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The amelioration of aluminium toxicity by silicon in wheat (*Triticum aestivum* L.): malate exudation as evidence for an *in planta* mechanism

Kay M. Cocker*, David E. Evans, Martin J. Hodson

School of Biological and Molecular Sciences, Oxford Brookes University, Gipsy Lane, Headington, Oxford, OX3 0BP, UK

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Abstract. Two wheat (*Triticum aestivum* L.) cultivars, one aluminium tolerant (Atlas 66) and one sensitive (Scout 66), were grown in a continuous-flow culture system (≤pH 5.0) containing aluminium (0–100 μM) and silicon (0–2000 μM) in factorial combination. Treatment with silicon resulted in a highly significant amelioration of aluminium toxicity as assessed by root growth in both cultivars. Amelioration was influenced by wheat cultivar and silicon concentration, as 2000 μM silicon significantly ameliorated the toxic effects of 100 μM aluminium in Atlas 66, and only 5 μM silicon alleviated the effect of 1.5 μM aluminium on Scout 66. Nutrient medium pH was critical, as an amelioration by silicon was apparent only at pH > 4.2 for Atlas 66, and at pH > 4.6 for Scout 66. Silicon neither reduced levels of toxic aluminium species in the growth solutions, nor the amount of aluminium taken up by roots. In experiments to assess exudation of malate by Atlas 66 roots treated with 100 μM aluminium, the presence of 2000 μM silicon (pH 4.6) was found to have a negligible effect on exudation. In contrast, citrate, a known aluminium chelator, reduced aluminium-induced exudation of malate at 5–40 μM and completely inhibited it at 100 μM citrate. The results indicate that silicon does not reduce aluminium phytotoxicity as a result of aluminium/silicon interactions in the external media, and that the mechanism of amelioration has an *in planta* component.

Key words: Aluminium toxicity – Hydroxyaluminosilicate – Malate – Root length – Silicon – *Triticum* (Al toxicity)

Introduction

The chemical form of aluminium in solution determines its toxicity to plants, and rhizotoxic species include Al^{3+}, $Al(OH)^{2+}$, $Al(OH)_2^+$, and polymeric Al_{13} (Parker et al. 1989). The speciation of aluminium in solution is pH dependent, and solubility is increased under acidic (pH < 6.0) and alkaline (pH > 8.0) conditions (Driscoll and Postek 1995). Whereas aluminium is renowned in plant biology for its toxicity, silicon is a beneficial element (Epstein 1994). Below pH 9.0, silicon exists as silicic acid ($Si(OH)_4$), the form of silica absorbed by plant roots (Jones and Handreck 1967). As only biologically available forms of aluminium are toxic, the ability of silicon to influence speciation and render aluminium non-toxic due to the formation of aluminium/silicon species is potentially important. Recently, the chemistry of aluminium and silicon interactions in solution has received much attention (Lumsdon and Farmer 1995; Exley and Birchall 1996). Aluminosilicate speciation is also determined by pH (Lindsay and Walthall 1995), and in solution it is believed that soluble species of hydroxyaluminium ions and silicic acid can form sub-colloidal hydroxyaluminosilicates (Exley and Birchall 1992, 1993). Silicon can ameliorate the toxic effects of aluminium in hydroponically grown sorghum (Galvez et al. 1987; Hodson and Sangster 1993), teosinte (Barceló et al. 1993), soybean (Baylis et al. 1994), and barley (Hammond et al. 1995), but not in rice, cotton, wheat or pea (see Hodson and Evans 1995).

Aluminium-stimulated increase in organic acid efflux from roots has been implicated as an aluminium-tolerance mechanism in a number of species. Foy and Lee (1987) proposed that superior aluminium tolerance in barley was correlated with the ability to maintain higher internal concentrations of organic acids (including malate) in the presence of aluminium. Delhaize et al. (1993) argued that exudation of malate by aluminium-tolerant genotypes of wheat may result in detoxification of aluminium.

