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Abstract: Aluminum (Al) has been suggested to be an important stress factor in forest decline due to its mobilization in soil following atmospheric deposition of acidic pollutants. A major goal of our research is to develop physiological and biochemical markers of stress in trees using cell cultures and whole plants. Needles of red spruce (*Picea rubens*) collected from several sites in the northeastern United States and red spruce cells grown in suspension cultures were examined for polyamine and inorganic-ion content. The cells in culture were exposed to various concentrations of Al for different lengths of time. Exposure to Al increased putrescine biosynthesis and lowered the concentrations of cellular Ca, Mg, Mn, and K. No treatments were applied to the trees but some of the sites were known to be under "general environmental stress" as indicated by a large number of dead and dying red spruce trees. All of the sites, while differing in geochemistry, had a soil pH value below 4.0. Data collected from field studies enabled us to categorize these sites on the basis of cellular levels of putrescine and soil chemistry. Needles from trees growing on Ca-rich soils (organic horizon) with low exchangeable Al:Ca ratios had lower levels of putrescine than those from trees growing on Ca-poor soils with high Al:Ca ratios.

INTRODUCTION

The negative effects of acidic deposition on soil fertility, possibly due to the mobilization of Aluminum (Al) and leaching of bases, are of major concern because such processes can impact forest growth over large areas. Although exposure to low levels of pollutants under natural conditions may not result in immediate visible injury, subtle physiological and biochemical changes may still be quantifiable. The ability to detect these changes at an early stage would enable us to predict future forest damage and may suggest means to reduce the severity of such damage. Therefore, it is necessary to develop a set of early physiological and biochemical indicators to assess possible adverse effects of soil Al and Calcium (Ca) concentrations on forest growth.

Changes in the Al:Ca ratio of the soil solutions are correlated with Al stress and nutrient imbalances in sensitive tree species (Cronan and Grigal 1995). Among the effects of Al on plants are inhibition of cell division, DNA synthesis, needle biomass, root growth, and seedling height (McQuattie and Schier 1990, Schier et al. 1990). Al also affects the uptake of Ca and other inorganic ions (Minocha et al. 1992, Zhou et al. 1995). Earlier work by our group suggests that changes in the Al:Ca ratio in fine roots of red spruce growing under stress may be linked to increased vulnerability and mortality of trees (Shortle and Smith 1988). However, the primary sites of Al toxicity and the chain of biochemical and molecular events through which Al exerts its toxic effects are not well understood (Delhaize and Ryan 1995).

Recently, considerable attention has been focused on the study of changes in the metabolism of aliphatic polyamines (putrescine, spermidine, and spermine) in plants subjected to various kinds of environmental stress. The cellular polyamine content is highly regulated and stimuli such as Ca and magnesium (Mg) deprivation, high salinity, sulfur dioxide (SO₂) fumigation, pathogenesis, osmotic stress, ozone, and acid stress lead to an accumulation of one or more of the polyamines (Galston 1989, Flores 1991). This increase in polyamines generally is accompanied by

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increased activity of their biosynthetic enzymes. Studies with cell cultures of a woody plant, *Catharanthus roseus*, showed that treatments with Al caused changes in cellular polyamines, particularly putrescine, while also causing inverse effects on cellular Ca levels (Minocha et al. 1992, Zhou et al. 1995). Therefore, we hypothesized that changes in levels of cellular putrescine or putrescine/spermidine ratios could be used as an early indicator of the stress response not only in cell cultures but also in mature forest trees.

The objectives of this study were to: 1) analyze changes in putrescine and putrescine/spermidine molar ratios in response to Al stress using cell cultures of red spruce; 2) determine cellular polyamine levels in needles of mature red spruce trees growing under stress; and 3) determine if there is a correlation between putrescine in needles of mature red spruce trees and Ca levels in soil solutions of the Oa and B horizons or exchangeable Al:Ca charge ratios in the Oa horizon.

MATERIALS AND METHODS

Cell Culture Studies

Culture conditions. Suspension cultures of *Picea rubens* were obtained from Dr. Krystyna Klimaszewska, Petawawa National Forestry Institute, Chalk River, ON, and maintained in half-strength Litvay's medium (Litvay et al. 1981) as modified by Klimaszewska (personal commun.). Modifications included the addition of 0.5 g/L glutamine, 1.0 g/L casein hydrolysate, 2 percent sucrose (rather than 3 percent), 9.05 μ M 2,4-D, and 4.44 μ M BA. In addition, iron-EDTA was replaced by 40 mg/L of a plant product called sequestrine containing 7 percent iron chelate (Plant Products Co., Brampton, Ontario L6T1). The medium was adjusted to pH 5.7 before it was autoclaved. Cells were subcultured at intervals of seven days by transferring 15 ml of cell suspension into 45 ml of fresh medium in 250-ml Erlenmeyer flasks. The flasks were kept in darkness at 25 °C $\pm$ 2 on a gyratory shaker at 120 rpm.

