Effects of foliar application of organic acids on alleviation of aluminum toxicity in alfalfa

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Abstract

Organic acids (OA) may affect plant resistance to aluminum (Al) toxicity in acidic soils. However, limited information is available on the effects of different organic acids on Al resistance in alfalfa. We investigated the effects of foliar application of organic acids (succinic acid, citric acid, malic acid, and oxalic acid) to alfalfa (*Medicago sativa* L.) under Al stress. Seedlings were grown in pH 4.5 nutrient solution containing Al at 0 or 100 μM, and were sprayed with water or 100 μM of oxalic acid, malic acid, citric acid, or succinic acid every 3 d during a 10 d experiment. Aluminum stress caused significant reduction in alfalfa growth (reflected by above-ground biomass, root weight and root length), root activity, mineral nutrient concentrations (Ca, K, Mg, Mn and Zn), and a significant increase in leaf membrane lipid peroxidation. Foliar application of the four organic acids, especially succinic acid, alleviated Al toxicity, as demonstrated by the increase in plant growth and root activity, as well as reduction in lipid peroxidation. Oxalic acid and malic acid treatments significantly increased oxalate exudation and decreased Al concentration in roots exposed to Al stress. Succinic acid treatment significantly increased accumulation of all four organic acids in roots, accumulation of Ca, K, Mg, Mn and Zn, and up-regulated the gene transcription of malate dehydrogenase (MDH) and phosphoenolpyruvate carboxylase (PEPC) in roots. Our results suggest that the promotion of oxalate exudation from roots through exogenous application of oxalate and malate could contribute to the improvement in Al resistance of alfalfa, and the positive effects of exogenous application of succinate on Al resistance may be associated with the increased endogenous accumulation of all four organic acids in roots, which may constitute an organic-acid detoxification system in alfalfa.

Key words: acidic soils / detoxification / exudation / oxalic acid / succinic acid

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1 Introduction

Free aluminum (Al) is highly abundant under acid soil conditions. When the soil pH drops below 5, toxic species of aluminum, $AlOH^{2+}$ and Al^{3+}, are released from aluminosilicate clay minerals into soil solution (*Kochian* et al., 2004). Aluminum toxicity represents one of the most important constraints for agricultural production in areas with acidic soils (*Kochian* et al., 2005). On a global scale, acidic soils cover an estimated 37.8 million km^2 of the earth's surface and comprise up to 50% of the world's potentially arable soils.

High concentrations of $AlOH^{2+}$ and Al^{3+} in soil solutions inhibit root development at the organ, tissue, and cellular levels (*Čiamporová*, 2002; *Ramírez-Benítez* et al., 2008). Aluminum toxicity may target the distal part of the transition zone of the root apex (*Ryan* et al., 1993), decrease H^+ influx in the distal elongation zone, or induce H^+ efflux in the mature zones, consequently leading to cytoplasmic alkalization in both zones (*Bose* et al., 2010), increase callose formation (*Stass* et al., 2008), inhibition of root growth by restricting cell elongation (*Barcelo* and *Poschenrieder*, 2002), limiting water and nutrient absorption from soils, and leading to nutrient deficiencies and plant growth reduction (*Ma* et al., 2001; *Delhaize* et al., 2007; *Olivares* et al., 2009). The reduced plant growth of roots and shoots may also be associated with Al stress enhancing the production of reactive oxygen species (ROS), resulting in oxidative stress to root and leaf tissues (*Zheng* and *Yang*, 2005; *Panda* et al., 2008; *Giannakoula* et al., 2010) leading to lipid peroxidation (*Giannakoula* et al., 2008) and loss of membrane integrity and cell death (*Tamás* et al., 2005).

The primary aluminum resistance mechanisms in plants involve Al exclusion or internal detoxification (*Kochian* et al., 2005). Aluminum-induced toxicity results from a high binding affinity of Al with extracellular and intracellular substances. Organic anions (OA), such as citrate, malate, and oxalate play an important role in plant resistance to Al stress. Root exudation of different organic anions is correlated with Al resistance of different plant species (*Ma* and *Furukawa*, 2003; *Kochian* et al., 2004, 2005). The enhanced exudation

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of organic acids in response to Al stress can lead to non-toxic complexes with Al ions in the root apoplast and/or rhizosphere (*Kinraide* et al., 2005), thus avoiding interaction of Al ions with root cellular components and the entry of ions to the root symplast (*Ma* et al., 2001; *Ryan* et al., 2001; *Delhaize* et al., 2007). The genes responsible for Al-activated malate and citrate efflux have been cloned from many plants, including wheat (*Triticum aestivum*; *Sasaki* et al., 2004), barley (*Hordeum vulgare*; *Furukawa* et al., 2007), and sorghum (*Sorghum bicolor*; *Magalhaes* et al., 2007). Overexpression of the citrate synthase gene in *Brassica napus* cv. Westar led to increased citrate synthesis and exudation, which conferred improved resistance to Al toxicity in transgenic plants (*Wang* et al., 2013).

Another Al resistance mechanism has been proposed that may involve Al-inducible changes in organic acid synthesis and compartmentation of Al–OA complexes into the vacuole (*Piñeros* et al., 2002; *Delhaize* et al., 2007). In Al-resistant maize (*Zea mays*), wheat, and buckwheat (*Fagopyrum esculentum*), the Al–OA complexes in the forms of Al–citrate (1 : 1) and Al–oxalate (1 : 1, 1 : 2, and 1 : 3) complexes, respectively, have been identified (*Barcelo* and *Poschenrieder*, 2002; *Ma*, 2005). Certain organic anions (such as citrate and malate) have also been reported to remove Al bound to cell-wall pectins, which might alleviate Al toxicity (*Wehr* et al., 2003; *Li* et al., 2009; *Rangel* et al., 2009).

