Citrate exudation from white lupin induced by phosphorus deficiency differs from that induced by aluminum

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Received: *8 May 2007*
Accepted: *3 July 2007*

Summary

• Both phosphorus (P) deficiency and aluminum (Al) toxicity induce root exudation of carboxylates, but the relationship between these two effects is not fully understood. Here, carboxylate exudation induced by Al in *Lupinus albus* (white lupin) was characterized and compared with that induced by P deficiency.

• Aluminum treatments were applied to whole root systems or selected root zones of plants with limited (1 μM) or sufficient (50 μM) P supply.

• Aluminum stimulated citrate efflux after 1–2 h; this response was not mimicked by a similar trivalent cation, La^{3+}. P deficiency triggered citrate release from mature cluster roots, whereas Al stimulated citrate exudation from the 5- to 10-mm subapical root zones of lateral roots and from mature and senescent cluster roots. Al-induced citrate exudation was inhibited by P limitation at the seedling stage, but was stimulated at later growth stages. Citrate exudation was sensitive to anion-channel blockers. Al treatments did not affect primary root elongation, but inhibited the elongation of lateral roots.

• The data demonstrate differential patterns of citrate exudation in *L. albus*, depending on root zone, developmental stage, P nutritional status and Al stress. These findings are discussed in terms of possible functions and underlying mechanisms.

Key words: aluminum, citrate exudation, cluster root, phosphorus deficiency, white lupin (*Lupinus albus*).

Introduction

Crop growth and yields are greatly reduced when crops are grown on acidic soils where phosphorus (P) deficiency and aluminum (Al) toxicity are two major factors limiting plant growth (Kochian *et al.*, 2004). In plants, numerous adaptive strategies have evolved in response to P deficiency and toxic Al concentrations in acidic soils. Root exudation of organic anions (e.g. citrate and malate) that mobilize sparingly available P forms and chelate toxic Al^{3+} in the rhizosphere has been identified as an effective mechanism by which to cope with P deficiency and Al toxicity (Ma *et al.*, 2001; Ryan *et al.*, 2001; Kochian *et al.*, 2004). White lupin (*Lupinus albus*) develops specialized bottlebrush-like lateral roots, referred to

as cluster roots, when grown under conditions of P deficiency (Gardner *et al.*, 1982), and this species has been widely used as a model system to study the morphology and physiology of cluster roots (Johnson *et al.*, 1994; Dinkelaker *et al.*, 1995; Keerthisinghe *et al.*, 1998; Neumann *et al.*, 1999; Pearse *et al.*, 2006). Cluster roots are characterized by a high capacity to release organic anions, such as citrate and malate (Neumann *et al.*, 1999; Watt & Evans, 1999a; Shen *et al.*, 2004). The organic anions, and citrate in particular, increase the availability of P to plants by mobilizing P bound to cationic sorption sites in the soil (Neumann & Martinoia, 2002; Shane & Lambers, 2005). Citrate exudation from cluster roots is stimulated by P deficiency and exhibits spatial and temporal variability in different stages of cluster root

development (Keerthisinghe *et al.*, 1998; Neumann *et al.*, 1999; Watt & Evans, 1999b). There is no close relationship between citrate efflux from cluster roots and intracellular citrate concentrations in white lupin (Keerthisinghe *et al.*, 1998; Neumann *et al.*, 1999), suggesting that membrane transport processes, rather than the internal concentrations, are the key steps controlling the exudation of citrate.

Organic acids, such as malic acid and citric acid, occur predominantly as divalent and trivalent anions at the cytosolic pH of 7.0–7.5, and movement of these anions out of the root cells is an energetically passive process resulting from a steep gradient in carboxylate concentration and the electrochemical potential across the plasma membrane. Therefore, exudation of organic anions from root cells is likely to be mediated by activation of anion channels permeable to malate and citrate in the plasma membrane of root cells, facilitating carboxylate efflux. The observation that citrate efflux from cluster roots of white lupin is sensitive to anion-channel blockers (Neumann *et al.*, 1999) is consistent with this proposition. The identification of whole-cell current carried by citrate efflux in cluster roots provides evidence in support of the involvement of anion channels in release of carboxylates (Zhang *et al.*, 2004). The enhanced exudation of citrate from cluster roots of white lupin is consistent with increased efflux of hydrogen (H^+), as a result of up-regulation of H^+-ATPases in the plasma membrane (Yan *et al.*, 2002) as well as stimulated efflux of other cations, including potassium (K^+), sodium (Na^+) and magnesium (Mg^{2+}) (Zhu *et al.*, 2005).

