

Changes in polyamines, inorganic ions and glutamine synthetase activity in response to nitrogen availability and form in red spruce (*Picea rubens*)

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Summary We analyzed effects of nitrogen availability and form on growth rates, concentrations of polyamines and inorganic ions and glutamine synthetase activity in in-vitro-cultured red spruce (*Picea rubens* Sarg.) cells. Growth rates, concentrations of polyamines and glutamine synthetase activity declined when either the amount of nitrate or the total amount of N in the culture medium was reduced. When total N in the medium was increased, cell mass increased without significant changes in glutamine synthetase activity or polyamine concentration. Reductions in the amount of nitrate or total N in the culture medium resulted in increased accumulations of Ca, Mn and Zn in the cells, and K accumulation decreased in response to decreasing nitrate:ammonium ratios. The data indicate that changes in total N availability as well as the forms of N play important roles in the physiological responses of in-vitro-grown red spruce cells that mimic the observed responses of forest trees to soil N deficiency and N fertilization.

Keywords: *acidic deposition, nitrogen assimilation.*

Introduction

Whether surplus N has positive or negative effects on plant growth depends on the cellular N status, soil N status, and the rate and duration of N deposition (Aber 1992, Aber and Driscoll 1997, Aber et al. 2003, Miller et al. 2007). If the additional N available to plants does not exceed their capacity for N uptake and assimilation, net primary productivity and carbon (C) sequestration may be enhanced. However, if N availability exceeds the capacity for N uptake and assimilation, resulting in N saturation, plant growth may be reduced (Nihlgard 1985, Agren and Bosatta 1988, Aber et al. 1989, 2003).

Cellular metabolites that are commonly affected by soil N availability and culture media include polyamines (PAs), amino acids and total cellular proteins (Minocha et al. 2000, 2004, Miller et al. 2007). Polyamines (putrescine, Put; spermidine, Spd; and spermine, Spm) play important roles in

growth and development and accumulate in cells in response to various stresses; e.g., low pH, osmotic shock, nutrient imbalances, low temperature, and high aluminum (Evans and Malmberg 1989, Minocha et al. 1996, Minocha and Long 2004). Polyamines, along with the amino acids arginine (Arg), proline and asparagine, serve as cellular N-storage compounds and act as sinks for excess $\text{NH}_4^+/\text{NH}_3$, thereby reducing its toxicity in the plant. In response to experimental N additions, field-grown pine, maple and oak trees show increases in foliar total N and PA concentrations (Minocha et al. 2000) and an increase in Arg, a precursor of Put (Bauer et al. 2004). The increases in PAs and Arg may be related to their role in detoxification of excess NH_3 in the cell. In poplar cells, increased accumulation of PAs as a result of genetic manipulation of Put biosynthesis is associated with increased accumulation of total N (Mohapatra 2008). An increase in foliar Put concentration is a common response to a variety of abiotic stresses (Alcázar et al. 2006); however, in combination with changes in soil chemistry, it may serve as an indicator of specific stresses, such as Ca deficiency (Minocha et al. 1997) and excess N deposition (Minocha et al. 2000).

Glutamine (Gln) synthetase (GS; E.C. 6.3.1.2.) and glutamate (Glu) synthase (GOGAT; E.C. 1.4.7.1.) are the entry portals for almost all N assimilated in plants. The immediate product of the GS-GOGAT cycle is Glu, which is a precursor of three major N-rich metabolites (proline, Put and γ -aminobutyric acid) that often co-accumulate in response to a variety of stresses. Another amino acid that accumulates in response to stress is Arg, which with ornithine is the major substrate for Put, the first PA made in all cells.

Given the potential importance of polyamines in alleviating toxicity in plants subjected to N saturation, we investigated how limited N, excess N and the form of N (NH_4^+ and NO_3^-) affect concentrations of PAs, total soluble proteins and inorganic ions and glutamine synthetase activity in suspension cultures of embryogenic cell masses of red spruce (*Picea rubens* Sarg.). We hypothesized that limiting N, excess N, and the

$\text{NO}_3^-:\text{NH}_4^+$ ratio affect not only the cellular concentrations of these metabolites but also the concentration of total soluble proteins and cellular ionic balance. In a culture medium saturated in total N, we found that the extra N was tolerated by the cells without harm. However, a reduction in total N or in the amount of NO_3^- in the medium (which lowers the $\text{NO}_3^-:\text{NH}_4^+$ ratio) caused reductions in growth and GS activity, decreased concentrations of PAs and K, and increased concentrations of Ca, Zn, Mn and Mg. These results are analogous to the observed responses of field-grown forest trees to soil N deficiency and N supplementation, demonstrating the potential usefulness of cell cultures as a model experimental system for biochemical and physiological studies of mature trees.

Materials and methods

Red spruce cell cultures

Suspension cultures of red spruce embryogenic cell masses were maintained in modified half-strength Litvay's medium (Litvay et al. 1981) as described by Minocha et al. (1996). The modifications included 7.25 μM Fe-EDTA, 0.1% (w/v) casein hydrolysate, 3.42 mM L-Gln, 9.05 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 4.44 μM benzyladenine and 2% (w/v) sucrose; adjusted to pH 5.7 before autoclaving. Suspension cultures were subcultured weekly by transferring 15 ml of the 7-day-old cell suspension to 45 ml of fresh medium in a 250-ml Erlenmeyer flask. The flasks were kept on a gyratory shaker at 120 rpm in the dark at 25 °C. The cell line RS-Z5 used for all experiments has been maintained at the USDA Forest Service Northern Research Station, Durham, NH, for several years.

