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Effects of aluminum on organic acid metabolism and secretion by red spruce cell suspension cultures and the reversal of Al effects on growth and polyamine metabolism by exogenous organic acids

RAKESH MINOCHA[1,2] and STEPHANIE LONG[1]

[1] *USDA Forest Service, Northeastern Research Station, P.O. Box 640, 271 Mast Road, Durham, NH 03824, USA*

[2] *Author to whom correspondence should be addressed (rminocha@hopper.unh.edu)*

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Summary In the absence of added Al, the concentration of succinate in cultured red spruce (*Picea rubens* Sarg.) cells was 15–20 times higher (> 600 nmol g_{FW}^{-1}) than that of citrate or oxalate and 4–6 times higher than that of malate. Addition of $AlCl_3$ (effective monomeric Al concentrations of 0.23 and 0.48 mM) to 3-day-old suspension cultures significantly increased cellular succinate concentrations with a concomitant decrease in cellular oxalate concentrations. However, in the medium of Al-treated cell cultures, both succinate and oxalate concentrations were significantly higher than in the medium of cell cultures without added Al, and oxalate concentrations were several times higher than succinate concentrations. Aluminum did not significantly affect the cellular concentrations of malate, ascorbate and citrate, and none of these organic acids was present in detectable quantities in the medium. Exogenous succinate alone or with Al had no effect on cellular free polyamine concentrations or cell mass. Aluminum caused a significant increase in cellular putrescine concentrations. Addition of malate had a positive effect on growth and completely reversed the effects of Al on cell physiology. In contrast, the addition of oxalate and citrate only partly reversed the effects of Al.

Keywords: citrate, inorganic ions, malate, oxalate, phosphorus, putrescine, succinate.

Introduction

Aluminum, when present in soluble form in acidic soils, is phytotoxic, inhibiting plant growth and productivity. Plants have evolved various tolerance mechanisms to cope with acidic rhizosphere environments where Al concentrations are high (Ma 2000, Ryan et al. 2001). However, the biochemical processes and molecular mechanisms through which Al exerts its phytotoxic effects and the mechanisms of Al resistance in plants are not well understood.

Aluminum causes a variety of physiological, biochemical and molecular effects in whole plants and cell cultures. For example, it induces nutrient deficiencies, especially Ca deficiency (Jones et al. 1998), and oxidative stress (Yamaguchi et al. 1999, Yamamoto et al. 2002) in tobacco cell cultures. It also causes nutrient deficiencies in leaves of mature conifer and hardwood tree species (Minocha et al. 1997, 2000). Other reported effects of Al include changes in ethylene production in maize leaves (Barreiro and Henriques 1995), polyamine metabolism in periwinkle and red spruce cell cultures and in the leaves of many hardwood and conifer tree species (Minocha et al. 1992, 1996, 1997, 2000), organic acid metabolism (reviews by Ma 2000, Ryan et. al. 2001 and references therein), lignin deposition in wheat roots (Sasaki et al. 1996), plasma membrane lipid composition in wheat (Zhang et al. 1997), cell viability in several plant species (Ishikawa and Wagatsuma 1998), protein metabolism in mungbean leaves (Hua and Min 2001) and mitochondrial activity in red spruce (*Picea rubens* Sarg.) (Minocha et al. 2001) and tobacco cell cultures and pea roots (Yamamoto et al. 2002). Minocha et al. (2001) also observed effects of Al on viability and protein metabolism in red spruce cell cultures. The major symptoms of short- and long-term Al treatment of plants and cell cultures include inhibition of DNA synthesis and cell division, reduced root growth, and loss of biomass (McQuattie and Schier 1990, Schier et al. 1990, Minocha et al. 1992, 1996, Kinraide 1998, Lazof and Holland 1999).

The aliphatic polyamines (putrescine, spermidine and spermine) play an important role in growth and development. They are metabolically derived from arginine and ornithine, and at cellular pH, carry a net positive charge (Cohen 1998). Abiotic stress factors such as low pH, high SO_2, high salinity, osmotic shock, nutrient deficiency, low temperature and exposure to Al can all result in an increase in cellular putrescine concentrations (Flores 1991, Minocha et al. 1992, 1996, Bouchereau et al. 1999). In foliage of mature conifer and hardwood forest trees, putrescine accumulates in response to environmental stresses including acidic deposition, chronic N fertilization, nutrient deficiencies and Al solubilization (Dohmen et al. 1990, Santerre et al. 1990, Minocha et al. 1997, 2000, Wargo et al. 2002).

Correlations between organic acid metabolism and plant tolerance to environmental stress have been reported (López-Bucio et al. 2000, Ryan et al. 2001). Organic acids such as oxa-

late, citrate and malate play a role in alleviating Al toxicity in higher plants (Delhaize and Ryan 1995, Ma 2000, Ryan et al. 2001 and references therein). Ryan et al. (1995) reported that the amount of malate released by an Al-tolerant genotype of wheat was 5- to 10-fold greater than that released by an Al-sensitive genotype. Tolerance to Al was conferred on transgenic tobacco and carrot cells by overexpression of the citrate synthase gene and on alfalfa by overexpression of the malate dehydrogenase gene (de la Fuente et al. 1997, Koyama et al. 1999, Tesfaye et al. 2001), indicating that organic acids are involved in the mechanism of Al tolerance in higher plants. However, Ishikawa et al. (2000) found no correlation between Al tolerance and citric or malic acid secretion by roots or protoplasts of a number of plant species, and concluded that these species must possess a more effective tolerance mechanism than the secretion of malic and citric acid.

