Boron-aluminum interactions affect organic acid metabolism more in leaves than in roots of *Citrus grandis* seedlings

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Abstract

Sour pummelo (*Citrus grandis*) seedlings were irrigated with nutrient solution containing four boron concentrations (*i.e.*, 2.5, 10, 25 and 50 μM H_3BO_3) and two aluminum concentrations [*i.e.*, 0 (-Al) and 1.2 mM $AlCl_3 . 6 H_2O$ (+Al)]. It was found that B did not affect, but Al increased, the Al content in the roots. The Al and citrate contents in the -Al leaves either did not change or slightly increased with increasing B concentration. On the other hand, the Al and citrate contents in the +Al leaves rapidly decreased as B concentration increased from 2.5 to 50 μM, then decreased at the highest B concentration. The Al and citrate contents were higher in the +Al than in the -Al leaves, except for at 25 μM B when they were similar. The leaf malate content did not change in response to B or Al, except for an increase in the +Al leaves and a decrease in the -Al leaves at 2.5 μM B. Similarly, the root malate and citrate contents did not change in response to B with or without Al, except for a decrease in the malate and citrate contents in the +Al roots at 50 μM B and an increase in the citrate content in the -Al roots at 50 μM B. The activities of acid-metabolizing enzymes were less affected by B-Al interactions in the roots than in the leaves.

Additional key words: acid-metabolizing enzymes, citrate, malate, sour pummelo.

Introduction

Aluminum (Al) toxicity is a major factor limiting crop productivity in acid soils. The primary symptom of Al toxicity is an immediate inhibition of root growth. Al first inhibits root cell expansion and elongation rather than cell division (Kochian *et al*. 2004). Al is assumed to exert its toxic effect in the apoplast through interaction with the negative binding sites of the cell walls, primarily pectin of root epidermal and cortical cells (Schmohl and Horst 2000).

Evidence exists for the exclusion of Al from the root apices *via* root exudation of organic acid (OA) anions as a major mechanism of Al tolerance in plants (Ma *et al*. 2001). Although a rapid release of OA anions was observed in tobacco (Delhaize *et al*. 2001) and wheat (Ryan *et al*. 1995), experiments with rye (Li *et al*. 2000) and soybean (Yang *et al.* 2001) showed that OA anion secretion was delayed for several hours after exposure to Al. Delayed exudation of OA anions could be due to the alteration of gene expression and protein synthesis involved in OA metabolism or in the transport of anions (Ma *et al*. 2001). In *Citrus junos* (Deng *et al*. 2009) and rye (Li *et al*. 2000) roots, Al-induced citrate exudation was accompanied by the increased citrate content and citrate synthase (CS; EC 2.3.3.1) activity. Recently, our study showed that Al decreased the contents of malate and citrate in *Citrus grandis* roots in the presence of 50 μM phosphorus, but did not affect the activities of CS, aconitase (ACO, EC 4.2.1.3), phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31), NADP-isocitrate dehydrogenase (NADP-IDH, EC 1.1.1.42), NAD-malate

Received 2 October 2009, *accepted* 15 March 2010.

Abbreviations: ACO - aconitase; CS - citrate synthase; NAD-MDH - NAD-malate dehydrogenase; NADP-IDH - NADP-isocitrate dehydrogenase; NADP-ME - NADP-malic enzyme; OA - organic acid; PEPC - phospho*enol*pyruvate carboxylase; PEPP - phospho*enol*pyruvate phosphatase; PK - pyruvate kinase; Pyr - pyruvate.

Acknowledgements: This study was financially supported by the National Natural Science Foundation of China (No. 30771487), the Agriculture Commonweal Industrial Special Fund Program of Department of Agriculture, China (nyhyzx07-023) and the Natural Science Foundation of Fujian, China (Nos. B0710011 and 2007J0050). The authors wish to thank Professor Frank Huang, Fujian Agriculture and Forestry University, for language correction and constructive comments on this manuscript.

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dehydrogenase (NAD-MDH, EC 1.1.1.37) and NADP-malic enzyme (NADP-ME, EC 1.1.1.40). In contrast, Al decreased the activities of CS, ACO, PEPC and NADP-IDH in the roots at 500 μM P, but did not affect the contents of malate and citrate (Chen *et al*. 2009).

OA metabolism in the leaves of some plant species is also affected by Al. Our study showed that both the malate and citrate contents in 1.2 mM Al-treated sour pummelo leaves increased with decreasing P supply, but the contents in 0 mM Al-treated leaves did not change in response to P (Chen *et al*. 2009). Watanabe and Osaki (2002) reported that Al increased the contents of citrate, oxalate and malate in *Melastoma malabathricum* leaves. Goodwin and Sutter (2009) observed that Al induced the expression of three transcripts involved in the OA metabolism pathway, MDH, 2-oxoglutarate dehydrogenase (EC 1.2.4.2), and alanine aminotransferase (EC 3.6.1.2) in *Arabidopsis thaliana* seedlings.

