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## Cold Acclimation of Norway Spruce Roots and Shoots after Boron Fertilization

Mikko Räisänen, Tapani Repo and Tarja Lehto

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Boron deficiency, manifested as shoot dieback, is a problem in conifer stands growing on soils with high nitrogen availability in Fennoscandia. Earlier observations on Norway spruce (*Picea abies* L. Karst.) suggest that freezing tolerance is decreased by boron deficiency. Here, the effect of boron fertilization on cold acclimation of Norway spruce was studied in a young stand with initially low boron status two years after fertilization. Buds, stems, needles and roots were collected at five sampling times during cold acclimation and subsequently exposed to series of freezing temperatures. Lethal temperatures of organs were assessed by electrolyte leakage method (EL) and visual scoring of damage (VS). Freezing tolerance of buds was measured also by differential thermal analysis (DTA).

The mean boron (B) concentration in needles was 4 mg kg<sup>-1</sup> in unfertilized and 21 mg kg<sup>-1</sup> in B-fertilized trees while critical level of B deficiency is considered to be 5 mg kg<sup>-1</sup>. The risk for increased freezing injuries in the low-B trees was not evident since all trees achieved cold hardiness that would be sufficient in central Finland. At two sampling times out of five, shoots or stem of B-fertilized trees were slightly more freezing tolerant than non-fertilized trees. However, the present study does not give strong evidence for the hypothesis that decreased freezing tolerance in B deficiency would be a triggering factor for leader dieback in Norway spruce at the B levels studied.

**Keywords** boron deficiency, dieback, freezing tolerance, mineral nutrition

**Addresses** *Lehto & Räisänen*: University of Joensuu, Faculty of Forest Sciences, P.O.Box 111, 80101 Joensuu, Finland; *Repo*: Finnish Forest Research Institute, Joensuu Unit, P.O. Box 68, 80101 Joensuu, Finland; *Räisänen* (present address): FAForest, 83480 Ahonkylä, Finland

**E-mail** tarja.lehto@joensuu.fi

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# 1 Introduction

Dieback of apical shoots caused by boron (B) deficiency is a common problem in forestry in Fennoscandian countries both in mineral soils and peatlands (Braekke 1979, Saarsalmi and Mälkönen 2001). In peatland forestry, B fertilization is standard practice especially in connection with macro-nutrient fertilization. In mineral-soil forests, N limitation is usually the major nutrient issue, but on sites with inherently high N fertility, B deficiency is the next one to manifest, particularly in areas remote from the sea (Tamminen and Saarsalmi 2004). A dilution effect on B has been found following N fertilization in several studies (Möller 1983, Mälkönen et al. 1990, Jalkanen 1990).

Freezing damage in wintering buds has been suggested to be one reason for dieback of conifer trees in B deficiency (Pietiläinen 1984). In a previous field study, we attempted to test this by determining the freezing tolerance of Norway spruce buds and stems in stands where part of the trees had B deficiency symptoms: loss of apical dominance and structural damage in buds (Räisänen et al. 2006a). If the buds were structurally normal, their freezing tolerance increased properly during the cold acclimation in the autumn. However, if the buds were deformed because of B deficiency, they were not able to deep supercool and were unable to cold harden well accordingly (Räisänen et al. 2006a). The deformation was visible (under a stereomicroscope) as poor development of the primordial shoot, collenchymatic plate and bud cavity in buds cut in half, even though all buds looked normal outwards. Deep supercooling is the mechanism of survival of winter temperatures in Norway spruce buds. It means cooling of the buds to temperatures down to even  $-40^{\circ}\text{C}$  without ice crystal formation in the primordial shoot. Deep supercooling of buds is dependent on the structures within the bud, as the collenchymatic plate in the bud axis functions as a barrier for the spread of ice (Bilkova et al. 1999, Räisänen et al. 2006b). Possible mechanisms that can affect the properties of this barrier include changes in the pectic compounds of the collenchymatic plate (Fleischer et al. 1999) or membrane-cell wall interactions (Bassil et al. 2004). Such changes

might occur due to B deficiency even in buds that are not visibly deformed.