Previously, we showed that the addition of $AlCl_3$ to 3-day-old red spruce suspension cultures caused a significant increase in cellular putrescine concentration and a reduction in cell mass, mitochondrial activity, cell viability and uptake of inorganic cations such as Ca and Mg. There were also changes in cell ultrastructure. Aluminum was localized within the cytoplasm of Al-treated cells, which seemed to be without membrane lesions. Our study objectives were to determine: (1) if organic acids are accumulated or secreted by red spruce cell cultures in response to Al treatment; and (2) if exogenous organic acids reverse effects of Al on growth and metabolism.

Materials and methods

Culture conditions

Embryogenic suspension cultures of *Picea rubens* (cell line RS 61.1, 03-92; Minocha et al. 1996) were maintained in modified half-strength Litvay's medium (Litvay et al. 1981) as described by Minocha et al. (1996). Briefly, the modifications included addition of 3.42 mM glutamine, 9.05 μM 2,4-dichlorophenoxyacetic acid, 4.44 μM N^6-benzyl adenine and 1.0 g l^{-1} casein hydrolysate, and a reduction in sucrose concentration to 2% (w/v). In addition, Fe-EDTA was provided as 40 mg l^{-1} Sequestrine containing 7% (w/v) iron chelate (Plant Products, Brampton, ON, Canada). The medium was adjusted to pH 5.7 before autoclaving. Cells were subcultured at 7-day intervals by transferring 15 ml of cell suspension to 45 ml of fresh medium in 250-ml Erlenmeyer flasks. The flasks were incubated in the dark at 25 ± 2 °C on a gyratory shaker at 120 rpm.

All studies, unless specified otherwise, were conducted in growth medium containing Sequestrine that precipitated a portion of free Al. In order to determine the effective monomeric Al concentration in this situation, Al was added either to the cell-free medium or to 3-day-old cultures at pH ≤ 4.2. In either case, about 55% (w/w) of the Al became insoluble. The precipitate was removed by centrifugation at 31,400 *g* for 20 min and the supernatant analyzed for monomeric Al by the procedure of Driscoll (1984). The effective concentrations of monomeric Al in the medium were 0.09, 0.23 and 0.48 mM for Al additions of 0.2, 0.5 and 1.0 mM, respectively (Minocha et al. 1996).

For the exogenous malate addition studies, Sequestrine was replaced with an equal amount of iron in the form of Fe-EDTA (7.25 μM), and chemical analysis of these solutions confirmed that the effective monomeric Al concentration was the same as the added concentration. Because the pH of the medium was always below 4.5, we assume that the monomeric Al existed primarily as Al^{3+}.

Experimental treatments

Seven-day-old cell suspensions from 5–9 culture flasks (each containing 60 ml of cell suspension) were combined and mixed with an equal volume of fresh medium. To obtain uniform distribution, the cells were constantly stirred at low speed during transfer of 10 ml of diluted cell suspension to each 50-ml experimental flask containing 10 ml of fresh medium to provide a final cell density comparable with routine subcultures on Day 0. On Day 3, $AlCl_3$ or organic acids, or both, were added to the cell suspensions. The pH of the medium was not adjusted during an experiment, because by Day 2 the pH of the medium had dropped to 4.2 ± 0.3 and stayed within this range during Al addition on Day 3 and for the next 4–5 days. Aluminum stock solution was prepared at a concentration of 100 mM and the pH of the stock was not adjusted before use. Organic acid stock solutions were prepared at 250 or 500 mM and the pH was adjusted to 4.0 before use. The pH of control and treated cultures was in the range of 4.0 to 4.1 at the time of cell collection at 24 h or 48 h after Al addition.

All experiments were repeated three to five times, with each treatment replicated three to four times unless specified otherwise. Various concentrations of several organic acids were tested for amelioration of Al effects. However, if several concentrations of a specific organic acid had no effect or similar effects, only one concentration was repeated three times. The data presented are from a single representative experiment from the set of repeat experiments.

Collection of cells

At 24 or 48 h after Al addition, cells were collected by vacuum filtration onto Miracloth (Calbiochem-Behring, La Jolla, CA) and washed twice with an equal volume of distilled and deionized water (ddH_2O). After determining total pellet fresh mass, cells were subdivided into fractions for analyses of cellular free polyamines, inorganic ions and organic acids. For some experiments, the spent medium from the cell cultures was collected for organic acid (only for cultures that received no additional organic acids) and inorganic ion analyses (for malate addition experiments).

Free polyamines and inorganic ions

Cells (200 mg) were collected in 5% perchloric acid (PCA) and the contents extracted by freezing and thawing three times (Minocha et al. 1994). Extracts were centrifuged at 13,000 *g* for 10 min at 4 °C. The supernatant was diluted 20× with ddH_2O for inorganic ion analyses. Inorganic ion content was determined with a Varian Vista CCD simultaneous inductively

coupled plasma-axial emission spectrometer. Ninety µl of the undiluted PCA extract was dansylated as described by Minocha et al. (1990). The dansylated polyamines were extracted with 400 µl of toluene. A 200-µl sample of the toluene phase was evaporated under vacuum and the residue dissolved in 1 ml of methanol. Free polyamines were separated by liquid chromatography with a gradient of acetonitrile and heptane sulfonate (10 mM, pH 3.4). The liquid chromatographic system comprised a Perkin-Elmer series 200 pump, a Hitachi AS-4000 auto-sampler fitted with a 20-µl loop (10 µl actual injection volume), a Perkin-Elmer Pecosphere C18 column (3 µm particle size, 33 × 4.6 mm i.d.) and a fluorescence detector (LC240, Perkin-Elmer). The excitation and emission wavelengths were set at 340 and 515 nm, respectively (Minocha et al. 1990).