Nitrogen treatments

Seven-day-old cell suspensions were transferred to sterile 50-ml conical tubes. The cells were allowed to settle to the bottom, washed twice with N-free medium by decantation (centrifugation was not used because it damaged the cells), and transferred to a Florence flask. While stirring slowly with a magnetic stirrer, 15-ml aliquots were transferred to 250-ml Erlenmeyer flasks containing 45 ml of medium modified for each N treatment. In the control medium, the inorganic N sources were 10.31 mM NH_4NO_3 and 9.40 mM KNO_3 , with a $\text{NO}_3^-:\text{NH}_4^+$ ratio of 2:1. Three sets of inorganic N treatments were tested. In the first set, KNO_3 was excluded from the medium and the amount of NH_4NO_3 was altered to test the effect of different amounts of total N in the presence of equimolar ratios of NO_3^- and NH_4^+ in contrast to the ratio of 2:1 in the control medium. The treatments consisted of (a) 2.57 mM (0.25 \times) NH_4NO_3 ; (b) 5.15 mM (0.5 \times) NH_4NO_3 ; (c) 10.31 mM (1.0 \times) NH_4NO_3 ; and (d) the control medium, i.e., 10.31 mM NH_4NO_3 and 9.40 mM KNO_3 . Potassium acetate was used to compensate for K in these treatments. The second set of N treatments had reduced amounts of total N in the medium (i.e., 0.25 \times and 0.5 \times control values), with the $\text{NO}_3^-:\text{NH}_4^+$ ratio held constant at 2:1. The treatments were: (a) 2.57 mM (0.25 \times) NH_4NO_3 and 2.35 mM (0.25 \times) KNO_3 ; (b) 5.15 mM (0.5 \times) NH_4NO_3 and

4.70 mM (0.5 \times) KNO_3 ; and (c) the control medium. The third set of N treatments had excess total N in the growth medium (i.e., 1.5 \times and 2 \times control values), with the ratio of NO_3^- to NH_4^+ held constant at 2:1. The treatments were (a) 15.46 mM (1.5 \times) NH_4NO_3 and 14.10 mM (1.5 \times) KNO_3 ; (b) 20.61 mM (2 \times) NH_4NO_3 and 18.79 mM (2 \times) KNO_3 ; and (c) the control medium.

All treatments consisted of three replicates and each experiment was repeated two or three times unless specified otherwise. Cells were collected at 24, 48 and 72 h after culture in the control or treatment media by vacuum filtration on Miracloth (Calbiochem).

Glutamine synthetase (GS) enzyme assays

Extraction of GS from cell cultures followed a modification of the procedure described by Peat and Tobin (1996). Cells were homogenized in extraction buffer (250 mM Tris, pH 7.2, 1 mM dithiothreitol, 1 mM EDTA, 1 mM 2-mercaptoethanol, 1 mM reduced glutathione, 10 mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, and 5 mM Glu) with a Polytron (PT 3000, Eppendorf) at 20,000 rpm for 30 s at 4 °C. The homogenate was centrifuged (10 min, 14,000 g, 4°C) and the supernatant used for enzyme assay as described by Rhodes et al. (1975), with minor modifications. The 2-ml reaction mix contained 18 mM ATP (pH 7.2), 6 mM hydroxylamine, 92 mM Glu, 50 mM imidazole-HCl (pH 7.2), 45 mM $\text{MgSO}_4\cdot \text{H}_2\text{O}$, 400 μl of the extract and 700 μl of extraction buffer. The reaction mixture was incubated at 30 °C for 60 min and stopped by adding 1.0 ml of ferric chloride reagent (8 g FeCl_3 and 10 g trichloroacetic acid in 250 ml of 0.5 M HCl). The absorbance of the product glutamyl- γ -hydroxamate was measured at 540 nm. Protein concentration was determined by the procedure of Bradford (1976) with Bio-Rad dye reagent.

Analyses of polyamines and inorganic ions

To extract PAs from cultured cells, samples comprising a 1:4 (w/v) ratio of cells (200 mg fresh mass) and 5% perchloric acid were frozen (-20 °C) and thawed (room temperature) three times to extract PCA-soluble PAs. The procedure for dansylation and quantification of PAs was as described by Minocha et al. (1994), with heptanediamine as internal standard. The dansyl-PAs were analyzed by HPLC using a gradient of acetonitrile (40 to 100%) and 10 mM heptanesulfonic acid, pH 3.4, on a reversed-phase Pecosphere C_{18} column (Minocha et al. 1990). Data were integrated using TotalChrom Workstation software (Perkin Elmer Instruments LLC, Shelton, CT).

The PCA extracts for PA analyses were also analyzed for inorganic ions. Each sample was diluted 50 \times with double deionized water, and analyzed by simultaneous inductively coupled plasma-axial emission spectrophotometry (Varian). For each sample, the pump rate was set at 15 rpm and three replicate readings were taken. All emission readings were analyzed against a prepared set of five standards containing the ions of interest, using the Varian computer software, ICP Vista PRO Version 3.0.

Statistical analysis

For all experiments involving quantitative analyses, the data were subjected to a two-way analysis of variance (ANOVA) with treatment and time as blocking factors. Tukey's test was performed after ANOVA to determine significance between treatments at $P = 0.05$.

Results

Cell growth

In all treatments where KNO_3 was absent or where total N was reduced, cell mass per flask was significantly lower than in the control cultures (Figures 1A and 1B). The reduction was detectable within 24 to 48 h, and there was a more than 50% reduction in cell mass per flask at 48 h. The adverse effect of reduced NO_3^- on cell growth increased with increasing amount of NH_4NO_3 in the medium. The greatest reduction in cell mass in response to reduced total N was in the medium with the lowest total N content. When total N in the medium was twice that of the control medium, there was a small but significant increase in cell mass at 48 and 72 h, which was not proportional to the increase in medium N concentration (Figure 1C).

Glutamine synthetase (GS) activity

Figure 2A shows that GS activity was significantly reduced in cells grown in the absence of KNO_3 , the effect being detectable at 48 h and thereafter. The lowest GS activity was observed at 72 h in cells growing in the medium with the highest NH_4NO_3 concentration. In cells grown in control medium, GS activity was 4- to 10-fold higher than in cells in the other treatments. Enzymatic activity either increased or did not change significantly with time in the control cultures, whereas it decreased with time in cells grown in the absence of KNO_3 .

Cells grown in media containing a reduced amount of total N had significantly reduced GS activity compared with control cells, at all sampling times (Figure 2B). Furthermore, the difference in GS activity between the control and treated cells increased with time of incubation in media containing reduced amounts of total N. In media with a high total N content, there were no significant changes in GS activity at any time, with GS activity showing saturation in cells grown in medium containing the full N complement (Figure 2C).