The aims of this study were to investigate the conditions under which silicon reduces aluminium

* *Present address*: Crop and Disease Management Department, IACR-Rothamsted, Harpenden, Hertfordshire, AL5 2JQ, UK

Abbreviation: HAS = hydroxyaluminosilicate

Correspondence to: M.J. Hodson;
E-mail: mjhodson@brookes.ac.uk; Fax: 44 (1865) 483242

toxicity in two wheat cultivars, one aluminium tolerant (Atlas 66) and one sensitive (Scout 66). The effects of silicon and citrate (a known Al chelator) on aluminium-induced stimulation of malate exudation from the roots of Atlas 66 were also investigated. The results indicate that amelioration of aluminium toxicity by silicon is not due to a reduction in aluminium levels due to alumino-silicate formation in the bulk nutrient solutions, but has an *in planta* component.

Materials and methods

Plant culture. Seeds of *Triticum aestivum* L. (cv. Atlas 66, aluminium-tolerant, and cv. Scout 66, aluminium-sensitive) were sterilised with 5% sodium hypochlorite for 30 min, and washed with deionised water. Germination occurred in seed trays on absorbent paper saturated with deionised water, in darkness at 25 °C for 72 h. After 3 d, seedlings with roots 4.5 cm long were suspended in lids of darkened plastic tubs (12 plants per tub, 8 replicate tubs per treatment). Tubs contained 6.5 l of a background solution of 500 μM $Ca(NO_3)_2$ and 31 μM KCl. Appropriate test concentrations of $AlCl_3$ (0–100 μM) and $Na_2SiO_3 \cdot 5H_2O$ (0–2000 μM) were added and the pH was adjusted to either 4.2, 4.6 or 5.0. Solutions were prepared according to the method of Li et al. (1989). Tubs were aerated and supplied with a continuous flow of pH-adjusted test solution at the rate of approximately $3\,l \cdot d^{-1}$ via a multi-channel peristaltic pump (Watson-Marlow, Falmouth, Cornwall, UK). Tub pH was tested twice daily and readjusted if necessary with 0.1 M NaOH or 0.1 M HCl. Plants were grown for 4 d in an environmental growth cabinet (Swan Environmental, Worthing, UK) set at 25 °C on a 16 h/8 h light/dark cycle after which plants were harvested, and the lengths of the longest roots were measured.

Solution aluminium analysis. Growth solutions were monitored for concentrations of free aluminium using the morin method of Browne et al. (1990). Morin (2, 3, 4, 5, 7-pentahydroxy-flavone), is a pentaprotic acid with five functional groups which binds to monomeric aluminium species in solution. The resulting aluminium-morin complex fluoresces, and the intensity is dependent on free aluminium and morin concentration. All samples and standards were of an identical background composition (500 μM $Ca(NO_3)_2$ and 31 μM KCl and pH 4.2–5.0 depending on the experiment). Samples and standards were immediately analysed in a silica quartz cuvette on an F-2000 fluorescence spectrofluorimeter (Hitachi, Tokyo, Japan; excitation wavelength: 420 nm; emission wavelength: 510 nm; slit setting: 2 nm). The fluorescence intensity of the test sample solutions in comparison with the standards gave an indication of the concentration of free aluminium in solution.

Plant aluminium analysis. The technique of Allen et al. (1986) was modified to assess aluminium content. Aluminium was analysed using a Jobin Yvon 70c inductively coupled plasma (J-Y, Longjumeau, France).

MINTEQA2 – a chemical speciation model. MINTEQA2 (Allison et al. 1990) is a program used to predict the speciation of metals at equilibrium, and is run in association with PRODEFA2, an extensive database. Here PRODEFA2 was modified by inputting data concerning the pH and chemical composition of the nutrient media in which plants were grown. After modification and the subsequent creation of an input file, the program was initiated and speciation data generated.