The Al levels (0.2, 0.5, and 1.0 mM of AlCl₃) used in cell-culture experiments were based on earlier work on the effects of Al on growth. The pH of the medium at the time of Al addition was 4.2 or lower. To study Al speciation in this medium Al was added to the cell-free medium or to three-day-old cell cultures. In either case, about half of the added AlCl₃ was found to be precipitated. Before analysis for monomeric Al, the precipitate was removed by centrifuging the medium.

For experimental treatments, filter-sterilized AlCl₃ was added to a final concentration of 0.2, 0.5, or 1.0 mM to 20 ml of three-day-old cells maintained in 50-ml flasks. The flasks were kept on a gyratory shaker at 120 rpm until analysis. The pH of the medium, which remained around 4.2 $\pm$ 0.3 after 24 h of subculture, was not adjusted during the incubation period. Each treatment was run in triplicate and each experiment was run at least three times. At the end of the treatment period, cells were collected and analyzed for polyamines and inorganic ions by methods described in the section that follows.

Inorganic ion analysis. Cells were collected on Miracloth by vacuum filtration, washed thoroughly with deionized distilled water, and weighed. One hundred mg of cells were frozen and thawed (3X) in 10 ml of 0.01 N HCl (Minocha et al. 1994). Extracts were centrifuged at 18,000 g for 20 min at 4 °C or filtered through a 45- μ m nylon syringe filter. The supernatant solutions or the filtrates were analyzed for inorganic ion content with a Beckman Spectrospan V ARL DCP (Direct Current Plasma Emission Spectrometer, Beckman Instruments, Inc., Fullerton, CA) using the Environmental Protection Agency's method number 66-AE0029 (1986).

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Polyamine analysis. Cells were collected and extracted in 5 percent perchloric acid (PCA) in the manner described for inorganic ions. Extracts were centrifuged at 18,000 g for 20 min at 4 °C. The supernatant fractions were used for dansylation and quantification of polyamines by high performance liquid chromatography (HPLC) (Minocha et al. 1990).

Whole-Plant Studies

Sites

Six sites from the northeastern United States were selected for collection of soil, root, wood-core, and needle samples, as part of a collaborative effort funded under the USDA Forest Service Global Change Program. The sites were located at Howland, Lead Mountain, and Kossuth, ME; Crawford Notch, NH; Groton, VT; and Big Moose, NY. Seventy-two red spruce trees were selected randomly and tagged at each site at the beginning of the study. Each site was sampled twice a year for two years except for needle samples from Kossuth and Big Moose, which were sampled once a year. Soil and needle samples were collected within one year of each other. Visual observations at each site indicated that Howland, Kossuth, and Groton had no dieback or unusual mortality but that stands at the Big Moose and Crawford Notch were experiencing dieback. The stand at Lead Mountain, also known as Bear Brook, is relatively younger and did not exhibit dieback.

Needle Samples

Extraction of acid soluble polyamines and inorganic cations. Ten of the 72 flagged trees were selected from each site for collection of needle samples. In some instances, because of difficulty in reaching branches of tall trees, untagged trees in the vicinity of tagged trees in the same stand were selected for needle samples. Needles from current- and previous-year growth were collected from freshly cut branches in the field and immediately placed in individual preweighed microfuge tubes containing 1 ml of 5 percent PCA. The tubes were kept on ice during transportation to the laboratory. The tubes were stored at -20 °C until they were processed. The samples were weighed, frozen and thawed (3X), and centrifuged at 14,000 rpm for 10 min. The supernatant was used directly for polyamine analysis or for inorganic-ion analysis after proper dilution with distilled, deionized water (final concentration of PCA 0.01 or 0.02 N) by the procedures described earlier.

Soil Samples

At each sampling time, three clusters of three trees each were selected from the 72 tagged trees. Soil samples were collected from the Oa horizon and upper 10 cm of the B horizon near each of the nine selected trees and combined for each cluster. Thus, 12 pooled samples from the Oa and B horizons were collected at each site over the four sampling periods.