Organic acids

Cells were collected in 1 *N* HCl (150 mg cells ml^{-1}) and homogenized for 2 × 45 s with a Brinkmann polytron. Spent medium from the samples was also collected. The extracts and media were autoclaved at 121 °C and 103.42 kPa for 20 min and centrifuged at 13,000 *g* for 15 min at room temperature, filtered through a 0.45-µm filter and stored at –20 °C. The liquid chromatographic system consisted of a Dionex 4000i ion chromatograph, a Beckman 164 variable wavelength UV detector set at 215 nm, and a Spectra Physics integrator (SP 4270). After dilution with ddH_2O (20× for cell extracts and 200× for medium), the samples were analyzed for organic acids by chromatography on an Alltech Alpha Bond C18 column (300 × 3.9 mm, 10 µm particle size) with 100 mM potassium phosphate buffer, pH 2.5, at a flow rate of 0.7 ml min^{-1}. The total run time was 15 min for cell extracts and 10 min for spent media.

Statistical analyses

Data for each variable were analyzed as a series of one-way analyses of variance (ANOVA) to determine whether statistically significant differences occurred between the control and Al-treated cells. When *F*-values for one-way ANOVA were significant, differences in treatment means were tested by Tukey's multiple comparisons test. The ANOVA and Tukey's tests were performed with Systat for Windows, Version 7.01 (SYSTAT, Evanston, IL) and a probability level of 0.05 was used for most tests unless specified otherwise.

Results

Cellular concentrations of organic acids and their secretion in the spent medium

Figure 1 shows that, in 3-day-old red spruce cultures, cellular concentrations of succinate (> 600 nmol g_{FW}^{-1}) were 15–20-fold higher than those of oxalate (> 30 nmol g_{FW}^{-1}) and citrate (> 40 nmol g_{FW}^{-1}) and 4–5 times higher than that of malate (> 120 nmol g_{FW}^{-1}, data not shown). Cellular ascorbate concentration was less than 5 nmol g_{FW}^{-1} (data not shown). Addition of 0.2 mM Al (effective monomeric Al concentration 0.09 mM)

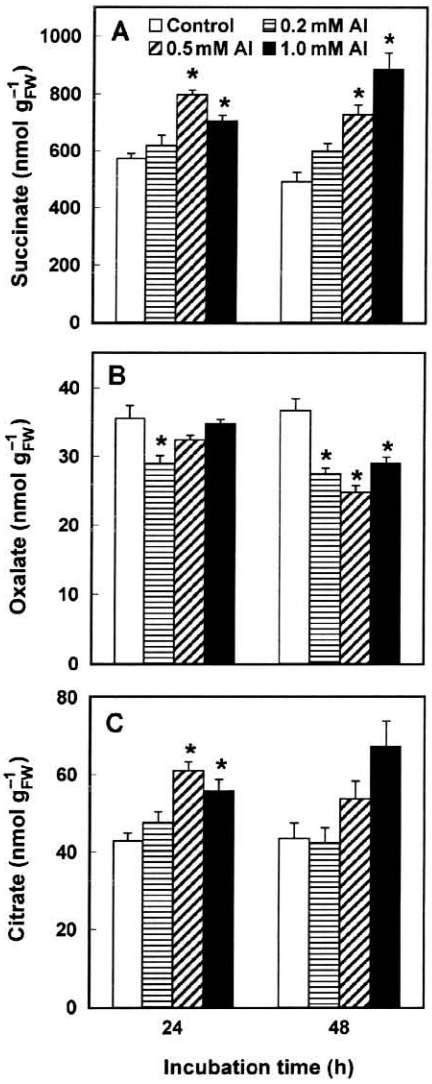

Figure 1. Effects of addition of 0.2, 0.5 and 1.0 mM $AlCl_3$ (effective concentrations 0.09, 0.23 and 0.48 mM) to 3-day-old red spruce cell cultures on cellular concentrations of succinate (A), oxalate (B) and citrate (C). Data are means + SE of three replicates. An asterisk denotes significant treatment differences at $P < 0.05$.

significantly decreased oxalate concentration but had no significant effect on the concentrations of other organic acids. Addition of 0.5 and 1.0 mM Al (effective monomeric Al concentrations of 0.23 and 0.48 mM) to 3-day-old cultures increased the cellular concentration of succinate with a concomitant decrease in oxalate concentration at 24 and 48 h (Figures 1A and 1B). The increase in succinate concentration was dose-dependent only at 48 h. Effects of Al on cellular concentrations of succinate and oxalate were generally less pronounced at 24 h than at 48 h. The cellular concentration of citrate increased at 24 and 48 h after Al addition, although the increase at 48 h was not statistically significant (Figure 1C). Furthermore, the Al-induced changes in cellular citrate concentration observed at 24 h did not always follow the same pattern in other experiments. Aluminum did not affect cellular

concentrations of malate and ascorbate (data not shown).

Forty-eight hours after treatment, both oxalate and succinate were present in the spent medium of cell cultures treated with 0.5 and 1.0 mM Al in significantly greater quantities than in the medium of control cultures (Figures 2A and 2B). At 24 h, the differences were neither significant nor dose-dependent. Oxalate concentration (> 250 nmol g_{FW}^{-1}) in the medium was about 4–5-fold higher than inside the cells and 15–20 times higher than the concentration of succinate in the medium. Succinate concentrations in the culture medium were only about 2.5% of cellular concentrations. Malate, ascorbate and citrate were not detectable in the spent medium of either control or Al-treated cultures (data not presented).

