S. capitata* and from 27 to 79 % in *S. guianensis*. Similar decreases in root elongation have been observed in other species and genotypes of *Stylosanthes* treated with Al such as *S. scabra* (71 %), six genotypes of *S. guianensis* (24 to 42 %, Li *et al.* 2009), *S. capitata* cv. Fine-stem (43 %), and *S. guianensis* genotype TPRC2001-1 (9 %, Du *et al.* 2009). However, no significant difference between the Al-tolerant TPRC2001-1 and Al-sensitive Fine-stem genotypes is detected when they are treated with 100 µM Al for 24 h (Sun *et al.* 2014). Such different responses indicate that Al resistance in *Stylosanthes* and other plant species may be determined not only by genetic variability but also by experimental factors such as pH, nutrient solution composition, and the strength of Al stress (Cambraia and Cambraia 1995).

The Al-induced inhibition of root elongation was rapid, reaching values above 70 % in *S. capitata* and 48 % in *S. guianensis* after only 12 h of the treatment. This decrease corresponds to more than 92 % of the inhibition obtained after 48 h of exposure to Al indicating that the deleterious effects of Al occur in less than 12 h. Some studies have corroborated our data showing that a significant inhibition of root elongation can be detected in Al-sensitive species after only 30 min of exposure to Al (Barceló and Poschenrieder 2002, Doncheva *et al.* 2005). Although the precise mechanism of Al toxicity is not yet fully understood, it appears that exposure to Al even for short periods of time can affect not only cell elongation but also cell division (Doncheva *et al.* 2005, Ma 2007).

One of the most important and frequently cited mechanisms proposed to explain Al resistance is based on secretion of OAs by roots into the external nutrient solution (Ma 2000). In the present experiment, citric acid was the only OA detected in the nutrient solution in an amount and secretion pattern consistent with its participation in the proposed mechanism of Al resistance in plants. The amount of citric acid produced up to 8 h of the treatment was quite small, suggesting the absence of pre-existing anion channels to transport it to the external environment (Ma 2000). However, after this lag phase, rapid and intense Al-induced secretion of citric acid was observed, and it lasted for 48 h. Although the intensity differed, the two species studied here displayed basically the same secretion profile. The results are similar to those obtained by Li *et al.* (2009) and Zuo *et al.* (2010) with different genotypes of *Stylosanthes* and with other plant species such as *Cassia tora* (Ma *et al.* 1997), *Phaseolus vulgaris* (Shen *et al.* 2004), *Glycine max* (Yang *et al.* 2001), and *Vigna unguiculata* (Yu *et al.* 2012).

The profiles of citric acid secretion observed in the two studied species of *Stylosanthes* (Fig. 3) reflect their different Al resistance in terms of root elongation inhibition they exhibited (Fig. 1*A*, Table 1). *S. guianensis* secreted more citric acid and displayed a higher root elongation than *S. capitata*. Parallel to this response, Al content was always lower in roots of *S. guianensis* than of *S. capitata* during the time of exposure to Al (Fig. 1*B*). Together, these factors indicate that secretion of citric acid decreased penetration of Al into roots, resulting in less cell damage and therefore allowing a greater root elongation in *S. guianensis*. In addition to being a ubiquitous molecule in cells, citric acid can be synthesized efficiently (Ryan and Delhaize 2010) and form a complex with Al, which is much stronger than that with other OAs. Therefore, citric acid is considered one of the most effective OAs in the process of Al exclusion from sensitive sites in roots (Ryan *et al.* 2001).