Culture conditions for the malate study. The two wheat cultivars were cultured according to the method of Delhaize et al. (1993). Seeds were surface-sterilised in 100 ml 20% sodium hypochlorite containing 0.05% w/v sodium dodecyl sulphate for 2 h, and agitated on an orbital shaker (120 rpm). They were then transferred to a sterile environment and aseptic conditions were observed throughout the following treatment procedure. Seeds were thoroughly rinsed with ten 30-ml washes of deionised water. Six seeds were placed into a 50-ml conical flask that contained 30 ml of 500 μM $Ca(NO_3)_2$ and 31 μM KCl at pH 4.2, 4.6 or 5.0. Flasks were sealed and incubated at 25 °C for 5 d while rotating on an orbital shaker (120 rpm). After 5 d, flasks were transferred to a sterile environment and the control nutrient solution was decanted. Seedlings were rinsed with 40 ml of control media followed by 40 ml of appropriate filter-sterilised treatment solution, prior to the addition of a final 40 ml of treatment solution. Treatment solutions were $AlCl_3$ (0–100 μM) and $Na_2SiO_3 \cdot 5H_2O$ (0–2000 μM) in factorial combination [500 μM $Ca(NO_3)_2$ and 31 μM KCl comprised the background solution]. The effect of citrate (5–2000 μM), an aluminium chelator, on aluminium-stimulated exudation of malate was also investigated. The pH of all treatment solutions was assessed before addition to flasks and adjusted with sterile HCl or NaOH if required. Conical flasks were covered with aluminium foil and replaced onto the orbital shaker for 24 h.

Malate measurement. Malate secreted from root apices was measured after 24 h incubation by the method of Delhaize et al. (1993) and assessed on a Lambda 3B UV/VIS spectrophotometer using PECSS software (Perkin-Elmer, Beaconsfield, Bucks., UK). This method is a malate dehydrogenase assay, and the increase in A_{340} due to the production of NADH (as a result of NAD reduction) was monitored with time, and was directly proportional to the amount of malate in the sample. To verify the specificity of the malic acid assay it was tested for a response to citrate (0–2000 μM) and results were negative.

Data analysis. All experiments were repeated at least three times and the data analysed using analysis of variance and a least significant difference test ($P < 0.01$).

Results

Solution analysis. Bulk solutions were measured daily, and the stability of free aluminium levels indicated that biologically available aluminium is in solution even in the presence of silicon (Table 1).

MINTEQA2. The chemical speciation model MINTEQA2 indicated that precipitating aluminium/silicon compounds [those showing high positive (+) saturation indices] should be present only in the aluminium/silicon solutions of the Atlas 66 treatments, and not in the solutions bathing Scout 66 roots (Table 2).

Effects on root growth. In Atlas 66 the toxic effects of 100 μM Al were apparent at both pH 4.2 and 4.6 as roots were reduced in length (relative to controls) by

Table 1. Morin analysis of biologically available aluminium in solution. Data represent the mean remaining biologically available aluminium content of culture solutions (4 replicate tubs ± SE) after wheat plants had been grown for 4 d. The cultivar Scout 66 was grown in 1.5 μM Al (pH 5.0) or 1.5 μM Al + 5.0 μM Si (pH 5.0) and the cultivar Atlas 66 in 100 μM Al (pH 4.6) or 100 μM Al + 2000 μM Si (pH 4.6)

Treatment	Al only	Al + Si
Scout 66	1 μM ± 0.165	1.2 μM ± 0.21
Atlas 66	88 μM ± 3.69	91 μM ± 5.1

Table 2. Saturation indices of potentially relevant Al/Si mineral complexes. Information concerning pH, and the concentration of basal nutrients and aluminium and silicon solutions were entered into PRODEFA2 (the MINTEQA2 database) and the speciation of elements and compounds was calculated. Only those compounds with a high positive saturation index are shown

Treatment solutions	Minerals				
	Halloysite $Al_2Si_2O_5(OH)_4$	Kaolinite $Al_2Si_2O_5(OH)_4$	Leonhardite $Ca_2Al_4Si_8O_{24} \cdot 7H_2O$	Muscovite $KAl_2[AlSi_3O_{10}](OH)_2$	Pyrophyllite $Al_2Si_4O_{10}(OH)_2$
1.5 μM Al/5 μM Si pH 4.6	−4.293	−1.025	−16.648	−5.867	−4.303
1.5 μM Al/5 μM Si pH 5.0	−2.4	0.1	−11.2	−2.6	−2.4
100 μM Al/2000 μM Si pH 4.2	2.16	5.42	4.979	3.4	7.35
100 μM Al/2000 μM Si pH 4.6	4.4	7.7	11.1	7.2	9.6