Reversal of Al effects on growth and polyamine concentration by organic acids

Aluminum significantly inhibited growth (measured by changes in fresh mass) and significantly increased the concentration of cellular free putrescine (Figures 3 and 4). Spermidine and spermine concentrations in these cultures did not fluctuate significantly, irrespective of the treatment applied (data not shown).

Succinate Alone, exogenous succinate (5 mM) had no significant effect on cellular putrescine concentration or cell mass (Figures 3A and 3B). Exogenous succinate at 2.5, 5 or 10 mM, added either 0 or 4 h after addition of 1 mM Al (effective Al concentration of 0.48 mM), did not reverse the effects of Al on cellular free putrescine concentration or cell mass (data not shown for 2.5 and 10 mM succinate).

Oxalate Addition of 5 mM oxalate to the cultures significantly increased free putrescine concentrations without affecting cell mass (Figures 3C and 3D). When oxalate (2.5 mM (data not shown) or 5 mM) was added with Al, the effect of Al on fresh mass was completely reversed, whereas its effect on free putrescine was only partially reversed whether oxalate was added at the time of Al addition or 4 h later.

Citrate Five millimolar citrate alone had no significant effects on cell mass or free putrescine concentration. However, citrate added with Al caused partial reversal of the effects of Al on putrescine concentration and full reversal of the Al effects on cell mass (Figures 3E and 3F). The addition of 10 mM citrate with Al resulted in partial recovery of cell mass but increased cellular putrescine concentrations beyond the values attributable to Al alone (data not presented).

Malate The addition of Al (effective monomeric Al concentration of 0.5 mM) caused a significant (3- to 4-fold) increase in free putrescine concentrations. Addition of malate alone, at a concentration of 1 mM or higher, significantly decreased free putrescine concentrations compared with the concentration in control cells (Figure 4A). Addition of 1 mM malate with Al caused only partial reversal of the effects of Al on free putrescine concentrations. In contrast, addition of 2, 5, 10 or 15 mM malate caused a complete reversal of the Al effects on free putrescine concentrations (Figure 4A), which declined to values below those of controls in most cases. Malate addition,

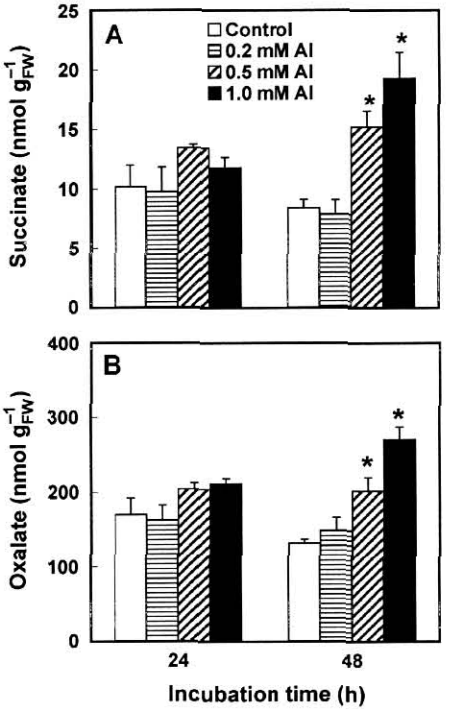

Figure 2. Effects of addition of 0.2, 0.5 and 1.0 mM $AlCl_3$ (effective concentrations 0.09, 0.23 and 0.48 mM) to 3-day-old red spruce cell cultures on exudation of succinate (A) and oxalate (B) in the culture medium. Data are means + SE of three replicates. An asterisk denotes significant treatment differences at $P < 0.05$.

with or without Al, had no effects on cellular free spermidine and spermine concentrations (data not shown).

The addition of 2, 5 or 15 mM malate alone caused a statistically significant increase in cell mass (Figure 4B). Malate at 1 mM partially reversed, and malate at 2, 5, 10 and 15 mM fully reversed, the inhibition of cell growth by 0.5 mM Al.

Inorganic ions in cell extracts

Figure 5 shows the effects of 0.5 mM Al (effective monomeric Al concentration of 0.5 mM) on cellular inorganic ions and the reversal of some of these effects by malate. Aluminum had no significant effect on cellular Ca concentrations. The addition of 1, 2 and 5 mM malate, either alone or with 0.5 mM Al, had variable effects on cellular Ca concentration, in some cases causing a slight decrease, in others having no effect.

Exogenous Al significantly increased cellular Mg concentrations, and the effect was reversed by 1, 2 or 5 mM malate. Alone, malate had no effect on Mg concentration.

Cellular Mn concentrations were unaffected by either Al or malate (1, 2 and 5 mM) alone. However, malate and Al together reduced cellular Mn concentrations relative to controls.