Boron deficiency is a widespread problem for many agricultural crops, including citrus (Han *et al*. 2008). Like Al, B also primarily inhibits root growth through limiting cell elongation rather than cell division. The predominant function of B is in the formation of primary cell walls, where it cross-links the pectic polysaccharide rhamno-galacturonan II (RG-II). The cross-linking RG-II by B ester results in a stable network of cell walls with decreased pore sizes (O'Neill *et al*. 2004), thus hampering Al from getting in contact with sensitive targets on the plasma membrane and/or symplasm (Corrales *et al*. 2008). It has been shown that B can alleviate Al toxicity in squash (LeNoble *et al*. 1996), common bean (Stass *et al*. 2007), cucumber, maize (Corrales *et al*. 2008), pea (Yu *et al*. 2009) and sour pummelo (Jiang *et al*. 2009a). Recently, Stass *et al.* (2007) observed that citrate exudation from common bean roots after a long Al treatment was lower in B-sufficient than in B-deficient plants. Therefore, B may affect long-term Al-induced changes in root as well as leaf OA metabolism. There is little information available on the effects of B-Al interactions on OA metabolism in roots and leaves.

Citrus are evergreen subtropical fruit trees cultivated in humid and subhumid tropical, subtropical and temperate regions of the world, mainly on acid soils. High Al and low B are frequently observed in citrus plantations (Han *et al*. 2008, Jiang *et al*. 2009b). Our study with sour pummelo seedlings showed that P could alleviate the Al-induced inhibition of growth and photosynthesis by increasing Al immobilization in roots and decreasing Al level in shoots (leaves). Both the contents of malate and citrate and the activities of acid-metabolizing enzymes were less affected by P-Al interactions in the roots than in the leaves, which might be attributed to the smaller changes in the root Al than in the leaf Al (Chen *et al*. 2009). OA metabolism can be less affected by B-Al interactions in the roots than in the leaves, as it exerted less effect on the Al level in the roots than in the leaves (Jiang *et al*. 2009a,b). In this paper, we investigated the effects of B-Al interactions on the contents of B, Al, malate and citrate as well as the activities of acid-metabolizing enzymes in the leaves and roots of an Al-sensitive sour pummelo. The objectives of this study were to determine how B-Al interactions affect the OA metabolism in the roots and leaves, and test if B-Al interactions affect OA metabolism more in the leaves than in the roots.

Materials and methods

This study was conducted from February to November 2007 at Fujian Agriculture and Forestry University. Sour pummelo [*Citrus grandis* (L.) Osbeck] seedlings were germinated in plastic trays with sand. Five weeks after germination, uniform seedlings with a single stem were selected and transplanted to 6 dm^3 pots with sand. Seedlings, three to a pot, were grown in a greenhouse under natural photoperiod. Each pot was supplied with 500 cm^3 of nutrient solution every two days. The nutrient solution was formulated with macronutrients [1 mM KNO_3, 1 mM $Ca(NO_3)_2$, 0.1 mM KH_2PO_4 and 0.5 mM $MgSO_4$] and micronutrients [10 μM H_3BO_3, 2 μM $MnCl_2$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.065 μM $(NH_4)_6Mo_7O_{24}$ and 20 μM Fe-EDTA]. Six weeks after transplanting, treatments were initiated and applied for 18 weeks. There were eight treatments in total, including four B levels (*i.e*., 2.5, 10, 25 and 50 μM H_3BO_3) × two Al levels [*i.e*., 0 (-Al) and 1.2 mM $AlCl_3 \cdot 6 H_2O$ (+Al)]. The pH of the nutrient solution was adjusted to 4.1 - 4.2 using HCl or NaOH solution. At the end of the experiment, the fully expanded (about 7- week-old) leaves and white new roots from different replicates and treatments were used for measurements. Leaf discs (0.61 cm^2 in size) and 10 mm long root apices were excised from the seedlings at noon, immediately frozen in liquid nitrogen and stored at -80 °C until extraction.

At the end of the experiment, 8 - 15 plants per treatment from different pots were harvested. The roots and shoots were separated from the plants, dried at 80 °C for 48 h and dry mass was measured. Root and leaf samples were digested in a HNO_3/HCl/$HClO_4$ mixture. B and Al in the solution were determined by the modified curcumin method (Kowalenko and Lavkulich 1976) and the aluminon method Hsu (1963), respectively.