In the previous study with a B fertilizer treatment, the fertilizer was applied mid June (early summer) and the freezing tolerance was studied during the following autumn (Räisänen et al. 2006a). However, it is known that the development of Norway spruce buds starts already at the time when the needles are in the early stage of elongation, and adequate B supply is needed already at that stage, late May in Finland (Sutinen et al. 2007). Hence the fertilizer did not fully affect the structure of the buds on the year of the field study. The results suggest that B fertilizer did not induce direct metabolic effects on freezing tolerance (Räisänen et al. 2006a). However, it remained to be shown whether B fertilization would actually improve the freezing tolerance if applied early enough to fully affect the structural development of buds. It was not shown either if B fertilization would improve the freezing tolerance of other plant organs. Boron may affect the function of the plasma membrane either directly or indirectly through changes in the cell wall (Muhling et al. 1998, Brown et al. 2002, Bassil et al. 2004). Instability of cell membranes has also been hypothesized to decrease the chilling tolerance of crop species in B deficiency (Huang et al. 2005). Plasma membrane is a primary site of freezing injury (Steponkus 1984). Therefore, B deficiency may increase the susceptibility of cells to freezing damage also in other organs than buds.

In a long-term field study in a Norway spruce stand with low B status, the proportion of dead fine roots decreased due to B fertilization (Mötönen et al. 2003). This may have been due to 1) increased growth, 2) decreased mortality at different times of the year, or 3) decreased decomposition rate of dead roots. In the present study, the focus is in the possibility of the increased mortality of roots during soil freezing and thawing cycles due to low-B conditions.

There are no earlier field studies on B effects on the freezing tolerance of stems, needles and roots. Therefore, we studied this property not only in buds but also stems, needles and roots at low B status in a young Norway spruce stand two years after fertilization. The aims were to study 1) the freezing tolerance of Norway spruce in different

stages of cold hardening and 2) the effect of boron and nitrogen availability on freezing tolerance. We hypothesize that freezing tolerance of trees is decreased in B deficiency and that B deficiency is exacerbated by N fertilization.

## 2 Material and Methods

### 2.1 Site Description and Fertilization Treatments

The experimental site in Eastern Finland (62°25'N, 29°55'E, 90 m asl.) was very fertile, varying from grass-herb to *Oxalis-Myrtillus*-forest type (Cajander 1949), about 25-year-old stand of Norway spruce (*Picea abies* L. Karst.) with abundantly admixed grey alder (*Alnus incana* (L.)). The experiment was a single-tree factorial fertilization experiment, with randomized blocks. Trees were allocated to 18 blocks of eight trees according to topography of area. Within each block, the trees were randomly allocated to the fertilization treatments (two trees per treatment combination). Spruce trees were fertilized in all factorial combinations of nitrogen (N) and boron (B): unfertilized control (0), B fertilized (B), N fertilized (N), N+B fertilized (NB). The fertilizers were applied as urea, 180 kg N ha<sup>-1</sup> (Urea, Kemira Growhow Oyj, Helsinki, Finland) and borax, 2 kg B ha<sup>-1</sup> (Borax Decahydrate, Borax Europe Ltd, Brance, France) in June 2000. The height of fertilized trees was 3–8 meters and the minimum distance between experimental trees was 7 m. Fertilizers were evenly spread on the soil in a circle with 2.5 m radius around each tree. For this study, one tree from each fertilization treatment was chosen randomly from nine blocks each.

Air and soil temperature was recorded during the sampling period (Campbell CR10X-logger with four T105-thermocouples). Temperature sensors in the soil were placed under the canopy of Norway spruce (shallow snow cover) or under the canopy of grey alder in an adjacent pure stand (thick snow cover) at two depths: under the litter layer and at the depth of 5 cm. The temperature under grey alder was recorded to assess the effect of the spruce canopy on the soil temperature.

