



RESEARCH PAPER

Aluminium resistance requires resistance to acid stress: a case study with spinach that exudes oxalate rapidly when exposed to Al stress

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Abstract

Spinach is a vegetable with a high oxalate concentration in its tissues. Oxalate efflux from spinach (*Spinacia oleracea* L. cv. Quanneng) roots was rapidly stimulated (within 30 min) by aluminium (Al) treatment. The efflux was constant within 6 h, but increased with increasing Al concentration. The efflux was confined to the root tip (0–5 mm), which showed a 5-fold greater efflux than the root zone distal to the tip (5–10 mm). Oxalate efflux could not be triggered by treatment with the trivalent cation lanthanum or by phosphorus deficiency, indicating that the efflux was specific to the Al treatment. All this evidence suggested that spinach possesses Al-resistance mechanisms. However, spinach was found to be as sensitive to Al toxicity as the Al-sensitive wheat line ES8, which had no Al-dependent organic acids efflux. The Al accumulated in the apical 5 mm of the roots of spinach which was also similar to that in the Al-sensitive wheat after 24 h treatment with 50 μ M AlCl_3 , indicating a non-exclusion mechanism. In addition, root elongation in spinach was significantly inhibited at pH 4.5, compared with that at pH 6.5. Based on this evidence, it is concluded that the sensitivity to acid stress in spinach could mask the potential role for oxalate to protect the plant roots from Al toxicity.

Key words: Aluminium resistance, organic acids, proton toxicity, soil acidity, *Spinacia oleracea* L.

Introduction

Spinach is a desirable leaf vegetable with good cooking qualities and a high nutritive value, containing many

important vitamins and minerals such as iron and magnesium. Although spinach generally favours non-acidic soils the pH of a large proportion of arable land, including much of southern China, is below pH 5.5 (Lin and Wang, 1993). Furthermore, the area of land affected by soil acidity is increasing due to atmospheric inputs of natural nitric and sulphuric acids, anthropogenic pollutants, and certain fertilization practices (Marschner, 1995).

Al toxicity is the most deleterious factor associated with soil acidity when the soil pH is decreased below 5.5. The toxic trivalent cations can rapidly inhibit root growth and reduce plant production in a large number of species investigated (Kochian, 1995). On the other hand, some plant species and cultivars of the same species have developed strategies to adapt to Al toxicity, and one of these strategies involves the efflux of organic acids (Ma *et al.*, 2001; Ryan *et al.*, 2001). The Al-dependent stimulation of organic acid efflux from roots has now been reported in more than ten species, and this response has been associated with an increase in Al resistance. These anions of organic acids (citrate, malate, and oxalate) released by roots are thought to chelate the toxic Al cations, and thus prevent them from interacting with the root apices. Consistent with this hypothesis is the observation that the application of organic acids to a solution containing toxic concentrations of Al will reduce Al toxicity and maintain root growth (Hue *et al.*, 1986; Ryan *et al.*, 1995a; Zheng *et al.*, 1998a).

The specific stimulation of oxalate efflux from the roots in response to Al stress plays an important role in the Al resistance of an Al-resistant buckwheat cultivar (*Fagopyrum esculentum* Moench cv. Jianxi). Moreover, since the oxalate concentration in buckwheat is very high, the amount of oxalate secreted only comprised 15% of the total

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soluble oxalate content in the root apex (Zheng *et al.*, 1998a). This led to the hypothesis that the higher oxalate content in other oxalate-accumulating plants could also contribute to enhance Al resistance. Spinach plants contain substantial quantities of oxalate (Eheart and Massey, 1962), but the physiological role of oxalate in spinach is uncertain, with some suggestions of possible involvement in osmoregulation or Ca homeostasis (Libert and Franceschi, 1987). In the present study, spinach was used to investigate the Al-stimulated oxalate secretion and its contribution to Al resistance. It was demonstrated, for the first time, that Al also triggers the specific efflux of oxalate from spinach roots, although spinach appeared to be very sensitive to Al stress. Spinach showed a high sensitivity to proton toxicity. It was concluded that resistance to both proton and Al toxicity are necessary for a plant to survive in acidic, aluminium-toxic soils.