Malate and citrate were extracted and determined according to Chen and Nose (2002). ACO, PEPC, NAD-MDH, NADP-ME, NADH-IDH, pyruvate (Pyr) kinase (PK, EC 2.7.1.40), phospho*enol*pyruvate phosphatase (PEPP, EC 3.1.3.60) and CS were extracted and determined according to Chen *et al*. (2009).

There were 30 pot seedlings per treatment in a completely randomized design. Experiments were performed with 4 - 15 replicates. Differences among treatments were separated by the least significant difference (LSD) test at $P < 0.05$ level.

Results

The root and shoot dry masses in the -Al seedlings did not significantly change in response to B addition, except for a slight decrease in root dry mass at 50 μM B. On the other hand, the root and shoot dry masses in the +Al seedlings increased as B increased from 2.5 to 25 μM, then decreased at the highest B concentration (Fig. 1*A,B*).

B did not significantly affect the root Al content in plants grown with or without Al, while Al addition increased the root Al content (Fig. 1*C*). The leaf Al content did not significantly change in response to B when plants grew without Al, but under Al stress it decreased with increasing B concentration from 2.5 to 25 μM, and then increased at 50 μM B. The leaf Al content was higher in the +Al than in the -Al plants, except for similar content under 25 μM B (Fig. 1*D*).

The root and leaf B content increased with increasing B concentration similarly in plants grown with or without Al (Fig. 1*E*,*F*).

The leaf malate content did not change significantly in response to B or Al, with exception of a decrease in the -Al leaves and an increase in the +Al leaves under 2.5 μM B (Fig. 2*A*). The order of citrate content in the +Al leaves was 2.5 μM B > 50 μM B > 10 and 25 μM B. The citrate content in the -Al leaves slightly