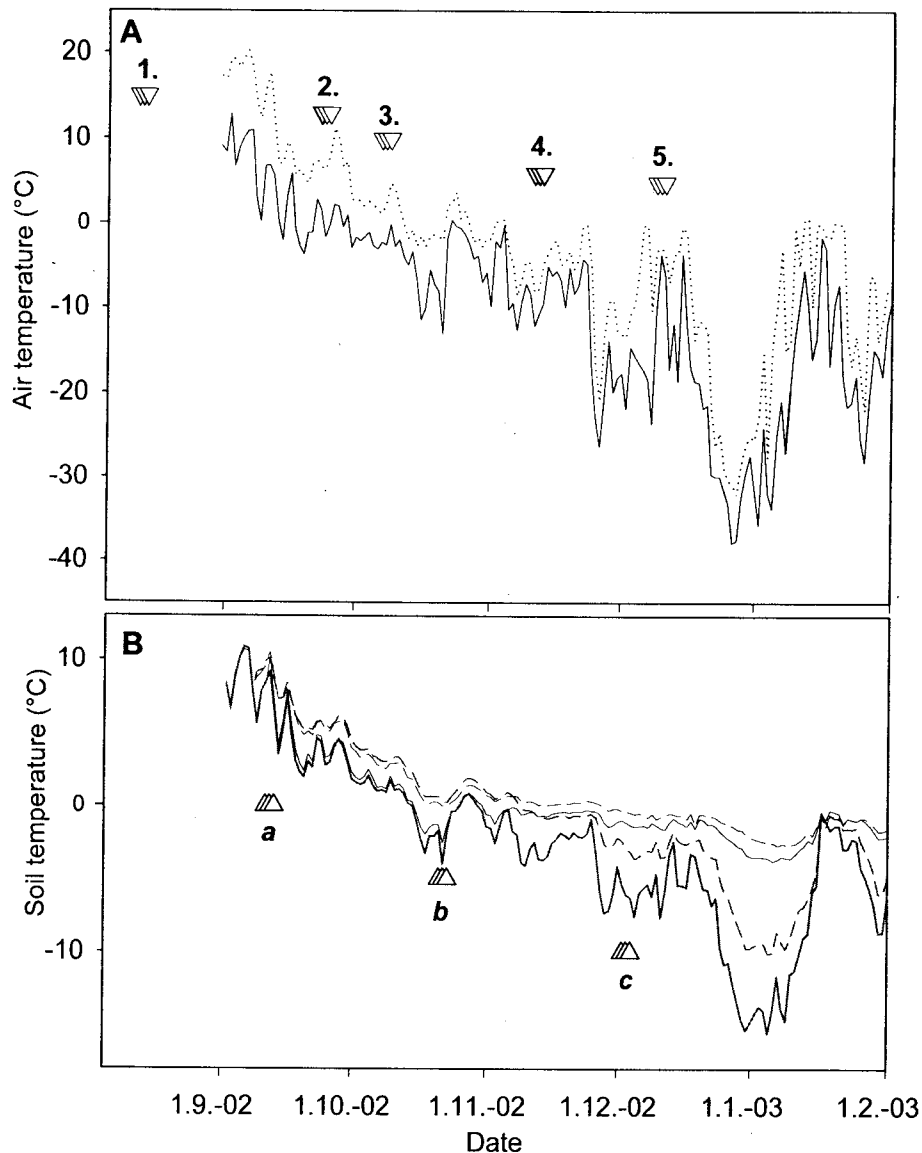
Needle samples for nutrient analysis were collected from new shoots from the uppermost third of the live canopy in February 2003. Boron concentrations were measured from dry-ashed samples after HNO<sub>3</sub> extraction (ICP-OES, IRIS Intrepid II XSP, Thermo Jarrel-Ash Corporation, Franklin, USA). N concentrations were determined by the Kjeldahl method (Halonen et al. 1983).

### 2.2 Sampling of Branches

For studies of freezing tolerance, current-year shoots from the uppermost third of the canopy were sampled at five times between 12th of August and 9th of December (Fig. 1A). At each time, branches of nine trees in each fertilization treatment (i.e. total of 36 trees) were sampled. Initially, each sampling took place on three subsequent days, i.e. three trees per fertilization treatment (total of 12 trees) each day. When the air temperature was below zero (see Fig. 1A), samples were thawed at +5°C before preparing them for freezing tests. At the fifth sampling (9th of December), all trees were sampled in one day and the branches were stored outdoors in plastic bags.

For differential thermal analysis (DTA, see details below), the terminal bud of each branch was excised by cutting the stem 1 cm below the bud cavity. Then needles, shoots and lateral buds were sampled for electrolyte leakage test (EL). Lateral whorls of branches were sampled for freezing test and visual scoring (VS) of damage.