Analysis of exchangeable Ca and Al in soil. Soil samples were extracted with 1 M ammonium chloride and the extracts were analyzed for Ca by the procedure of Blume et al. (1990). Exchangeable Al was determined in 1 M KCl extracts (Thomas 1982).

Ca in soil solution. Each soil sample was placed in a sealed cylinder and an artificial throughfall (chemically similar to Howland site throughfall) was added to attain a moisture content that approximated field capacity. The soil solution was expelled by positive air pressure from the soil that was placed in the sealed cylinder. This procedure for extraction of soil solution was developed by Lawrence et al. The soil solution was analyzed for aqueous Al species and other ions according to the procedures of Driscoll (1984) and Lawrence et al. (1995).

⁶Lawrence, G. B. and M. B. David. A new method for collecting soil-solution in forest soils. In preparation.

RESULTS

Cell Culture Studies

Adding AlCl_3 to the cell-free medium or cell cultures resulted in precipitation of 50 to 60 percent of the added Al even when the medium pH was 4.2 or lower. This medium contains a nursery product called "sequestrine", which has 7 percent iron-EDTA. The remaining undefined component(s) of sequestrine in half-strength Litvay's medium was largely responsible for the precipitation of Al (data not presented). All of the soluble Al was present in monomeric form at final concentrations of 0.085, 0.22, and 0.510 mM, respectively for the 0.2, 0.5, and 1.0 mM AlCl_3 added to the cultures. More than 75 percent of this total monomeric Al was present as inorganic monomeric Al.

A significant negative growth effect was observed with the 1.0 mM Al treatment, as early as one day after the addition of Al. The 0.2 mM Al treatment had no significant effect on growth for the first three days. A dose-dependent inhibition of growth increased with increasing incubation time for treatments after four days (Fig. 1).

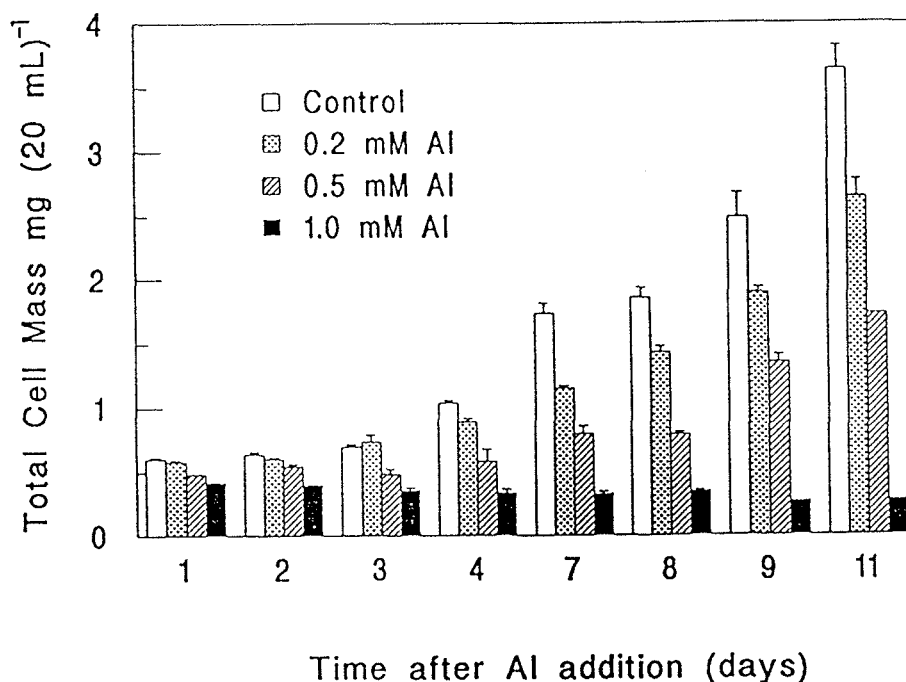


Figure 1. Effects of aluminum chloride on total cell mass in three-day-old cell cultures of red spruce (data are mean $\pm$ SE of three replications).

Effects of Al on cellular putrescine metabolism were observed as early as 4 h after treatment (Fig. 2). In general, Al caused a dose-dependent elevation of cellular putrescine in these cells at all times ($\alpha = 0.05$ for 0.5 and 1.0 mM Al). Spermidine levels were not affected or showed a slight increase (Fig. 3). This effect was not always dose-dependent.

