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# Effects of aluminium treatment on Norway spruce roots: Aluminium binding forms, element distribution, and release of organic substances

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**Abstract**

In order to investigate if Al resistance in Norway spruce (*Picea abies* [L.] Karst.) can be attributed to similar exclusion mechanisms as they occur in several crop plants, three-year-old Norway spruce plants were treated for one week in hydroculture with either 500 $\mu M$ $AlCl_3$ or $CaCl_2$ solutions at pH 4. Sequential root extraction with 1 *M* $NH_4Cl$ and 0.01 *M* HCl and EDX microanalysis revealed that Al and Ca in cell walls and on the surface participated in exchange processes. About half of the Al extracted by the sequential extraction was not exchangeable by 1 *M* $NH_4Cl$. Phenolics and phosphate present in the root extracts are possible ligands for Al adsorbed to or precipitated at the root in a non-exchangeable form. In both treatments, C release during the first period of 2 d was much higher than during the remaining time of the experiment. Al treated plants released less total C, carbohydrates and phenolics than did Ca treated plants. Acetate was the only organic acid anion that could be detected in some samples of both treatments. Free amino acids were present at micromolar concentrations but as hydrolysis did not increase their yield, there was no evidence of peptide release. One to two thirds of the released C were large enough not to pass a 1 kDa ultrafilter. The results suggest that exudation of soluble organic complexors is not a major Al tolerance mechanism in Norway spruce, although complexation of Al by phenolic substances released by the root could be detected by fluorescence spectroscopy. Aluminium tolerance could rather be attributed to immobilization in the root apoplast, where strong binding sites are available or precipitation may occur.

## Introduction

Norway spruce (*Picea abies* [L.] Karst.) is a common forest tree in Northern and Central Europe, which is found mostly on acid soils of pH 4-5 (Leibundgut, 1984). At such acidic sites proton buffering by the mineral phase leads to a release of $Al^{3+}$, which can result in high concentrations of toxic Al species in the soil solution. Free $Al^{3+}$ ions potentially are toxic to plants as they can inhibit root growth and impair nutrient uptake (Delhaize and Ryan, 1995; Kinraide, 1990; Kochian, 1995). However, in contrast to agricultural crops, where $Al^{3+}$ concentrations at the low micromolar level cause growth reductions in sensitive wheat and maize varieties (Pellet et al., 1995, 1996), Norway spruce is able to tolerate concentrations of up to 0.3 m*M* $Al^{3+}$ before growth is reduced. Lethal concentrations are even 30 times higher (Göransson and Eldhuset, 1991). Only very little is known on the mechanism of the Al resistance in Norway spruce as studies focused rather on nutritional and growth effects of high Al concentrations (e.g. Godbold and Jentschke, 1998; Göransson and Eldhuset, 1991). Several plant species have developed strategies to avoid or tolerate Al toxicity. The proposed mechanisms of Al resistance can be classified into internal tolerance mechanisms and exclusion mechanisms (Kochian, 1995; Taylor, 1991). The main difference between these two mechanisms is the site of Al detoxification: symplasm (internal)

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or apoplasm (exclusion). The internal tolerance mechanism immobilizes, compartmentalizes or detoxifies Al entering the symplasm. Recently, complexation by citrate in hydrangea and by oxalate in buckwheat has been shown to detoxify Al internally (Ma et al., 1997, 1998). By contrast, exclusion mechanisms prevent Al from entering the symplasm, where sensitive intracellular sites are located (Taylor, 1991). A proposed exclusion mechanism is the excretion of chelating organic substances as these can form stable complexes with $Al^{3+}$ ions in the soil solution, which are less phytotoxic than free $Al^{3+}$ ions (Hue et al., 1986). For Al resistant varieties of wheat (Delhaize et al., 1993), maize (Pellet et al., 1995), and buckwheat (Zheng et al., 1998), exudation of organic acids was found as a reaction to Al exposure. Beside organic acids, polysaccharides and polyuronic acids secreted by the root cells and forming the mucilage around root tips have been proposed to be involved in Al tolerance of cowpea (Horst et al., 1982). It is not known if these processes have any importance for the Al resistance of Norway spruce. There is generally very little and partly contradictory information on exudation of organic substances by forest trees. While Smith (1969) reported detailed patterns of organic acids exuded by seedlings of various pine species, Eltrop (1993) found exudation of organic acids by Norway spruce seedlings in semi-hydroponic culture to be below the detection limit of modern analytical tools. However, organic acid exudation of a given tree species may increase with age (Smith, 1970). In forest soils, organic acids have been detected in varying concentrations depending on soil type and vegetation, but their origin (i.e. plant or microbial) is unknown (Fox and Comerford, 1990; Jones, 1998; Shen et al., 1996).

An exclusion mechanism does not necessarily have to work completely outside the root. In the apoplast, binding of Al to non-sensitive sites can be equally efficient in excluding $Al^{3+}$ ions from sensitive symplastic sites (Horst, 1995). It is known that Al mainly is retained in the roots of Norway spruce and only very little is translocated to the shoot (Göransson and Eldhuset, 1991; Hentschel et al., 1993). However, only little information is available on the compartmentation and chemical form of Al accumulated in the root (Jentschke, 1990). Dahlgren et al. (1991) discussed co-precipitation of Al with oxalate and phosphate as possible retention mechanisms of Al in fine roots of a Northwest American conifer stand of *Abies amabilis* (Dougl.) Forbes with *Tsuga mertensiana* (Bong.) Carr. as an associated species. Mycorrhizal infection could also be involved in preventing Al transport to the shoot as inoculation with the mycorrhizal fungus *Pisolithus tinctorius* increased Al resistance in pitch pine seedlings (Cumming and Weinstein, 1990) and Al retention in Norway spruce roots was increased by inoculation with *Paxillus involutus* (Hentschel et al., 1993).

The aim of this study was on one hand to examine if root exudation of organic substances is likely to play a role in the Al resistance of Norway spruce, and on the other hand to get more insight into the distribution and chemical form of Al accumulated in the roots of this plant species. Treatments of three-year-old soil-grown tree individuals in hydroponic culture were chosen because organic acid release from older trees can be expected to be substantially higher than from few month old seedlings (Smith, 1970). The use of single salt solutions eliminated unwanted amelioration of Al toxicity by nutrient cations (Grauer and Horst, 1992). Since changes in the exudation pattern and reactions of Al with the root surface are likely to occur within a few hours or days after the stress is imposed (Delhaize et al., 1993; Pellet et al., 1995), one week treatments were considered sufficient. Within such short periods it can be expected that no nutrient deficiencies and only limited microbial growth occur.