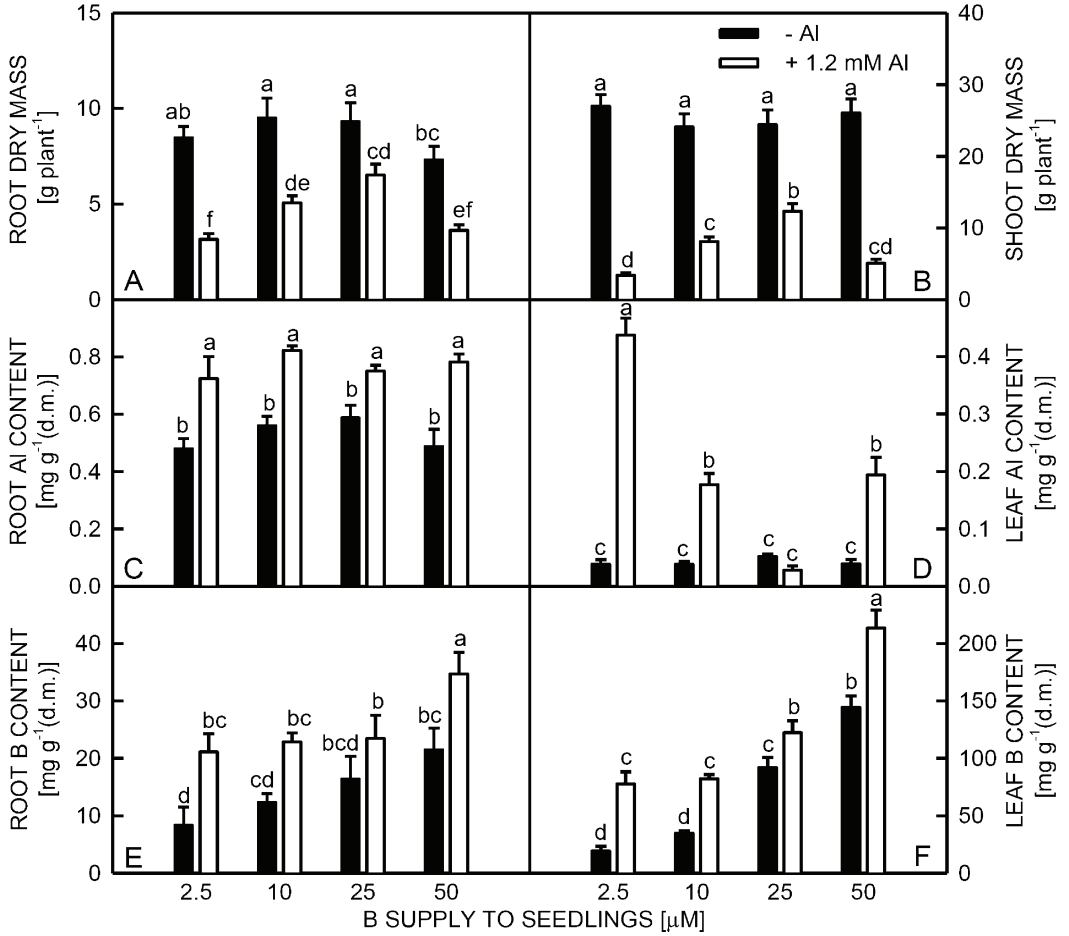

Fig. 1. Effects of boron (B)-aluminum (Al) interactions on root (*A*) and shoot (*B*) dry masses, Al (*C* and *D*) and B (*E* and *F*) contents in roots and leaves of *Citrus grandis* seedlings. Means ± SE ($n = 4$ - 15). Different letters indicate significant differences among eight treatments at $P < 0.05$.

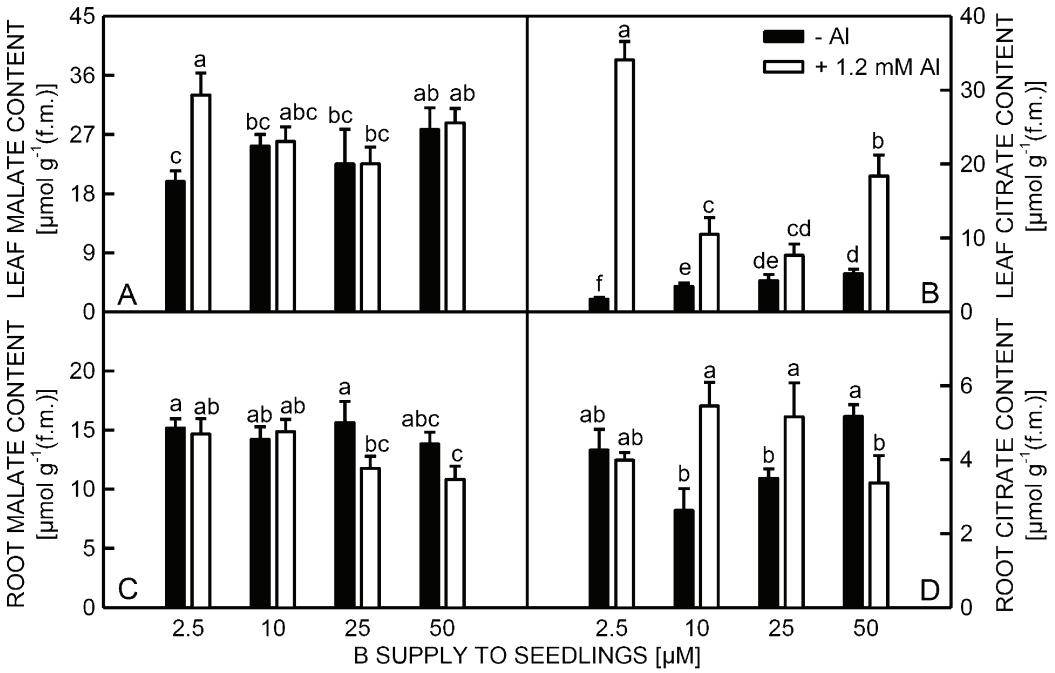

Fig. 2. Effects of boron (B)-aluminum (Al) interactions on malate (*A* and *C*) and citrate (*B* and *D*) contents in roots and leaves of *Citrus grandis* seedlings. Means ± SE (n = 5 - 6). Different letters indicate significant differences among eight treatments at $P < 0.05$.

increased with increasing B concentration. The citrate content was higher in the +Al than in the -Al leaves, except that the content was similar at 25 μM B (Fig. 2*B*).

The root malate content did not significantly differ among B and Al combinations, except that a slight decrease under 25 μM B + 1.2 mM Al and 50 μM B + 1.2 mM Al was observed (Fig. 2*C*). B did not significantly affect the root citrate content in plants grown with or without Al, other than an increase in the -Al roots and a decrease in the +Al roots under 50 μM B. The citrate content was higher in the +Al than in the -Al roots under 10 and 25 μM B, but it was higher in the -Al than in the +Al roots under 50 μM B (Fig. 2*D*).