Alfalfa is an important legume used as a forage crop worldwide. Aluminum toxicity is a major factor limiting alfalfa production in soils with low pH. Organic anions play an important role in plant resistance to Al stress. However, limited information is available for the effect of different organic anions on Al resistance of alfalfa. We hypothesized that Al resistance of alfalfa is: (1) associated with the exclusion of Al from the symplast or with the intracellular chelation of Al by accumulated organic anions within root tips; (2) that different organic anions may have different functions in Al resistance *via* Al exclusion or internal detoxification.

Exogenous foliar application is an effective strategy to investigate the effects of organic compounds on plant resistance to environmental stresses (*Rombola* et al., 2002; *Zaki* and *Radwan,* 2011). However, no studies have examined the effect of foliar application on Al toxicity. In the present study, we tested our hypotheses by spraying one of four organic acids onto alfalfa leaves. We measured plant growth, lipid peroxidation, and root activity as indicators of Al resistance. We also evaluated potential mechanisms of Al resistance, including Al accumulation, mineral nutrient accumulation, root tip organic anion accumulation, and exudation, and gene expression.

2 Materials and methods

2.1 Plant material and growth conditions

The alfalfa (*Medicago sativa* L.) genotype used in this study was WL-525HQ, an aluminum-resistant cultivar (*Pan* et al., 2008). Ten-day-old seedlings of WL-525HQ were wrapped with sponge and then placed in holes in foam boards (40 cm × 30 cm). Each foam board was placed into one of 18 plastic containers filled with half Hoagland's solution, pH 4.5. Two days later, 100 μM $AlCl_3$ were added to 15 containers (pH 4.5 + Al); the remaining three containers were the control (pH 4.5). The 15 Al-added containers were divided into five groups of three containers each. Every three days, plants in the four groups were sprayed with 20 mL, pH 4.0, 100 μM of oxalic acid, DL-malic acid, citric acid, or succinic acid (Sigma, MO, USA), respectively, or with water (pH 6.0), the latter as the Al-stressed control. The foam boards were removed from each container before organic acids or water were sprayed onto leaves. In addition to these treatments, another four treatments received half Hoagland's solution, pH 4.5, and foliar spraying of one of the organic acids (20 mL, 100 μM) onto alfalfa leaves to test the effects of organic acids in the absence of Al stress. The pH of 4.5 of the nutrient solution was adjusted by adding 1 M HCl or 0.4 M NaOH. The nutrient solution was aerated and renewed every 2 d. All containers were placed in environmentally controlled growth rooms with day/night temperature of 25/20°C and a 12 h photoperiod of 600 μmol m^{-2} s^{-1}.

After 10 d of treatment with aluminum and four organic acids, ten plants of each treatment were separated into shoots and roots. The shoots were weighed directly as above-ground biomass. The roots were blotted dry with paper towels and then weighed. Root length was measured from the root tip to the base of the main roots.

2.2 Extraction and analysis of organic acids

Organic anion extraction from roots was performed as described by *Pellet* et al. (1995) with some modifications. One gram of root tips was ground in liquid N_2 and then extracted with ethanol in a chilled mortar. The resulting mixture was centrifuged for 30 min at 15,000 *g* (Eppendorf 5810R, Hamburg, Germany). The supernatant was saved, and the pellet was resuspended in ethanol and spun again. This procedure was conducted four times for each sample. Combined samples were dried to constant weight in a vacuum (Labconco Corp., Kansas City, MO, USA). Residues were redissolved in 5 mL double-distilled water, filtered through a 0.45 μm filter, and 20 μL were analyzed using high-performance liquid chromatography (HPLC; Waters 600E, Milford, MA, USA). Separation was conducted on a 250 mm × 4.6 mm reversed-phase column (Hibar column RT C18, Merck Chemicals Corp., Germany). The mobile phase was 0.05 mM sulfuric acid with a flow rate of 1 mL min^{-1} at 30°C. Individual components were detected at 210 nm using a Waters 2996 photodiode array detector.

Organic anion exudation was performed as described by *Dong* et al. (2008) with some modifications. On days 3 and 10, fifteen seedlings of each treatment were transferred to 10 mL of filter-sterilized exudation solution (pH 4.5) with or without 100 Al μM for 6 h. The collected root exudates were then passed through cationic and anionic chromatography columns to remove Al^{3+} and other inorganic anions that could interfere with the measurement of organic anions. The organic anions retained on anion-exchange resin were eluted with 2 M HCl, and the eluate was concentrated to dryness at 40°C using a rotary evaporator. After the residue was redis-

solved in 1 mL water, the concentration of organic anions was analyzed with HPLC as described above.

2.3 Mineral concentration in plants

After 10 d treatment with aluminum + organic acids, roots were oven-dried for 24 h at 80°C and ground to a fine powder. Samples were digested in a 4 : 1 (v/v) nitric acid/perchloric acid solution (HNO_3/$HClO_4$). Mineral nutrient concentrations (Al, Ca, Mg, K, Mn, and Zn) were determined using an inductively coupled plasma emission spectrometer (ICP-AES; Iris Advantage 1000, Jarrell Ash Corp. Franklin, MA, USA; *Giannakoula* et al., 2008).

2.4 Determination of lipid peroxidation

Lipid peroxidation in leaves after 3, 7, and 10 d of treatment with aluminum + organic acids was measured as malondialdehyde (MDA), which was determined by reaction with 2-thiobarbituric acid (TBA)-reactive substances as described previously (*Giannakoula* et al., 2008).