During sampling, samples of three replicate trees in each sampling day were pooled for assessment of one freezing tolerance value. Samples for freezing exposure and electrolyte leakage (EL) tests comprised either, three stem sections (4 mm long each) or 10 needle sections (10 mm). The stems and needles were taken from the middle part of the terminal shoot of each branch. In addition, seven lateral buds were collected from each of three branches and cut below the bud cavity. Samples were rinsed in deionised water and distributed into test tubes. Three needle sections, three stem sections, or one bud was put into each test tube. There were three test tubes for each



**Fig. 1.** A) Daily maximum (dotted line) and minimum (solid line) air temperatures and sampling times of shoot samples (triangle down, 1–5). B) Daily minimum soil temperatures under litter layer (solid line) and in 5cm depth of humus layer (dashed line) in soil under canopies of Norway spruce (thick grey lines) and grey alder (black thin lines); triangle up (*a,b,c*) show root sampling times.

organ, each fertilization treatment and for each of seven exposure temperatures. Samples were kept at +5°C until the frost exposures commenced.

### 2.3 Root Sampling

Root in-growth bags were used to obtain root samples with only new roots generated after fertilization. The bags were set into soil in the

experimental site in June 2001. The litter layer was removed and hollows were drilled into humus layer by a soil corer (5 cm diameter, 5 cm depth) 75 cm from trunk of tree. Flexible nylon mesh bags (mesh size 5.5 mm) were set into the hollow and filled with pre-fertilized humus. The humus was collected from a non-fertilized place on the edge of the experimental site and homogenized by sieving it through a 6mm sieve. In pre-fertilization, the same amount of fertilizers was mixed

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in the humus that was applied to the experimental plots in field, based on the cross section area of root in-growth bag. There were five bags around each of the 9 sampling trees per treatment.

The root in-growth bags were lifted at three samplings (*a*, *b* and *c*) during autumn 2002 (Fig. 1B). First the roots in a 10 cm deep soil layer were cut around the bags with a soil corer (Ø 10 cm), and a sharp knife and edged planting spade. Thereafter, the bags were gently lifted up and packed into plastic bags. The samples were transported in insulated boxes into a refrigerator at +5°C until the next morning when the preparation for the frost exposures started. Each sampling day, one bag for each of 12 trees was collected, i.e. three bags per treatment. In December, all of the 36 trees were sampled on one day and the in-growth bags were stored in a freezing chamber at the soil temperature of the experimental site (see Fig. 1B). Each day, twelve samples were set to thaw overnight at +5°C for preparation in next day.

In preparation of the samples for freezing tests, the soil around the root in-growth bags was removed by cutting with scissors and knife. Pulling roots out of the in-growth bags was avoided. Roots were separated and washed gently with tap water, and the cleaning was completed under a stereo microscope. For a series of frost exposures, 10 mm fine root sections (diameter < 1 mm) with 5 mm long branches were sampled. All three root in-growth bags from the same fertilization treatment were prepared at one time. For each of 21 test tubes, one root section was picked up from each in-growth bag, i.e. three sections into each tube.

## 2.4 Frost Exposures

The samples from different plant parts were subjected to freezing temperatures in programmable freezing chambers (ARC 300/-55+20, Arctest, Espoo, Finland) and in one N<sub>2</sub>-gas-cooled chamber (GCC-30, Carbolite, Chelmsford, UK). Samples for electrolyte leakage tests were distributed into six different freezing exposure temperatures while samples for visual scoring were exposed to three to five temperatures. In addition, non-frozen control samples were stored for the time of frost

exposures at +5°C. The minimum temperature used was -110°C. The initial and final temperature of each freezing cycle was +5°C. The cooling and warming rates were 5°C h<sup>-1</sup>, and the duration of the exposure temperature (the minimum temperature of freezing cycle) was 4h. Exposure temperatures varied depending on the expected freezing tolerance of the organs.

## 2.5 Measuring of Electrolyte Leakage (EL)

After frost exposure of EL-samples, 4 ml of deionised water was added into tubes with root samples; 7.5 ml to bud and needle samples; and 12.5 ml to stem samples. After 22 hours shaking on bench top shakers (200 rpm), the conductivity of solution in test tubes ( $L_1$ ) was measured (CDM92-conductivity meter with CDC641T-electrode, Radiometer, Copenhagen, Denmark). Subsequently, the samples were heat-killed in a water bath at 92°C for 20 minutes, shaken another 22 hours, and the conductivity was measured again ( $L_2$ ). The relative electrolyte leakage from tissues was calculated as in Eq. 1.

$$REL = \frac{L_1}{L_2} \quad (1)$$

Freezing tolerance of each organ per sampling day and fertilization treatment was calculated as the inflection point,  $C$ , of the sigmoid function shown in Eq. 2 (Repo and Lappi 1989).