Total soluble proteins

A reduction in the $\text{NO}_3^-:\text{NH}_4^+$ ratio in the medium (i.e., in the absence of KNO_3) resulted in a decline in total soluble protein concentration at 48 h, particularly in cells grown in medium containing $0.5\times \text{NH}_4\text{NO}_3$ (Figure 3A), whereas no significant effect was found at $0.25\times \text{NH}_4\text{NO}_3$. The amount of protein decreased with time of incubation in cells cultured in the presence of $0.5\times \text{NH}_4\text{NO}_3$. At 48 and 72 h in medium containing $1.0\times \text{NH}_4\text{NO}_3$, total cell mass was insufficient for protein analysis; thus there are no data for these times. Lowering the amount of total N in the medium caused a reduction in total soluble protein concentration of the cells of up to 50–60% (Figure 3B); the effect was seen within 24 h and increased

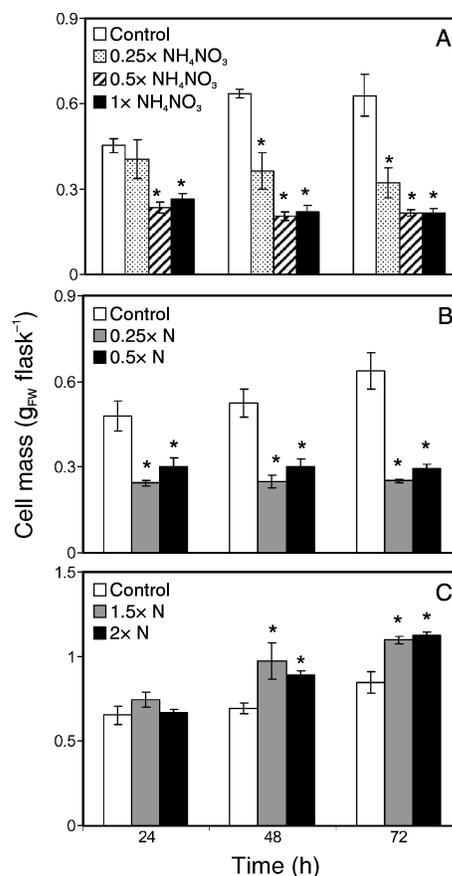


Figure 1. Effects of the concentration and form of N on the yield (cell mass, g_{FW} per flask; 60 ml culture) of red spruce cell cultures at different times following transfer to fresh medium. (A) Different concentrations of NH_4NO_3 in the absence of KNO_3 ; (B) reduced total N in the medium; and (C) increased total N in the medium. The total N in the control medium was $10.31 \text{ mM NH}_4\text{NO}_3 + 9.40 \text{ mM KNO}_3$. Values are means \pm SE of six replicates from two experiments. An asterisk (*) indicates the treatment was significantly different ($P = 0.05$) from the control.

with time. Increased amounts of total N in the medium did not significantly alter the total soluble protein concentration (Figure 3C).

Polyamines

Figures 4A–C show the concentrations of the three major PAs at various times in cells cultured in media containing different concentrations of NH_4NO_3 in the absence of KNO_3 . At all times, Put was the predominant PA in the cells followed by Spd and Spm. At 24 h there was no significant effect of the absence of KNO_3 on Put concentration (except at the highest NH_4NO_3 concentration, where a small but significant increase was seen); however, by 48 h, a significant reduction in Put concentration was observed in cells in all treatments in which KNO_3 was absent (Figure 4A). All three concentrations of NH_4NO_3 tested in the absence of KNO_3 had similar effects on Put at 48 and 72 h. The deleterious effect on Put concentration

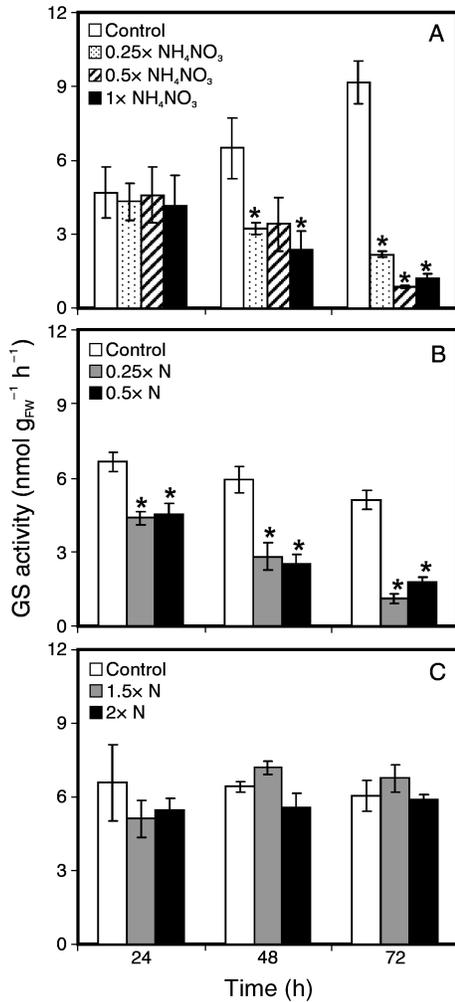


Figure 2. Glutamine synthetase (GS) activity ($\text{nmol glutamyl-}\gamma\text{-hydroxamate g}_{\text{FW}}^{-1} \text{h}^{-1}$) in red spruce cell cultures at different times following transfer to fresh medium with different concentrations and forms of N. (A) Different concentrations of NH_4NO_3 in the absence of KNO_3 ; (B) reduced total N in the medium; and (C) increased total N in the medium. The total N in the control medium was $10.31 \text{ mM NH}_4\text{NO}_3 + 9.40 \text{ mM KNO}_3$. Values are means \pm SE of six replicates from two experiments. An asterisk (*) indicates the treatment was significantly different ($P = 0.05$) from the control.

of excluding KNO_3 from the culture medium increased over time.