2.5 Root activity analysis

Root activity after 3, 7, and 10 d of treatment was measured using the triphenyltetrazolium chloride (TTC) reduction method as described by *Li* (2000). 0.5 g root samples were rinsed twice with de-ionized water and were incubated with mixture reagent (5 mL 0.4% TTC and 5 mL 0.1 mM phosphate buffer solution; pH 7.0) for 2 h in the dark at 37°C. Then, 2 mL of 1 M H_2SO_4 was added to the tube to stop the reaction. The roots were thoroughly ground in 10 mL ethyl acetate to extract 1,3,5-triphenyltetrazolium formazan (TPF). The root activity was expressed as the amount of TPF deoxidized by TTC. Absorbance was read at 485 nm.

2.6 Quantitative-PCR analysis

Six genes related to regulation of organic acid synthesis through the tricarboxylic acid cycle (TCA) were detected with quantitative PCR (Q-PCR) after 3 and 10 d of Al exposure with or without succinic acid treatment. Total RNA in roots was extracted using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was generated with a RETROscript Kit (Applied Biosystems/Ambion Inc., Austin, TX, USA) using oligo (dT) as primer. Primers for the markers were synthesized from published sequences (Genbank accessions XM_003590421, XM_003608279, XM_003621771, XM_003607534, TC172910 in DFCI, XM_003611938). Sequences of the primers were: (1) Isocitrate dehydrogenase (ISD; EC 1.1.1.42) marker 5′-ACACAGCGTAATGGCTTCTCA-3′ (forward) and 5′-GGGTCAACTTCAGTCCCTACA-3′ (reverse); (2) malate dehydrogenase (MDH; EC 1.1.1.37) marker 5′-CCATTCAACTTCAGCACACAC-3′ (forward) and 5′-ACAACATCACCTCCTTCCTGG-3′ (reverse); (3) α-oxoglutarate dehydrogenase (OGD, EC 1.2.4.2) marker 5′-ATCCAATGTCTGGCAGGTCT-3′ (forward) and 5′-TGCGTGAAAGATGGTTCGTC-3′ (reverse); (4) phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.38) marker 5′-AAGAGGAGGGACCGTAGGAA-3′ (forward) and 5′-CATTCTGGCTTTGGTGAGACA-3′ (reverse); (5) succinyl-CoA ligase (SCS; EC 6.2.1.4) marker 5′-GCTATCTTCACCTCCCTGTC-3′ (forward) and 5′-TACACAATACTGCCCAACTCG-3′ (reverse); (6) succinate dehydrogenase (SDH; EC 1.3.99.1) marker 5′-TCTTCTCCAGTGAGTCTTACAC-3′ (forward) and 5′-CCTCCAGTCATCTTCAGTCATA-3′ (reverse). Q-PCR amplification mixtures (25 μL) contained 25 ng template cDNA, 2X SYBR Green I Master Mix buffer (12.5 μL) (Applied Biosystems), and 300 nM forward and reverse primer. Reactions were run on an ABI PRISM 5700 Sequence Detector (Applied Biosystems). The cycling conditions comprised 4 min polymerase activation at 94°C and 40 cycles at 94°C for 30 s , 58°C for 30 s and 72°C for 30 s. The EF1-KLMF primer pairs 5′-GCACCAGTGCTCGATTGC-3′ (forward) and 5′-TCGCCTGTCAATCTTGGTAACAA-3′ (forward) were used for amplifying an internal control. Each gene was detected three times. All PCR efficiencies were above 95%. Sequence Detection Software (version 1.3; Applied Biosystems) results were exported as tab-delimited text files and imported into Microsoft Excel for further analysis. The median coefficient of variation (based on calculated quantities) of duplicated samples was 6%.

2.7 Statistical analysis

The main effects and interactions of treatments were calculated by analysis of variance (ANOVA) in SAS 9.0 (SAS Institute Inc., Cary, NC, USA). Treatment differences were tested using a mean-separation test named least significant difference (LSD) at the $P = 5\%$ level.

3 Results

Aluminum stress significantly inhibited plants growth. Root length, above-ground biomass, and root weight of plants exposed to Al for 7–10 d significantly decreased compared to plants grown without Al (Fig. 1a–c). However, foliar spraying of oxalic acid, malic acid, citric acid and succinic acid improved the growth of Al-stressed plants. Root length (Fig. 1a), above-ground biomass (Fig. 1b), and root weight (Fig. 1c) significantly increased after 7 and 10 d of Al exposure, respectively, compared with Al treatment alone, except for above-ground biomass and root weight of plants treated with malic acid and oxalic acid, respectively, after 7 d. Of the four organic acids tested, succinic acid had the most pronounced effects on improving the growth of Al-exposed plants. In the absence of aluminum, plants sprayed with organic acids showed no significant differences in root length, above-ground biomass, and root weight compared with pH 4.5 treatment alone (data not shown).

The malondialdehyde (MDA) concentrations in leaves significantly increased after 7 and 10 d of Al exposure, respectively, compared to plants grown without Al (Fig. 2). However, MDA concentrations were reduced by foliar sprays of the four organic acids. The differences were significant for oxalic acid and succinic acid treatments after 7 d, and for succinic acid, citric acid and malic acid treatments after 10 d compared with Al treatment alone. Succinic acid treatment was the most