The leaf CS and PK activities did not significantly differ among B and Al combinations, except for a slight increase in the CS activity under 10 μM B + 0 mM Al and a slight decrease in the PK activity under 25 μM B + 0 mM Al (Fig. 3*A*,*H*). B did not significantly affect the leaf ACO activity. The ACO activity did not significantly differ between the Al treatments except for a decrease in the +Al leaves under 50 μM B (Fig. 3*B*). The PEPC activity did not significantly change in response to B combined 0 or 1.2 mM Al, except for a slight increase in the +Al leaves and a decrease in the -Al leaves under 2.5 μM B. The PEPC activity did not significantly differ between the +Al and -Al leaves, except for an increase in the +Al leaves under 2.5 μM B (Fig. 3*C*).

The NADP-IDH activity did not significantly change in response to B with or without Al, except for an increase in the -Al leaves under 50 μM B. This enzyme's activity was significantly higher in the +Al than in the -Al leaves, except that the activity was similar under 2.5 μM B (Fig. 3*D*).

The PEPP activity increased with increasing B without Al, but it was significantly higher in the +Al leaves treated with 2.5 and 50 μM B than with 10 and 25 μM B. The PEPP activity was significantly higher in the +Al than in the -Al leaves, except that the activity was similar under 10 μM B (Fig. 3*E*).

The NAD-MDH activity did not significantly change in response to B with or without Al, except for a decrease in the -Al leaves under 2.5 μM B and an increase in the +Al leaves under 25 μM B. The activity of NAD-MDH was significantly lower in the +Al than in the -Al leaves, except that the activity was similar under 25 μM B (Fig. 3*F*).

B did not significantly affect the leaf NADP-ME activity with or without Al. This enzyme's activity was significantly lower in the +Al than in the -Al leaves under 10 or 50 μM B, but similar under 2.5 or 10 μM B (Fig. 3*G*).

The activities of CS, ACO, NADP-IDH, PEPP, NAD-MDH, NADP-ME and PK in roots did not significantly differ among B and Al combinations, except for decreases in the CS and NAD-MDH activities under 25 μM B + 1.2 mM Al, in the ACO and NADP-IDH activities under 50 μM B + 1.2 mM Al and in the PK activity under 2.5 μM B + 0 mM Al, and increases in the CS, ACO and NADP-IDH activities under 10 μM B + 0 mM Al (Fig. 4*A*,*B*,*D*,*H*).

The PEPC activity in roots did not significantly change in response to B under Al stress, except for a decrease under 50 μM B. It was higher in the -Al roots treated with 2.5 and 25 μM B than with 10 and 50 μM B.

The root PEPC activity did not differ between the Al treatments (Fig. 4*C*).

The leaf malate and citrate contents linearly increased with increasing leaf Al content. Under Al stress, these contents decreased in a curvilinear manner with increasing shoot dry mass. The citrate content in the -Al leaves linearly or curvilinearly increased with increasing PEPC or PEPP activity (data not shown).