There was an increase in cellular concentrations of Al and P in response to Al additions at all times tested (Fig. 4). This increase always was dose-dependent for Al. There was no change in cellular levels of Al between 4 and 48 h when cultures were incubated with 0.2 mM Al. However, the concentration of Al in 1.0 mM Al-treated cells increased from $11.4 \mu\text{mol (g FW)}^{-1}$ at 4 h to $23.94 \mu\text{mol (g FW)}^{-1}$ at 24 h after incubation. The content of cellular potassium (K) showed a dose-dependent decrease with all concentrations of Al. Both manganese (Mn) and Mg decreased following treatment with 0.5 and 1.0 mM Al, while Ca was significantly reduced only by 1.0 mM Al.

A comparison of putrescine/spermidine ratios showed a significant increase in these ratios with Al treatment (Fig. 5). This increase generally was dose-dependent.

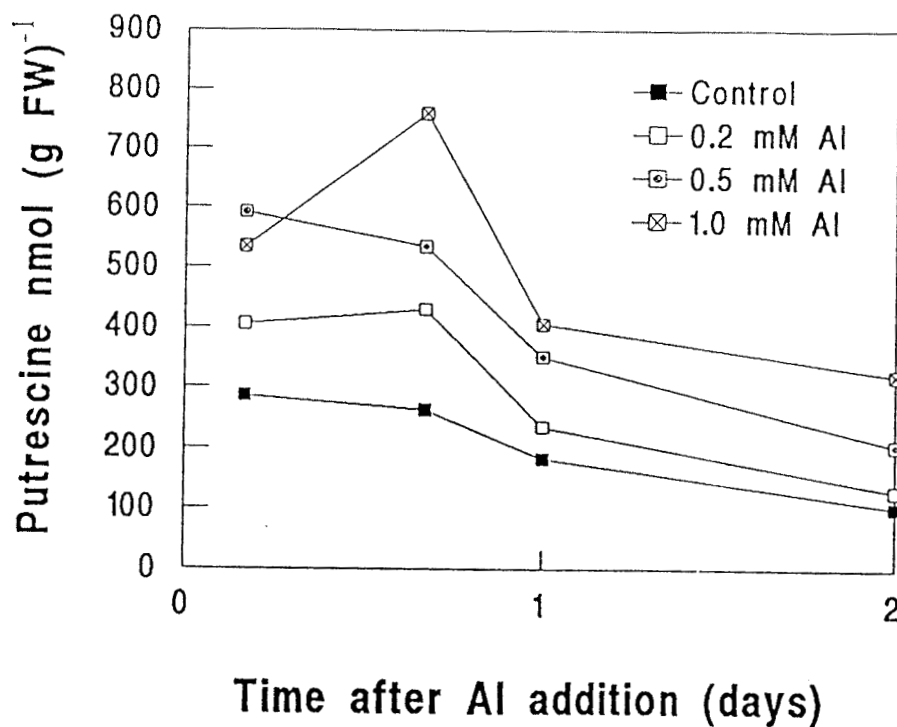


Figure 2. Effects of aluminum on cellular levels of putrescine in three-day-old cell cultures of red spruce (data are mean $\pm$ SE of three replications).