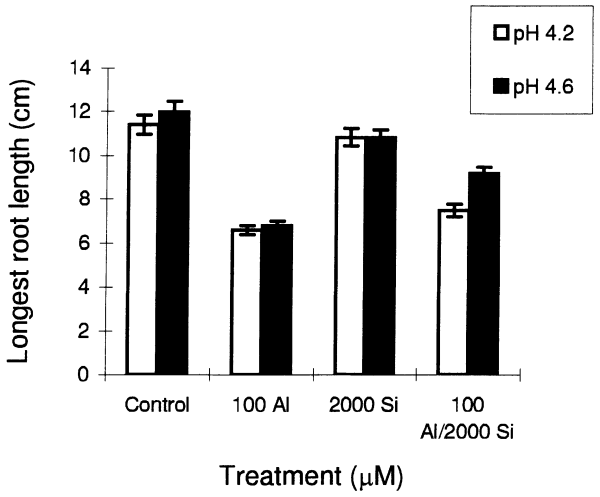

Fig. 1. Longest root length of Atlas 66 seedlings in response to 100 μM Al, 2000 μM Si and 100 μM Al/2000 μM Si at pH 4.2 and pH 4.6. Prior to treatment all roots were 4.5 cm in length. Data represent the mean values (±SE) of at least 96 seedlings (8 replicate tubs)

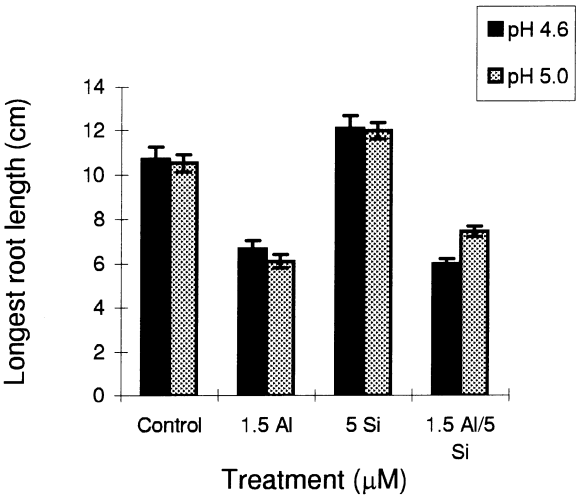

Fig. 2. Longest root length of Scout 66 seedlings in response to 1.5 μM Al, 5 μM Si and 1.5 μM Al plus 5 μM Si at pH 4.6 and pH 5.0. Prior to treatment all roots were 4.5 cm in length. Data represent the mean values (±SE) of at least 96 seedlings (8 replicate tubs)

42.1% at pH 4.2, and 43.3% at pH 4.6 (Fig. 1). There was no significant amelioration of aluminium-induced reduction in the longest root length at pH 4.2. At pH 4.6, however, roots of seedlings grown with 100 μM Al/2000 μM Si at pH 4.6 were 19.9% longer than aluminium-treated seedlings, and this increase was highly significant ($P < 0.01$). Figure 2 shows that Scout 66 required only 1.5 μM aluminium in order to inhibit root elongation and these roots were shorter than controls by 37.7% at pH 4.6, and 41.9% at pH 5.0. A highly significant ($P < 0.01$) amelioration of this toxicity was induced by 5 μM silicon at pH 5.0, where aluminium/silicon-treated roots were 12.7% longer than aluminium-treated roots, but this was not seen at pH 4.6. As silicon was added to solutions in the form of $Na_2SiO_3 \cdot 5H_2O$, the influence of sodium on the root growth of aluminium-treated plants was also assessed, and was found to be insignificant (Cocker 1997).

Aluminium analysis of roots. Aluminium content of roots increased in aluminium treatments, but there was no significant difference ($P < 0.01$) between the total aluminium content of aluminium and aluminium/silicon-treated roots irrespective of pH (Fig. 3).