## Material and methods

### *Plant material*

Norway spruce (*Picea abies* [L.] Karst.) seedlings from a parent tree near Bremgarten (canton of Aargau, Switzerland) were grown for three years in a tree nursery and for another half year in a greenhouse in pots filled with a fertilised mixture of peat and wood chips. Upon transplantation to the pots, the root system was cut back to about half of its size to induce formation of new roots.

### *Treatments*

For the treatments the plants were removed from the pots and a shower fed with tap water was used to remove substrate adhering to the roots. The root system was bathed in $10^{-4}$ *M* HCl for 10 min in order to neutralize carbonates originating from the tap water. The plants were transferred to 250 mL Erlenmeyer flasks where the root system was treated with 250 mL of treatment solution while the shoot remained outside the flask. The treatment solutions were either 0.5 m*M*

$AlCl_3$ or $CaCl_2$ (control) solutions acidified to pH 4.0 with HCl and sterile filtered before use. Treatments were done in triplicates.

In both treatments, the pH of the system was kept between 3.8 and 4.0 by a titroprocessor (Metrohm 670). While in the Al treatments only negligible amounts of HCl needed to be added to keep pH at this level, about 100 $\mu$M HCl had to be added in the Ca treatments. At this pH, Al speciation is dominated by the trivalent $Al^{3+}$ ion. During the experiment, the solutions were aerated with 0.2 $\mu$m filtered air. The treatments lasted for one week, which was divided into three periods of 2, 2, and 3 d. After each of these periods the treatment solutions were replaced by fresh ones. The purpose of this pattern was to separate rapid effects caused by the treatment shock at the beginning of the experiment from longer-term plant reactions. The experiments were carried out in a growth chamber (20 °C, 50% relative humidity, 16 h photoperiod).

*Sampling*

Solution samples were taken three times a day during the first and second period and once a day during the third period. Ten mL were sampled at each sampling date, and the liquid in the Erlenmeyer flask lost by sampling and evapotranspiration replaced with sterile distilled water. The samples were filtered immediately through 0.45 $\mu$m syringe filters (Spartan 30 B; Schleicher & Schuell) and stored at −20 °C until analysis. At the end of each treatment period the remaining solutions also were 0.45 $\mu$m filtered and stored frozen.

*Solution analysis*

On all solutions the following analyses were performed: Cations were measured by capillary electrophoresis (CE) (BioFocus 3000, BioRad, 40 cm × 50 $\mu$m fused silica capillary) using the metol buffer of Göttlein and Blasek (1996). Dissolved organic carbon (DOC) was determined with a TOC-Analyzer (Shimadzu TOC-500). Blank values of DOC due to release from the syringe filters were determined separately (5 replicates) and subtracted from the measured values. UV absorption was measured at 215 nm, 254 nm and 280 nm with a UV-VIS spectrophotometer (Shimadzu UV-240), and total phenolics were analysed with a colorimetric method according to Swain and Hillis (1959) using phenol as standard.

On the solutions remaining at the end of each treatment period, additional analyses were performed. Forty mL were freeze-dried and redissolved in 1 mL of 0.1 *M* HCl. Chloride and cations in the concentrated solutions were removed by passing over a cation exchange resin saturated with $Ag^+$ ions, and organic acids were analysed using CE (buffer: 10 m*M* potassium hydrogen phthalate; 2.5% Waters OFM Anion-BT; pH 5.6). Oxalate was determined separately by CE using the anion method of Göttlein and Blasek (1996). During lyophilization calcium oxalate can precipitate. In order to dissolve such precipitates, after removal of the concentrated sample solutions the bottles used for freeze-drying were shaken with 1 mL of 1 m*M* EDTA. Organic acids and oxalate in these solutions were analysed by CE as mentioned above. Experiments with standards showed that this also improves malate and citrate recovery in the presence of Al. Total carbohydrates were determined in 10-fold concentrated solutions by the phenol-sulfuric acid assay according to Chaplin (1994) using glucose as standard. Total amino acids were determined in 5-fold concentrated solutions before and after alkaline hydrolysis using the ninhydrin method (Allen, 1981). Twelve mL of the non-concentrated final solutions were filtered through a 1 kDa filter (Macrosep™ centrifugal concentrator, Pall Filtron, USA) at 5000 $min^{-1}$ for about 2.5 h. Retentate and filtrate were analysed for DOC and for total phenolics as above. The amounts of high molecular C and high molecular phenolic substances were calculated as difference between retentate and filtrate amounts. The fluorescence spectra of selected samples were recorded using quartz cells (path length 1 cm) on a Shimadzu RF 5000 spectrometer. Excitation and emission monochromator entrance and exit slit widths were set to 3 nm. Excitation wavelength was varied from 250 nm to 550 nm.

*Root extraction*

At the end of each treatment, a four-step sequential extraction of the roots was performed. One hundred mg of fresh fine roots were extracted for 30 min with 10 mL of 1 *M* $NH_4Cl$ on an end-over-end shaker to yield exchangeable cations. The supernatant was sampled by pipetting. Since a small volume of extract still adhered to the roots, it was necessary to perform a second extraction with fresh $NH_4Cl$ in order to obtain a complete recovery of exchangeable cations and to avoid carry-over into the following extract. In a third step, 10 mL of 0.01 *M* HCl were added to the roots and the bottles were shaken again for 30 min and the super-

natant sampled with a pipette. This extract was chosen to dissolve potential precipitates of Al (Dahlgren et al., 1991). Finally, the HCl extraction procedure was repeated once to obtain complete recovery of the acid soluble fraction.

In all extracts total element concentrations were determined using inductively-coupled plasma atomic emission spectrometry (Optima 3000, PE), and total phenolics were determined with the method described above. After removal of chloride, oxalate was determined with the method described above.