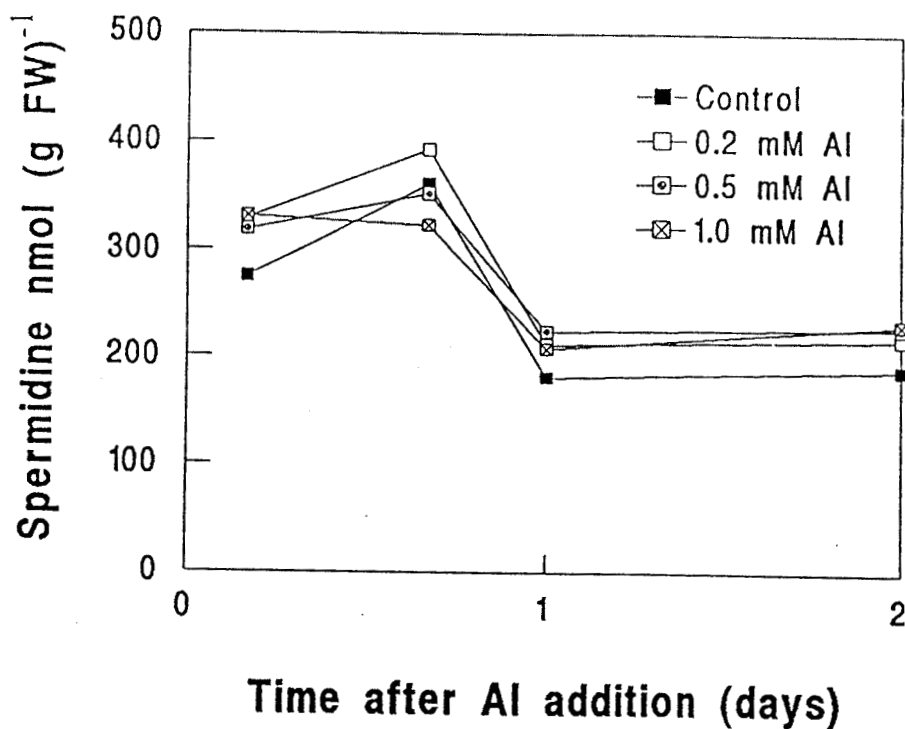


Figure 3. Effects of aluminum on cellular levels of spermidine in three-day-old cell cultures of red spruce (data are mean $\pm$ SE of three replications).

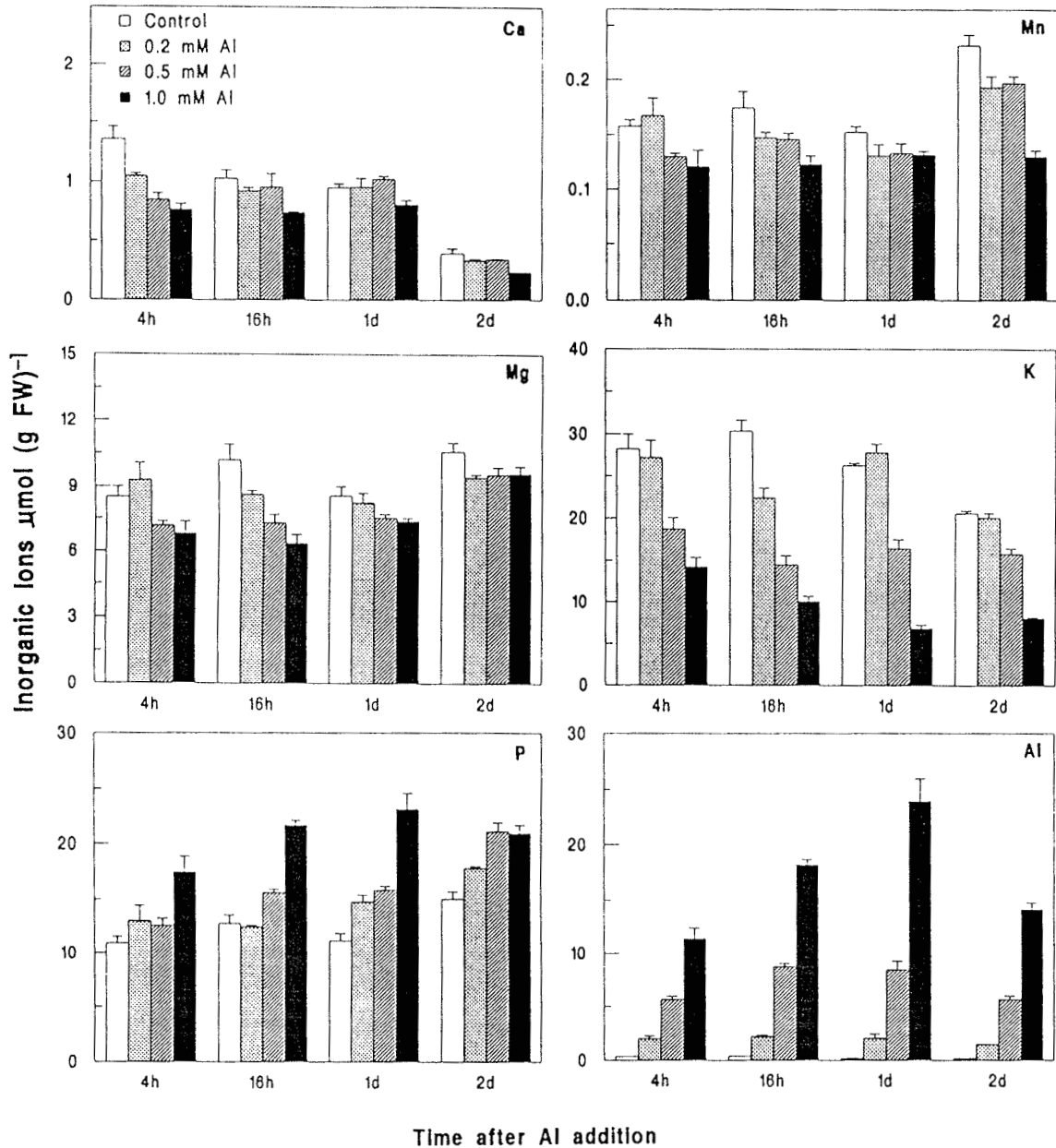


Figure 4. Effects of aluminum on cellular levels of inorganic ions in three-day-old cell cultures of red spruce (data are mean $\pm$ SE of three replications).