Aluminum caused a significant decrease in cellular K concentration, whereas 5 mM malate caused a small but statistically significant increase. The effect of Al on cellular K concentration was partially reversed by 1 mM malate, and fully reversed by 2 and 5 mM malate. Exogenous Al significantly increased cellular Al concentrations. Exogenous malate

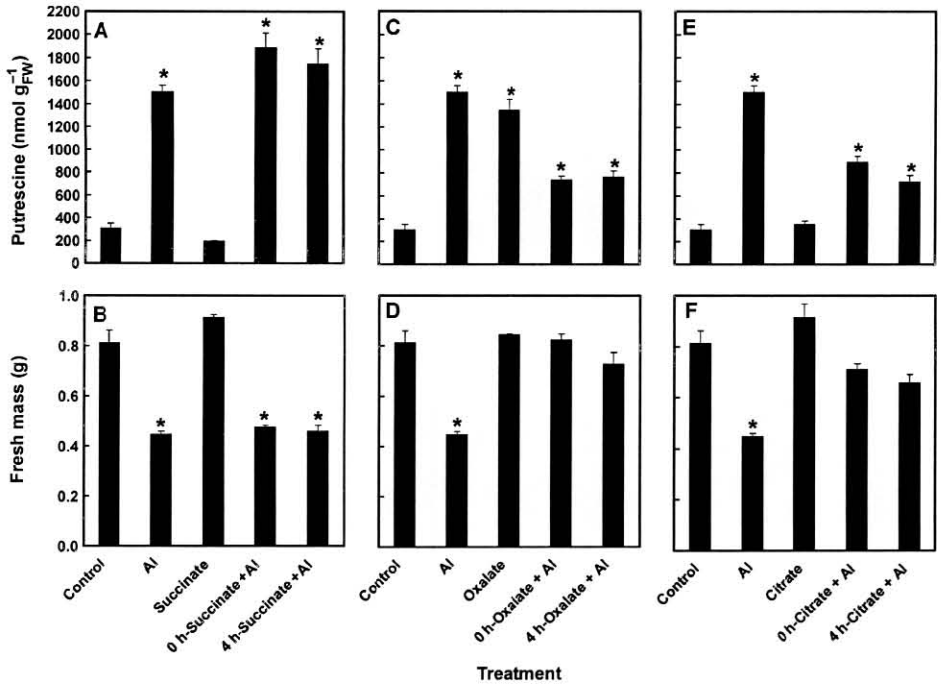

Figure 3. Reversal of effects of addition of 1.0 mM $AlCl_3$ (effective concentration 0.48 mM) to 3-day-old red spruce cell cultures on cellular concentrations of putrescine and growth (fresh mass) by the addition of 5 mM succinate (A, B), oxalate (C, D) and citrate (E, F) at either 0 or 4 h after the addition of Al. Cells were collected at 48 h after Al addition. Data are means + SE of three replicates. An asterisk denotes significant treatment differences at $P < 0.05$.

(1, 2 and 5 mM) alone had no effect on cellular Al concentrations, but exogenous malate in the presence of exogenous Al reduced cellular Al concentrations in a dose-dependent manner.

Exogenous Al significantly increased cellular P concentrations. Malate (1, 2 and 5 mM) alone and with Al significantly decreased cellular P concentrations in all cases but one.

Inorganic ions in spent medium

Figure 6 shows the effects of 0.5 mM Al (effective monomeric Al concentration of 0.5 mM) on concentrations of inorganic ions in the spent medium and the reversal of some of these ef-

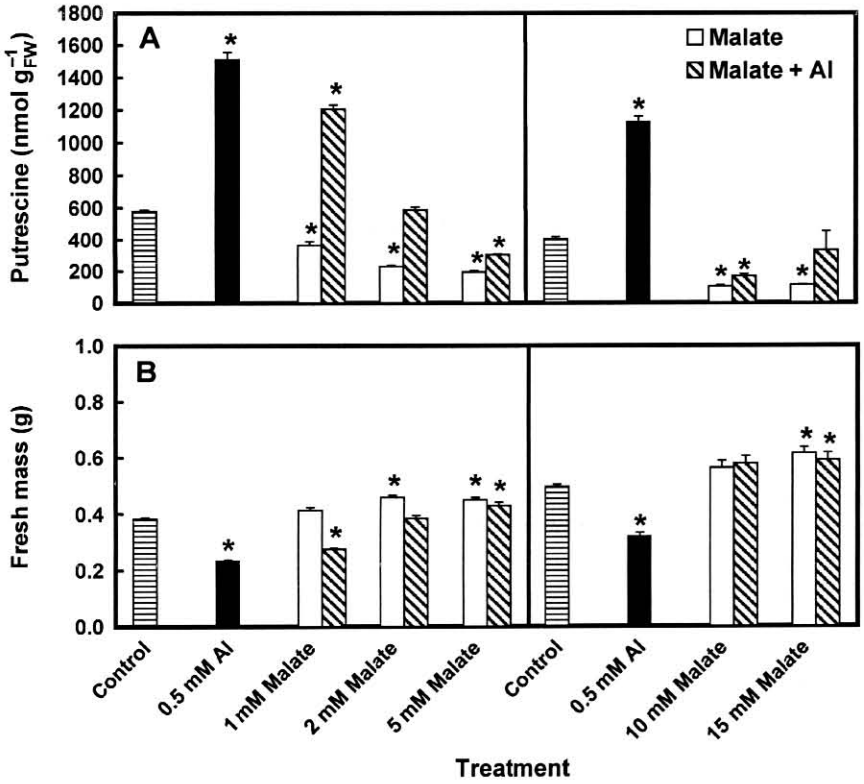

Figure 4. Effects of addition of 1, 2, 5, 10 and 15 mM malate with or without 0.5 mM $AlCl_3$ (effective concentration 0.5 mM) to 3-day-old red spruce cell cultures on cellular putrescine concentrations and growth (fresh mass). Data were collected in separate experiments in different weeks: 1, 2 and 5 mM malate treatments in one experiment, and 10 and 15 mM malate treatments in the other experiment. Cells were collected 48 h after addition of Al. Data are means + SE of five replicates. An asterisk denotes significant treatment differences at $P < 0.05$.