*Electron microscopy and X-ray microanalysis*

Fine root samples of newly formed lateral roots were taken from treated plants and from a control plant in a pot. Lateral roots of approx. 5 mm length were fixed vertically in slits of supporting brass stubs using a cryo glue, frozen in liquid nitrogen and freeze-fractured with a rotating microtome at −90 °C in the preparation chamber (Balzers SCU 020) of the cryo scanning electron microscope (Philips 515). For surface analyses, the samples were fixed horizontally with double-sided adhesive tape and frozen in liquid nitrogen. Elemental concentrations on the root surface, in cell walls and lumina of epidermis and cortex, and in the stele were assessed by energy-dispersive X-ray microanalysis in the cryo scanning electron microscope (Brunner et al., 1996). The microscope was operated at an acceleration voltage of 20 kV with a beam current of 80 $\mu$A and a take-off angle of 15°. Working distance was 12 mm. All spectra were acquired for 120 s (live time) and a dead time of 20%. Spot analysis was carried out with a maximum magnification of 10 000. Spectra were analysed using the Voyager software package and results for individual elements are presented as background corrected net counts. These net counts are semi-quantitative measures of concentration, but as analysis conditions and the etching process were not controlled rigorously and because of the problems of obtaining fully quantitative results from bulk-frozen hydrated samples (Van Steveninck and van Steveninck, 1991), they were not converted to absolute concentrations. Three samples per treatment were analysed; on each sample 4–5 analyses per compartment were performed.

*Statistical analysis*

In order to compare treatment effects in a given period, one-way ANOVA tests were performed. The unequal variances between first and later treatment periods did not allow a two-way analysis over all data on the factors treatment and period. Statistical analysis was done using Data Desk 6.0.2. for Macintosh (Data Description Inc.).

## Results

*Ion exchange*

During the first 2-day period, the concentration of $Al^{3+}$ in the Al treatment solutions, as measured by CE, decreased very rapidly from the initial level of 500 $\mu M$ to approximately 100 $\mu M$ (Figure 1). A less pronounced decrease in $Al^{3+}$ concentrations was observed during the second and third period. Minimum $Al^{3+}$ concentrations were about 250 $\mu M$ in the second period and only in one case dropped below 400 $\mu M$ during the third period.

Figure 2 compares the concentrations of $Al^{3+}$ ions with the sum of the concentrations of the major nutrient cations ($Ca^{2+}$, $Mg^{2+}$, $K^{+}$) in all samples of the Al treatments. Concentrations are given in micromoles of cationic charge per L in order to allow easy comparison. As only $Al^{3+}$ was added to the treatment solutions, all nutrient cations must have been exchanged from the root surface. The line indicates a value of 1500 $\mu M$ total cationic charge, which should be reached if only equivalent exchange happened between solution cations and adsorbed cations. This was the case during the entire experiment. Sodium and $NH_4^{+}$ ions were present at concentrations below the limit of quantification Together, they did not exceed 3% of total cationic charge and could be neglected for the calculation of total cationic charge.

*Sequential extraction*

When comparing exchangeable cations at the root surface (Figure 3) at the end of the experiment, there were marked differences between the treatments. In Ca treatments, Al was mostly below the detection limit and never exceeded 5% of the exchangeable cations, whereas in Al treatments it represented about 30% of the exchangeable cations. Accordingly, there was about 2.5 times more exchangeable Ca in Ca treatments than in Al treatments. A higher amount of exchangeable K was found for Al-treated roots. Due to the large variability of exchangeable K values, this difference is not significant, however. No significant differences between treatments could be found for exchangeable Mg and Na. The amount of acid soluble

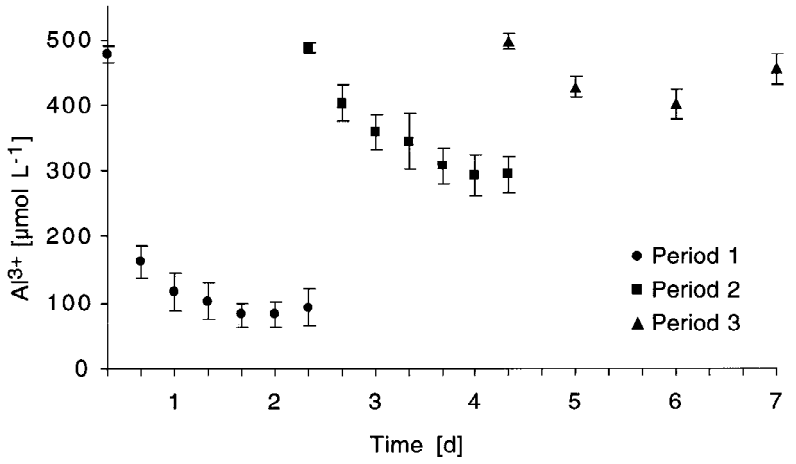


*Figure 1.* Concentrations of $Al^{3+}$ in Al treatment solutions as measured by CE (mean of three replicates ± SE).

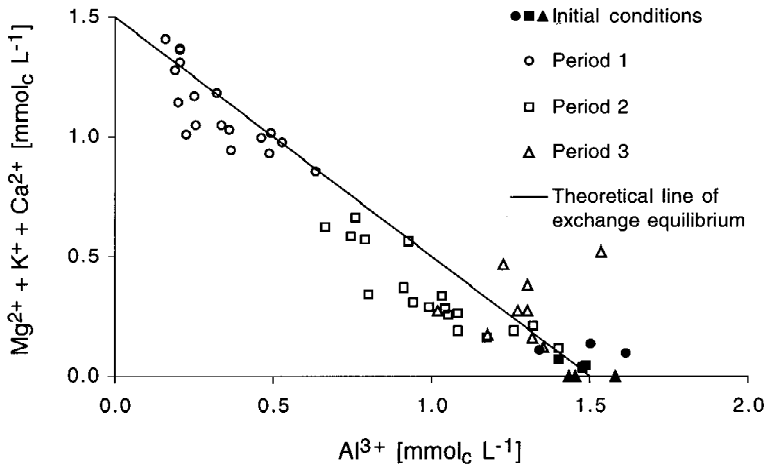


*Figure 2.* Exchange of nutrient cations against $Al^{3+}$ ions at the root surface: Solution equivalent concentration of $Mg^{2+}$ + $K^{+}$ + $Ca^{2+}$ *vs.* solution equivalent concentration of $Al^{3+}$.

but non-exchangeable Al found in the 0.01 *M* HCl extract of Al treated roots (Figure 4) was similar to the amount of $NH_4Cl$-exchangeable Al in these roots. In Ca treatments, acid extractable Al was at the detection limit. There were no large differences in acid extractable Ca between the treatments. Acid extractable K was below the detection limit in most samples of the Al treatment whereas in the Ca treatments concentrations of exchangeable and acid extractable K were similar. There were no significan differences in acid extractable Mg and Na between the treatments. When comparing total extractable amounts of the cations, significan differences between the treatments could be found for Al and Ca only. The total amount of phenolics extractable from the root surface by the sequential extraction did not differ between the treatments (Figure 5). However, while in Al treatments between 50 and 67% of all extracted phenolics were extractable with the f rst $NH_4Cl$ extraction step, this percentage was only 10–33% in Ca treatments. More than 60% of the phenolics at the surface of Ca-treated roots could not be extracted with 1 *M* $NH_4Cl$, but only with 0.01 *M* HCl.