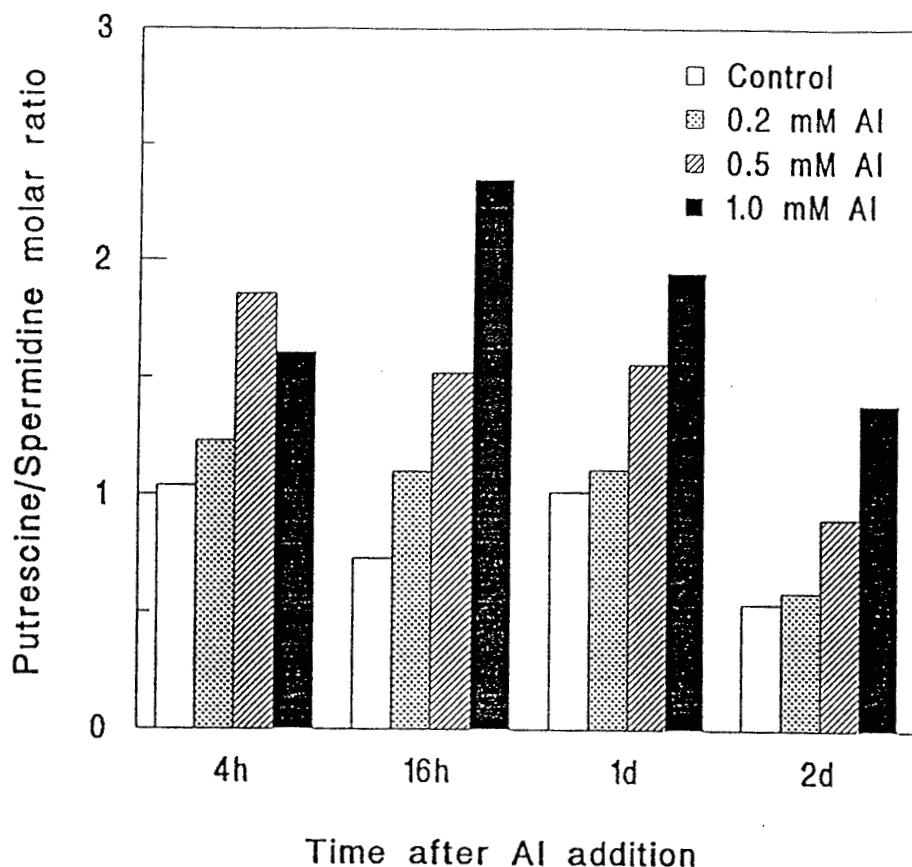


Figure 5. Effects of aluminum on putrescine/spermidine molar ratio in three-day-old cell cultures of red spruce (data are mean $\pm$ SE of three replications).

Whole-Plant Studies

All field sites sampled had a soil pH (measured in 0.01 M CaCl_2) of 2.56 to 3.11 and 3.58 to 4.46 for the Oa and B horizons, respectively. However, these sites differed in soil Ca and Al levels as well as general tree stress. While Groton and Howland showed no obvious signs of dieback, Crawford Notch and Big Moose exhibited higher general stress as indicated by a high rate of dieback and mortality. Levels of cellular putrescine generally were higher in needles of red spruce trees growing at sites with higher exchangeable Al:Ca ratios (Fig. 6). Differences in putrescine levels between sites were statistically significant ($P < 0.01$). Putrescine levels in the needles were significantly correlated to the charge ratio of exchangeable Al:Ca in the Oa horizon of the forest floor ($r^2 = 0.68$, $P = < 0.02$) (Fig. 6). Whereas putrescine levels in the needles showed a significant correlation with Ca in the soil solution of the Oa horizon of the forest floor ($r^2 = 0.58$, $P = < 0.05$), there was no correlation with the Ca concentration in the soil solution of the upper 10 cm of the B horizon of the mineral soil ($r^2 = .03$) (Table 1).