Aluminium induced secretion of Oas, such as citric or malic acids, to the nutrient solution is mediated by anion channels (Yang *et al.* 2013). The involvement of these channels has been assessed with the use of specific inhibitors of either activity or synthesis of proteins that form these channels (Yu *et al.* 2012). The application of inhibitors of anion channels NIF and 9AC strongly decreased the amount of citric acid secreted into the nutrient solution in both the species (Fig. 4*A*). This result confirms the participation of anion channels in Al-induced secretion of citric acid in *Stylosanthes*, as suggested by other authors (Yang *et al.* 2006a, Li *et al.* 2009). The plant treatments with these inhibitors caused a proportional increase in absorption of Al by roots with a decrease in secretion of citric acid into the nutrient solution (Fig. 4*B*), supporting a hypothesis that Al-citrate complexes formed in the nutrient solution are not significantly absorbed by roots (Kochian *et al.* 2004). These results confirm the hypothesis of the involvement of citric acid in Al resistance in these two species of *Stylosanthes*, corroborating results of other authors (Li *et al.* 2009).

Aluminium induced secretion of citric acid was also strongly reduced by the non-specific protein synthesis inhibitor CHM confirming that *de novo* synthesis of proteins that form anion channels is required in both the species of *Stylosanthes*. Plant treatment with CHM also eliminates efflux of citric acid and malic acid in *Vigna unguiculata* (Yu *et al.* 2012) and *Cassia tora* (Ma *et al.* 1997), but it does not affect secretion of oxalic acid in *Fagopyrum esculentum* (Yang *et al.* 2006b). Therefore, citric acid efflux to the external solution requires *de novo* synthesis of proteins.

Two strategies have been suggested to explain detoxification of Al by plants. One is exclusion of Al from root tips by secretion of OA into the external solution. This mechanism proved to be essential for the *Stylosanthes* species studied here. Some plant species, however, cannot avoid the entry of significant amounts of Al into the symplasm. In this case they may use an internal detoxification mechanism involving production of OAs, which would form complexes with Al and would undergo subsequent transport/storage into vacuoles (Singh and Chauhan 2011). In this case, an increase in content of OAs in plant tissues is expected. An elevated content of OAs has been found in roots of resistant genotypes of sorghum (Cambraia *et al.* 1983, Gonçalves *et al.* 2005), maize (Chaffai and Marzouk 2009), *Plantago algarbiensis*, and *P. almogravensis* (Martins *et al.* 2013) indicating operation of an internal Al detoxification mechanism. In the present study, however, the content of the two more abundant OAs in roots decreased or was not affected by Al, and so it is likely that an internal Al detoxification is not an important mechanism of Al resistance in *Stylosanthes*. A decrease in malic and citric acid content after Al treatment has been reported in wheat (Andrade *et al.* 2011), *Citrus grandis*, and *C.* sinensis (Yang *et al.* 2011). Because enzymes associated with biosynthesis of malic acid are hardly influenced by Al, the observed decline in root content of this acid might be the result of an increase in its oxidation (Andrade *et al.* 2011). On the other hand, the decrease in citric acid content in roots of *S. capitata* seems to be mainly the result of an Al-induced decrease in activity of the enzyme involved in its biosynthesis, *i.e.*, CS. In *S. guianensis*, however, the root citric acid content remained unchanged whereas the CS activity increased with the Al treatment. The influence of Al on CS activity seems to be dependent on species and/or factors in a nutrient solution. Activity of CS increases in soybean (Yang *et al.* 2001), *Cassia tora* (Yang *et al.* 2004), and *P. algarbiensis* (Martins *et al.* 2013), remains unchanged in *P. almorgavensis* (Martins *et al.* 2013) and *Citrus grandis* (Yang *et al.* 2011), and decreases in *Citrus sinensis* (Yang *et al.* 2011). Considering the high rate of citric acid secretion into the nutrient solution, CS activity probably played a crucial role in the maintenance of citric acid content in roots of *S. guianensis*.

In conclusion, the higher Al resistance of *S. guianensis* was probably due to the higher Al-induced secretion of citric acid into the external solution. Anion channels are likely involved in secretion of citric acid, and transcription of a channel protein is likely to be up-regulated by exposure to Al in *Stylosanthes*.

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