### *Localization of elements*

Lateral roots investigated in the electron microscope showed no mycorrhizal structures, although single fungal hyphae were present. Elemental analysis revealed that a large proportion of Al was bound to the root surface (Table 1). In Al treated roots, Al was also found in cell walls of the epidermis and cortex, but not

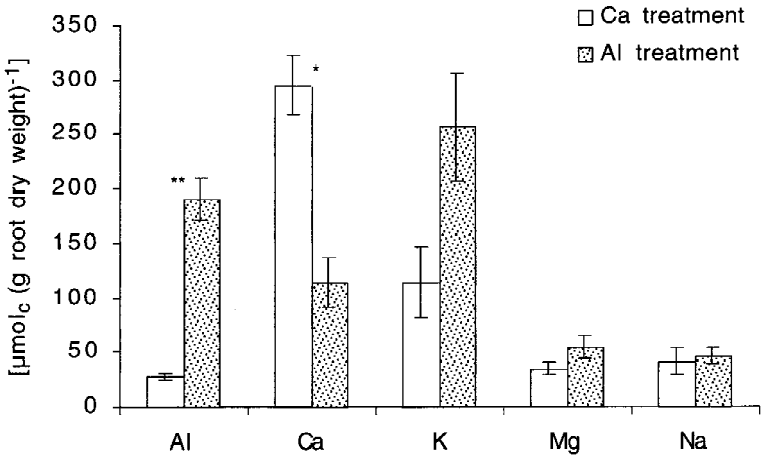


*Figure 3.* Cations exchangeable from Norway spruce roots by 1 *M* $NH_4Cl$. Values are means of three plants per treatment and three replicate extractions per plant. Bars indicate SE. Differences between the treatments: * = significan at $p < 0.05$; ** = significan at $p < 0.01$.

in the stele (Table 2). In the lumen of epidermal and cortical cells of Al treated plants, Al was present at concentrations near the detection limit. Calcium was found throughout the root, with the highest counts in the cell walls of Ca treated and untreated control plants. In cell walls of Al treated plants, however, Ca was drastically reduced. Phosphorus was slightly more abundant in the lumen than in cell walls, especially in control and Ca treated plants. In epidermal cells of Al-treated plants the count rate in the lumen was as low as in cell walls. However, surface analysis revealed an increase of P on Al treated roots compared with Ca treated or control plants. Potassium was found mainly in the lumina and less often in cell walls. Net counts in epidermal cells and in the stele of samples from Ca treated plants were very high. Elevated Cl concentrations were found in cortical lumina of both treatments when compared to the control. In Al treated plants both epidermal lumina and cell walls were low in Cl, while in Ca treated plants epidermal lumina reached f ve times more Cl net counts than the cell walls.

*Amount of released organic substances*

The amount of C released by individual plants varied strongly. On a fresh weight basis, the roots of the Ca treated plants released more C than those of the Al treated plants (Table 3). For periods 1 and 2, the differences were signif cant. In both treatments, the amount of released C during the f rst period was higher than during the two later periods. This resulted in mean solution concentrations of 1 m*M* C (Ca treatments) and 0.5 m*M* C (Al treatments) during the f rst period and about four times lower concentrations during the second and third period.

UV absorption data of all sampling dates are shown in Figure 6. UV absorption usually correlates closely with DOC for samples of the same origin, and thus, absorption data can be used as a measure of DOC (Buff e et al., 1982). In our experiment, we found similar molar absorptivity at 280 nm in both treatments (Table 3). At the low levels of DOC in the second and third period, absorption data were more sensitive than DOC measurements. For both treatments, UV absorption at 280 nm increased during all periods. During the f rst periods, this occurred rapidly during the f rst hours, after which absorption remained almost constant for the next two days. In the following periods, this increase was less pronounced and more gradual.

*Characterization of released C*

Total carbohydrates represented between 25% and 45% of released C over all samples. The percentage of carbohydrates did not differ signif cantly between the treatments. Thus, the release of total carbohydrates per g root fresh weight followed a similar pattern as total C (Table 3). For periods 1 and 2, carbohydrate release was significa tly lower in Al treatments.

Concentrations of total phenolics amounted to 5–10% of released C in the f rst periods and 10–20% in the second and third periods. The percentage of phenolics was signif cantly higher for Al treated plants during the second period only. As with total C, the solution concentrations in the f rst periods of both treatments were highest, ranging between 6 and 14

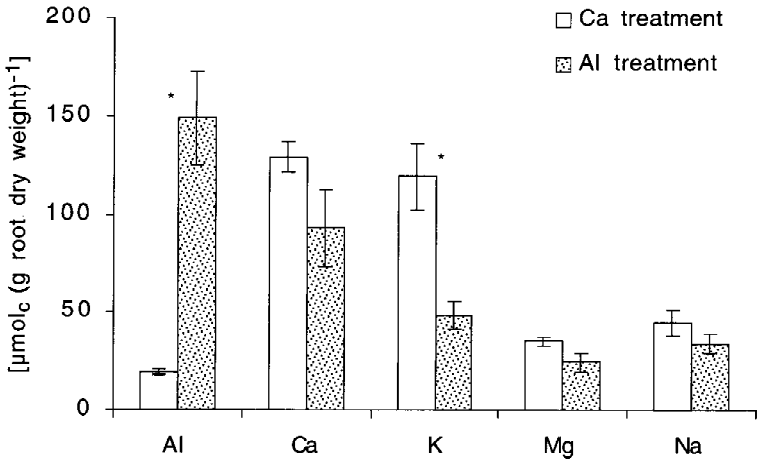


*Figure 4.* Cations extractable from Norway spruce roots by 0.01 *M* HCl during a sequential extraction. Values are means of three plants per treatment and three replicate extractions per plant. Bars indicate SE. For some samples, Al, K or Na were below the detection limit. In these cases, the detection limit was used for calculations. Differences between the treatments: * = significan at $p < 0.05$.