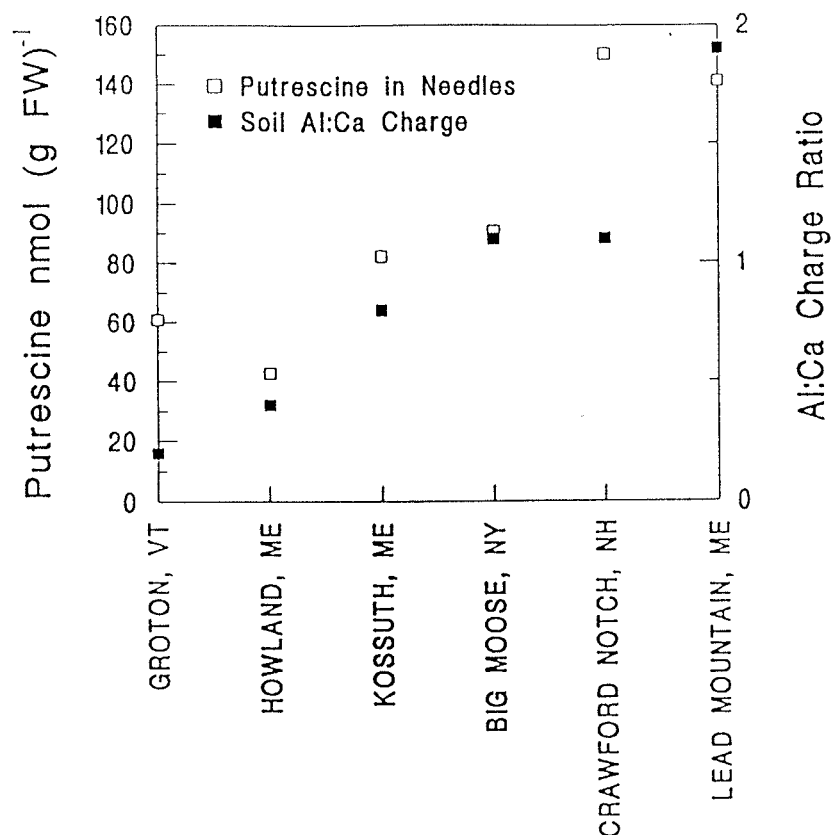


Figure 6. Comparison of putrescine in needles of red spruce trees and charge ratio of exchangeable Al:Ca in Oa horizon of the forest floor for the six sites studied. Data for putrescine are mean of 80 replicate analyses (except for Kossuth, ME, and Big Moose, NY, for which $n = 40$). Each value for soil data represents 36 samples collected individually and combined into 12 samples for analysis. (Data for Al:Ca are a mean of 12 replicate analyses).

Table 1. Comparison of putrescine in needles of red spruce trees, Ca in soil solution of Oa horizon of forest floor, and Ca in soil solution of upper 10 cm of B horizon of mineral soil for six sites studied. Data for putrescine are mean $\pm$ SE of 80 replicate analyses (except for Kossuth, ME; and Big Moose, NY; for which $n = 40$). Each value for soil data represents 36 samples collected individually and combined into 12 samples for analysis (data for Ca are mean $\pm$ SE of 12 replicate analyses).

Site	Putrescine in needles	Ca in soil solution of Oa	Ca in soil solution of B
	(nmole/g FW)	-----(μ mol/L)-----	
Groton, VT	60.9 $\pm$ 5.2	182 $\pm$ 43	6 $\pm$ 1
Howland, ME	42.6 $\pm$ 3.3	143 $\pm$ 28	5 $\pm$ 1
Kossuth, ME	82.3 $\pm$ 7.7	85 $\pm$ 27	5 $\pm$ 1
Big Moose, NY	90.4 $\pm$ 7.1	22 $\pm$ 2.9	5 $\pm$ 1
Crawford Notch, NH	150.2 $\pm$ 12.3	47 $\pm$ 13	4 $\pm$ 1
Lead Mountain, ME	140.9 $\pm$ 13.1	30 $\pm$ 3.9	8 $\pm$ 1

DISCUSSION

Joslin and Wolfe (1988) reported a significant reduction in root and foliar biomass with an increase in the levels of inorganic monomeric Al in the soils, and Ohno et al. (1988) found a negative correlation between biomass of needles and concentration of Al in the needles of red spruce. The data presented here using suspension cultures of red spruce are consistent with these reports. The inhibition of DNA synthesis and cell division had been previously associated with a reduction in the growth of Al-treated cells or plants (Maniwaki et al. 1992, Ulrich and Clarkson 1992).