The exclusion of KNO_3 from the medium caused a significant reduction in Spd concentration within 24 h in all treatments with NH_4NO_3 (Figure 4B). As with Put, the concentration of Spd progressively declined in the absence of KNO_3 ; however, it did not change much in the control cultures and so Spd concentrations were almost 10-fold lower in cells grown for 72 h in the absence of KNO_3 . The effect of excluding KNO_3 was qualitative in that the three concentrations of NH_4NO_3 tested had similar effects on cellular Spd concentration at all times. Spermine, the least abundant of the three PAs, showed minimal variation in response to the treatments; however, its concentration declined over time in cells grown in the absence

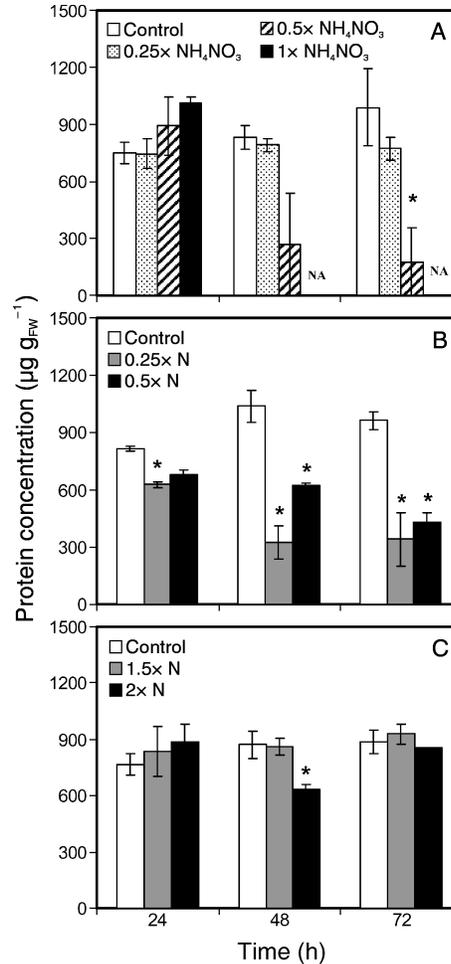


Figure 3. Soluble protein concentrations in red spruce cell cultures at different times following transfer to fresh medium with different concentrations and forms of N. (A) Different concentrations of NH_4NO_3 in the absence of KNO_3 ; (B) reduced total N in the medium; and (C) increased total N in the medium. The total N in the control medium was $10.31 \text{ mM NH}_4\text{NO}_3 + 9.40 \text{ mM KNO}_3$. Abbreviation: NA = data unavailable because of the low yield of cell mass. Values are means \pm SE of six replicates from two experiments. An asterisk (*) indicates the treatment was significantly different ($P = 0.05$) from the control.

of KNO_3 (Figure 4C).

A reduction in total N in the medium had effects similar to those observed in cells cultured in the absence of KNO_3 . Thus, there was a several-fold decrease in cellular Put concentration at 48 and 72 h; the effect was not proportional to the reduction in total N in the medium, and the effect increased with time of incubation in cells grown at the two lower concentrations of N (Figure 4D). The concentrations of Spd and Spm also declined in response to a reduction in total N in the medium; in both cases the effects were seen within 24 h of treatment and they increased with the time of incubation (Figures 4E and 4F). Increasing total N in the growth medium had no effect on any of the PAs studied, except for a small decline in Put concentration at 72 h (Figures 4G–I).

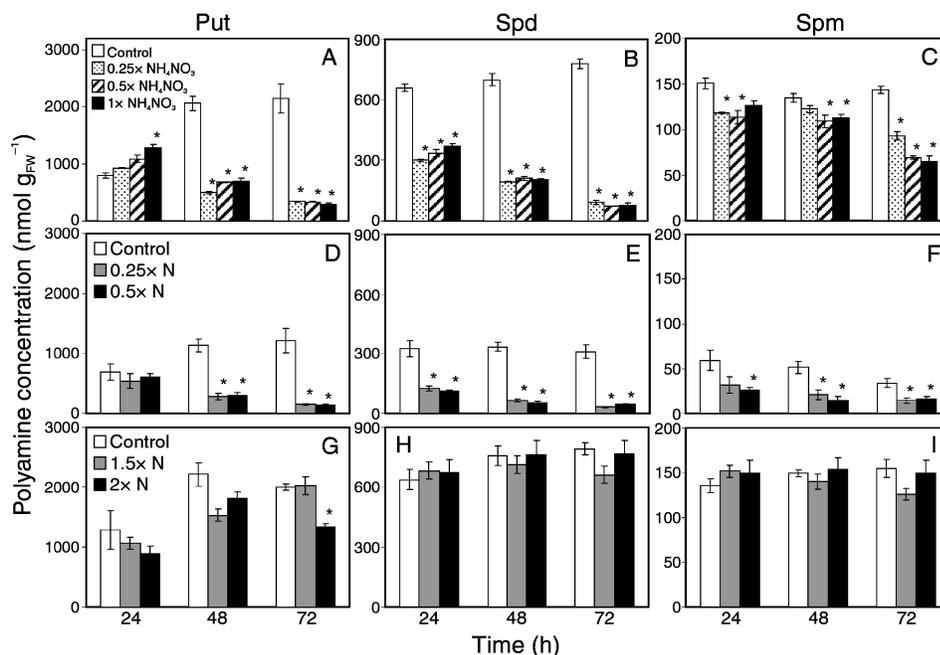


Figure 4. Cellular polyamine concentrations in red spruce cell cultures at different times following transfer to fresh medium with different concentrations and forms of N. (A–C) Different concentrations of NH_4NO_3 in the absence of KNO_3 ; (D–F) reduced total N in the medium; and (G–I) increased total N in the medium. The total N in the control medium was 10.31 mM NH_4NO_3 + 9.40 mM KNO_3 . Values are means \pm SE of three replicates from a representative experiment. An asterisk (*) indicates the treatment was significantly different ($P = 0.05$) from the control.