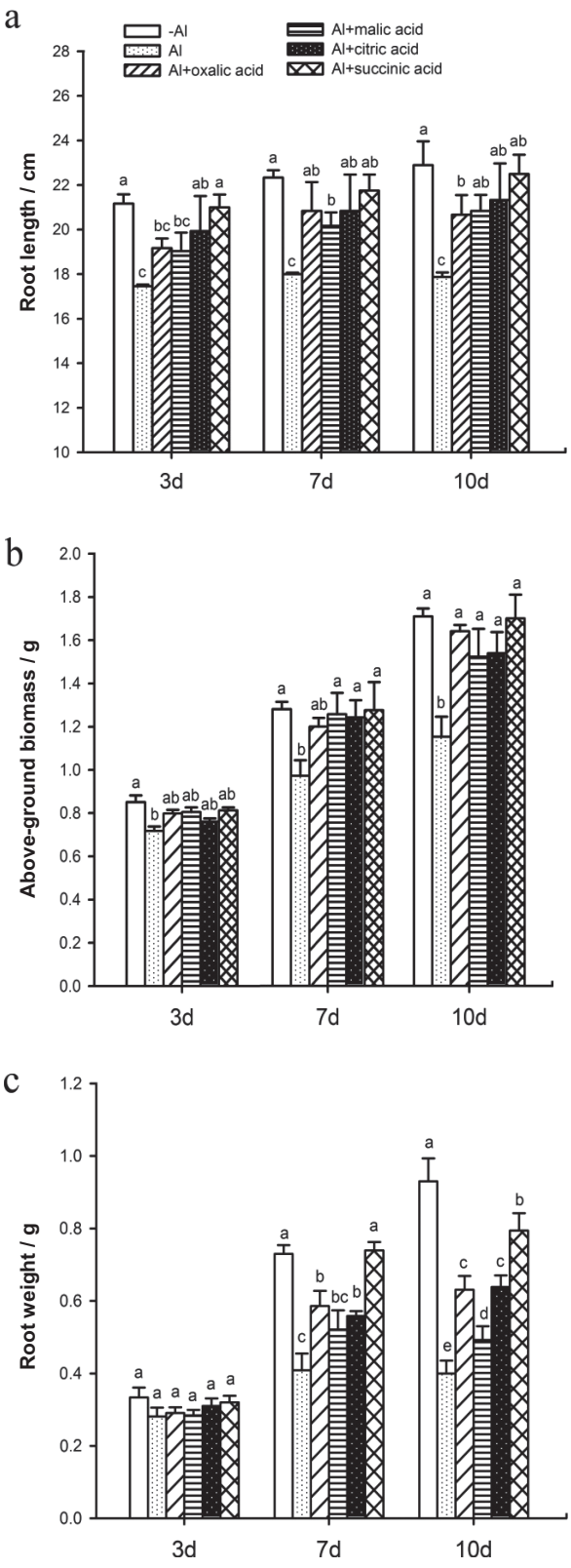

Figure 1: Root length (a), above-ground biomass (b), and root weight (c) of alfalfa seedlings after 3 d, 7 d and 10 d treatment. The seedlings were grown in pH 4.5 nutrient solution containing 0 or 100 μM Al, and were sprayed with water or 100 μM oxalic acid, malic acid, citric acid or succinic acid every 3 d during a 10 d experiment. Data are means ± SE (n = 3). Means with different letters are statistically significant at P = 5% on a given day.

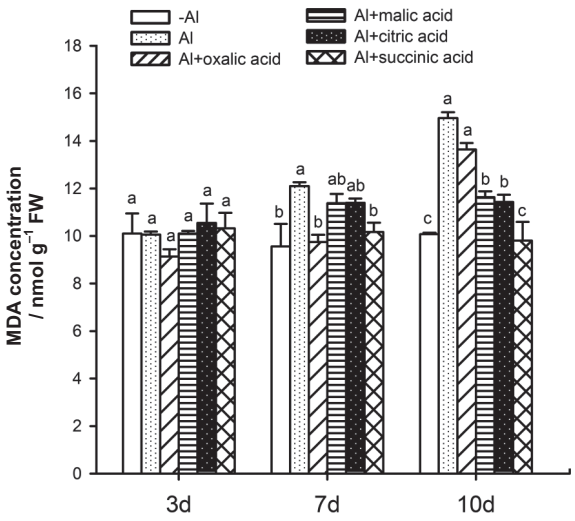

Figure 2: Malondialdehyde (MDA) concentration of leaves of alfalfa seedlings after 3 d, 7 d and 10 d treatment. The seedlings were grown in pH 4.5 nutrient solution containing 0 or 100 μM Al, and were sprayed with water or 100 μM oxalic acid, malic acid, citric acid or succinic acid every 3 d during a 10 d experiment. Data are means ± SE (n = 3). Means with different letters are statistically significant at P < 5% on a given day.

effective in reducing MDA concentrations of all organic acid treatments.

The impacts of aluminum on root activity were pronounced after 3–10 d of Al exposure. The root activity significantly decreased after 3, 7 and 10 d of Al exposure, respectively, compared with plants grown without Al (Fig. 3). The root activity under Al exposure was significantly increased by foliar spraying of succinic acid throughout the 10 d experimental period, by oxalic acid after 3 and 7 d, respectively, and by malic acid and citric acid after 10 d. Of the four organic acids, succinic acid had the most pronounced positive effects on root activity.

Aluminum stress caused a significant increase in Al concentration in roots after 10 d of Al exposure (Fig 4). However, foliar spraying of the four organic acids to Al-stressed alfalfa decreased Al concentration in roots, except citric acid treatment. Oxalic acid treatment had the lowest Al concentration among all the Al treatments. Although the Al concentrations in roots were decreased by foliar spray of oxalic acid, malic acid, and succinic acid, the total amount of Al was still increased. Aluminum uptake by roots treated with oxalic acid, malic acid, citric acid, and succinic acid increased by 6.2%, 4.1%, 72.4%, and 65.1%, respectively, compared with Al treatment alone.

Aluminum stress caused a significant decrease in mineral nutrient concentrations of Ca, K, Mg, Mn, and Zn in roots after 10 d of Al exposure (Table 1). The nutrient concentrations of roots increased significantly, with the exception of Ca, when Al-stressed plants were sprayed with succinic acid, but decreased significantly when Al-stressed plants were sprayed with oxalic acid.