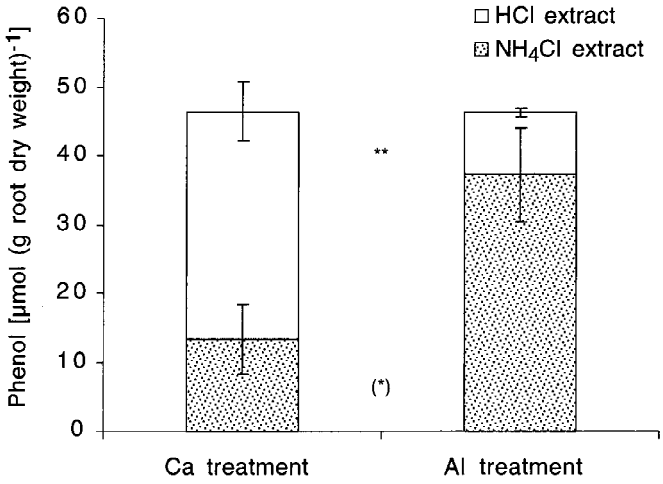


*Figure 5.* Fractions of phenolic substances extractable from the root surface of Norway spruce (means of three replicates per plant and three plants per treatment, error bars denote SE). Differences between the treatments for a given extract: (*) = significan at $p < 0.1$; ** = significan at $p < 0.01$.

*Table 1.* X-Ray net counts of selected elements on the surface of Norway spruce fin roots. Values are mean ± SD of 3-4 samples per treatment and of f ve spectra per sample

| | Net counts | | | | |
|---|---|---|---|---|---|
| | Al | Ca | K | P | Cl |
| Ca treatment | 215 ± 82 | 3220 ± 611 | 1306 ± 389 | 219 ± 71 | 358 ± 106 |
| Al treatment | 1431 ± 449 | 254 ± 96 | 667 ± 274 | 640 ± 287 | 916 ± 188 |
| Untreated control | 323 ± 67 | 2189 ± 590 | 1087 ± 590 | 311 ± 56 | 214 ± 71 |

*Table 2.* X-Ray net counts of selected elements in various cell types and compartments of freeze-fractured Norway spruce f ne roots. Values are mean ± SD of 3-4 samples per treatment and of four spectra per cell type, n.d. = not detected

| Cell type | Net counts | | | | |
|---|---|---|---|---|---|
| | Al | Ca | K | P | Cl |
| Ca treatment | | | | | |
| Epidermal cell wall | 151 ± 20 | 6125 ± 1084 | 2239 ± 296 | 258 ± 87 | 449 ± 135 |
| Epidermal lumen | n.d. | 1165 ± 238 | 5976 ± 781 | 592 ± 361 | 2292 ± 472 |
| Cortical cell wall | n.d. | 1731 ± 380 | 1398 ± 255 | 241 ± 65 | 228 ± 62 |
| Cortical lumen | n.d. | 254 ± 147 | 3378 ± 638 | 629 ± 271 | 781 ± 170 |
| Stele | n.d. | 1383 ± 275 | 6207 ± 508 | 511 ± 170 | 859 ± 211 |
| Al treatment | | | | | |
| Epidermal cell wall | 627 ± 138 | 188 ± 68 | 522 ± 91 | 278 ± 94 | 577 ± 208 |
| Epidermal lumen | 88 ± 27 | 117 ± 25 | 1961 ± 582 | 316 ± 70 | 391 ± 78 |
| Cortical cell wall | 552 ± 145 | 350 ± 77 | 1659 ± 388 | 295 ± 87 | 455 ± 176 |
| Cortical lumen | 24 ± 8 | 186 ± 53 | 2831 ± 605 | 480 ± 217 | 1269 ± 331 |
| Stele | n.d. | 174 ± 49 | 2199 ± 157 | 293 ± 58 | 1440 ± 142 |
| Untreated control | | | | | |
| Epidermal cell wall | 93 ± 42 | 2293 ± 436 | 702 ± 147 | 688 ± 297 | 75 ± 21 |
| Epidermal lumen | n.d. | 98 ± 30 | 1855 ± 528 | 1508 ± 603 | 34 ± 14 |
| Cortical cell wall | n.d. | 1027 ± 473 | 905 ± 216 | 246 ± 117 | 234 ± 66 |
| Cortical lumen | n.d. | 137 ± 66 | 2299 ± 725 | 891 ± 260 | 155 ± 79 |
| Stele | n.d. | 420 ± 128 | 3084 ± 609 | 376 ± 67 | 182 ± 76 |

*Table 3.* Release of organic substances by roots of 3-year-old Norway spruce during treatments in hydroculture (mean ± SE). Data were calculated from concentrations in the solutions remaining after each period. For a given period, release by Al treated plants was different from release by Ca treated plants at the following levels of signif cance: $^{**}$: $p < 0.01$; $^{*}$: $p < 0.05$; (*): $p < 0.1$; ns: not significan ($n$=3)