Inorganic ions

Changes in the concentration of total N and the $\text{NO}_3^-:\text{NH}_4^+$ ratio in the culture medium had variable effects on the accumulation of inorganic ions (Figure 5). The cellular concentration of Ca did not change over time in the control cultures. Cells cultured in media from which KNO_3 was omitted accumulated more Ca at all times than cells cultured in control medium; the maximum difference occurring at 72 h. At the highest concentration of NH_4NO_3 in the medium (1.0 \times) in the absence of KNO_3 , the increase in Ca accumulation was less than that at lower concentrations of NH_4NO_3 (Figure 5). The accumulation of both Zn and Mn showed trends similar to those seen for Ca; in cells grown in the absence of KNO_3 , Zn accumulation was up to 6-fold higher and Mn was almost 10-fold higher at 72 h (Figure 5). In contrast, K accumulation was adversely affected by lowering the NO_3^- concentration of the medium, even though the total amount of K in the medium was held constant. Except in cells grown for 72 h in medium containing the lower concentration (0.25 \times) of NH_4NO_3 where Mg accumulation was higher than in control cells, no significant difference was seen in P or Mg accumulation in cells grown in the various N treatments (Figure 5).

Lowering the amount of total N in the medium resulted in increased accumulations of Ca, Zn, Mn and to some extent Mg, whereas there were no effects on the accumulation of K (except a small reduction at 72 h in medium containing 0.25 \times total N) and soluble P (Figure 6). As in the set of experiments conducted in the absence of NO_3^- , the effect of lowering the amount of total N was greatest for Mn accumulation which increased 10- to 12-fold compared with control values. The effects for most ions tested were concentration dependent; i.e., the lower the N concentration the greater the accumulation. The changes with time were relatively small for each ion. Ele-

vated amounts of total N in the growth medium had no significant effect on the uptake of any of the ions analyzed at any time (Figure 7).

Discussion

We studied red spruce cell cultures as an experimental model system to examine the effects of altered N availability in the medium on the yield of cell mass, GS activity, and the concentrations of proteins, PAs and inorganic ions. It was envisioned that the results will help us understand the relationships of reduced and elevated N availability with cell physiology in red spruce, with the possibility of gaining insights into red spruce decline in the Northeastern United States (Holldampf and Barker 1993). We found that changes in the availability of total N as well as in the form of N, particularly the $\text{NO}_3^-:\text{NH}_4^+$ ratio, played important roles in the physiological responses of red spruce cells, and that these responses were analogous to those observed in long-term field studies with forest trees.

The preference for NH_4^+ over NO_3^- varies among species, with most conifers having a stronger preference for NH_4^+ as compared to angiosperms (Ingstad 1979, Lavoie et al. 1992, Kronzucker et al. 1996). Although the availability of NH_4^+ in most agricultural soils is low compared with that of NO_3^- , and plants utilize significantly more NO_3^- than NH_4^+ overall, the reverse situation may exist in some forest soils where NH_4^+ can sometimes be phytotoxic (Britto and Kronzucker 2002, Miller and Cramer 2004). In cell cultures, NH_4^+ is assimilated before NO_3^- and our results provide further support to earlier findings that high amounts of NH_4^+ can be phytotoxic (Farquhar et al. 1980, Durzan 1987, Schjoerring et al. 2000, Britto and Kronzucker 2002, Miller and Cramer 2004, Miller et al. 2007),

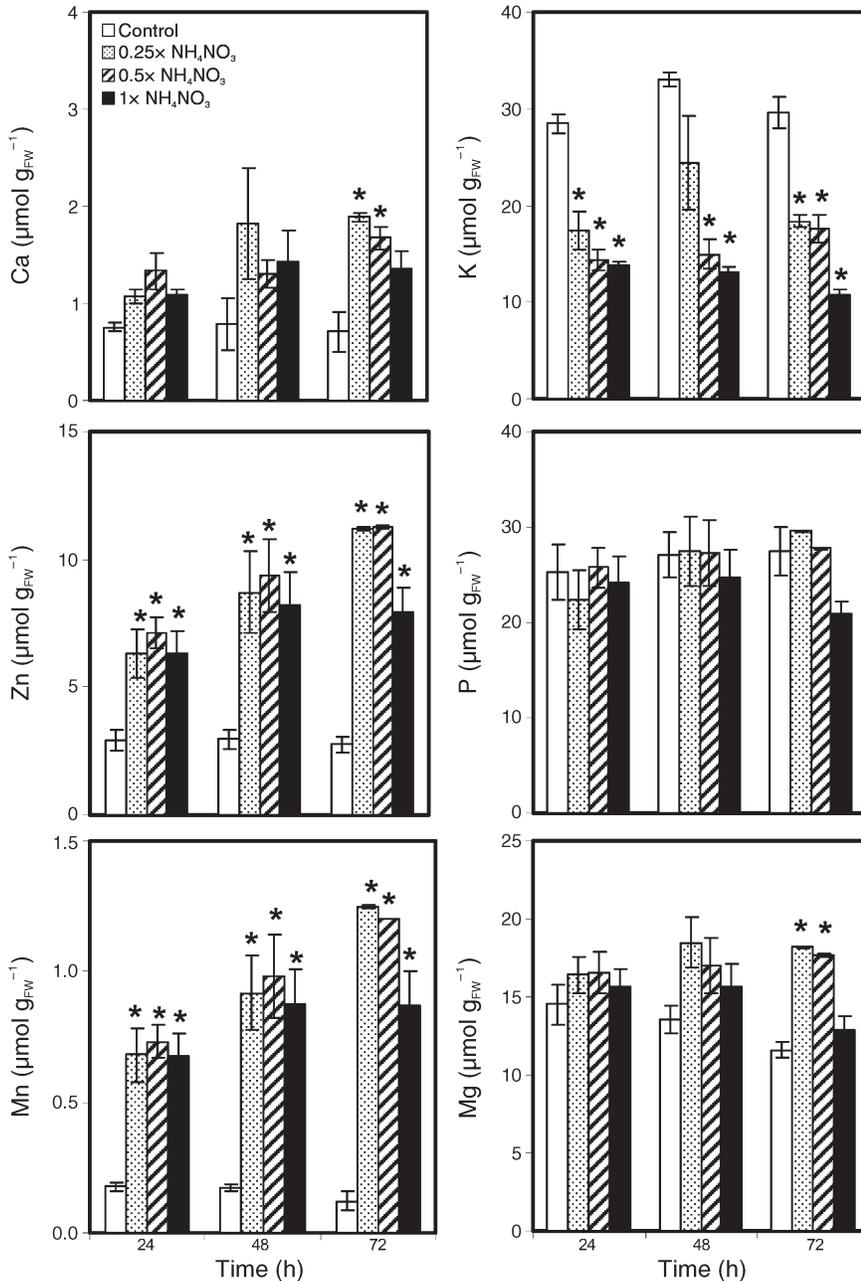


Figure 5. Effects of different concentrations of NH_4NO_3 in the absence of KNO_3 on the concentration of exchangeable ions in cultured red spruce cells at different times following transfer to fresh medium. Values are means \pm SE of six replicates from two experiments. An asterisk (*) indicates the treatment was significantly different ($P = 0.05$) from the control.