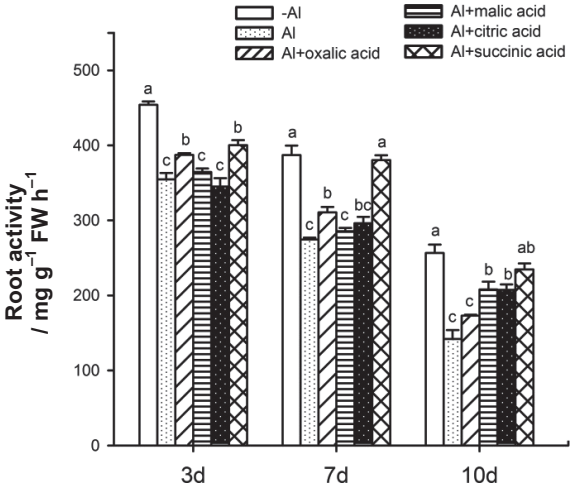

Figure 3: Root activities of alfalfa seedlings after 3 d, 7 d and 10 d treatment were measured using the triphenyltetrazolium chloride (TTC) reduction method. The seedlings were grown in pH 4.5 nutrient solution containing 0 or 100 μM Al, and were sprayed with water or 100 μM oxalic acid, malic acid, citric acid or succinic acid every 3 d during a 10 d experiment. Data are means ± SE (n = 3). Means with different letters are statistically significant at P = 5% on a given day.

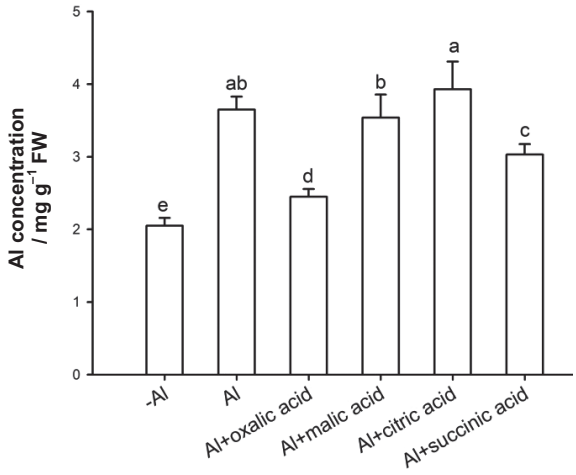

Figure 4: Aluminum concentrations of roots of alfalfa seedlings after 3 d, 7 d and 10 d treatment. The seedlings were grown in pH 4.5 nutrient solution containing 0 or 100 μM Al, and were sprayed with water or 100 μM oxalic acid, malic acid, citric acid or succinic acid every 3 d during a 10 d experiment. Data are means ± SE (n = 3). Means with different letters are statistically significant at P = 5% on a given day.

Aluminum stress reduced the total internal organic anion concentrations of roots (Table 2), but promoted oxalate exudation from roots after 3 and 10 d of Al exposure, respectively (Table 3). Exogenous application of organic acids to Al-stressed alfalfa altered organic anion accumulation (Table 2) and exudation from the roots (Table 3). Succinic acid sprayed to Al-stressed alfalfa caused largest increases in the concentrations of oxalate, malate, citrate, and the sum of the four organic anions in roots after 3 and 10 d of Al exposure, respectively. Citric acid applied to Al-stressed alfalfa significantly increased the concentrations of malate, succinate, and the sum of the four organic anions in roots (Table 2). Oxalic acid or malic acid applied to Al-stressed alfalfa did not significantly increase the contents of four organic acids in roots, but significantly increased oxalate exudation from roots. The exudation of malate and citrate were very small or not detected in roots of Al-stressed alfalfa with or without organic acid treatment (Table 3).

Exogenous application of succinic acid to Al-stressed alfalfa substantially up-regulated the transcription levels of malate dehydrogenase (MDH), phosphoenolpyruvate carboxylase (PEPC), isocitrate dehydrogenase (ISD), α-oxoglutarate dehydrogenase (OGD), succinyl-CoA ligase (SCS), and succinate dehydrogenase (SDH) in roots after 3 and 10 d of Al exposure, respectively, except succinyl-CoA ligase after 3 d. The up-regulation of gene transcription of MDH and PEPC was significant compared to Al treatment alone (Fig. 5a, b).

4 Discussion

The most prominent effect of Al toxicity in plants is the inhibition of root growth (*Delhaize* et al., 2007; *Valle* et al., 2009; *Cartes* et al., 2010). In the present study, Al inhibited alfalfa root growth within 3 d of Al exposure. However, spraying Al-stressed plants with oxalic acid, malic acid, citric acid, and succinic acid improved plant growth compared with Al treatment alone (Fig. 1). Organic anions serve critical functions in plant growth, including functioning as carbon source and che-

Table 1: The mineral concentrations (mg g^{-1} fresh weight) of roots of alfalfa seedlings after 10 d treatment. The seedlings were grown in pH 4.5 nutrient solution containing 0 or 100 μM Al, and were sprayed with water or 100 μM of oxalic acid, malic acid, citric acid or succinic acid every 3 d during a 10 d experiment. Data are means ± standard errors of three replicates. Means with different letters within columns are significantly different (P = 5%) among treatments.