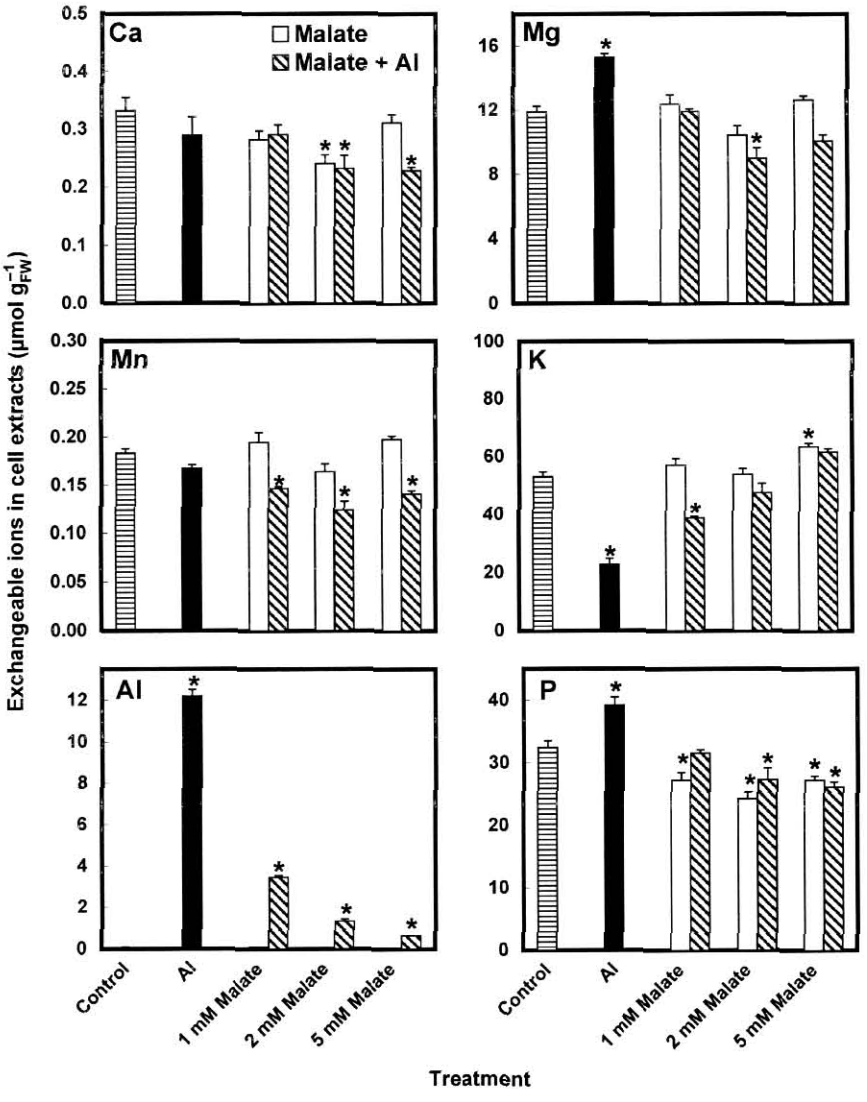

Figure 5. Effects of addition of 1, 2 and 5 mM malate with or without 0.5 mM $AlCl_3$ (effective concentration 0.5 mM) to 3-day-old red spruce cell cultures on concentrations of cellular inorganic ions. Cells were collected 48 h after addition of Al. Data are means + SE of five replicates. An asterisk denotes significant treatment differences at $P < 0.05$.

fects by malate. There were no significant effects on the concentrations of Ca, Mg and Mn in the spent medium of cells treated with either Al, malate, or Al plus malate. However, the addition of 0.5 mM Al alone caused a significant increase in K and P concentrations in the spent medium. Malate concentrations greater than 2 mM, added with Al, generally caused a complete reversal of Al effects on K concentration. Malate, when added alone or with Al, caused a decrease in P concentrations in a dose-dependent manner. However, malate addition caused a dose-dependent increase in Al concentrations in the spent medium, indicating that, at lower malate concentrations, more Al entered the cells, which secreted more K compared with cells treated with malate at higher concentrations. Control and malate-treated cells secreted no Al in the medium.

Discussion

Aluminum decreases growth, increases putrescine accumulation, and changes cellular concentrations of inorganic ions in proembryogenic red spruce cell cultures (Minocha et al. 1996). It also causes a loss of cell viability, a reduction in mitochondrial activity, changes in cellular ultrastructure and stereology, i.e., quantitative changes in the ratios between the size of an organelle (e.g., its volume or surface area) and the volume of the whole cell (Minocha et al. 2001), and significant changes in amino acid metabolism and antioxidant enzymes (authors' unpublished data). We have now shown that Al causes changes in organic acid metabolism of red spruce cell cultures. Because all the metabolites that we measured in Al-treated red spruce are interconnected through various biochemical pathways (see Figure 1 in Minocha et al. 2004), it can be postulated that a change in the concentration of a single metabolite (e.g., putrescine) may have caused changes in several metabolites of the same or other related pathways by currently unknown mechanism(s). The induction of changes in related metabolites by overproduction of only one metabolite induced by genetic manipulation has been reported in transgenic poplar in cell suspension culture (Bhatnagar et al. 2001, 2002, R. Minocha et al., unpublished data). These studies show that insertion of a single gene (cDNA for ornithine de-