particularly when the availability of NO_3^- is low. We found that a reduction in NO_3^- concentration in the medium caused reductions in GS activity, total soluble protein, cellular PAs and cellular K, and an increase in cellular Ca, Zn and Mn, resulting in a reduction in cell mass.

Nitrogen, cell growth and glutamine synthetase

Whether NH_4^+ or NO_3^- is the available N source, it must be assimilated into Gln, the formation of which is catalyzed by GS (Vance 1997, Cruz et al. 2006). A plant's tolerance of high N is related to its capacity for N assimilation, which in part depends on GS activity (Cruz et al. 2006). When available N exceeds the assimilatory capacity of the plant, GS activity can be inhibited,

resulting in an accumulation of NH_3 in the cells (Farquhar et al. 1980, Finnemann and Schjoerring 1999, Schjoerring et al. 2000), which becomes cytotoxic (Cruz et al. 2006). Our results are consistent with the findings of Finnemann and Schjoerring (1999) showing that there is an upper limit for GS expression and activity. In red spruce cells, GS activity declined with decreasing total N concentration in the medium but did not increase with higher than normal N concentration in the medium, indicating that the culture medium was probably N saturated. Activity of GS was also affected by the NO_3^- : NH_4^+ ratio, possibly leading to NH_3 toxicity, which was responsible for the observed reduction in cell mass.

Studies with Norway spruce showed that the accumulation

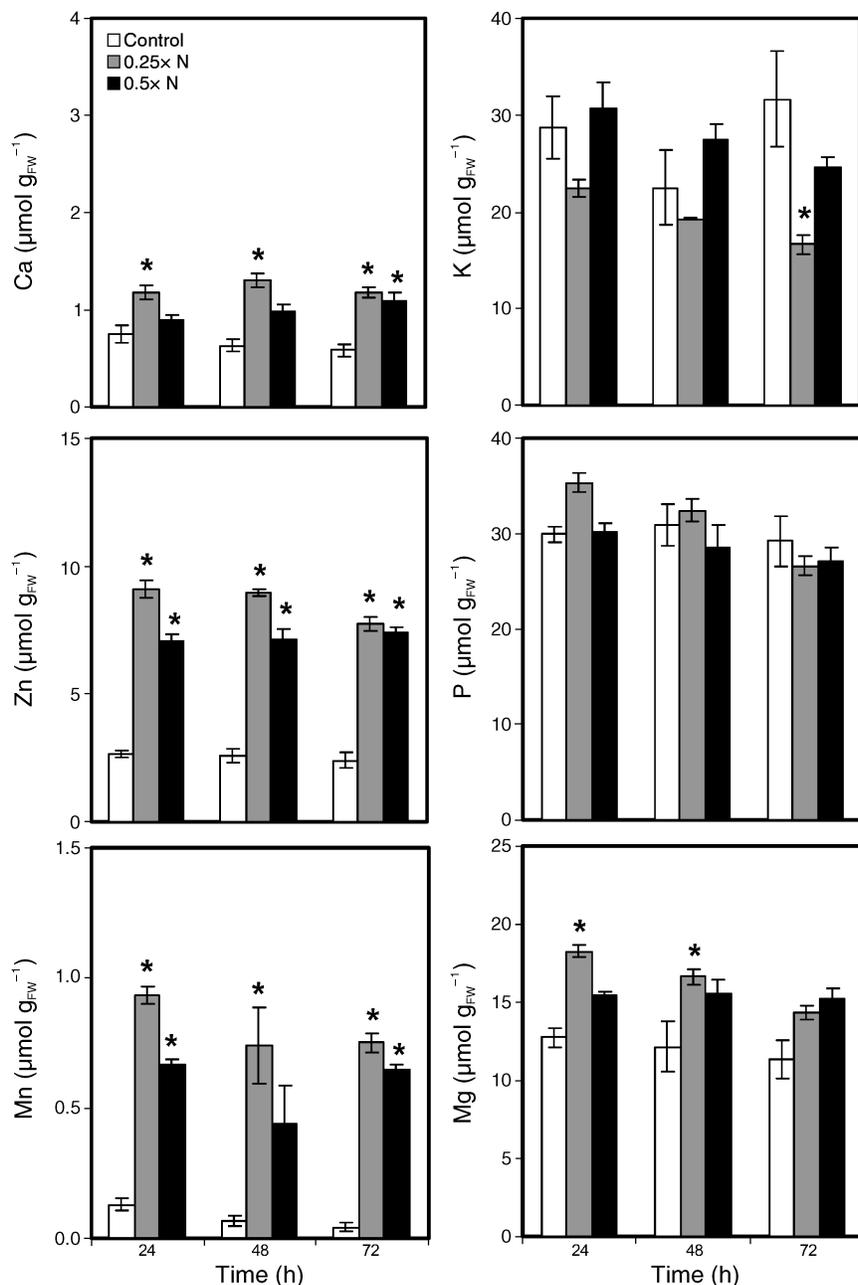


Figure 6. Effects of reduced total N on the cellular concentration of exchangeable ions in red spruce cell cultures at different times following transfer to fresh medium. Values are means \pm SE of six replicates from two experiments. An asterisk (*) indicates the treatment was significantly different ($P = 0.05$) from the control.

of NH_4^+ in seedlings increased when the $\text{NO}_3^-:\text{NH}_4^+$ ratio was lowered (Aarnes et al. 1995). Our finding of reduced cell mass yield per flask in treatments involving a reduction in the $\text{NO}_3^-:\text{NH}_4^+$ ratio (i.e., in the absence of KNO_3) corroborates the response seen in Norway spruce seedlings. Our results further showed that it was not the absolute concentration of NH_4^+ in the medium that determined its toxicity, because NO_3^- in the medium reduced or alleviated NH_4^+ toxicity, when present at higher concentrations than NH_4^+ .