| | Ca treatment | | | Al treatment | | |
|---|---|---|---|---|---|---|
| | 1st period Day 2 | 2nd period Day 4 | 3rd period Day 7 | 1st period Day 2 | 2nd period Day 4 | 3rd period Day 7 |
| | $\mu$mol C (g root fresh weight)$^{-1}$ | | | | | |
| Total carbon | 20.2 ± 2.3 | 5.3 ± 0.2 | 4.3 ± 0.1 | 9.8 ± 1.5 $^{*}$ | 2.7 ± 0.3 $^{**}$ | 2.8 ± 0.7 ns |
| Total carbohydrates | 8.0 ± 1.1 | 1.8 ± 0.1 | 1.6 ± 0.1 | 3.5 ± 0.4 $^{*}$ | 0.9 ± 0.1 $^{**}$ | 0.9 ± 0.2 (*) |
| Total phenolics | 1.3 ± 0.1 | 0.6 ± 0.0 | 0.6 ± 0.0 | 0.8 ± 0.1 $^{*}$ | 0.5 ± 0.1 ns | 0.5 ± 0.1 ns |
| Amino acids unhydrolysed | 1.2 ± 0.3 | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.7 ± 0.1 ns | 0.4 ± 0.1 ns | 0.2 ± 0.0 ns |
| hydrolysed | 1.3 ± 0.2 | 0.4 ± 0.1 | 0.4 ± 0.1 | 0.5 ± 0.1 (*) | 0.5 ± 0.1 ns | 0.3 ± 0.1 ns |
| HMW $^{a}$ carbon | 14.3 ± 2.2 | 2.3 ± 0.6 | 1.3 ± 0.4 | 4.3 ± 1.1 $^{*}$ | 0.6 ± 0.3 ns | 0.6 ± 0.1 ns |
| HMW phenolics | 0.8 ± 0.0 | 0.3 ± 0.0 | 0.2 ± 0.0 | 0.3 ± 0.1 $^{**}$ | 0.1 ± 0.0 $^{*}$ | n. a.$^{b}$ |
| | L (mol C)$^{-1}$ cm$^{-1}$ | | | | | |
| Molar absorptivity | 158 ± 26 | 211 ± 25 | 216 ± 34 | 135 ± 5 ns | 177 ± 21 ns | 173 ± 14 ns |

$^{a}$High molecular weight (> 1kDa); $^{b}$ not analysed.

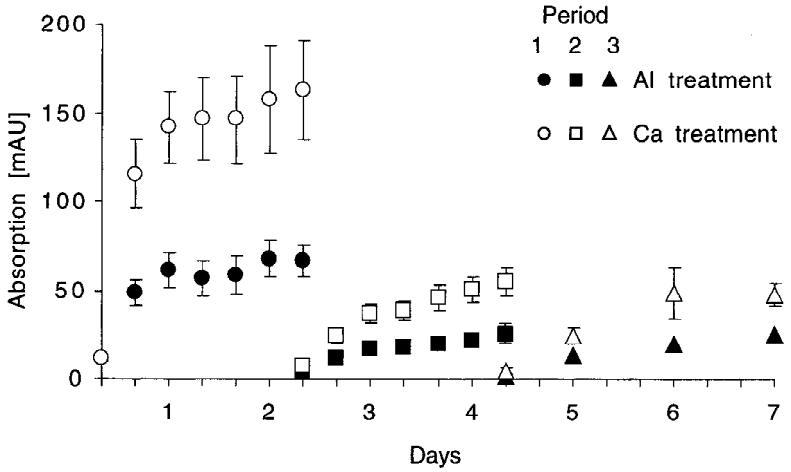


*Figure 6.* UV absorption of Norway spruce treatment solutions at 280 nm (mean of three replicates ± SE).

$\mu M$, while they reached only 3–6 $\mu M$ in the following periods. In the first period, the release of phenolics in the Al treatment was significantly lower than in the Ca treatment (Table 3), while in the later periods there were no differences.

Total amino acids represented between 4 and 20% of total C, if a mean number of 5 C atoms per molecule is assumed. Alkaline hydrolysis did not significantly increase amino acid concentrations in the samples. In the first and third periods, Ca treated plants tended to release more amino acids than did Al treated plants, but the difference was only slightly significant for hydrolysed samples of the first period.

Organic acids analysis included oxalate, tartrate, malate, citrate, succinate, glutarate, glycolate, pyruvate, acetate and lactate. Taking into account the concentration procedure, the detection limit for these acids was in the range of 1–1.5 $\mu M$ in solution and 0.1–0.2 $\mu$mol per g root dry weight. Except for traces of acetate in some samples of both treatments, all organic acids were below the detection limit. Compared with total C released by the plants, this is less than 2-5% for a single acid, depending on its number of C atoms per molecule.

*Molecular weight of released C*

A large portion of the dissolved C did not pass a 1 kDa filter (Table 3). In the first period, significantly more high molecular weight C was released by the Ca treated plants than by those treated with Al. During the experiment, the percentage of high molecular weight C decreased from 70 to 30% in Ca treatments and from 45 to 25% in Al treatments. In periods 2 and 3, differences between the treatments were statistically not significant.

A similar pattern was observed for high molecular weight phenolics. When compared with total phenolics, their percentage decreased from 60 to 30% in Ca treatments, from 30 to 20% in Al treatments. Differences between the treatments were significan in the first and second periods.

*Fluorescence spectra*

All samples used for fluorescence measurements showed a peak at an excitation wavelength of 330 nm and an emission wavelength of about 440 nm, which is characteristic for simple phenolic structures (Blaser et al., 1999; Wolfbeis, 1985). If phenolic substances are complexed with Al, a second peak appears at the same excitation wavelength but a lower emission wavelength (about 410 nm) (Blaser et al., 1999; Luster et al., 1996). Such a peak can be clearly seen in the spectra of the Al treatment solutions as shown for one example (Figure 7).

## Discussion

*Processes at the root*

The rapid removal of $Al^{3+}$ ions from solution can be explained by exchange processes occurring at the root surface. Due to the substrate within which the plants were raised, their root exchange complex was almost saturated with nutrient cations at the beginning of the experiment. In the Al treatments, nutrient cations were