Phytotoxic Al species (Al^{3+}) are solubilized from aluminum silicates and aluminum oxides/hydroxides when soil pH drops below 5.5, and become major factors limiting crop growth (Kochian, 1995; Matsumoto, 2000). A number of plant species and genotypes within the same species display an inheritable resistance to Al (Ma *et al.*, 2001; Ryan *et al.*, 2001). Exudation of Al-chelating ligands such as carboxylates is an important mechanism of resistance to Al (Ma *et al.*, 2001; Ryan *et al.*, 2001; Kochian *et al.*, 2004, 2005). The Al-induced exudation of carboxylates is restricted to root apices, which are major sites for Al toxicity (Ryan *et al.*, 1995). Al-resistant species or genotypes exude a number of organic anions in the presence of external Al, including citrate, malate and oxalate (see reviews by Ryan *et al.*, 2001; Kochian *et al.*, 2004). Among the carboxylates released under Al stress (i.e. citrate, malate and oxalate), citrate forms the most stable complexes with Al, at a 1 : 1 ratio (Ryan *et al.*, 2001). Al–carboxylate complexes are nontoxic to plants, thus protecting root apices from Al damage (Kochian, 1995; Ma *et al.*, 2001). Similar to P limitation in *L. albus*, Al activates anion channels that are permeable to chlorine (Cl^-), malate and citrate in the plasma membrane of subapical root zones in Al-resistant wheat (*Triticum aestivum*) (Ryan *et al.*, 1997; Zhang *et al.*, 2001) and maize (*Zea mays*) (Kollmeier *et al.*, 2001; Piñeros & Kochian, 2001; Piñeros *et al.*, 2002). Furthermore, the Al-activated malate-permeable channels display comparable pharmacological profiles to Al-induced malate exudation from intact wheat roots (Ryan *et al.*, 1995; Zhang *et al.*, 2001). These findings indicate that activation of anion channels permeable to organic anions is a key event conferring Al resistance in wheat. A gene encoding the Al-activated malate transporter (*TaALMT1*) has been cloned (Sasaki *et al.*, 2004). Expression of *TaALMT1* in Xenopus oocytes and in rice (*Oryza sativa*), tobacco (*Nicotiana tabacum*) and barley (*Hordeum vulgare*) led to an Al-induced malate efflux in all these transgenics and increased Al resistance of the tobacco cells and barley plants (Delhaize *et al.*, 2004; Sasaki *et al.*, 2004).

White lupin is a plant species that is well adapted to moderately acidic soils with low P availability (Gladstones, 1970; Römer, 1994; Braum & Helmke, 1995) and has been widely used to characterize P-deficiency-induced exudation of organic anions (Neumann & Martinoia, 2002; Vance *et al.*, 2003; Shane & Lambers, 2005). However, little information is available about the interactions between P deficiency and Al toxicity in terms of exudation of organic anions. In the present study, we evaluated the sensitivity to Al of *L. albus* (cv. Kiev Mutant), which is well adapted to acidic soils in Western Australia, under conditions of varying P supply, and compared the patterns of carboxylate exudation induced by Al with those induced by P deficiency.

Materials and Methods

Plant growth

White lupin seeds (*Lupinus albus* L. cv. Kiev Mutant) were germinated and grown in plastic pots containing 6 l of an aerated nutrient solution (five plants per pot). The solution was composed of (µM): $Ca(NO_3)_2$ (2000), K_2SO_4 (700), $MgSO_4$ (500), KCl (100), H_3BO_3 (10), $ZnSO_4$ (0.5), $MnSO_4$ (0.5), $CuSO_4$ (0.2), $(NH_4)_6Mo_7O_{24}$ (0.01) and Fe-ethylenediaminetetraacetic acid (EDTA) (20). Phosphorus was supplied at 1 µM (P_1, limited) or 50 µM (P_{50}, sufficient) as KH_2PO_4. The pH of the solution was adjusted daily to 5.6, and the solution was renewed every 3 d. Plants were grown in a controlled environment with a light:dark regime of 14 : 10 h, a temperature of 28 : 18°C and a light intensity of 230 µmol m^{-2} s^{-1}.

Effect of Al and P deficiency on root elongation

The 7-d-old lupin plants, precultured at either 1 or 50 µM P, were exposed to the basal salt solution containing 0.5 mM $CaCl_2$ at pH 4.5 (BSS) with or without 20 µM $AlCl_3$ for 24 h, and elongation of primary and lateral roots was directly measured using callipers before and after the start of the treatments.

Collection of root exudates

To investigate the interactive effects of P deficiency and Al on citrate exudation, four experiments were conducted. In the

following experiments, four replicates for each treatment were used. Before collection of root exudates, roots were rinsed in BSS and incubated in BSS overnight in order to avoid Al complexation with other nutrients. A 10-ml root exudate solution was subsampled after collection of each replicate, and stored at –20°C for analysis of organic anions.

Expt 1. Al-induced citrate exudation at different developmental stages

White lupin plants grown for 10, 20 and 30 d at 1 or 50 μM P were exposed to 0 or 50 μM AlCl$_3$ in BSS and incubated in a 100-ml collection solution for 12 h (starting at 10:00 h) to collect root exudates. Afterwards, plants were harvested and separated into shoots, noncluster roots and cluster roots. The numbers of cluster roots were recorded. Cluster roots were defined as those portions of primary lateral roots bearing 'bottlebrush-like' clusters with a density of 10 or more rootlets per 10 mm (Johnson *et al.*, 1996). The harvested plant material was dried in an oven at 70°C for 1 wk and weighed.

Expt 2. Time course of Al-induced citrate exudation

The experiment was conducted to examine the time course of Al-induced citrate exudation, as described by Ma *et al.* (2001). The 25-d-old plants grown in 1 μM P were exposed to BSS with or without 50 μM AlCl$_3$ for collection of root exudates, and the solution was renewed every 3 h. Subsamples (10 ml) of root exudate solution were collected at 3, 6, 9 and 12 h after the start of the Al treatment. In addition, in order to determine whether Al-induced exudation of organic anions requires continuous exposure of roots to Al, an Al pulse was used to treat selected roots. Roots were exposed to 50 μM AlCl$_3$ in BSS for 3 h, and the root exudate solution was sampled, and then incubated in BSS for another 9 h after the roots had been thoroughly washed with BSS. As described for the first part of the experiment, the BSS collection solution without Al was renewed every 3 h and root exudate solution was collected at 6, 9 and 12 h.