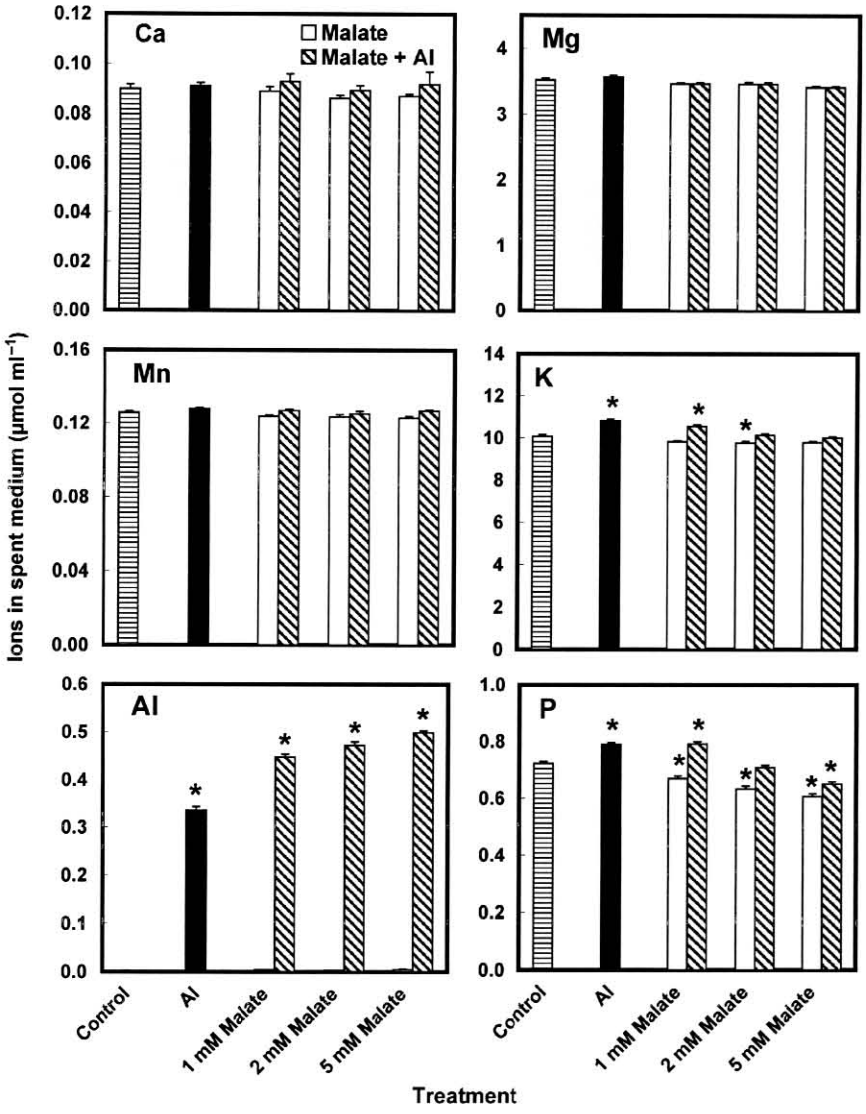

Figure 6. Effects of addition of 1, 2 and 5 mM malate with or without 0.5 mM $AlCl_3$ (effective concentration 0.5 mM) to 3-day-old red spruce cell cultures on concentrations of inorganic elements in the spent medium. Spent medium was collected at 48 h after addition of Al. Data are means + SE of five replicates. An asterisk denotes significant treatment differences at $P < 0.05$.

carboxylase) leading to the overproduction of putrescine causes changes in a number of metabolites in the polyamine pathway and related pathways.

Nutrient imbalances, such as P deficiency, exposure to toxic metals (e.g., Al), or anoxia (Keerthisinghe et al. 1998, Neumann et al. 1999, Watt and Evans 1999) cause secretion of organic acids by plant roots. The process may: (1) detoxify the extracellular environment by binding toxic metals such as Al^{3+} and reducing accumulation of toxic ions in the cytoplasm (Ryan et al. 1995, Jones 1998, Ma et al. 2001) or (2) increase the availability of nutrients for root uptake (Jones 1998). Ma (2000) has proposed two patterns of Al-induced organic acid secretion. In the first, there is no lag phase between Al exposure and organic acid secretion, and activation of anion channels has been implicated. In the second, there is a marked lag phase between Al exposure and organic acid secretion, and metabolism may be affected by gene activation. Our results with red spruce cell cultures, where a more pronounced and often dose-dependent response to Al was observed after a lag phase of > 24 h, appear to fit the second pattern.

Succinate and oxalate were secreted by control cultures, and the secretion increased with the addition of Al in a dose-dependent manner. Succinate (and to a lesser extent citrate) accumulated and oxalate declined inside the cells in response to Al. Although succinate was a major component of the cellular organic acids examined, oxalate was the main component of the secreted organic acids. As in previous studies with roots of wheat and maize plants (Delhaize et al. 1993, Pellet et al. 1995), there was no correlation between intracellular concentrations of specific organic acids and their secretion by red spruce cell cultures.

Exogenous succinate did not reverse the effects of Al on growth and polyamine metabolism. The accumulation and secretion of succinate in response to Al may be explained by the hypothesis that increased putrescine production in response to Al exposure is accompanied by an increase in the rate of putrescine degradation, as observed in putrescine-overproducing transgenic poplar cell lines (Bhatnagar et al. 2001, 2002).

The degradation products of this reactions are hydrogen peroxide (H_2O_2) and γ-aminobutyric acid (GABA); the latter breaks down to produce succinate (see Figure 1 in Minocha et al. 2004). Aluminum may damage the integrity of the plasma membrane (Minocha et al. 2001), allowing some of the succinate produced during putrescine degradation to leak from the cell. In the present study, only a relatively small portion (2.5%) of cellular succinate was secreted into the culture medium. In addition, the lack of reversal of Al effects by exogenous succinate supports the hypothesis that succinate secretion may be only a side effect of putrescine breakdown and may not play a role in Al detoxification in this species. Additional data on changes in activities of enzymes involved in the degradation of putrescine and GABA are needed to prove this hypothesis. Current data are insufficient to determine whether succinate is used to bind Al within cells and thus detoxify the internal environment.