Under normal cell culture conditions, the pH of the growth medium tends to decline as NH_4^+ is assimilated: the pH does not increase until NO_3^- consumption begins (Durzan 1987, Minocha 1987). It can thus be argued that the uptake of NO_3^-

which can reduce NH_4^+ toxicity, occurs by a low-efficiency transport system that operates constitutively even in the presence of NH_4^+ —the existence of such systems is well established in plants (Kronzucker et al. 1996, Miller et al. 2007). Part of the survival mechanism for plant cells in culture is to maintain an ionic balance to create pH-buffering conditions. A doubling in the concentration of total N, as long as the $\text{NO}_3^-:\text{NH}_4^+$ ratio was high (2:1), had no deleterious effects on the cultures. However, when KNO_3 was omitted from the medium, which lowered the $\text{NO}_3^-:\text{NH}_4^+$ ratio, it may have altered the buffering capacity of the medium, causing the pH to drop even further, thus affecting ion uptake, including the uptake of cations, as well as cell growth. Disturbed assimilation of cations

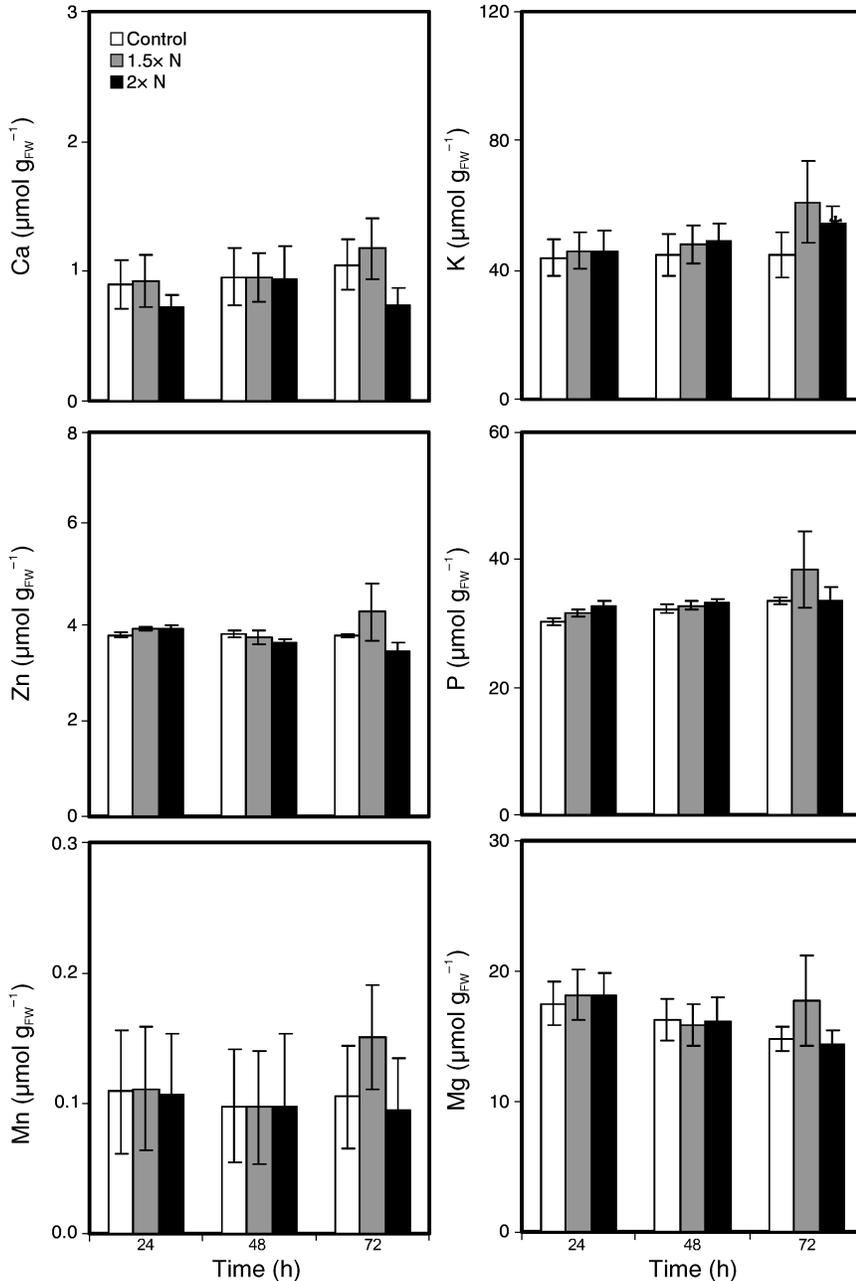


Figure 7. Effects of increased total N on the cellular concentrations of exchangeable ions in red spruce cell cultures at different times following transfer to fresh medium. Values are means \pm SE of six replicates from two experiments. An asterisk (*) indicates the treatment was significantly different ($P = 0.05$) from the control.

was indicated by the observed increase in Mn uptake and decrease in K uptake, which together negatively affected the yield of cell mass. Ammonium toxicity in the absence of NO_3^- is further indicated by the decrease in concentrations of PAs and soluble proteins and the reduction in GS activity. At the physiological level, a possible explanation for these reductions may be associated with the observed increase in Mn concentration, which is known to be cytotoxic in plants (Kabata-Pendias 2001).