Materials and methods

Plant materials and culture

Seeds of spinach (*Spinacia oleracea* L. cv. Quanneng) were fully imbibed with deionized water for 24 h, and then germinated at 26 °C in the dark. After germination, the seeds were transferred to a floating tray with a net bottom suspended in 5.0 l solution containing 0.5 mM CaCl₂ (pH 4.5). The solution was renewed daily. On day 4, seedlings of a similar size were removed to assess Al resistance. The remaining seedlings were transplanted after another 2 d to plastic pots containing 1.0 l aerated nutrient solution (8 seedlings per pot). The nutrient solution contained the following macronutrients (in mM): KNO₃, 1.0; Ca(NO₃)₂, 1.0; MgSO₄, 0.4; NH₄H₂PO₄, 0.2, and the following micronutrients (in μM): NaFeEDTA, 20; H₃BO₃, 3; MnCl₂, 0.5; CuSO₄, 0.2; ZnSO₄, 0.4; (NH₄)₆Mo₇O₂₄, 1. The solution was adjusted to pH 4.5 with 1 M HCl and renewed every 3 d. All the experiments were carried out in an environmentally controlled growth room with a 14/10 h 26/23 °C day/night regime, a light intensity of 150 μmol photon m⁻² s⁻¹, and a relative humidity of 70%.

Aluminium treatment

Prior to each experiment, the roots were rinsed by soaking in aerated 0.5 mM CaCl₂ solution at pH 4.5 overnight. In the dose-response experiment, 20-d-old seedlings were exposed to 0.5 mM CaCl₂ containing 0, 10, 25, 50, or 100 μM AlCl₃. All treatment solutions were adjusted to pH 4.5 with 0.1 M HCl. Root exudates were collected after a 6 h treatment. In the time-course experiment, seedlings were exposed to 0.5 mM CaCl₂ with 50 μM AlCl₃ (pH 4.5) and root exudates collected after 0.5, 1, 3, and 6 h.

Other treatments

Experiments were performed to determine whether phosphorus (P) deficiency or LaCl₃ treatment could stimulate the efflux of oxalic acid. For the P-deficiency experiment, 14-d-old seedlings were grown in nutrient solution devoid of P. Every other day seedlings were transferred to 0.5 mM CaCl₂ (pH 4.5) for 9 h to collect root exudates. After 8 d of P deprivation the seedlings were subjected to 0.5 mM CaCl₂ solution containing 50 μM AlCl₃ (pH 4.5) for 9 h and root exudates were collected. For the LaCl₃ experiments, 20-d-old seedlings were subjected to 0.5 mM CaCl₂ with 50 μM LaCl₃ (pH 4.5) and root exudates collected after 6 h.

Location of oxalic acid secretion site

In experiments designed to study the spatial aspects of oxalate efflux, excised root segments were taken from 4-d-old seedlings. The apical 0–5 mm region and the next 5 mm segment farther back (5–10 mm from the root apex) were excised and collected in Petri dishes containing 20 ml Milli-Q water. After 1 h (wash 3 times each of 20 min) the root apices were transferred to a 10 ml centrifuge tubes containing 5 ml 0.5 mM CaCl₂ solution, pH 4.5. Al treatment was initiated by replacing the solution with 5 ml 0.5 mM CaCl₂ solution containing 50 μM AlCl₃. The treatment was conducted in the dark for 3 h, during which the tubes were gently shaken by hand at 10 min intervals. After the treatment period, the organic acids concentrations in the solution and in the root tissue were measured according to Zheng *et al.* (1998a).

Collection and analysis of root exudates

Exudates collected from either intact roots or excised root tissues were passed through a cation-exchange column (16 mm × 14 cm) filled with 5 g of Amberlite IR-120B resin (H⁺ form, Muromachi Chemical, Tokyo, Japan), followed by an anion-exchange column (16 mm × 14 cm) filled with 2 g of Dowex 1 × 8 resin (100–200 mesh, formate form) in a cold room. The organic acids retained on anion-exchange resin were eluted with 15 ml of 1 M HCl, and the eluate was concentrated to dryness by a rotary evaporator (40 °C). After the residue was redissolved in 50 mM HClO₄ solution, the concentration of organic acids was analysed by HPLC. Aliquots (50 μl) were analysed on a Shodex RSpak KC-811 cation exchange column (300 × 8 mm) using 50 mM HClO₄ solution as mobile phase at 1.0 ml min⁻¹ and 50 °C.