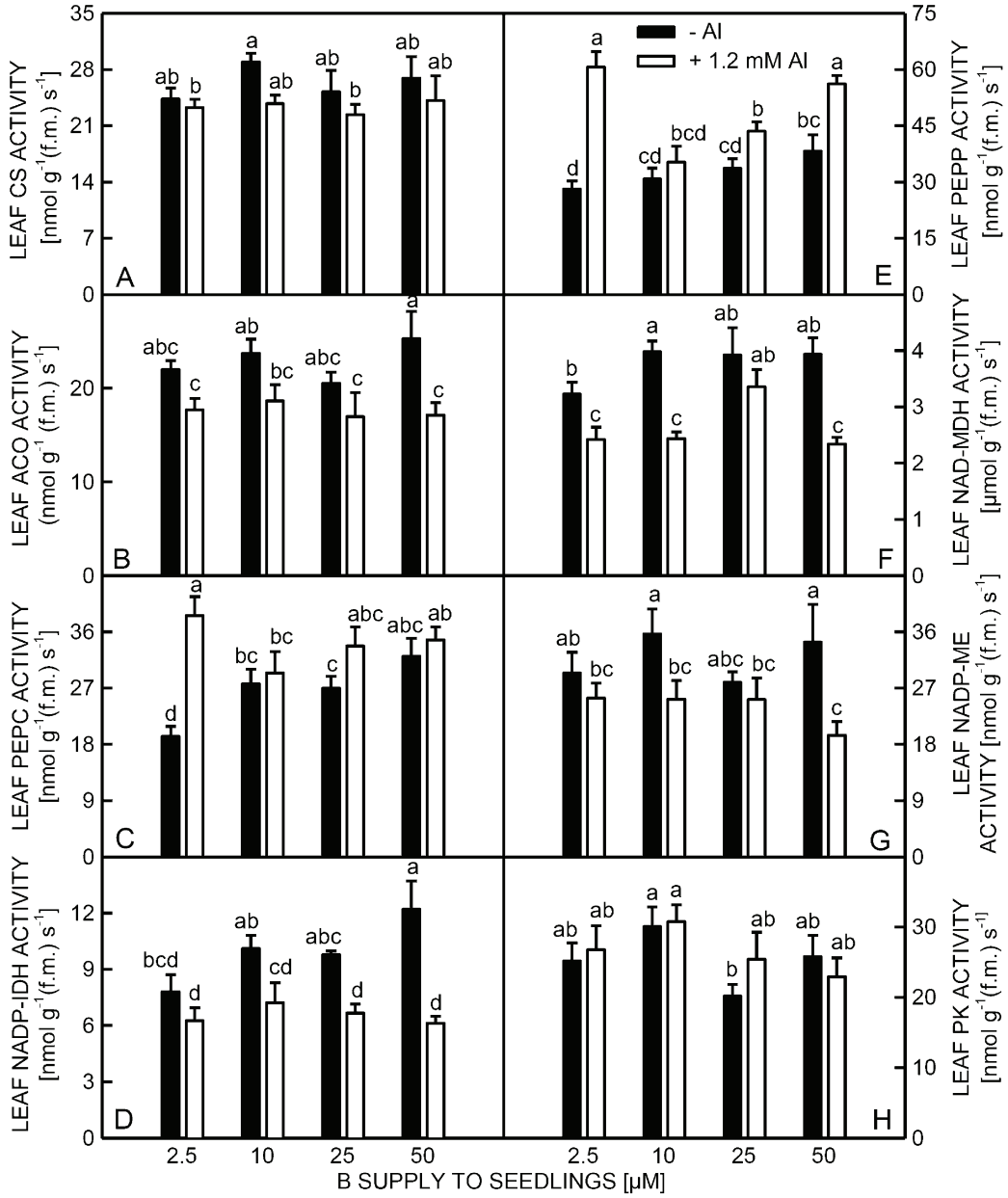

Fig. 3. Effects of boron (B)-aluminum (Al) interactions on citrate synthase (CS, *A*), aconitase (ACO, *B*), phospho*enol*pyruvate carboxylase (PEPC, *C*), NADP-isocitrate dehydrogenase (NADP-IDH, *D*), phospho*enol*pyruvate phosphatase (PEPP, *E*), NAD-malate dehydrogenase (NAD-MDH, *F*), NADP-malic enzyme (NADP-ME, *G*) and pyruvate kinase (PK, *H*) activities in leaves of *Citrus grandis* seedlings. Means ± SE (n = 5 - 6). Different letters indicate significant differences among eight treatments at $P < 0.05$.