$$y = \left( \frac{A}{1 + e^{B(C-x)}} \right) + D \quad (2)$$

where  $y$  is REL,  $x$  is exposure temperature,  $A$  and  $D$  define the asymptotes, and  $B$  is slope at the inflection point  $C$ . The parameter  $C$  was taken as the lethal temperature for 50% of the samples (LT50), and subsequently it is called the freezing tolerance by EL. For a proper convergence of the fitting function, the parameters  $A$  and  $D$  had to be fixed in some cases according to the mean REL by the lowest and highest exposure temperatures, respectively.

## 2.6 Visual Scoring (VS) of Injury

Sampled lateral shoots were dipped in water, put into plastic bags, and subjected to the same freezing temperatures as the EL samples. At each temperature, there were one to three shoots from each fertilization treatment. The highest number of shoots was at the temperatures of expected lethal freezing temperature. After frost exposures, needle and stem samples of each tree were put on moist paper and kept at room temperature for two weeks for visual injuries to appear. The stems were split longitudinally in two pieces. Samples were scored visually for browning of needles and stems by different temperatures. At the fifth and fourth sampling times, freezing injuries of primordial shoots in the apical buds were also scored (no damage/damage). The lethal freezing temperature was determined as 50% of injury ( $LT_{50}$ ) for each organ and tree by linear interpolation between scored values by the exposure temperatures.

## 2.7 Differential Thermal Analysis (DTA)

Differential thermal analysis (DTA) is a method for determining the freezing point of tissues (Burr et al. 2001). Tissues were cooled within specifically constructed blocks (see Räsänen et al. 2006a for details). The actual-temperature of the block, and the differential temperatures ( $\Delta T$ ) for sample blocks and individual reference for each sample were recorded. At the freezing point heat is released, and this can be seen as an exotherm in the DTA curve. The actual temperature at the point where the exotherm is found indicates the temperature for the beginning of ice crystallization.

For DTA, the terminal bud of the branch was prepared from each tree sampled. Needles and lateral buds were excised, and a thermocouple was set into a hole punctured in the pith of each stem. The samples were dipped in water, wrapped into aluminium foil and placed into the DTA device (for details, see Räsänen et al. 2006a). The sample was cooled from  $+5^{\circ}\text{C}$  to  $-48^{\circ}\text{C}$  at a rate of  $5^{\circ}\text{C h}^{-1}$ , kept at the destination temperature for three hours, and warmed again at the same rate as cooled. The freezing point of the primordial

shoot, i.e. lethal temperature, was determined on the basis of the low temperature exotherm (LTE) (Räsänen et al. 2006a).