An additional experiment was conducted to investigate the Al-induced citrate exudation pattern during the first 3 h of exposure to Al. Plants grown at 1 μM P were continuously exposed to 50 μM AlCl$_3$ in BSS for 3 h, and root exudates were collected at 0, 10, 20, 30, 60, 120 and 180 min after Al treatment. To determine whether Al-induced citrate exudation in white lupin is specific to Al, a similar trivalent cation, La^{3+}, was also used to treat white lupin roots under conditions identical to those used in the Al treatment.

Expt 3. The site of Al-induced citrate exudation

The plants were precultured in nutrient solution with 1 or 50 μM P for 25 d. Root segments (0–5 and 5–10 mm from the root apex) of lateral roots or mature and senescent cluster roots (approx. 1 cm in length) grown at 1 μM P were excised. As controls, the apices of lateral roots grown at 50 μM P were also excised from plants. The excised root segments were washed thoroughly with BSS as described by Ryan *et al.* (1995), and root exudates were collected after incubation of the excised roots in 1 ml of BSS with or without 50 μM AlCl$_3$ for 6 h. There were 15 root segments or four cluster roots in each replicate.

Expt 4. Effect of anion-channel antagonists on citrate exudation

Two typical anion-channel inhibitors, anthracene-9-carboxylic acid (A-9-C) and niflumic acid, were used to investigate the responses of citrate exudation induced by P deficiency and Al to these inhibitors. To avoid interaction between the anion-channel inhibitors and Al, the plants were first incubated in deionized water supplemented with A-9-C (50 μM) or niflumic acid (5, 20 or 50 μM) for 1 h, and then rinsed using deionized water to remove the inhibitors. Afterwards, the plants were immediately transferred into 100 ml of BSS with 0 or 50 μM AlCl$_3$ for 3 h to collect root exudates.

Analysis of organic anions

Organic anions in root exudates were analysed using a reversed-phase high-performance liquid chromatography (HPLC) system according to a previous report (Wang *et al.*, 2006, modified from Cawthray, 2003). Separation was conducted on a 250 × 4.6 mm reversed-phase column (Alltima C18, 5 Micron; Alltech Associates, Inc., Deerfield, IL, USA). The mobile phase was 25 mM KH$_2$PO$_4$ (pH 2.5) with a flow rate of 1 ml min^{-1} at 28°C, and detection of organic anions was carried out at 214 nm.

Results

Effect of P on biomass and of Al on root elongation

Root and shoot dry masses of white lupin grown in 1 μM P (P$_1$) did not differ from those of plants grown in 50 μM P (P$_{50}$) at 10 d (Fig. 1a,b). At 20 d, P supply at 50 μM P enhanced shoot biomass in comparison with P deficiency, but did not affect root biomass. However, at 30 d, the root and shoot dry masses of the P$_{50}$ plants increased by 58 and 150%, respectively, compared with those of the P$_1$ plants (Fig. 1a,b). Short-term (24-h) Al treatment had no impact on the biomasses of shoot and root (data not shown). Because biomass became dependent on the external P supply from 20 to 30 d (Fig. 1), this being associated with the formation of numerous cluster roots (Fig. 1b, inset), plants at this developmental stage were used to characterize the exudation of organic anions.

To examine the resistance of white lupin to Al, the effect of Al on elongation of primary and lateral roots of 7-d-old plants
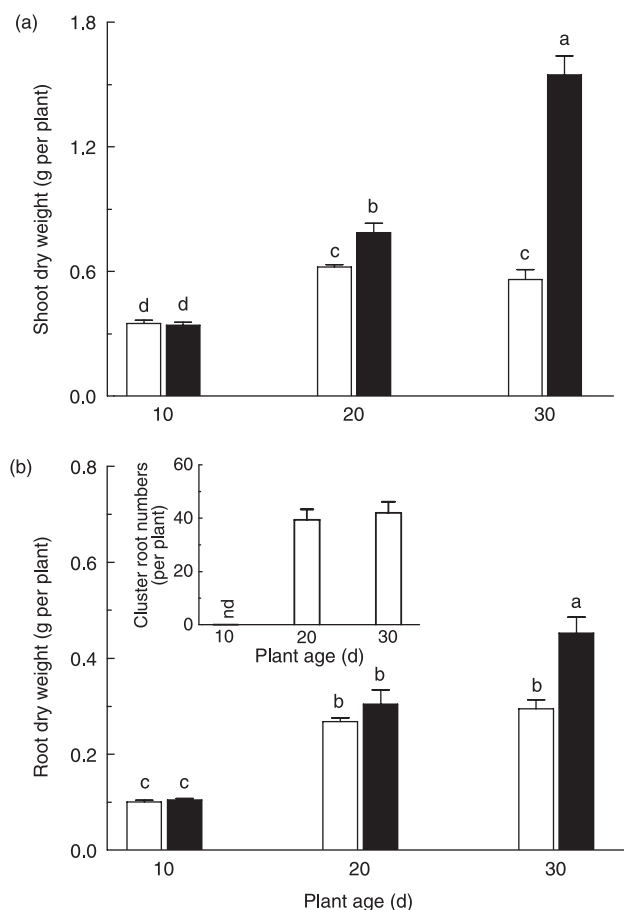
Fig. 1 Effect of phosphorus (P) supply on (a) shoot and (b) root dry weight. White lupin (*Lupinus albus*) plants were grown in nutrient solution containing 1 μM P (P_1, open bars) or 50 μM P (P_{50}, closed bars) for 10, 20 and 30 d. Data are means + standard errors of four replicates. Inset: P-deficiency-induced formation of cluster roots (nd, not detected). Means with different letters are significantly different ($P < 0.05$) with regard to P treatments.