It is not known if the H_2O_2 produced during the degradation of putrescine is responsible for the changes in antioxidant enzymes observed in Al-treated red spruce cell cultures (authors' unpublished data). Aluminum can cause oxidative stress in *Arabidopsis thaliana* (L.) Heynh. and tobacco cell cultures (Yamamoto et al. 1997, 2002, Richards et al. 1998, Yamaguchi et al. 1999). Yamamoto et al. (2002) concluded that Al causes mitochondrial dysfunction in tobacco cell cultures, leading to the production of reactive oxygen species.

The 10-fold increase in oxalate concentration in the spent medium of Al-treated cell cultures, compared with cellular oxalate concentration, may indicate that oxalate has a role in Al detoxification. Oxalate has been identified in root exudates of Al-treated Norway spruce seedlings (Heim et al. 2001), and may restrict Al uptake by forming a stable complex with Al. Aluminum-induced oxalate exudation has also been observed in taro (*Colocasia esculenta* L.) (Ma and Miyasaka 1998) and in mycorrhizal as well as non-mycorrhizal Scots pine (*Pinus sylvestris* L.) (Ahonen-Jonnarth et al. 2000). However, in buckwheat, oxalate was shown to detoxify Al internally (Ma et al. 1998). Although the amount and kind of organic acid secreted, and its elution pattern in response to Al exposure, is stress- and species-specific (Jones 1998, Zheng et al. 1998, Li et al. 2000, Ma et al. 2001), the overall mechanism of Al detoxification is presumed to be independent of which acids a species secretes.

Exogenous oxalate and citrate at concentrations of 5 mM or higher only partially reversed the toxic effects of Al, whereas malate at concentrations greater than 2 mM did so completely. Although exogenous citrate and malate are either as effective or more effective than oxalate in reversing the toxic effects of Al, only oxalate was secreted in significant quantities by red spruce cells. The cellular concentrations of citrate, ascorbate and malate showed little or no change in response to exogenous Al and are unlikely, therefore, to have a role in Al detoxification.

Malate may reverse the physiological and growth effects of Al through the inhibition of Al uptake. Inhibition of Al uptake by malate was dose-dependent (Figures 5 and 6) and may have resulted from the formation of an Al–malate complex, making Al unavailable for uptake. The increase in Al concentration in the spent medium with an increase in the amount of added malate implies that the putative Al–malate complex was soluble, as an insoluble complex would have been removed by the centrifugation before inorganic ion analyses. Therefore, the quantitation of inorganic ions in the spent medium may not necessarily measure what is available for uptake by the plant.

As well as reversing effects of Al, exogenous malate promoted cell growth, perhaps by providing an additional carbon source. However, as there was 2% sucrose in the modified LM medium, this hypothesis seems unlikely. An alternative explanation for the positive growth effects of malate addition is that some nutrients in the 1/2 LM medium used for red spruce proembryogenic cultures may have been present in excess. Added malate may have complexed these excess nutrients, thereby reducing nutrient concentrations to those optimal for growth, leading to the improved growth and reduced cellular putrescine concentrations observed in this study.

The efflux of large amounts of organic acids from cells in response to Al must be counterbalanced by the activation of other transporters, such as those that transport K^+ or H^+, to stabilize the membrane potential. Ryan et al. (1995) demonstrated that K^+ was the counter-ion for the Al-induced malate efflux from wheat roots. The efflux of malate and K^+ was shown by Osawa and Matsumoto (2002) to occur through different channels in wheat roots, leading these authors to suggest that the release of K^+ coupled with malate plays an important role in stabilizing intracellular pH. In our study, red spruce cultures secreted K^+ along with oxalate and succinate, possibly thereby stabilizing intracellular pH.

The decrease in cellular P concentrations accompanied by an increase in cell mass in response to exogenous malate indicates that our cells were not P-limited under control conditions and that the changes in organic acids in response to Al treatment were not a result of P deficiency. Similar observations have been made in taro, where only the addition of Al, and not P deficiency, induced oxalic acid exudation (Ma and Miyasaka 1998). Phosphorus uptake in these cultures increased with Al addition, as also observed in *Zea mays* L. (Gaume et al. 2001). Our observations are in line with previous findings showing that Al and P co-localized in vacuoles (Vázquez et al. 1999, Minocha et al. 2001). Concomitant with a decrease in cellular P concentration, there was a dose-dependent decrease in P concentration in the medium when malate was added alone or with Al. These results can only be explained if one assumes that P precipitated in a dose-dependent manner in the culture medium with the addition of malate.

We found that Al exposure caused a significant increase in oxalate secretion by red spruce cell cultures. When oxalate was added exogenously to the cell cultures, it partially reversed the negative effects of Al on growth and polyamine metabolism, indicating that it plays a role in Al detoxification in red spruce cells. Although malate complexed with Al, thus preventing Al from entering the cells and thereby reversing Al phytotoxicity, malate was neither secreted nor accumulated by

red spruce cells in culture. Therefore, malate may not be involved in the Al tolerance of this species. Increased succinate secretion in red spruce cells treated with Al may be a by-product of increased putrescine degradation rather than part of an Al tolerance mechanism in red spruce.

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