Al resistance, Al accumulation and proton toxicity

Aluminium resistance was assessed by measuring root length with a ruler before and after an Al treatment. The Al-dependent inhibition of root growth in spinach was compared with a wheat cultivar (ES8, an Al-sensitive line frequently reported in the literature). Plant roots of 4-d-old seedlings were exposed to CaCl₂ solution, pH 4.5, containing 0 or 50 μM AlCl₃. After 24 h treatment, 5 mm root tips were excised and placed in a 1.5 ml Eppendorf tube containing 2 M HCl for total Al measurement (Osawa and Matsumoto, 2001). The Al concentrations in the solutions were determined by Inductively Coupled Plasma (ICP) (AIRIS/AP, TJA, Thermo, USA).

To assess the effect of different pH values on root growth, 4-d-old spinach plants were subjected to 0.5 mM CaCl₂ solution with the pH value adjusted to 6.5, 5.5, 5.0, or 4.5. Root lengths were measured with a ruler, both before and after treatments (24 h).

Results

Efflux of oxalate from spinach roots

Aluminium treatment stimulated the efflux of oxalate from the roots of spinach seedlings, and no other organic acids was detectable in the exudates. The magnitude of the oxalate efflux increased with increasing Al concentration, reaching about 12 μmol g⁻¹ DW after 6 h at 100 μM (Fig. 1). The roots showed severe visible Al-induced damage at concentrations above 50 μM AlCl₃, and so in all further experiments 50 μM Al was used. Oxalate was detected in the bathing solution within 30 min of adding Al, and efflux was relatively constant for at least 6 h (Fig. 2).

The spatial variation in oxalate efflux was investigated using excised root tissue (Fig. 3). Al-stimulated efflux of

oxalate from the apical 0–5 mm region was $0.76 \text{ nmol tip}^{-1}$ which was 5-fold greater than from the next contiguous 5 mm region. This pattern is consistent with previous reports on wheat (Ryan *et al.*, 1993, 1995a). Furthermore, the amount of oxalate exuded was much lower than that in the root tissue (only about 5% and 2.7% of soluble oxalate in the 0–5 mm and 5–10 mm segments were secreted during 3 h, respectively) (Fig. 3).

Specificity of Al-induced oxalate efflux

It was investigated whether other toxic trivalent cations or the onset of P deficiency could also stimulate oxalate efflux from spinach roots. No oxalate efflux was detected after 8 d of P deprivation. However, when the P-deficient roots were exposed to Al treatment on day 9, a significant amount of oxalic acid was released from the roots (Fig. 4). Furthermore, Al-induced efflux of oxalate from roots continued even after 10 d of intermittent Al treatment (Fig. 4). Exposure to $50 \text{ }\mu\text{M La}^{3+}$ failed to induce any significant oxalate efflux (Fig. 5), whereas addition of $50 \text{ }\mu\text{M Al}$ to the same roots induced an efflux of oxalate of approximately $8 \text{ }\mu\text{mol g}^{-1} \text{ DW}$ after 6 h.

Al-induced inhibition of root growth

The inhibition of root elongation by a 24 h treatment in $50 \text{ }\mu\text{M AlCl}_3$ was not significantly different for spinach and wheat (83% and 89% inhibition, respectively) (Fig. 6A). The Al content in the apical 5 mm of roots in spinach plants reached $0.25 \text{ }\mu\text{g tip}^{-1}$ which was similar to that in the Al-sensitive wheat line ES8 (Fig. 6B).

Proton toxicity on root growth

The root elongation was 23.1 mm after 24 h when spinach roots were grown in 0.5 mM CaCl_2 at pH 6.5 for 24 h. However, root growth was drastically decreased with decreasing growth medium pH, and the root elongation was inhibited by 78% at pH 4.5 compared with that at pH 6.5 (Fig. 7).

Discussion

The specificity of oxalate efflux in spinach

It was shown that Al stimulated the efflux of oxalate from spinach roots in a concentration-dependent manner (Fig. 1). The rates observed at $100 \text{ }\mu\text{M AlCl}_3$, i.e. $12 \text{ }\mu\text{mol g}^{-1} \text{ DW}$ after 6 h are equivalent to approximately $200 \text{ nmol g}^{-1} \text{ FW h}^{-1}$ which is higher than oxalate efflux from buckwheat and similar to the efflux of citrate from tobacco and *Cassia tora* (Ryan *et al.*, 2001). Time-course data (Fig. 2) indicate that oxalate efflux was a quick response with no lag phase of induction. This rapid activation of organic acids efflux by Al is characteristic of a Type 1 response (Ma *et al.*, 2001), which has been observed in a number of other plant species