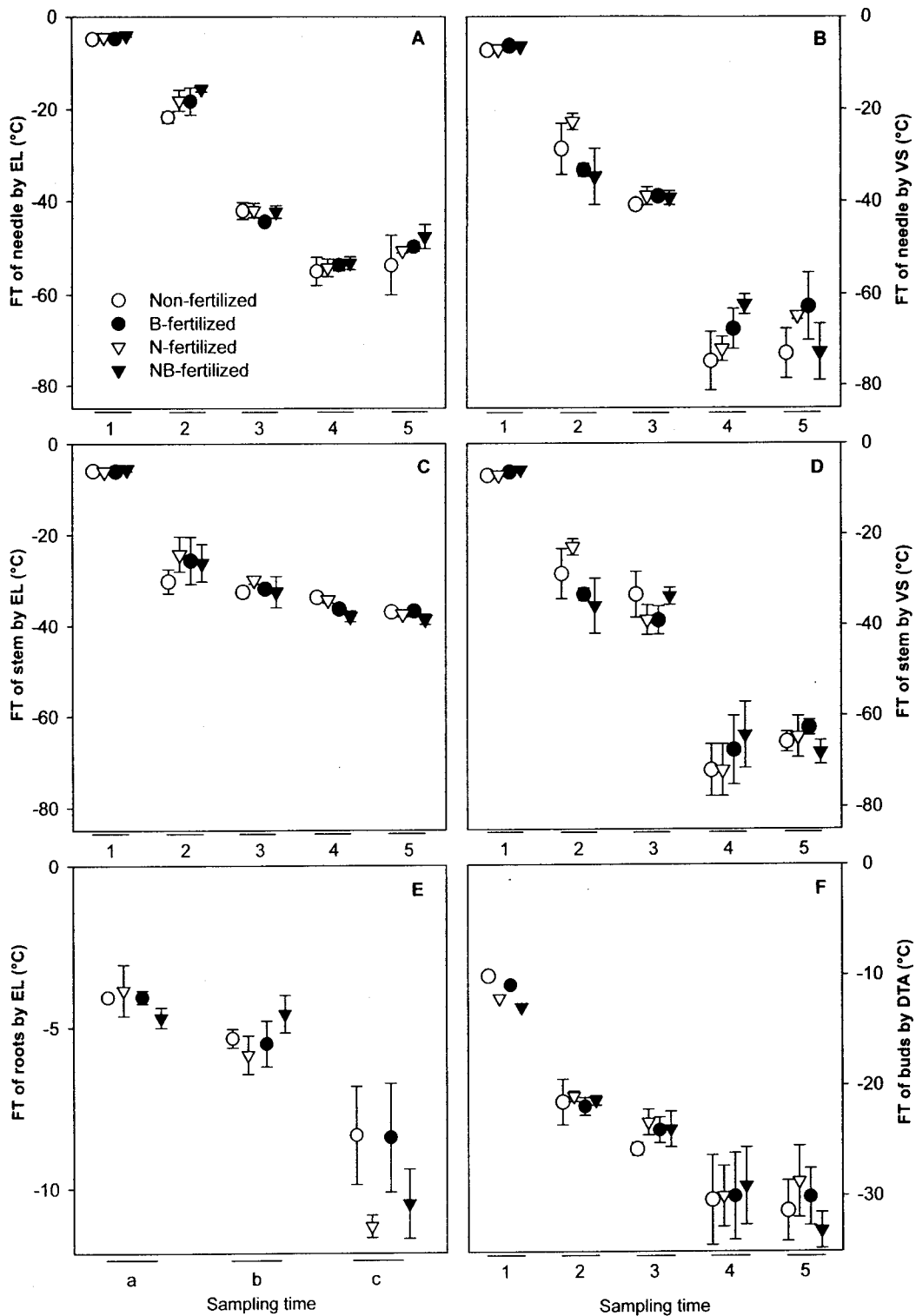
## 2.8 Data Analysis

Data on freezing tolerance of each tissue from all the five sampling times with two factors of fertilization (N and B) were subjected to repeated measures analysis of variance with blocks. Sampling day was used as block factor in analysis. When any effect of the factors B or N ( $P < 0.10$ ), or their interaction was found, data within each sampling were analysed in ANOVA. Significant differences between fertilization treatments were analysed by Tukey's test when interaction of B and N was found. Correlation of needle nutrient concentrations and freezing tolerance of bud by DTA in the same tree was studied with Pearson's correlation. All statistical analyses were carried out using SPSS (SPSS v. 15.0.1., SPSS Inc., Chicago, IL, U.S.A.).

## 3 Results

Boron fertilization had a significant effect on the B concentration of needles ( $P < 0.001$ ) but the effect of N or interaction of N and B were not significant. The mean B concentration (S.E.) was  $4.9 (0.8) \text{ mg kg}^{-1}$  in non-fertilized trees,  $21.2 (1.3) \text{ mg kg}^{-1}$  in B-fertilized,  $3.0 (0.4) \text{ mg kg}^{-1}$  in N-fertilized, and  $21.8 (2.0) \text{ mg kg}^{-1}$  in NB-fertilized trees. Nitrogen concentrations of needles ranged from 12 to  $19 \text{ g kg}^{-1}$ . There were no significant effects of factors N and B or their interaction on N concentrations in needles. The N/B ratio in needles was  $5900 \pm 2200$  (mean  $\pm$  standard error) in non-fertilized,  $6100 \pm 980$  in N-fertilized,  $740 \pm 33$  in B-fertilized and  $710 \pm 120$  in NB-fertilized trees. There was a significant effect of B ( $P < 0.001$ ) only on N/B-ratio, no effect of N-fertilization was found.