	Ca	K	Mg	Mn	Zn
– Al	5.39 ± 0.41a	10.88 ± 0.91b	4.46 ± 0.32 b	0.117 ± 0.007a	0.219 ± 0.011a
+ Al	3.23 ± 0.21c	4.97 ± 0.40e	2.49 ± 0.18c	0.032 ± 0.001d	0.178 ± 0.014b
Al + oxalic acid	2.74 ± 0.18d	2.75 ± 0.12f	1.51 ± 0.10e	0.023 ± 0.001e	0.103 ± 0.008c
Al + malic acid	4.41 ± 0.31b	6.71 ± 0.51d	1.97 ± 0.13d	0.027 ± 0.002de	0.188 ± 0.009b
Al + citric acid	3.63 ± 0.25c	4.17 ± 0.25e	1.79 ± 0.12de	0.030 ± 0.002d	0.178 ± 0.011b
Al + succinic acid	3.54 ± 0.24c	9.34 ± 0.71c	4.54 ± 0.34b	0.059 ± 0.003c	0.230 ± 0.015a

Table 2: Organic-anion concentrations (mg g^{-1}) in root tips of alfalfa seedlings after 3 d and 10 d treatment. The seedlings were grown in pH 4.5 nutrient solution containing 0 or 100 μM Al, and were sprayed with water or 100 μM oxalic acid, malic acid, citric acid or succinic acid every 3 d during a 10 d experiment. Data are means ± standard errors of three replicates. Means with different letters within columns are significantly different (P = 5%) among treatments. nd: organic acid not detected.

	Treatment	Oxalate	Malate	Citrate	Succinate	Sum
3 d	– Al	6.83 ± 0.31bc	4.00 ± 0.14c	1.44 ± 0.06ab	1.50 ± 0.05b	13.77
	+ Al	5.63 ± 0.15c	2.33 ± 0.06d	1.15 ± 0.02c	1.05 ± 0.07c	10.16
	Al + oxalic acid	6.59 ± 0.18c	3.08 ± 0.11cd	1.24 ± 0.06bc	0.09 ± 0.00d	11.00
	Al + malic acid	8.28 ± 0.24ab	2.87 ± 0.13d	1.37 ± 0.03bc	0.14 ± 0.01d	12.66
	Al + citric acid	7.41 ± 0.21bc	5.58 ± 0.23b	0.84 ± 0.01d	1.40 ± 0.04b	15.23
	Al + succinic acid	8.54 ± 0.19ab	6.84 ± 0.25a	1.69 ± 0.08a	2.71 ± 0.14a	19.78
10 d	– Al	10.16 ± 0.68b	0.53 ± 0.12de	0.08 ± 0.00d	0.21 ± 0.07c	10.98
	+ Al	3.59 ± 0.21d	0.64 ± 0.15dc	0.31 ± 0.04dc	0.21 ± 0.08c	4.75
	Al + oxalic acid	3.77 ± 0.34d	0.28 ± 0.06e	0.09 ± 0.01d	nd	4.14
	Al + malic acid	4.85 ± 0.18d	0.88 ± 0.09c	0.60 ± 0.13bc	nd	6.33
	Al + citric acid	4.16 ± 0.12d	1.20 ± 0b	0.65 ± 0.05c	1.09 ± 0.10b	7.09
	Al + succinic acid	6.47 ± 0.27c	1.39 ± 0b	0.87 ± 0.01ab	0.99 ± 0.06b	9.72

Table 3: Organic anion exudation (g L^{-1}) from roots of alfalfa seedlings after 3 d and 10 d treatment. The seedlings were grown in pH 4.5 nutrient solution containing 0 or 100 μM Al, and were sprayed with water or 100 μM of oxalic acid, malic acid, citric acid or succinic acid every 3 d during a 10 d experiment. 15 seedlings of each treatment were transferred to 10 mL of filter-sterilized exudation solution (pH 4.5) with or without 100 Al μM for 6 h on days 3 and 10. Data are means ± standard errors of three replicates. Means with different letters within columns are statistically significant at P = 5% among treatments. nd: organic acid not detected.

	Treatment	Oxalate	Malate	Citrate
3 d	– Al	0.011 ± 0.001d	0.038 ± 0.001d	nd
	+ Al	0.029 ± 0.004c	0.065 ± 0.005bc	0.065 ± 0.002b
	Al + oxalic acid	1.561 ± 0.043b	0.058 ± 0.001c	0.070 ± 0.001b
	Al + malic acid	2.485 ± 0.094a	0.080 ± 0.002a	0.085 ± 0.001a
	Al + citric acid	0.027 ± 0.008c	0.077 ± 0.007ab	0.090 ± 0.005a
	Al + succinic acid	0.028 ± 0.091c	0.084 ± 0.001a	0.089 ± 0.003a
10 d	– Al	0.584 ± 0.021d	0.006 ± 0.002	nd
	+Al	0.872 ± 0.026c	nd	nd
	Al + oxalic acid	2.815 ± 0.142a	nd	nd
	Al + malic acid	1.371 ± 0.087b	nd	nd
	Al + citric acid	0.128 ± 0.001e	0.007 ± 0.0002	nd
	Al + succinic acid	0.067 ± 0.001f	0.009 ± 0.0001	nd

lates for detoxification of inorganic ions (*Schulze* et al., 2002). In our study, applying organic acids to leaves of alfalfa plants that were not Al-stressed did not increase plant growth, but resulted in significant improvement of shoot and root growth when plants were exposed to Al stress (Fig. 1), suggesting that the four organic acids played important roles in the alleviation of Al toxicity in alfalfa.