Nitrogen and polyamines

Polyamines are often found in millimolar quantities in plants; their high concentrations make them strong sinks for excess N

within the cell, thus helping to reduce cytotoxicity due to NH_3 . The biosynthesis of PAs relies in part on how much N can be assimilated by the cell, which makes the activity of GS a major factor in PA synthesis. Bhatnagar et al. (2001) observed a reduction in PAs in poplar cells in the presence of methionine sulfoximine, an inhibitor of GS activity. Furthermore, NH_4^+ and NO_3^- have different effects on the accumulation of PAs. When NH_4NO_3 was removed from the growth medium of tobacco cell suspension cultures, there was a significant decline in Put (Altman and Levin 1993); whereas there were marked increases in Put and Spd when KNO_3 was omitted from the culture medium. Our results were different, however, with a reduction in NO_3^- concentration in the medium causing a slight increase in Put at 24 h, although by 48 h, Put had declined by

almost 50%. Thus in red spruce cells, in the absence of KNO_3 , Put seems to respond first by increasing; i.e., a (stress) response to the absence of NO_3^- and the ensuing NH_4^+ toxicity, then by decreasing due to reduced total N uptake and the sustained effects of NH_4^+ toxicity. The finding that there was no increase in GS activity or in PAs when N availability was higher than normal further supports the suggestion that the limiting factor for PA accumulation is N assimilation via GS. This suggestion is consistent with our earlier work with poplar cells, where supplementing the medium with NH_4NO_3 did not enhance N sequestration in PAs (Minocha et al. 2004). It is also consistent with the reduction in soluble amino acid pools observed in red spruce cells grown under similar conditions (M.J. Serapiglia, R. Minocha, and S.C. Minocha, unpublished data).

Our control cell culture medium, with its high concentrations of both NH_4^+ and NO_3^- , may be considered N saturated, whereas the forest soils in the northeastern United States are N limited, and thus should respond to N additions in a manner opposite to that seen in our study with cell cultures. However, even though the environments (the culture medium and the forest soil) differ in N saturation, it is likely that in both, PAs are reliable indicators of cellular N status. Long-term field studies have demonstrated that the accumulation of PAs, amino acids, and soluble proteins are enhanced in foliage of mature trees of several species (*Pinus resinosa* Ait, *Quercus rubra* L., *Acer rubrum* L.) in response to NH_4NO_3 fertilization of N-limited soil (Minocha et al. 2000, Bauer et al. 2004). Thus the results of a study of mature trees in a long-term experiment lasting over a decade are analogous to our findings from short-term cell culture experiments.

Several-fold changes in Put concentration are commonly observed in response to a variety of stress responses, whereas Spd is a tightly regulated metabolite in plant cells even when its precursor Put shows major changes in response to a variety of chemical treatments like Al (Minocha et al. 1996) and Cd (Thangavel et al. 2007), or overproduction by genetic manipulation (Bhatnagar et al. 2001, 2002). However, small changes in Spd concentration have been observed in response to excess N as a result of acidic deposition across northern New England (Minocha et al. 1997), and in response to chronic N exposure at Harvard Forest (Minocha et al. 2000). In our study, large declines in cellular Spd concentration were seen under conditions of lower total N availability as well as to changes in the $\text{NO}_3^-:\text{NH}_4^+$ ratio; no change in Spd concentration was seen, however, in response to excess N. Spermine, the least abundant of the three PAs also showed greater declines in response to lower total N availability and changes in the $\text{NO}_3^-:\text{NH}_4^+$ ratio than are commonly seen in response to most treatments in plant cells.

Nitrogen and inorganic ions

A large portion of forest decline research has implicated acid precipitation (lower soil pH) as the cause of Ca deficiency as a result of Al solubilization (Shortle and Smith 1988, Cronan and Grigal 1995, McNulty et al. 1996, Minocha et al. 1997). Declines in exchangeable Ca in organic soil horizons and in

the foliage of conifers in response to acid precipitation and excess N deposition have been observed (Ericsson et al. 1993, Minocha et al. 1997). Putrescine seems to be the major PA involved in this response and is often inversely correlated with soil or foliar soluble Ca, or both (Minocha et al. 1997, 2000, Wargo et al. 2002, R. Minocha et al. unpublished data). Based on these findings, we speculate that the increased uptake of the divalent cations Ca, Mn and Zn (Figures 5 and 6) by our red spruce cells was a direct effect of a lowering in total N concentration or a changing $\text{NO}_3^-:\text{NH}_4^+$ ratio (when NO_3^- was lower), perhaps associated with a more rapid decrease in pH of the medium as a result of NH_4 depletion. The inverse relationship between Ca accumulation and accumulation of PAs is consistent with previous findings in mature trees, including red spruce (Reggiani et al. 1993, Minocha et al. 1997, 2000). We speculate that the decrease in K accumulation when KNO_3 was replaced by K acetate in the culture medium permits cells to recover in some degree from an ionic imbalance caused by the form of N in the medium.

The observed increases in Ca and Mn accumulation and concomitant decrease in K accumulation together point to an increase in the production of reactive oxygen species (ROS) in cells cultured in a medium with low NO_3^- and high NH_4^+ (Demidchik et al. 2003, Mori and Schroeder 2004, Cuin and Shabala 2007). Increased ROS formation could also explain the negative effects on cell mass and reductions in soluble protein concentration of culturing cells in the presence of low NO_3^- and high NH_4^+ concentrations.

In conclusion, although an understanding of the metabolic reactions in mature tissues of trees is needed to fully comprehend the effects of N deficiency and N excess and N form at the cellular level, cell cultures provide many unique features as model experimental systems for biochemical and physiological studies compared with mature trees. For example, the observation that GS activity and PAs did not increase in red spruce cell cultures under conditions of supplemental N indicates that, when the cells are growing in N-saturating conditions, the cells can tolerate extra N without harm. This could be similar to the situation of N saturation in the field. Analogous to the findings of field studies with forest trees subjected to N depletion or long-term fertilization with NH_4NO_3 , decreases in N concentration in the cell culture medium were accompanied by increased cellular concentrations of Ca, Zn, Mn and Mg. The reduction in NO_3^- concentration in the medium was probably responsible for the symptoms of NH_4^+ toxicity in the cell cultures, which also occurs in the field. Ammonium toxicity in the field is further exacerbated by the declining pH that accompanies NH_4^+ uptake, particularly when NO_3^- leaching occurs in response to acid precipitation.

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