Fig. 2 Effect of aluminum (Al) on elongation of (a) primary and (b) lateral roots of 7-d-old white lupin (*Lupinus albus*) plants. Plants grown in 1 or 50 μM P for 7 d were exposed to 0 or 20 μM $AlCl_3$ in 0.5 mM $CaCl_2$ (pH 4.5) for 24 h. The lengths of primary and lateral roots were measured before and after the Al treatment. Data are means + standard errors. Means with different letters are significantly different ($P < 0.05$) with regard to P and Al treatments.

grown at 1 and 50 μM P was investigated. There was no significant difference in the elongation rates of primary roots between the P_1 and P_{50} treatments (Fig. 2a). Treatment of roots with 20 μM $AlCl_3$ did not significantly reduce the elongation of primary roots in either P_1 or P_{50} plants (Fig. 2a). The lateral roots elongated much more slowly than the primary roots irrespective of P supply (Fig. 2b). Like the primary roots, elongation of 7-d-old lateral roots was relatively independent of the external P supply (Fig. 2b). However, unlike the primary roots, the growth rate of the young lateral roots of 7-d-old P_1 and P_{50} plants was inhibited by 47 and 60%, respectively, when they were exposed to the same concentrations of $AlCl_3$ as used for treatments of primary roots (Fig. 2b).

Exudation of organic anions in intact roots

To investigate exudation of organic anions in response to Al, interactive effects of P and Al on exudation of organic anions
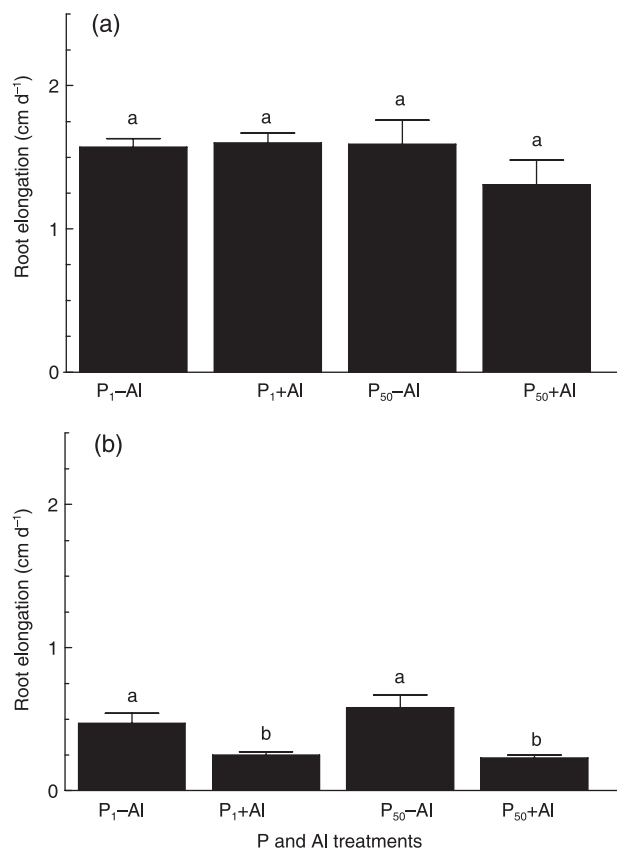
from white lupin roots at different developmental stages were studied. As shown in Fig. 3, no exudation of organic anions was detected in white lupin roots grown for 10 d in either P_1 or P_{50} solutions. However, when exposed to 50 μM $AlCl_3$, citrate exudation was observed in both 10-d-old P_1 and P_{50} plants, with the exudation rate being greater in P_{50} plants than in P_1 plants. No malate or oxalate was exuded from roots challenged with $AlCl_3$ (data not shown). P-deficiency-induced exudation of citrate was observed in the 20- and 30-d-old P_1 plants. Similar to the 10-d-old plants, Al stimulated efflux of citrate from both the 20- and 30-d-old P_1 and P_{50} plants (Fig. 3). The Al-induced citrate exudation was approximately twofold greater in the 30-d-old P_1 plants than in the 10-d-old P_1 plants (Fig. 3). By contrast, Al-induced citrate exudation showed the opposite pattern for the P_{50} plants, i.e. citrate exudation in the 10-d-old P_{50} plants was about twofold greater than that in the 30-d-old P_{50} plants (Fig. 3).