Air temperature in the experimental site was mostly above zero until the mid of September (Fig. 1A). Thereafter it decreased to subzero temperatures being only occasionally above zero. Soil temperature was about  $+5^{\circ}\text{C}$  at the first root



**Fig. 2.** Freezing tolerance (FT) of Norway spruce needles (A and B), stems (C and D) and roots (E) as determined by electrolyte leakage test (EL) (on left) and by visual scoring (VS) (on right) in autumn 2002. Freezing tolerance of buds (F) was determined by differential thermal analysis (DTA). Above ground parts were sampled between August 12 and December 11, and on roots between September 10 and December 3 from a factorial fertilization experiment. Symbols for treatments: control (open circle), B (filled circle), N (open triangle), B+N (filled triangle). For exact sampling times of aboveground and belowground organs see Fig. 1A and 1B respectively. Error bars show S.E. of mean (n = 3).



sampling time *a* (11th of Sept), and it decreased to subzero temperatures under the litter layer, but was still about +1°C at 5cm depth, before sampling time *b* (21st of Oct) (Fig. 1B). By sampling time *c* it had decreased to -8°C under litter layer and to -3°C in 5 cm depth of soil. Under alder canopy, temperature was below zero only under the litter layer at sampling time *b* but by sampling time *c* (2nd of December), it decreased to -0.5°C and -1.3°C, and later in December to -4°C and -3°C, respectively.

During the study period, all sampled organs hardened significantly. Needles achieved the highest freezing tolerance, roots the least, and stems and buds were intermediate (Fig. 2). Before the end of September (1st and 2nd sampling weeks for aerial organs), there was no clear difference in lethal temperatures as assessed by EL or VS between stem and needle tissues but needles were more tolerant thereafter (Fig. 2). The LT<sub>50</sub> (lethal temperature for 50% of the samples) values of stems (-68°C) and needles (-69°C) assessed by VS indicated much higher freezing tolerance than that by the EL-method (-38°C and -52°C, respectively) in November and December (4th and 5th sampling times). Most of the buds scored visually were injured at -20°C in the fourth and -25°C in the fifth sampling week whereas the DTA resulted -30°C and -31°C, respectively. The mean LT<sub>50</sub> values for buds assessed by electrolyte leakage were -7°C, -22°C, -34°C, -38°C and -36°C at the first, second, third, fourth and fifth sampling times, respectively. For fine roots, the mean LT<sub>50</sub> values assessed by electrolyte leakage were -4.2°C, -5.3°C, -9.6°C for sampling times *a*, *b*, and *c*, respectively.

At the fourth sampling, the lethal temperature for half of the samples (LT<sub>50</sub>) values of stems by EL test were significantly lower in B- and NB-fertilized trees than in non-fertilized or N-fertilized trees (for B main effect,  $P=0.004$ ). On average, freezing tolerance of stem in B- and NB-fertilized trees was -37°C and in non-fertilized and N-fertilized trees -34°C, respectively. No such effect of B was found in the results of VS at fourth sampling, however (Fig. 2 B). On the other hand, freezing tolerance of shoot by VS, i.e. both needle and stem, in the second sampling was higher in B-fertilized trees than in non-fertilized and N-fertilized trees (on average -34°C and -25°C,

respectively, for B effect  $P=0.03$ ). However, the freezing tolerance of stem and needles by EL were highest in non-fertilized trees at the same sampling although differences were not significant by EL method (Fig. 2A). Otherwise, no significant effects of N and B fertilization treatments on the freezing tolerance of buds, roots and needles or stem were found. Regression analysis did not reveal significant correlations between N or B concentrations in needles and freezing tolerance of buds by DTA at any sampling time.

## 4 Discussion

After B fertilization, the B status of the trees increased to levels considered optimal (Jukka 1988). Without B fertilization the B status varied: some trees were clearly deficient (B concentration < 2.5 mg kg<sup>-1</sup>) (Braekke 1979) and some were near the deficiency limit (5 mg kg<sup>-1</sup>) (Jukka 1988). There were no differences in needle N concentrations between fertilization treatments, and there was no evidence of a dilution effect. This was contrary to the hypothesis that N fertilization would exacerbate the B deficiency. The likely explanation is that the site was naturally so fertile in terms of N that the added N did not further increase the growth. In earlier studies (Möller 1983, Mälkönen et al. 1990) dilution effect is the most probable mechanism for B deficiency after N fertilization. The N/B ratios in fertilized trees were high; however, a survey of growth disorders in fertile sites of Norway spruce in Finland showed that growth disturbances were more related to low B status of trees, not imbalance of N and B (Tamminen and Saarsalmi 2004). Furthermore, there is little physiological basis to suggestions about the particular importance of the N/B ratio, as opposed to direct effects of low B status.