Oxidative damage is a common consequence of Al toxicity. Reactive oxygen species (ROS) and lipid peroxidation were increased by Al stress, and caused inhibition of root growth of rice and maize (*Meriga* et al., 2004; *Giannakoula* et al., 2008; *Tahara* et al., 2008). Thus, the ability of a plant to improve its ROS-scavenging capacity is a key element in Al resistance. In the present study, the Al-induced increase of lipid peroxidation in leaves, estimated by MDA production, was reduced by exogenous application of the four organic acids after 7 and 10 d of Al exposure (Fig. 2), respectively, indicating that these organic-acid treatments decreased oxidative damage in Al-stressed alfalfa. *Doncheva* et al. (2006) reported that the addition of succinate to the nutrient solution prior to Cu treatment could lower the Cu-induced formation of ROS, decrease the levels of oxidative components induced by Cu toxicity, and improve plant growth. Based on these observations, the mechanism by which succinic acid, oxalic acid, malic acid, and citric acid alleviate Al-related impairment of alfalfa growth is likely associated with an increase in antioxidant system activity or a decrease in the Al-induced formation of ROS. As a result, the Al-induced decrease in root activity

was recovered by exogenously applied organic acids, especially succinic acid.

Aluminum stress leads to nutritional deficiencies in plants (*Poschenrieder* et al. 1995; *Silva* et al. 2010). However, the Al-induced inhibition of mineral nutrient absorption (K, Ca, Mg, Mn, and Zn) was significantly alleviated by exogenous application of succinic acids to Al-stressed alfalfa in the present study; levels of all five mineral nutrients measured were significantly higher in roots of the succinic acid treatment than in the Al-stressed treatment without organic acid spray (Table 1). These results indicate that succinate improved mineral nutrient absorption in Al-stressed alfalfa.

Potassium (K) is extremely important for osmotic balance and, therefore, for membrane function and cell extension (*Silva* et al., 2010). *Pavlovkin* et al. (2009) reported that Al stress resulted in rapid membrane depolarization in root cortical cells of *Lotus corniculatus*, and the membrane depolarization was followed by a large loss of K from roots of an Al-sensitive variety. The decreased K levels in Al-stressed alfalfa (Table 1) seen in this study may cause disorders in osmoregulation and ultimately in cell extensibility. Thus, the increased growth accompanied by higher K accumulation in succinate-treated Al-stressed alfalfa suggested the occurrence of a better osmotic balance relative to Al-stressed alfalfa. Calcium and magnesium have been shown to alleviate Al-induced reductions in root growth in wheat, rice bean, and Japanese cedar (*Hossain* et al., 2005; *Takami* et al., 2005; *Yang* et al., 2007). These effects were attributed to Ca maintaining the normal synthesis of hemicellulosic polysaccharides and phenolic compounds that stabilize cell wall properties and architecture in wheat (*Hossain* et al., 2005), to Mg promoting citrate efflux and blocking Al from entering roots in rice and bean (*Yang* et al., 2007), and to Ca and Mg stimulating antioxidant enzyme activities that reduced ROS in Japanese cedar (*Takami* et al., 2005). In the present study, succinic acid treatment increased growth of Al-stressed alfalfa, and this seemed to be related to the ameliorative effects of Ca and Mg on Al toxicity (Table 1).

Aluminum-stimulated efflux of organic anions has been considered an important mechanism leading to Al resistance in many plant species (*Ryan* et al., 2001; *Yang* et al., 2006; *Wang* et al., 2007; *Dong* et al., 2008), but the mechanisms of organic acid exudation by roots are not yet well understood. In the present study, Al-induced oxalate exudation was very high after 3 and 10 d of Al exposure, respectively, but exudation of malate and citrate only occurred after 3 d (Table 3), indicating that malate and citrate had little long-term effects on alleviating Al toxicity though exudation in Al-stressed alfalfa. Exogenous application of oxalic acid and malic acid significantly increased oxalate exudation compared with Al treatment alone, while Al concentrations in roots decreased significantly (Fig. 4), suggesting that the enhanced oxalate exudation could effectively prevent Al from entering roots. This result agreed with those of other studies showing that oxalate exudation was an important mechanism of Al resistance in *Polygonum aviculare* and buckwheat (*You* et al., 2005; *Kochian* et al., 2005; *Klug* and *Horst*, 2010). *Tesfaye* et al. (2001) reported that overexpression of malate dehydrogenase (MDH) in transgenic alfalfa resulted in a large amount of oxalate exudation, while there was no oxalate exudation in the untransformed control plants, supporting our conclusion. However, oxalate exudation might decrease nutrient uptake by buckwheat (*Chen* et al., 2010), as well as by alfalfa under Al stress in the present study. Our results indicate that increasing the level of endogenous oxalate and malate in Al stressed alfalfa may enhance plant resistance to Al toxicity through oxalate exudation from roots. However, the effect of oxalate exudation on Al resistance might be weakened by lower nutrient uptake.

When Al is absorbed by roots, it could chelate with organic anions to form non-phytotoxic complexes (*Kochian* et al., 2005) that are subsequently stored in root and shoot cell vacuoles (*Tolrá* et al., 2005; *Ramírez-Benítez* et al., 2008). The tricarboxylate citrate^{3-} anion is a better Al^{3+} chelator than the bicarboxylate malate^{2-} and oxalate^{2-} ion (*Kochian* et al., 2004), and the stability constants decrease in the order: Al-citrate > Al-oxalate > Al-malate (*Ryan* et al., 2001). In addition, Al-organic anion (Al-OA) complexes that contribute to Al resistance in plants may occur in different forms. For example, in roots, xylem, and leaves of Al-resistant buckwheat, Al was present as Al–oxalate, Al–citrate, and Al–oxalate complexes, respectively (*Ma* et al., 2001). *Klug* and *Horst* (2010) reported that Al and citrate transport rates from root apex to xylem were positively correlated in Al-stressed buckwheat, and citrate was the most likely ligand for Al in the xylem. In the present study, only when all four of the organic acids reached high concentrations in roots, the alfalfa did possess a high level of Al resistance in the succinic acid treatment (Table 2). The four organic acids may cooperate in alfalfa roots to alleviate Al toxicity by forming different Al-OA forms as reported in *Ma* et al. (2001). Thus, an organic anion system for internal Al detoxification seems to occur in Al-resistant species, and the organic acid detoxification system for alfalfa may include oxalate, malate, citrate, and succinate.