The pattern and time course of the Al-induced exudation rate of citrate were investigated using 25-d-old P_1 plants. The exudation of citrate from roots in response to 3 h of pulsed or
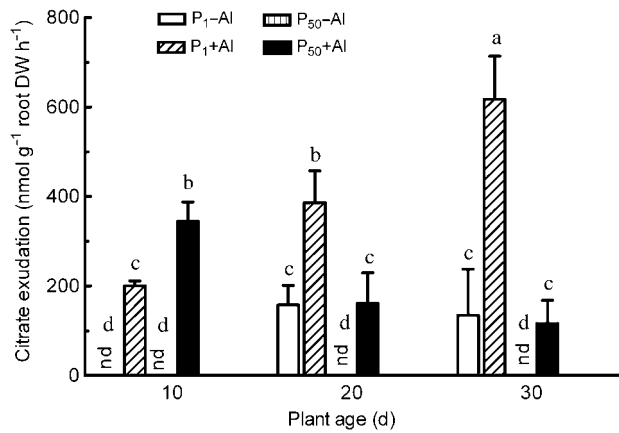
Fig. 3 Citrate exudation from white lupin (*Lupinus albus*) roots of different ages grown in P_1 (1 μM P) and P_{50} (50 μM P) solutions. Plants grown in nutrient solution supplemented with 1 or 50 μM P for 10, 20 and 30 d were exposed to 0 or 50 μM $AlCl_3$ in 0.5 mM $CaCl_2$ (pH 4.5) for 12 h, and root exudates were collected for analysis of organic anions. Data are means + standard errors of four replicates (nd, not detected). Means with different letters are significantly different (*P* < 0.05) with regard to aluminum (Al) and P treatments.

Table 1 Time course of citrate exudation from roots of white lupin (*Lupinus albus*) grown in P_1 (1 μM P) solution for 25 d in response to different aluminum (Al) treatments

Time course (h)	Citrate exudation (nmol g^{-1} root DW h^{-1})		
	−Al	+Al	3-h Al pulse
0−3	863.9 ± 226.8b	1334.1 ± 305.2a	1373.3 ± 249.6a
3−6	971.9 ± 163.9b	1528.6 ± 234.1a	1183.6 ± 26.3ab
6−9	802.6 ± 76.5b	1727.7 ± 234.4a	1140.1 ± 65.1ab
9−12	441.6 ± 115.9b	1300.4 ± 296.6a	1133.8 ± 411.7ab

For the 3-h Al pulse treatment, plant roots were first treated with $AlCl_3$ for 3 h, and thereafter incubated in 0.5 mM $CaCl_2$ solution without Al for the next 9 h. For the constant Al treatment (+Al), plant roots were continuously exposed to 50 μM $AlCl_3$ for 12 h. Citrate exudation from P_1 plants treated in the absence of Al was used as the control (−Al). Root exudates were collected at intervals of 3 h and the collection solution was renewed every 3 h. Citrate was analysed by high-performance liquid chromatography. Data are means ± standard errors of four replicates. Means with different letters within rows are significantly different (*P* < 0.05) with regard to Al treatments. DW, dry weight.

constant 50 μM $AlCl_3$ was higher than that without Al at 1 μM P. The Al-induced exudation of citrate in response to a 3-h pulsed Al treatment was reduced within 3 h following removal of Al from the incubation solution. Thereafter, the citrate release remained relatively constant, but was greater than that in the treatment without Al (Table 1). However, once activated by Al, the exudation rate of citrate reached maximal values when the roots were exposed continuously to Al throughout the experimental period (Table 1).

Monitoring of the citrate exudation pattern during the first 3 h of Al treatment also showed that a small amount of citrate was exuded from roots of P_1 plants after exposure to Al for 60 min (Fig. 4). Thereafter, a marked increase in the secretion rate of citrate was detected when the roots were treated with Al for 120 min, and Al-stimulated citrate exudation increased with continued exposure to Al treatment (Fig. 4). Note that citrate exudation from P_1 plants in the absence of Al at the beginning of this experiment was much lower than that found in the previous experiment (Fig. 4 vs Fig. 3). This difference is likely to be accounted for by the much shorter duration of collection of exudation in this experiment, as shown in Fig. 4 (i.e. 10, 20, 30 and 60 min vs 12 h), such that citrate exuded during the shorter collection period was not allowed to accumulate to the same extent.

The specificity of citrate exudation in response to Al was also studied by examining the effect of La^{3+}, as a similar cation, on the release of citrate (Zheng *et al.*, 1998). Citrate exudation from white lupin plants treated with 50 μM $LaCl_3$ was 99 ± 19% (mean ± standard error; *n* = 4) relative to the control in the absence of $LaCl_3$. Therefore, La^{3+} did not induce citrate exudation, demonstrating the specificity of the Al effect.
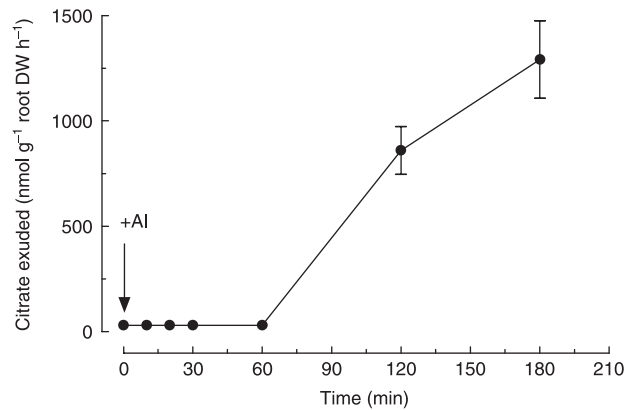

Fig. 4 Pattern of aluminum (Al)-induced citrate exudation with time. White lupin (*Lupinus albus*) plants grown in P_1 (1 μM P) solution for 25 d were exposed to 50 μM $AlCl_3$ in 0.5 mM $CaCl_2$ (pH 4.5) and root exudates were collected at different time intervals (10, 20, 30, 60, 120 and 180 min) within 3 h. Data are means ± standard errors of four replicates.