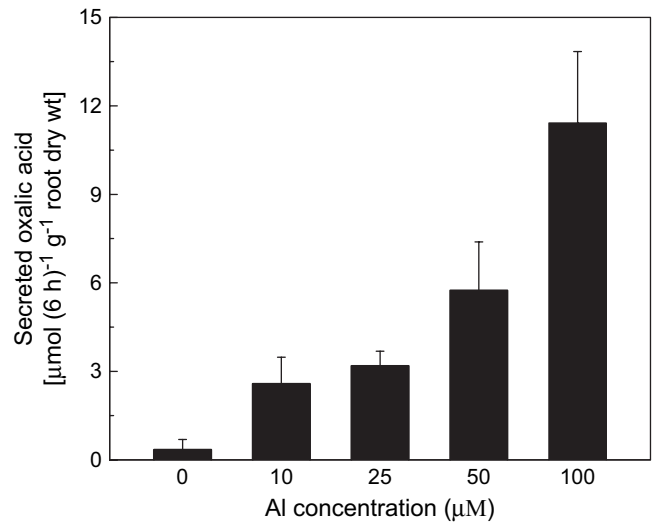


Fig. 1. Effect of Al concentration on oxalate efflux from spinach roots. Seedlings were subjected to 0.5 mM CaCl_2 solution containing 0 (control), 10, 25, 50, or $100 \text{ }\mu\text{M AlCl}_3$. All treatment solutions were adjusted to pH 4.5 with 1 M HCl . Root exudates were collected after a 6 h treatment and analysed by HPLC. Data are means \pm SD ($n=3$).

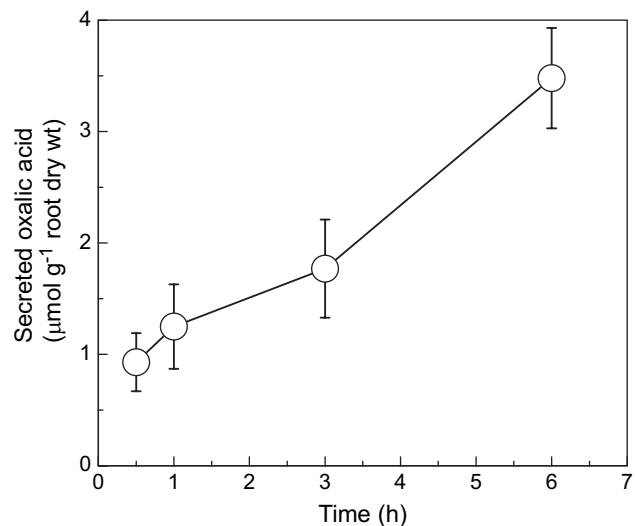


Fig. 2. Time-course of oxalate efflux from spinach roots in response to $50 \text{ }\mu\text{M AlCl}_3$ (pH 4.5). Root exudates were collected at time points of 0.5, 1, 3, and 6 h and analysed by HPLC. Data are means \pm SD ($n=3$).

including buckwheat (oxalate) (Zheng *et al.*, 1998a), tobacco (citrate) (Delhaize *et al.*, 2001), and an Al-resistant genotype of wheat (malate) (Ryan *et al.*, 1995b). Furthermore, neither P deficiency (Fig. 4) nor LaCl_3 (Fig. 5) stimulated oxalate efflux, indicating that the response was specific to Al.

These results suggest that Al-activated anion channels are probably present in spinach roots, just as they occur in wheat and maize (Kollmeier *et al.*, 2001; Ryan *et al.*, 1997; Zhang *et al.*, 2001). As far as is known, there are no reports describing oxalate-permeable channels from any system,