Citrate and malate synthesis in root tissues involves oxaloacetate. The generation of oxaloacetate involves phosphoenolpyruvate carboxylation by the enzyme phosphoenolpyruvate carboxylase (PEPC), and reduction of oxaloacetate to malate was catalyzed by the enzyme malate dehydrogenase (MDH) (*Ramírez-Benítez* et al., 2008). An increase in the enzymatic activities of MDH, PEPC and CS has been linked to synthesis and exudation of malate and citrate in response to Al stress (*Anoop* et al., 2003; *Mariano* et al., 2005). Foliar spraying of succinic acid to Al-stressed alfalfa significantly up-regulated the transcription of *MDH* (Fig. 5a) and *PEPC* (Fig. 5b), which are involved in the synthesis of malate and citrate in roots. The accumulation of oxalate, malate, and citrate in plants treated with succinic acid was highest in all Al-stressed treatments after 3 and 10 d of Al exposure (Table 2), respectively. *Yang* et al. (2001) speculated that succinate might play a key role in malate and citrate synthesis by maintaining the balance between citrate synthesis and release from the root apices of Al-stressed soybean, supporting our conclusion. The data in the present study suggest that succinic acid could be involved in modulation of the synthesis of the organic anion detoxification system by up-regulating

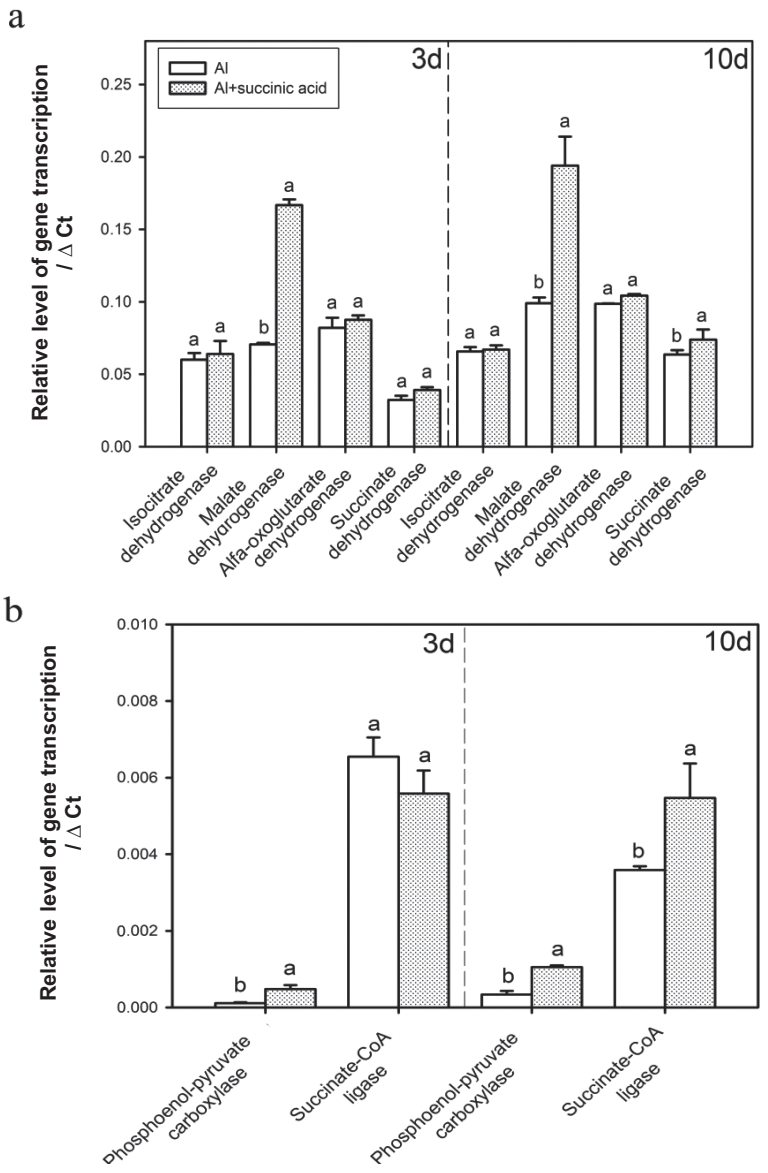

Figure 5: Gene transcription in roots of alfalfa exposed to nutrient solution (pH 4.5) with 100 μM Al, and with or without foliar sprays of succinic acids onto alfalfa for 3 and 10 d. Data are means ± SE (n = 3). Means with different letters are statistically significant at P = 5% on a given day.

both *MDH* and *PEPC* gene expressions in the roots of Al-stressed alfalfa. Thus, increased levels of endogenous succinate in roots exposed to Al might promote the synthesis of the organic acid system, leading to enhanced Al detoxification.

5 Conclusion

Exogenous application of oxalic acid, malic acid, citric acid, or succinic acid to Al-stressed alfalfa substantially alleviated Al toxicity as reflected by enhanced growth, improved physiological status, and mineral nutrient balance. The mechanisms by which the four organic acids alleviated Al toxicity were different. Oxalate and malate might alleviate Al toxicity through Al exclusion by increasing root exudation of oxalate, while citrate and especially succinate might alleviate Al toxicity through accumulation of organic anions for internal Al detoxification in roots. The organic-anion detoxification system in alfalfa may include oxalate, malate, citrate, and succinate, which may work together to enhance Al resistance.

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