Exudation of organic anions from excised root segments and cluster roots

To determine the location of Al-indced citrate exudation, different segments of lateral roots were excised from plants grown in the P_1 and P_{50} solutions and exposed to Al. There were no significant differences in citrate exudation from segments located at 0−5 or 5−10 mm from the root apex between P_1 and P_{50} plants in the presence of Al (Fig. 5). Moreover, citrate exudation induced by Al treatment from root segments at 0−5 mm was not significantly different from
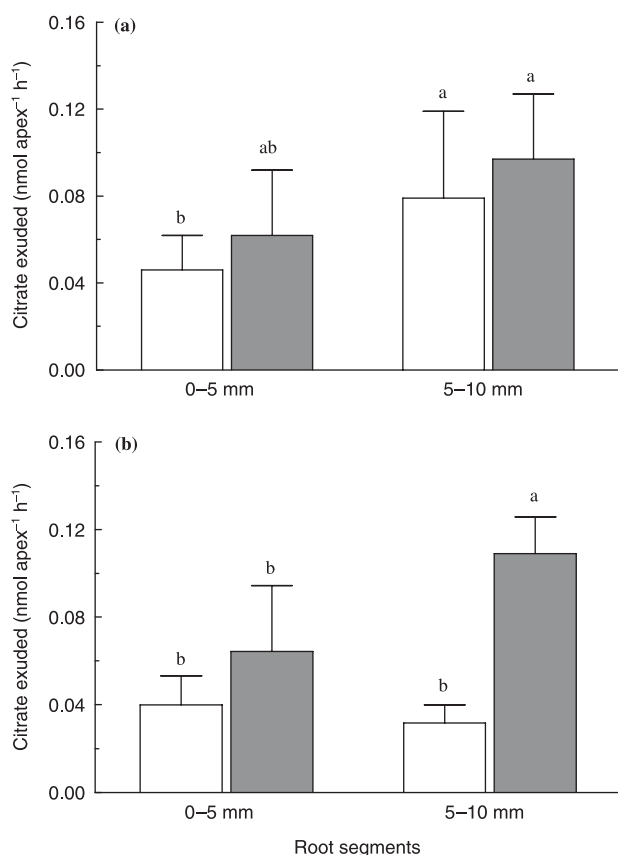
Fig. 5 Effect of phosphorus (P) deficiency and aluminum (Al) on citrate exudation from excised root segments of white lupin (*Lupinus albus*) grown in (a) P_1 (1 µM P) and (b) P_{50} (50 µM P) solutions. Plants were cultured in nutrient solution supplemented with 1 or 50 µM P for 25 d. Root segments (0–5 and 5–10 mm from the root apex) of lateral roots grown in 1 µM P were excised. As controls, the apices of noncluster lateral roots grown in 50 µM P were also excised from plants. After a thorough wash with 0.5 mM $CaCl_2$ (pH 4.5), the excised root segments were exposed to 0 µM $AlCl_3$ (–Al, open bars) or 50 µM $AlCl_3$ (+Al, closed bars) in 0.5 mM $CaCl_2$ (pH 4.5) for 6 h to collect root exudates for analysis of organic anions. Data are means + standard errors of four replicates. Means with different letters are significantly different ($P < 0.05$) with regard to Al treatments.

Fig. 6 Effect of aluminum (Al) on citrate exudation from excised cluster roots (CRs). Mature and senescent cluster segments (approx. 1 cm in length) were excised from white lupin (*Lupinus albus*) plants grown in P_1 (1 µM P) solution for 25 d, and incubated in 0 µM $AlCl_3$ (–Al, open bars) or 50 µM $AlCl_3$ (+Al, closed bars) in 0.5 mM $CaCl_2$ (pH 4.5) for 6 h after washing thoroughly with 0.5 mM $CaCl_2$ (pH 4.5). The root exudates were collected and organic anions in the collected root exudates were analysed. Data are means + standard errors of four replicates. Means with different letters are significantly different ($P < 0.05$) with regard to Al treatments.

Table 2 Effect of anion-channel blockers anthracene-9-carboxylic acid (A-9-C) and niflumic acid on phosphorus (P)-deficiency- and aluminum (Al)-induced citrate exudation of white lupin (*Lupinus albus*)

	Citrate exudation (% of control)	
Treatment	$P_1 - Al$	$P_1 + Al$
A-9-C	51.5 ± 16.1	135.8 ± 26.2
Niflumic acid	21.9 ± 4.0	38.0 ± 13.8

Twenty-five-day-old roots of plants grown in 1 µM P (P_1) were pretreated with 50 µM A-9-C and 5 µM niflumic acid in deionized water for 1 h. The plants were then exposed to 0 (–Al) or 50 µM $AlCl_3$ (+Al) in 0.5 mM $CaCl_2$ (pH 4.5) for 3 h, and root exudates were collected for analysis of citrate. Data are the percentage of citrate exuded from roots pretreated with A-9-C and niflumic acid relative to the controls (treatments without inhibitor). Data are means ± standard errors of four replicates.

that from root segments at 5–10 mm in P_1 plants (Fig. 5a). However, in contrast to P_1 plants, in P_{50} plants Al-induced citrate exudation from root segments at 5–10 mm was significantly ($P = 0.03$) greater than that from root segments at 0–5 mm (Fig. 5b). These results clearly demonstrate that Al induced citrate exudation in the 5–10-mm subapical root zone of P_{50} plants.