Malate exudation experiments. The effect of aluminium treatment on malate exudation in intact seedlings was determined over 24 h for Atlas 66, and results are expressed as μM malate per 40-ml flask (Fig. 4). Seedlings treated with control nutrient solution did not exude malate. The concentration of aluminium required to stimulate malate efflux was 10 μM, and malate exudation increased with aluminium concentration. Malate secretion was also investigated in Scout 66, but none was detected. To test the hypothesis that silicon can form complexes with aluminium which result in the reduction of biologically available levels of aluminium, Atlas 66 was treated with 100 μM aluminium combined with 2000 μM silicon (Fig. 5). There was no significant difference ($P < 0.001$) in the level of malate produced in the presence of aluminium alone, and in aluminium/silicon treatments either at pH 4.2 or pH 4.6. To assess the effect of chelation on aluminium-induced exudation of malate, plants were treated for 24 h with 100 μM

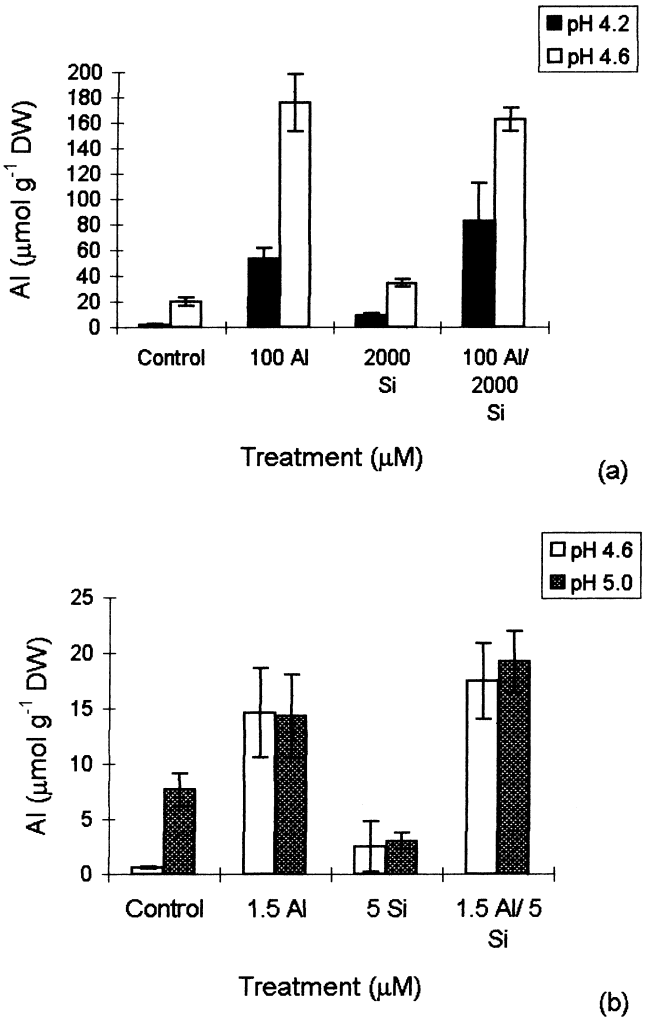

Fig. 3. **a** Aluminium content ($\mu mol \cdot g^{-1}$ DW) of Atlas 66 roots treated with 0–100 μM Al in factorial combination with 0–2000 μM Si at pH 4.2 and 4.6. **b** Aluminium content ($\mu mol \cdot g^{-1}$ DW) of Scout 66 roots treated with 0–1.5 μM Al in factorial combination with 0–5 μM Si at pH 4.2 and 4.6. Data represent the means (± SE) of five replicate seedlings

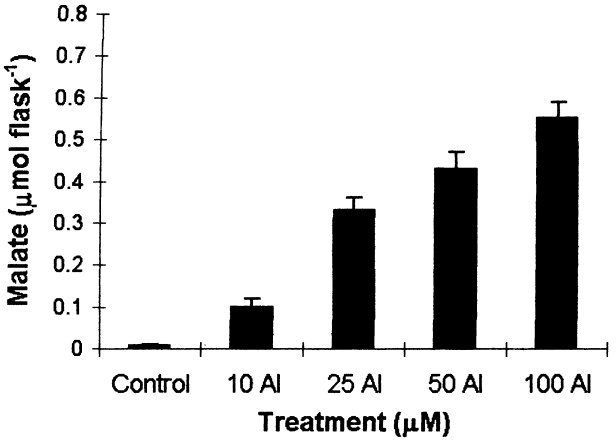

Fig. 4. Malate secreted from roots of Atlas 66 seedlings after 24 h treatment with Al (0–100 μM) at pH 4.6. Data represent the means of at least 12 replicate flasks (± SE) per treatment