The fine roots were considerably less frost tolerant than the aboveground organs in accordance with previous studies (Bigras and Dumais 2005). Slight hardening of the fine roots was observed in soil temperatures above zero but major hardening occurred at soil temperatures just below zero. In earlier studies on *Pinus sylvestris* and *Picea abies*, roots achieved freezing tolerance between -4°C

and  $-10^{\circ}\text{C}$  at above zero temperatures (Lindström and Nyström 1987, Sutinen et al. 1998). In the present study, the fine roots achieved the freezing tolerance of  $-9.6^{\circ}\text{C}$ , which still is less than the maximal tolerance for fine roots in other studies (Lindström and Nyström 1987, Sutinen et al. 1998). In the present study, the root samples included abundant mycorrhizal branches which may affect the results. According to previous studies, woody roots of coniferous trees are more freezing tolerant than their non-woody, white fine roots (Ryypö et al. 1998, Bigras and Dumais 2005) but there are no studies on the role of mycorrhizal fungi in the freezing tolerance.

At the end of the study period, the freezing tolerance of needles and stems assessed by visual scoring was higher than by the electrolyte leakage test which agrees with previous studies (Sutinen et al. 1992, Repo et al. 2000, Burr et al. 1990). On the other hand, the freezing tolerance of buds by visual scoring and differential thermal analysis were lower than by the electrolyte leakage test. This may be explained by difference in sample preparation. While the differential thermal analysis and visual damage scoring both assess the actual lethal temperature for the primordial shoot in buds, the result of the electrolyte leakage test is an integrated value for different organs, i.e. a piece of stem, primordial shoot, bud axis and bud scales. Furthermore, when the buds are sampled in winter, their freezing tolerance may decrease in a few hours after thawing (Räisänen et al. 2006b), which may have resulted in an underestimate of frost tolerance in this study. Therefore, the rather narrow difference between the minimum air temperature and the freezing tolerance of buds probably overestimates the risk for freezing injuries.

No clear effect of B deficiency was found on the freezing tolerance of fine roots, buds or needles, but there was some indication of B effect on stems. In the second sampling time in September, the freezing tolerance of shoots in B fertilized trees was higher than N fertilized trees (VS method). Later, in the fourth sampling time in November, the tolerance of stems in B fertilized trees was significantly higher (EL method). As these treatment differences were not consistent, but occurred only in some cases, there was no strong evidence for the initial suggestion that B fertilization would improve the freezing toler-

ance in these trees. Moreover, there was also an unsolved difference in the results of two methods (VS vs. EL) in relation to B effect. Nevertheless, the present results give a reason to focus further studies on the relations between stem structure and freezing properties in B deficiency.

It appears that in the present study, the B deficient trees had achieved sufficient freezing tolerance. This agrees with our earlier short-term findings in the field, as there was reduced freezing tolerance in buds only if they were already visibly structurally defect (Räisänen et al. 2006a). However, in a previous pot experiment with Norway spruce seedlings, the freezing tolerance of stem and bud in B deficient seedlings was decreased at all sampling times of hardened seedlings (Räisänen et al. 2007). In the pot experiment, the B status of seedlings was still somewhat lower in lowest B treatment level than in the present study. Although it is not well known whether the B requirements of Norway spruce seedlings differ from older trees, the comparison suggests that freezing damage may become more evident at still lower internal B levels than observed in this study. It should be considered also that boron deficiency may occur at a critical time for the development of an organ, and consequently it may manifest as irreversible structural deformation. As adequate B is needed for the structural development of Norway spruce needles and buds throughout the growing season, structural disorder may arise during transient low B availability (Sutinen et al. 2006, 2007). Vice versa, fluctuations in needle B levels may lead to very low B at the time of needle sampling even if the tissues have developed well due to adequate B at the critical times. Needle B concentration may rapidly change because of environmental factors, particularly soil moisture (Bell 2000, Möttönen et al. 2005, Sutinen et al. 2006).

To conclude, some effect of B on the freezing tolerance of stems of Norway spruce was found, but it was not very consistent. The increase in the risk of freezing injury was so small that it is not likely to be a significant mechanism for the loss of apical dominance in B deficient Norway spruce trees at the B levels studied here. At still lower B availability, structural damage and freezing injury may be more likely.

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