Mature cluster roots and senescent cluster roots differed in terms of citrate exudation under P deficiency; mature cluster roots released huge amounts of citrate, whereas senescent cluster roots hardly exuded any citrate in the absence of Al (Fig. 6). However, Al markedly stimulated citrate exudation particularly from mature but also from senescent cluster roots (Fig. 6). Furtherm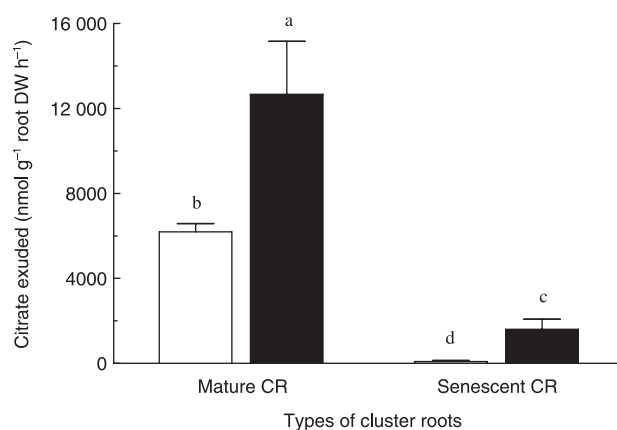ore, no exudation of malate or oxalate was observed from the mature and senescent cluster roots in the presence of Al (data not shown).

Effect of anion-channel inhibitors on citrate exudation

The anion-channel blocker anthracene-9-carboxylic acid (A-9-C) at 50 µM inhibited exudation of citrate from intact roots of the P_1 plants by *c.* 50% (Table 2). In contrast, the same treatment stimulated Al-induced citrate exudation (Table 2). Another widely used anion-channel antagonist in plant cells, niflumic acid, at 5 µM inhibited Al-induced and

P-deficiency-induced citrate exudation by *c.* 60 and 80%, respectively (Table 2). When niflumic acid at concentrations greater than 20 μM was used, both P-deficiency- and Al-induced citrate exudation was markedly stimulated (data not shown).

Discussion

Exudation of organic anions, particularly citrate, in response to P deficiency is well documented in white lupin (Keerthisinghe *et al.*, 1998; Watt & Evans, 1999a; Neumann & Martinoia, 2002). However, little is known about the patterns of carboxylate exudation from white lupin in response to Al toxicity and about possible interactions between these two factors.

No citrate was detectable in root washings of white lupin seedlings at 10 d after sowing (DAS), independent of the level of P supply (Fig. 3). In the later stages of plant development (20 and 30 DAS), citrate exudation was stimulated in the low-P treatment (P$_1$). This was associated with the formation of cluster roots, but citrate still remained undetectable in root washings of plants with sufficient P supply (P$_{50}$; Fig. 3). This finding is consistent with previous reports on white lupin, demonstrating that induction of citrate exudation occurs in the later stages of P limitation at 3–4 wk after sowing, and is mainly restricted to cluster roots (Neumann *et al.*, 1999; Watt & Evans, 1999a, Watt & Evans, 1999b).

In contrast, Al triggered root exudation of citrate but not of malate or oxalate, and the exudation was independent of the plant developmental stage as well as the level of P supply (Fig. 3). Al-induced citrate exudation was a rapid and specific response, which was detectable after a lag phase of 1–2 h (Fig. 4), and declined again after removal of Al^{3+} from the incubation medium (Table 1). Furthermore, the Al-induced citrate exudation was not mimicked by external application of La^{3+}, a cation with chemical similarity to Al^{3+}. This characteristic is typical of the so-called 'type II response' of Al-induced carboxylate exudation reported for many other plant species, such as soybean (*Glycine max*), rye (*Secale cereale*) and potato (*Solanum tuberosum*) (Ma, 2000; Neumann & Römheld, 2007). In contrast, the 'type I reaction' of Al-induced exudation of carboxylates is characterized by an instantaneous release of malate in wheat (Delhaize *et al.*, 1993) or of oxalate in buckwheat (*Fagopyrum esculentum*) (Zheng *et al.*, 1998) without a detectable lag phase after exposure to Al. However, alongside these similarities, white lupin also exhibits distinct differences in the patterns of carboxylate exudation as compared with other plant species.

1 Citrate exudation in white lupin was triggered by both P limitation and Al (Fig. 3), while in many other Al-resistant plant species and cultivars, carboxylate exudation is exclusively induced by Al (Nian *et al.*, 2003; Ligaba *et al.*, 2004a,b). P-deficiency-induced citrate exudation in white lupin is mainly restricted to mature cluster roots (Neumann *et al.*, 1999; What & Evans, 1999a,b) in the later stages of plant development (3–4 wk after sowing). By contrast,

Al-induced citrate exudation occurred in the subapical root zones all over the root system (Fig. 5), and was even found in mature and senescent cluster roots (Fig. 6).

2 Kollmeier *et al.* (2000) reported the highest rate of Al-induced carboxylate exudation in maize in the distal transition zone, located approx. 2 mm behind the root tip between the root meristem and the zone of root elongation, which was identified as the root zone with the highest sensitivity to Al. In white lupin, the highest rate of citrate release was located at least 5 mm behind the root tip (Fig. 5), suggesting differences in the anatomical organization of the root apex as compared with that of maize.