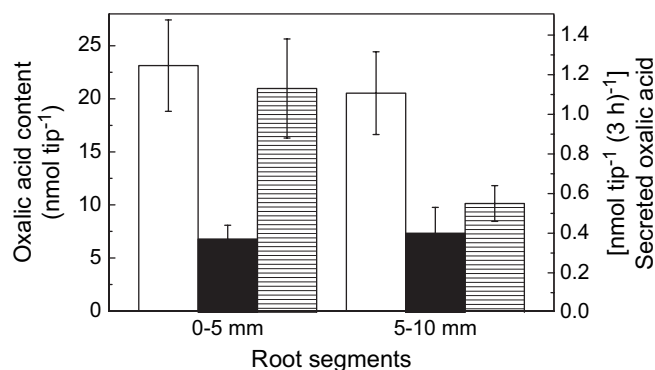


Fig. 3. Al-induced secretion of oxalate from different sections of spinach roots. Root segments excised from the root apex (0–5 mm) and the next segment (5–10 mm) were washed in 0.5 mM CaCl₂ solution, pH 4.5, and then transferred to a similar solution containing 0 or 50 μM AlCl₃. After 3 h the root exudates were collected. The oxalic acid content in root apices was analysed as described in the text. Values are means of three replicates ±SD. Shown are the oxalic acid content in roots (white bars), oxalic acid secreted by the roots not treated with Al (black bars), and oxalic acid secreted by roots treated during Al treatment (shaded bars).

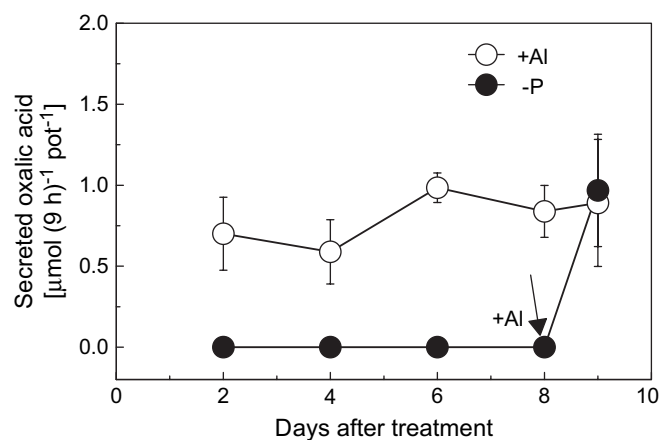


Fig. 4. Efflux of oxalate in response to P deficiency. Ten-day-old seedlings were removed from a full nutrient solution and transferred to a nutrient solution devoid of P (filled circles). Every other day the seedlings were exposed to 0.5 mM CaCl₂ solution (pH 4.5) for 9 h. At the end of the P-deficient treatment, the seedlings were exposed to 0.5 mM CaCl₂ containing 50 μM AlCl₃ (pH 4.5) for 9 h and root exudates collected (open circles). Seedlings were treated with Al every other day for 9 h by exposing them to 0.5 mM CaCl₂ with 50 μM AlCl₃ (pH 4.5). Root exudates were collected after each treatment and analysed by HPLC. Data are means ±SD ($n=3$).

and so characterizing this channel and comparing it with the Al-activated malate transporter (ALMT1) recently cloned from wheat (Sasaki *et al.*, 2004) would be an exciting area for future research. Interestingly, spinach plants only released oxalate during Al treatment, even though their roots contained significant amounts of water-soluble malate (about 20 μmol g⁻¹ root FW). This might indicate the transporter facilitating oxalate is permeable to oxalate only, and not to malate.

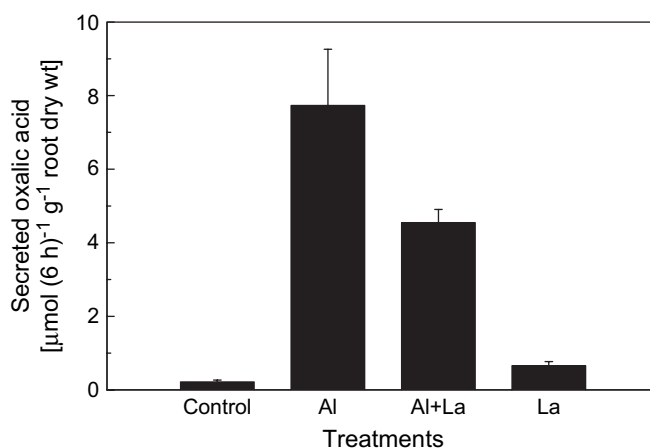


Fig. 5. Effect of Al and La on the oxalate efflux. Seedlings were subjected to 0.5 mM CaCl₂ without Al (control), with 50 μM AlCl₃, with 50 μM LaCl₃, or with 50 μM AlCl₃ and 50 μM LaCl₃. All treatment solutions were adjusted to pH 4.5 with 1 M HCl. Root exudates were collected after a 6 h treatment and analysed by HPLC. Data are means ±SD ($n=3$).