While the effects of Al on K uptake vary with plant species (Cummings et al. 1985, Godbold et al. 1988, Ohno et al. 1988, Schier et al. 1990); Al often causes a reduction in Mg and Ca in needles, roots, or shoots of spruce and pine seedlings (Asp et al. 1988, Ohno et al. 1988, Schroder et al. 1988, Schier et al. 1990, Jentschke et al. 1991). In our study with red spruce cultures, a decrease in accumulation of Ca, Mg, Mn, and K was coincident with an increase in putrescine. The observed decrease in K uptake in response to Al may be related to the efflux of malate and K, as seen in the root apices of wheat (Ryan et al. 1995). This has been suggested as a mechanism for Al detoxification (by chelation) around the critical growth region of the root (Delhaize and Ryan 1995). The observed increase of P in Al-treated cells is consistent with previous studies (Asp et al. 1988, Bengtsson et al. 1988, Maniwaki et al. 1992). See Zhou et al. (1995) for a discussion of the interaction between Al and P.

Various biological and chemical agents induce the accumulation of cellular putrescine in several plant species (Dohmen et al. 1990, Santerre et al. 1990, Flores 1991). The increase in putrescine generally was accompanied by an increase in arginine decarboxylase activity (Flores 1991). Treatment with Al also resulted in an increase in cellular putrescine in our study. This effect generally was dose-dependent and could be observed as early as 4 h after treatment. In most plants, while levels of both putrescine and spermidine change in response to growth, it is only putrescine that changes in response to stress. Thus, a molar ratio of putrescine/spermidine might be a better indicator of stress than putrescine concentration alone. In this study, the level of cellular putrescine was significantly higher in the needles of red spruce growing in areas of higher general stress such as Big Moose and Crawford Notch. An exception was at Lead Mountain, despite the higher level of cellular putrescine in needles at that site, the trees did not show any visible signs of stress. However, these trees are much younger than those at Crawford Notch and Big Moose, so they may be better able to cope with stress.

The data from Lead Mountain also indicate that along with high reactive Al concentrations, soil solutions at this site contain high concentrations of nitrate. In healthy coniferous stands nitrogen generally is expected to be growth limiting. This results in low to undetectable concentrations of nitrate in soil solutions. High concentrations of nitrate in soil solutions suggest that the trees at this site may be under early stages of stress that have not yet produced visible symptoms but this stress is detectable by changes in putrescine metabolism. These results are consistent with other studies with Norway spruce (*Picea abies*) that show that cellular putrescine levels and putrescine/spermidine ratios are higher in needles collected from trees growing in polluted areas (Tenter and Wild 1991, Villanueva and Santerre 1989).

An inverse correlation between cellular putrescine and Ca in *Catharanthus roseus* cell cultures treated with Al was reported by Maniwaki et al. (1992) and Zhou et al. (1995). A similar response was seen with the cell cultures of red spruce. This observation supports the view that under stress, putrescine production (a divalent organic cation) may substitute for Ca and Mg deficiency (Cohen and Zalik 1978, Cho 1983). Further analysis showed that there was a significant inverse correlation between putrescine levels of needles and Ca concentration in soil solution and between putrescine levels and exchangeable Al:Ca charge ratio of the Oa horizon of the forest floor at all six sites. The reason for the lack of a correlation between putrescine levels and Ca in the soil solution of the B horizon may be that most roots of red spruce grow only in the Oa horizon. As a consequence the soil chemistry of only this horizon primarily affects the health of the species. Our data also suggest that all parts of a tree need not be exposed to high levels of Al to respond to stress.

The Al/Ca ratio of the soil solution is one of several tools that can be used in assessing and predicting forest health (Cronan and Grigal 1995). Likewise, the molar ratio of Al/Ca rather than absolute amounts of these ions in root tips of spruce has been suggested as being important in determining the level of Al toxicity (Shortle and Smith 1988,

Schroder et al. 1988). Several studies have been reported where an Al/Ca molar ratio of greater than 1.0 in the soil solution, growth medium, or inside the cells had inhibitory effects on growth (Schier 1985, Stienen and Bauch 1988, Kruger and Sucoff 1989, Schulze 1989). On the basis of a critical review of the literature on Al stress, Cronan and Grigal (1995) estimated that there may be a 50, 75 and 100 percent risk of adverse impacts on tree growth or nutrition with molar Al:Ca ratios of 1, 2 and 5, respectively. We demonstrate here that an increase in cellular putrescine level in response to direct or indirect stress imposed on trees by Al exposure may be considered as another type of tool for assessing and predicting forest health. Further, the similarity of our results (on Al effects on growth and inorganic-ion uptake) using cell cultures of red spruce with the results obtained by others using seedlings of red spruce indicates that the cell cultures grown in vitro are highly suitable for studies of the mechanisms of Al effects on the metabolic processes of cells.

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