3 At the seedling stage (10 DAS), P limitation of white lupin was associated with a reduction in Al-induced citrate exudation by approx. 50%, compared with P-sufficient plants (Fig. 3). A similar impairment in expression of Al resistance mechanisms by P limitation has been previously reported for rape (*Brassica napus*) and cowpea (*Vigna savi*) (Ligaba *et al.*, 2004a; Akinrinde *et al.*, 2006). However, at later stages of plant development (20–30 DAS), P limitation in white lupin stimulated Al-induced citrate exudation per unit root biomass by 150–400% (Fig. 3). This could mainly be attributed to a particularly high rate of Al-induced citrate exudation in mature and senescent cluster roots (Fig. 6), which were formed in high abundance in P-deficient plants at 20 and 30 DAS (Fig. 1).

Interestingly, in white lupin seedlings, elongation of the main root was not affected by 20 μM AlCl$_3$ in the incubation medium, while lateral root elongation was inhibited by 50–60% (Fig. 2), demonstrating variability of Al sensitivity in different root types of the same plant. A similar differential sensitivity of primary and lateral roots to Al has been reported in soybean (Silva *et al.*, 2001). The exact mechanism underlying the difference in root elongation between primary and lateral roots in response to Al remains to be determined. Nevertheless, from the ecological point of view, higher Al sensitivity of the lateral roots could be a strategy to direct lateral root growth into the upper soil layers, where Al is usually more abundant in complexed and detoxified form with organic matter. Moreover, nutrient concentrations and mineralization rates are usually much higher in the top soil layers than in the subsoil. As cluster roots are formed along lateral roots (Neumann & Martinoia, 2002), preferential allocation of laterals to the upper soil layers would also direct cluster root formation into the soil zone giving the best chance of P acquisition. This may provide a sufficiently high P nutritional status to maintain the Al resistance of the main roots in deeper soil layers with higher concentrations of toxic Al forms.

The role of the high rate of Al-induced citrate exudation in cluster roots is not clear yet. An Al detoxification function would be plausible only for the growing juvenile clusters, as Al toxicity affects mainly the growth activity of root apices (Kollmeier *et al.*, 2001). However, mature or senescent cluster roots are highly differentiated root structures with very limited longevity, without growth activity and without active meristems

(Watt & Evans, 1999a; Neumann & Martinoia, 2002) that could act as targets for Al toxicity. Therefore, the high rate of Al-induced citrate exudation, particularly in mature root clusters, is probably not related to Al detoxification. Mature cluster roots are the major sites of citrate exudation under P limitation mediating mobilization of sparingly soluble soil P forms (Neumann & Martinoia, 2002). However, in acid soils with high soluble Al^{3+} concentrations, the formation of Al–citrate complexes will reduce the amount of citrate available for P mobilization in the rhizosphere of cluster roots. This would require a surplus of citrate exudation from mature root clusters to maintain efficient P acquisition, and in this case the additional Al-induced citrate exudation (Fig. 6) may be part of the P acquisition mechanism of cluster roots in acid soils, rather than a protective response to Al toxicity.

The mechanisms of citrate exudation in roots of white lupin are not well understood. Using a patch clamp approach, Zhang *et al.* (2004) recently identified anion channels probably responsible for P-deficiency-induced citrate release from cluster roots of white lupin, but a similar channel activity was also detectable in lateral roots of lupin plants with and without sufficient P supply. Accordingly, external application of certain anion-channel antagonists was able to inhibit citrate exudation (Table 2) both in P-deficient plants (anthracene-9-carboxylic acid and niflumic acid) and in plants exposed to Al (niflumic acid). However, the efficiency of the tested inhibitor compounds was not always consistent and was strongly dependent on the inhibitor concentration (niflumic acid) and on the physiological status of the test plants (anthracene-9-carboxylic acid). The channel inhibitor in some conditions stimulated citrate efflux, possibly as a result of the toxic nature of this drug, as mentioned previously by Zheng *et al.* (1998). This underlines the limitations of the use of ion-channel antagonists in whole-plant systems, which can provide only initial indications of an involvement of channels in membrane transport processes. However, the possibility of nonspecific side-effects means that conclusions cannot be drawn regarding the pharmacological characteristics of the transport mechanism.

Zhang *et al.* (2004) reported activation of the citrate channel in cluster roots of white lupin by hyperpolarization of the plasma membrane. Under P limitation, this may be mediated by the well-documented activation of the plasmalemma H^+-ATPase (Kania *et al.*, 2001; Yan *et al.*, 2002), which leads to rhizosphere acidification (Neumann *et al.*, 1999). Accordingly, root exudation of citrate in white lupin was also stimulated by artificial activation of the plasmalemma H^+-ATPase after fusicoccin application (Zhu *et al.*, 2005). Similarly, high concentrations of Al^{3+} in the external medium may contribute to hyperpolarization of the plasma membrane resulting from the effect of Al on H^+-ATPases (Rengel & Zhang, 2003), and thereby to channel activation under conditions of Al toxicity, as shown in the present study.

In conclusion, in this study differential patterns of citrate exudation were demonstrated in roots of white lupin depending on root zone, plant developmental stage, nutritional status and external stress factors, such as Al toxicity, P deficiency and their interactions. The physiological basis of these processes and the regulatory mechanisms involved in them warrant further investigation.

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