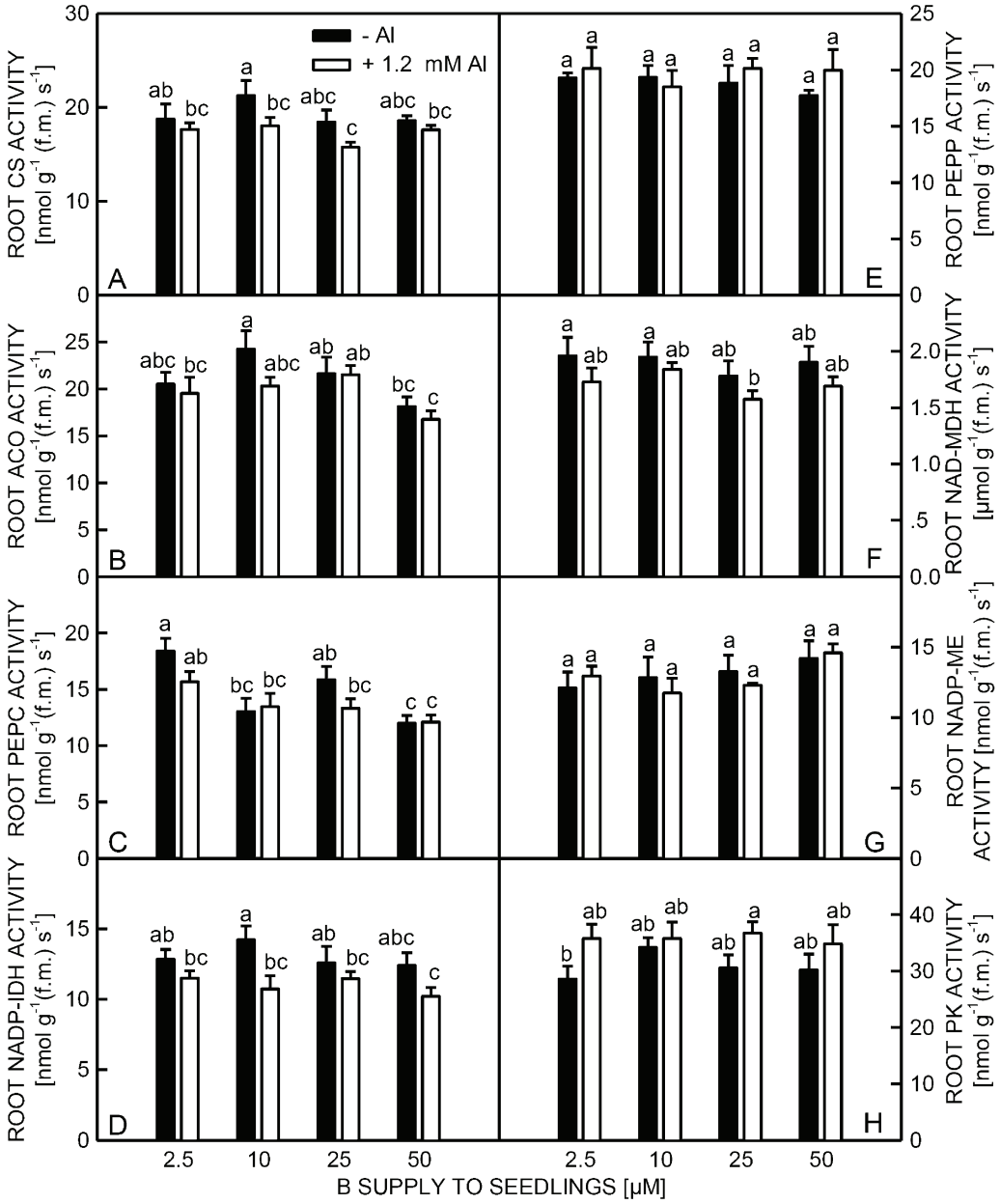

Fig. 4. Effects of boron (B)-aluminum (Al) interactions on citrate synthase (CS, *A*), aconitase (ACO, *B*), phospho*enol*pyruvate carboxylase (PEPC, *C*), NADP-isocitrate dehydrogenase (NADP-IDH, *D*), phospho*enol*pyruvate phosphatase (PEPP, *E*), NAD-malate dehydrogenase (NAD-MDH, *F*), NADP-malic enzyme (NADP-ME, *G*) and pyruvate kinase (PK, *H*) activities in roots of *Citrus grandis* seedlings. Means ± SE (n = 5 - 6). Different letters indicate significant differences among eight treatments at $P < 0.05$.

Discussion

We found that the leaves and roots from plants grown under 1.2 mM Al had a higher or similar B content as compared to the plants grown without Al (Fig. 1*E*,*F*). The order of the ameliorative effect of B on Al-induced growth inhibition was 25 µM B > 10 and 50 µM B > 2.5 µM B (Fig. 1*A*,*B*). It means that the Al-induced growth inhibition is not due to the Al-induced B-deficiency. Corrales *et al*. (2008) reported that Al enhanced the content of reduced glutathione (GSH) in the roots of maize plants growing with adequate B but not in those growing with either deficient or excess B. The decrease found in the root dry mass in 50 µM B + 0 mM Al-treated seedlings (Fig. 1*A*) indicated that these plants received excess B. This would explain why 50 µM B

negatively affected root and shoot growth (Fig. 1*A*,*B*) especially under Al stress, as the B content was significantly higher in the roots and leaves of +Al than –Al plants. The B-induced alleviation of root inhibition caused by Al was probably caused by different Al compartmentation in roots (Corrales *et al*. 2008) rather than the reduced Al accumulation, because the Al content in the +Al roots did not differ among the B treatments (Fig. 1*C*). On the other hand, the B-induced amelioration of shoot inhibition could be due to the reduced Al accumulation in stems (Jiang *et al*. 2009a,b) and leaves (Fig. 1*D*).

The low B-induced decrease in the malate content in the leaves of -Al plants (Fig. 2*A*) was probably caused by a decreased biosynthesis rather than the increased degradation, because it was accompanied by the decreased PEPC (Fig. 3*C*), PEPP (Fig. 3*E*) and NAD-MDH (Fig. 3*F*) activities with a similar NADP-ME activity (Fig. 3*G*). In contrast, the malate content was higher in the +Al leaves treated with 2.5 μM B than with 25 μM B (Fig. 2*A*). This might result from an increased biosynthesis as indicated by the increased PEPP (Fig. 3*E*) activity and the reduced dilution as the malate content in the +Al leaves decreased with increasing shoot dry mass (data not shown). In addition, the higher malate content in the +Al than in the -Al leaves under 2.5 μM B might result from an increased biosynthesis as indicated by the increased PEPC (Fig. 3*C*) and PEPP (Fig. 3*E*) activities and the reduced dilution as the shoot dry mass was largely decreased by Al at the lowest B supply (Fig. 1*B*).