Does oxalate efflux enhance Al resistance in spinach?

Two strategies for Al resistance in plants have been suggested (Taylor, 1991; Kochian, 1995). One is the exclusion of Al from the root tips (exclusion mechanism), and the other is tolerance of the Al that enters the plant (internal tolerance mechanism). Recently, the Al-induced efflux of organic acids from root apices has been widely considered as a mechanism whereby some species and genotypes can acquire a greater degree of resistance to Al toxicity (Ma *et al.*, 2001; Ryan *et al.*, 2001). Mariano and Keltjens (2003) found that Al resistance in eight maize genotypes was strongly related to citrate secretion rate by the root apex. Furthermore, a good correlation between organic acid secretion and Al resistance has been established in a range of wheat genotypes and other species as well (Ryan *et al.*, 1995a; Ma and Furukawa, 2003). On the other hand, both complexation of Al with organic compounds and sequestration into the vacuole have been identified as internal detoxification mechanisms (Shen *et al.*, 2002). In the present study, although Al stimulated a substantial and constant efflux of oxalate from spinach roots, it was found that spinach plants were relatively sensitive to Al stress; in fact spinach was as sensitive as the Al-sensitive wheat ES8 (Fig. 6A). This finding was surprising since previous reports on buckwheat (Zheng *et al.*, 1998a, b) have suggested that oxalate efflux contributes to greater Al resistance in that species due to the high stability constant for the Al:oxalate complexes. There are also other reports that do not support the hypothesis that organic acids efflux enhances Al resistance of plants (Ishikawa *et al.*, 2000; Parker and Pedler, 1998; Wenzl *et al.*, 2001). Citrate efflux could not explain the difference in Al resistance in some maize cultivars (Piñeros *et al.*, 2005). Recent finding showing no correlation

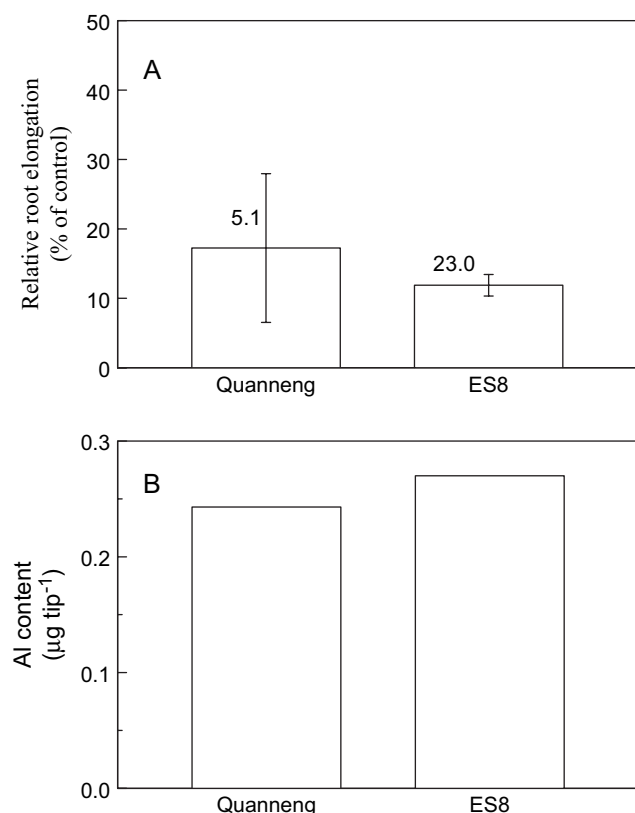


Fig. 6. Effect of 50 μM AlCl_3 on root elongation and Al accumulation in spinach and wheat (cv. ES8) roots. Seedlings were exposed to 0.5 mM CaCl_2 (pH 4.5) with or without 50 μM AlCl_3 for 24 h. Root lengths were measured and root tips excised for measurements of Al content. Absolute control growth rates ($\text{mm} [24 \text{ h}]^{-1}$) are indicated above the bars. Values in (A) are means \pm SD ($n=14$) for root elongation, and in (B) are means of total Al contents of 14 tips.

between Al tolerance and oxalate efflux in seven cultivars of buckwheat indicates that oxalate efflux plays only a minor role in the high Al tolerance of that species (Zheng *et al.*, 2004). Therefore, the role of organic acid secretion in Al resistance should not be overemphasized, as alternative mechanisms may play an equal or even more important role in some plants; further experimental support is required.