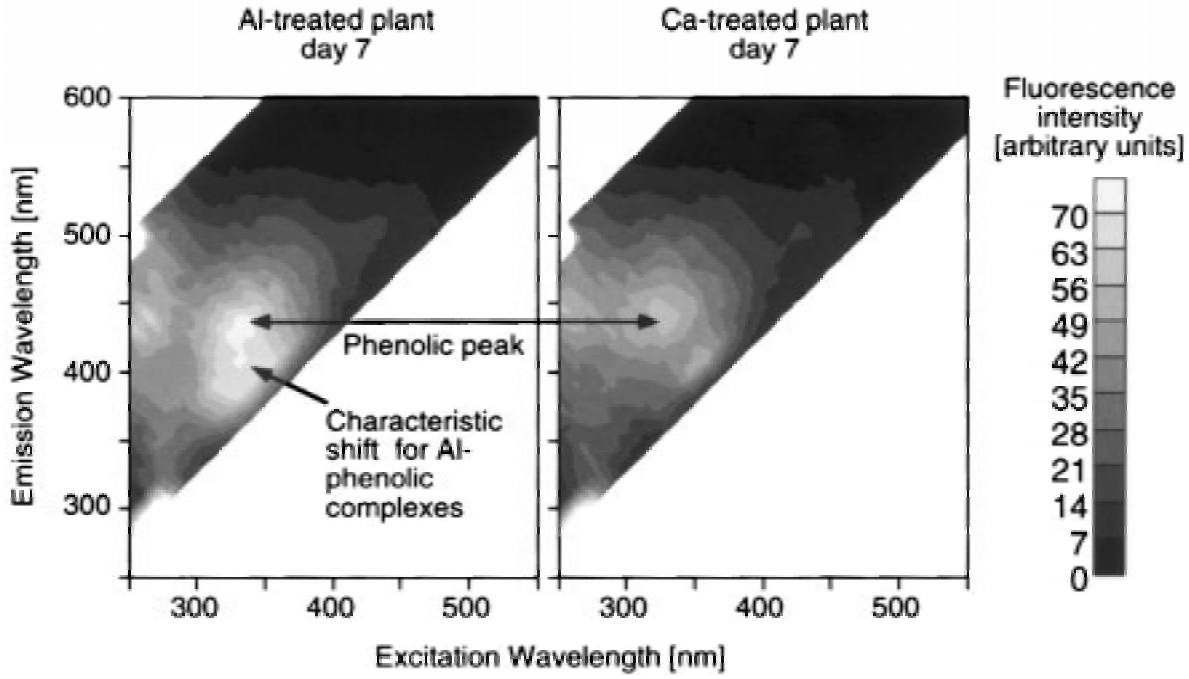


*Figure 7.* Fluorescence spectra of Norway spruce treatment solutions at the end of the experiment.

easily displaced by the highly charged $Al^{3+}$ ion with its higher affinity to the exchange sites. This effect was most pronounced when there was still little Al bound to the roots. As the Al saturation of the root exchange complex increased, there were less binding sites available which could release nutrient cations and remove $Al^{3+}$ ions from solution. The agreement between the theoretical 1:1 exchange line and measured cation concentrations in solution is strong evidence that cation exchange is the main process removing $Al^{3+}$ ions from solution.

These $Al^{3+}$ ions were adsorbed to exchange sites on the root surface. In Ca treatments, these sites were occupied by $Ca^{2+}$ ions as indicated by the fact that the equivalent sum of exchangeable $Ca^{2+}$ + $Al^{3+}$ did not differ between the treatments, whereas the equivalent ratio of adsorbed Ca/Al reached 11 (Ca treatment) and 0.6 (Al treatment).

The high percentage of $NH_4Cl$-extractable phenolics in Al treatments suggests that part of the exchangeable Al ions were not adsorbed as free $Al^{3+}$ ions but as Al-phenolic complexes. If a simple complex of one phenolic unit and one $Al^{3+}$ ion was assumed, there would be sufficient phenolic ligands present in these extracts to bind to two thirds of the adsorbed Al. The sequential extraction results indicate that at the end of the Al treatment a large percentage of the Al adsorbed during the treatment is more strongly bound at the root in an acid soluble form. These results are in agreement with the findings of Dahlgren et al. (1991) who examined Al forms in *Abies amabilis* roots. They found only 12–17% of root Al to be exchangeable and discussed co-precipitation of Al with phosphate or oxalate within roots or on root surfaces. Our data do not suggest that oxalate is involved in the formation of an acid-soluble Al precipitate, as no oxalate could be found in the HCl extracts (data not shown). Formation of aluminium phosphates might occur to some extent as EDX microanalysis revealed an increase in P at the surface of roots in the Al treatments. However, as in HCl extracts of roots of both treatments P was found at levels near the detection limit (data not shown), aluminium phosphates, if present, did not account for more than 30% of the acid soluble Al and thus are not the major form of acid soluble Al.

Total phenolics in the HCl extract of Al treated roots accounted for only 6% of the Al equivalents found in this extract. Therefore, it must be concluded that the nature of the HCl extractable Al could not be elucidated completely.

The behaviour of K may be explained by its specif c functions in plant cells. The cytoplasmic concentrations are generally very high and almost constant. Potassium easily leaks out of cells if membrane stability is compromised by either high proton infl x (Sasaki et al., 1994) or insuff cient Ca saturation (Mengel, 1991). The absence of strong binding forms of K in plant cells (Mengel, 1991) suggests that the HCl extractable K rather represents cytoplasmic K than strongly bound K. For Al treated roots, HCl extractable K was low, as most of the cytoplasmic K may already have leached across the plasmalemma membrane during the preceding $NH_4Cl$ extraction. In Ca treated roots, membranes remained intact during the $NH_4Cl$ extraction and K only leaked outside when high proton inf ux during HCl extraction caused depolarization of the membrane.

*X-ray microanalysis*

The accumulation of Al in the cell walls of peripheral cortex cells as confirme by the EDX microanalysis is in accordance with the results of Bauch and Schröder (1982), who investigated f ne roots of healthy and diseased silver f r and Norway spruce trees and found Al mainly in cortex cell walls and only very low concentrations in cell walls of the xylem.

Accumulation of Al and decrease of Ca in cell walls has been shown by several authors (Godbold et al., 1988; Godbold and Jentschke, 1998; Kuhn et al., 1995; Schröder et al., 1988). The decrease of Ca in cell walls of Al-treated plants is in accordance with the results of the $NH_4Cl$ extraction, as cation exchange sites are mainly located on pectins, proteins and phospholipids in the cell wall (Horst, 1995).

High X-ray net counts of K in Ca treated roots may be partly due to overlapping of the primary Ca peak (Ca K$\alpha$) and the secondary K peak (K K$\beta$, Lazof and Läuchli, 1991). Additionally, it is possible that the very mobile K ion (Marschner, 1995) was translocated from the shoot to the roots in order to compensate the dehydrating effects of high Ca concentrations (Bergmann, 1993). Chloride present in the treatment solutions was taken up by the treated plants to reach a higher level than in the control as confirme by EDX microanalysis. For reasons of charge balance, this should increase the pH of the treatment solutions if no equivalent amounts of cations are taken up. In accordance with this, in Ca treatments in which Cl concentrations in cell lumina were high, more HCl than in Al treatments had to be added during the experiment in order to keep the pH of the solution below 4.0 (data not shown).