The low-B-induced decrease in the citrate content in the -Al leaves (Fig. 2*B*) might result from a decreased biosynthesis of citrate as the citrate content in the -Al leaves decreased with decreasing PEPC or PEPP activity (data not shown) rather than from the increased degradation, as low B did not increase the ACO (Fig. 3*B*) and NADP-IDH (Fig. 3*D*) activities. Our results showed that the order of the citrate content in the +Al leaves was 2.5 μM B > 50 μM B > 10 and 25 μM B (Fig. 2*B*). Similar trends were found for the PEPC (Fig. 3*C*) and PEPP (Fig. 3*E*) activities in the leaves of +Al plants in response to B. However, the CS (Fig. 3*A*), ACO (Fig. 3*B*) and NADP-IDH (Fig. 3*D*) activities in the leaves of +Al plants did not differ with various B treatments. In +Al plants, the higher citrate content in the leaves under 2.5 and 50 μM B as compared with 10 and 25 μM B could be related to an increased biosynthesis and the reduced dilution. The higher citrate content in the leaves of +Al than in the -Al plants under 2.5, 10 or 50 μM B was attributed to a combination of several factors, such as an increased biosynthesis as indicated by the increased PEPC (Fig. 3*C*) and PEPP (Fig. 3*E*) activities, a decreased degradation as indicated by the decreased ACO (Fig. 3*B*) and NADP-IDH (Fig. 3*D*) activities and the reduced dilution as indicated by the decreased shoot dry mass (Fig. 1*B*).

Our results showed that the contents of malate and citrate (Fig. 2*C,D*) and the activities of acid-metabolizing enzymes (Fig. 4) in the roots did not change significantly in response to B in plants grown with or without Al, except for an increase in the citrate content and a decrease in the ACO activity under 50 μM B + 0 mM Al, an increase in the PEPC activity under 2.5 μM B + 0 mM Al and 25 μM B + 0 mM Al, and a decrease in the malate and citrate contents and in the ACO and PEPC activities under 50 μM B + 1.2 mM Al. It suggested that the OA metabolism in the roots was little affected by B. The lower contents of malate and citrate in the +Al roots with 50 μM B than with 2.5, 10 and 25 μM B (Fig. 2*C*) might relate to a decreased biosynthesis as indicated by the reduced PEPC activity (Fig. 4*C*). Our finding on the activities of PEPC, PEPP, NAD-MDH, NADP-ME and PK (Fig. 4*C* and 4*E-H*) and the content of malate, which did not differ between the +Al and -Al roots, except for a slight increase in the malate in the -Al roots under 25 μM B (Fig. 2*C*) suggested that the biosynthesis and utilization of malate could be at equilibrium through the action of acid-metabolizing enzymes. In contrast to the root malate, the content of citrate was higher under 10 and 25 μM B, but lower under 50 μM B in the roots of +Al than in the -Al plants (Fig. 2*D*). This cannot be explained either by an increased biosynthesis of citrate or by a decreased degradation as Al did not affect the activities of CS, ACO, PEPC, NADP-IDH and PEPP (Fig. 4*A-E*) and the ratio of CS to NADP-IDH (data not shown) except for a slight increase in the CS (Fig. 4*A*) and NADP-IDH (Fig. 4D) activities in the -Al roots under 10 μM B. Neumann and Römheld (1999) concluded that the ability to accumulate carboxylic acids in P-deficient chickpea (*Cicer arietinum*) and white lupin (*Lupinus albus*) roots depended on the partitioning of carboxylic acids or related precursors between roots and shoots. Our data showed that the partitioning of citrate between roots and leaves was altered (Fig. 2*C,D*). This would explain why the citrate content was higher under 10 and 25 μM B, but lower under 50 μM B in the +Al than in the -Al roots (Fig. 2*D*). Compared with the -Al roots, the higher citrate content under 10 and 25 μM B in +Al roots might also relate to the reduced dilution due to the decreased root growth (Fig. 1*A*). The lower citrate content under 50 μM B in the +Al roots might also be associated with Al-induced exudation of OA anions.

In conclusion, B supply could result in a more stable network of cell walls with decreased pore sizes in roots, thus hampering Al from getting into shoots. The B-Al interactions affected the leaf more than the root Al content. Consequently, OA metabolism was more affected by B-Al interactions in the leaves as compared to the roots.

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