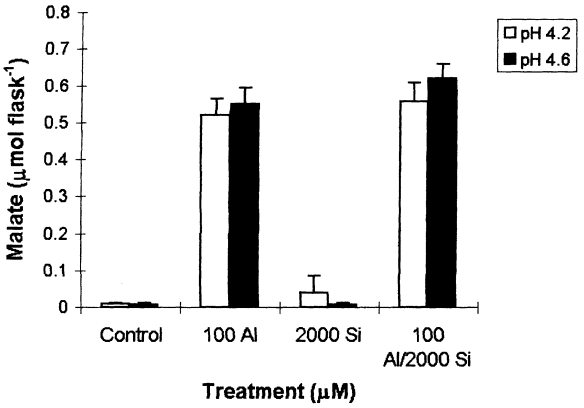

Fig. 5. Malate secreted from roots of Atlas 66 seedlings after 24 h treatment with 100 μM Al, 2000 μM Si and 100 μM Al plus 2000 μM Si at pH 4.2 and 4.6. Data represent the means of at least 30 replicate flasks (± SE) per treatment

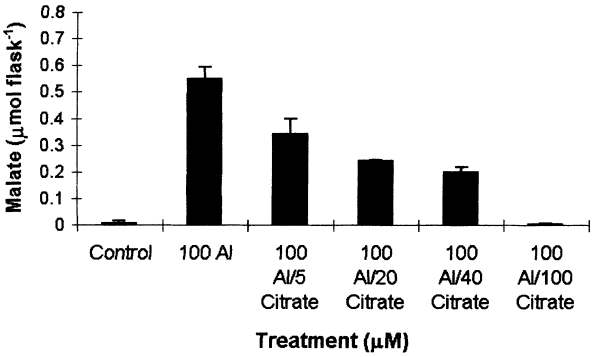

Fig. 6. Malate secreted from roots of Atlas 66 seedlings after 24 h treatment with 100 μM Al and 100 μM Al plus increasing citrate concentration at pH 4.6. Data represent the means of at least five flasks (± SE) per treatment

aluminium and various concentrations of citrate, a strong chelator of aluminium. Secretion of malate from the roots was inhibited by 5–40 μM citrate and completely stopped at 100 μM citrate (Fig. 6).

Discussion

Hydroxyaluminosilicate (HAS) formation has been implicated in the mechanism of silicon amelioration of aluminium toxicity, and this concept has recently been extended to plants (Hodson and Evans 1995). However, the reported conditions under which HASs form are controversial. Exley and Birchall (1992, 1993) supplied evidence for the existence of HASs, but Lumsdon and Farmer (1995) suggested that at pH 4.96 the majority of the aluminium in their solutions would be present as Al^{3+} and $AlOH^{2+}$, regardless of the presence of silicic acid. Rowatt and Williams (1996) demonstrated that, below pH 6.5, free Al^{3+} was not decreased in a solution containing 5 μM aluminium and 2 mM silicic acid. Nevertheless, Birchall et al. (1996) maintain that HASs can form in dilute aluminium solutions (<10 μM) containing high silicic acid concentrations at

pH 4.5–7.5, and are the precursors of amorphous solids such as proto-imogolite and allophane. Our experiments were conducted at pH ≤ 5.0 and Birchall et al. (1996) consider HAS formation to be of limited importance in this range. Chemical analysis of the solutions in which both wheat cultivars were grown indicated that biologically available aluminium levels were not reduced by silicon (Table 1). Similar aluminium levels were found in the roots of both aluminium- and aluminium/silicon-treated plants (Fig. 3), showing that aluminium continued to be available for uptake, and was not rendered unavailable due to precipitation or HAS formation in the bulk solutions.