The root apex is where cell division and elongation occur, and it is there that Al causes greatest damage (Ryan *et al.*, 1993). Thus, theoretically, organic acid efflux would need to be released from this region of the root to have any protective effect from Al stress. The present results demonstrate that the efflux of oxalate was confined to the root apex (Fig. 3) which is consistent with the sensitive site for Al toxicity. A close negative relationship between Al concentrations in root tips and relative root elongation (an index of Al resistance) has previously been reported for maize (Lluganyi *et al.*, 1994) and wheat (Rincon and Gonzales, 1992). Recently, similar results were also found in Chinese triticale breeding lines (Yang *et al.*, 2005). However, spinach still accumulated a substantial quantity of

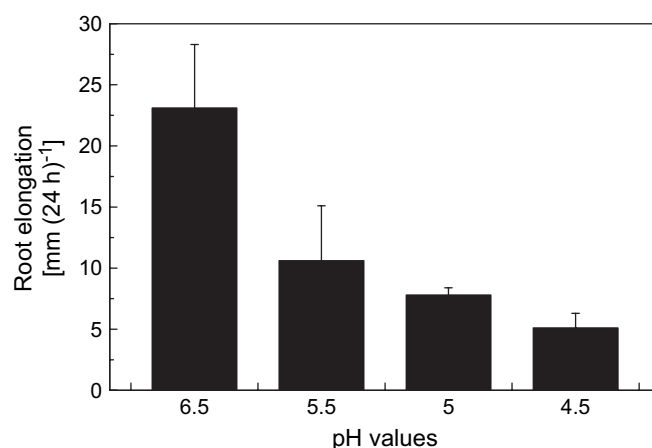


Fig. 7. Effect of different pH values on root elongation in spinach. Seedlings were exposed to 0.5 mM CaCl_2 solutions with different pH values for 24 h. Root lengths were measured before and after treatments with a ruler. Data are means \pm SD ($n=14$).

Al in the apical 5 mm of its roots which was similar to that in the Al-sensitive wheat ES8 (Fig. 6B) with much less secretion of organic acids. This result is contrary to the prevailing theory that organic acid efflux protects plants by preventing Al from entering the root tissues. No relationship between Al content and Al resistance in 37 maize cultivars differing in Al resistance was reported either (Horst *et al.*, 1997). Thus, the relationship between Al accumulation in root tips and Al resistance does not hold for all species.

Does proton toxicity mask the potential role of oxalate efflux?

Al toxicity is not the only stress in acid soils; among others, proton and manganese toxicity as well as phosphorus deficiency are also common (Marschner, 1995). Root growth in spinach was significantly inhibited at pH 4.5 compared with growth at more neutral conditions (Fig. 7), and it is possible that this high background stress prevented the protective effects of oxalate efflux from being fully realized. In contrast to the large amount of literature on Al-toxicity mechanisms in plants, little is known about how plants cope with proton stress. It was reported that proton toxicity inhibits root elongation in several plant species (Kinraide and Parker, 1987; Koyama *et al.*, 1995), and that Ca displacement by protons is part of the toxic action of proton toxicity (Kinraide *et al.*, 1994). Recently, Koyama *et al.* (2001) provided evidence that the primary target of proton toxicity may be linked to the disturbance of pectic-polysaccharide crosslinks, where Ca plays a key role. Given the Ca displacement by protons, it is not surprising that oxalate efflux does not exclude Al from roots, or enhance Al resistance in spinach plants, because one of the candidates of Al toxicity might also be linked to Ca displacement (Reid *et al.*, 1995), and a large proportion of

Al is adsorbed by the negatively charged cell-wall pectins. Further analysis is needed to test this possibility. Nonetheless, additional research on adaptation to proton stress is needed, because it is likely that resistance to Al and resistance to protons are controlled by separate mechanisms (Lazof and Holland, 1999; Kidd and Proctor, 2001).

In conclusion, it was found that the efflux of oxalate is a specific response to Al stress in spinach roots. Nonetheless, the spinach plants were relatively sensitive to Al stress. The influence of pH itself on root growth may have masked the potential for oxalate to protect the plants from Al toxicity. It was concluded that resistance to both proton and Al toxicity are necessary for a plant to survive in acidic, aluminium-toxic soils.

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