*Release of organic substances*

Our experimental system very likely included rhizospheric microorganisms living at the root surface that were transferred to the treatment flas together with the roots. Therefore, this setup did not allow calculation of total C released by the roots, as part of it may be metabolized by microorganisms (Marschner, 1995). However, it is well suited to characterize those organic substances that are not rapidly degraded. With respect to Al detoxificati n by complexation with organic ligands (Delhaize et al., 1993; Pellet et al., 1995; Zheng et al., 1998), only this fraction is considered efficient

If Norway spruce followed the strategy to exclude Al by exudation of organic substances with strong complexing properties, either an enhancing effect of Al on release of organic substances, or qualitative changes in their composition would be expected. In our experiment, Al treatment reduced total C release, and only during the second period the Al treated plants released a signif cantly higher percentage of phenolics than Ca treated plants. No other changes in composition were observed. The decrease in total C release could be the consequence of reduced C assimilation of Al treated spruce (Hentschel et al., 1993), or it could be due to changes in plasma membrane permeability. In various plant species, membrane permeability to electrolytes may increase by Al treatment (Calbo et al., 1997; Ishikawa and Wagatsuma, 1998) and permeability to nonelectrolytes may decrease (Parent et al., 1996; Zhao et al., 1987), but the membrane itself is considered to stay intact although specif c transport proteins may be blocked by Al (Kochian, 1995).

Organic acids are suitable ligands that could effectively detoxify Al (Hue et al., 1986). However, our results support the f nding of Eltrop (1993) that organic acid release by Norway spruce is generally very low. In contrast to the f ndings on maize (Pellet et al., 1995), wheat (Delhaize et al., 1993), and buckwheat (Zheng et al., 1998), where exudation of organic acids has been shown to be an effective detoxifying mechanism for Al, our results indicate that Norway spruce is not able to increase the release of organic acids to a level where effective detoxificatio of Al could occur. The conclusion, that organic acid exudation is not an important mechanism in alleviating Al toxicity in spruce, is indirectly supported by an investigation

of organic acid concentrations in Norway spruce f ne roots sampled in the humus layer and the upper mineral soil of a dystric cambisol (humus form: moder) in Germany (Nowotny et al., 1998). Upon acid irrigation, which very likely increased Al concentrations in the soil solution, citrate and malate concentrations in the roots decreased or remained constant, indicating that synthesis of these acids was not stimulated.

Beside organic acids, a role of polysaccharides and polyuronic acids in mucilage for Al tolerance has been proposed (Horst et al., 1982). However, excretion of carbohydrates and high molecular weight substances was not enhanced by Al treatment but reduced to the same degree as total C release, when compared with the results of the Ca treatments.

About 10% of the released C can be attributed to phenolic substances, a group that can form stable complexes with $Al^{3+}$ ions (Martell and Smith, 1977). The f uorescence spectra of our solutions show $Al^{3+}$ complexation by phenolic substances. Although during the second period phenolics represented a higher percentage of total C in Al treatment solutions, Al treatment decreased the amount of phenolics released during the f rst period and had no effect on the amount in the later ones. Thus, complexation of $Al^{3+}$ by phenolic substances exuded by Norway spruce roots cannot be regarded as an active protective mechanism. Furthermore, as total phenolics are only present at micromolar levels, only a minor part of the total Al in solution can be bound by these substances.

The low concentrations of free amino acids released do not suggest an important role for these compounds either. Amino acid release was of the same order of magnitude as release of phenolics. This is in good agreement with the results of Eltrop (1993) who found phenolics and amino acids at equal but low levels in the exudates of 5-month-old Norway spruce seedlings. Since hydrolysis did not increase amino acid yield, specif c root exudation of polypeptides in response to Al stress as has been demonstrated in wheat (Basu et al., 1994; 1997) is unlikely to occur in Norway spruce.

*Characterization of released C*

When summing up the percentage of analysed organic substances, at best 60% of the released C could be identif ed. However, the analysis of carbohydrates and phenolic substances was done in terms of the low-molecular weight substances glucose and phenol, respectively. Considering the high content of high-molecular weight substances indicated by the ultrafilt ation results, it is likely that the effective concentrations of released carbohydrate C and aromatic C were higher. Additionally, it should be noted that organic acids may constitute a significa t portion of total C, although all single acids were below the detection limit. The sum of several single acids, each of them being present at concentrations below 2% of total C, could easily account for 10–20% of total C. This could not be checked for, since there is no method available to assess total organic acids. Generally, the results suggest that only minor changes of the composition of the released C occur while quantity is more clearly affected by Al treatment. This is further supported by the molar absorptivity data, which can be used as an indicator of aromaticity (Chin et al., 1994). In contrast to absolute absorptions, they did not differ signif cantly between the treatments in the remaining solutions of each period, which indicates that quality of released C is less affected by treatment than quantity.

*Temporal changes in C release*

The temporal pattern of C release was independent of treatment and very similar for all C fractions observed. The pattern might be partly inf uenced by the experimental design. The higher C loss and the lower percentage of phenolics in the f rst period might be attributed to a treatment shock, when plants were transferred from soil to hydroculture. Treatment solutions were single salt solutions and thus differed signif cantly from soil solution. As a consequence, root chemistry, which, at the beginning of the f rst period, was still influence by the chemical conditions in the soil, had to be adjusted to the solution conditions. By contrast, in the following periods, the roots were preconditioned by the f rst treatment period. This explains why the results from the second and third periods for a given treatment differ much less from each other than they do from the results of the f rst period. The data from the second and third periods represent the C release which is characteristic of the given experimental conditions, while the f rst period must be considered to represent a transient state.

## Conclusions

The results of this study imply that root exudation of organic substances with complexing properties does not contribute significa tly to the relatively high Al

tolerance of Norway spruce. Uptake of Al is impeded by immobilization at the root surface and in the cell walls of epidermis and cortex. Complexation of Al with phenolic substances present at the root surface and precipitation as phosphate may play important roles in this immobilisation. If such a mechanism was to work over long periods of time, however, high turnover rates of the f ne root system would be necessary (Vogt et al., 1987).

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