We used MINTEQA2 to predict the speciation of precipitating aluminosilicate compounds. In the Scout 66 treatment solutions (1.5 μM Al/5 μM Si) the calculated saturation indices of aluminosilicate species were negative and indicated that they would not precipitate (Table 2). This is in agreement with our chemical analyses (Table 1), and we are not aware of any chemical investigations indicating Al/Si interactions at these low solution concentrations. In contrast, MINTEQA2 calculations of the speciation of Atlas 66 growth solutions resulted in high positive saturation indices for a number of aluminium/silicon compounds (Table 2). However, MINTEQA2 calculates chemical speciation at equilibrium (Li et al. 1989); precipitation of aluminium/silicon compounds is believed to be very slow, and is unlikely during our 4-d experiments.

Delhaize et al. (1993) reported higher malate exudation from near-isogenic lines of wheat that were aluminium-tolerant than from sensitive lines, and this trend was mirrored in our study. Seedlings of Scout 66 did not secrete malate when treated with 100 μM aluminium after 24 h, whereas it was stimulated by aluminium in Atlas 66, and the effect was concentration dependent (10–100 μM, Fig. 4). Ryan et al. (1995) demonstrated that malate exudation in tolerant wheat cultivars was correlated with monomeric aluminium concentration, and in particular with Al^{3+}.

We observed no significant difference between malate levels secreted in tolerant seedlings treated with aluminium (100 μM) and those seedlings treated with 100 μM aluminium/2000 μM silicon (Fig. 5). This indicates that there had been no reduction in biologically available aluminium levels resulting from HAS formation in our bulk solutions. If there had been HAS formation, we would have to assume that HAS and free aluminium could elicit the malate efflux response in a similar manner. It would also indicate that species other than monomeric aluminium (Ryan et al. 1995) are correlated with malate efflux in tolerant wheat plants. Our analyses of free aluminium indicate that these are unaffected by the presence of 2000 μM silicon at pH 4.6, and that monomeric aluminium species are predominant.

Malate secretion in seedlings of Atlas 66 treated with 100 μM aluminium and citrate (5–100 μM) was also assessed (Fig. 6). Citrate is a known chelator of aluminium and decreases aluminium toxicity under appropriate conditions (Ownby and Popham 1989). Malate secretion in aluminium/citrate-treated seedlings was reduced in comparison with those treated with 100 μM aluminium alone. Presumably, citrate decreased biologically active aluminium levels at the plasma membrane, thereby decreasing malate secretion. The concentration of citrate required to completely abolish malate efflux was 100 μM, whilst 2000 μM silicon had little effect. This indicates that silicon does not act in the same way as citrate in this system, and that its ability to chelate aluminium under our conditions is small. Thus, although we have demonstrated that 2000 μM silicon can partially ameliorate effects of aluminium in Atlas 66, it appears that the amelioration is not mediated by a chelation effect in the bulk solutions.

The concentration of silicon required before amelioration becomes apparent is critical for both cultivars (5 μM silicon in Scout 66 and 2000 μM silicon in Atlas 66; Cocker 1997). Under some conditions, aluminium and silicon levels within the root cell walls may exceed minimum concentrations required for HAS formation, leading to decreased entry of aluminium into the symplast. Hodson and Evans (1995) reviewed the evidence for aluminium/silicon co-localisation in plants, and this has been observed in the root cell walls of wheat, Norway spruce, and sorghum. The identity of these deposits is uncertain, but they may include HAS compounds.

The ability of silicon to ameliorate aluminium toxicity is dependent on the pH of the growth medium. Atlas 66 required a pH of 4.6 before the toxic effects of aluminium on root length were ameliorated by silicon, whereas in Scout 66 a pH value of 5.0 was required (Figs. 1, 2). Root apoplastic pH ranges from 4.0 to 7.0 (Grignon and Sentenac 1991), and is influenced by many factors, including external solution pH. The principal malate species released from the cytoplasm is the divalent anion, and this may tend to raise the pH of the rhizosphere (Ryan et al. 1995). This potential increase in apoplastic pH is of importance as Rowatt and Williams (1996) demonstrated that Al^{3+} is precipitated by silicic acid at pH greater than 6.5, and thus a near-neutral apoplastic pH may be required for HAS formation.

We believe that HAS formation in our bulk solutions was very limited, and free aluminium levels were unaffected. The amelioration mechanism could involve the formation of HAS within the root apoplast. Further work is required to investigate the presence, identity and potentially inert nature of such complexes within root cell walls.

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