

# Simulated snowmelt and infiltration into frozen soil affected root growth, needle structure and physiology of Scots pine saplings

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Climate warming scenarios predict a decreased snow cover and more rain instead of snow in boreal areas in winter. These conditions may affect soil freezing processes. We studied how watering of the soil before and after soil thawing affects fine roots and shoots of Scots pine saplings during the follow-up season. A growth chamber experiment was carried out with four treatments. During the dormancy water was applied onto frozen soil three times (3×FROSOIL) or once (1×FROSOIL) whereas in the beginning of the growing season the watering was started when the soil temperature reached +9 (START+9) and +15 °C (START+15). Fine root damage was noted in 3×FROSOIL, and on a smaller scale also in 1×FROSOIL. The root growth did not recover during the follow-up season. Due to the root damage, in 3×FROSOIL there was a reduced transport of water in the saplings, which induced xeromorphic features in the needles developed after the treatment. The fine root damage in 3×FROSOIL was most probably caused by mechanical breakage after the applied water froze in the soil. Our results suggest that even established saplings may suffer root damage if the climatic and soil conditions favour infiltration and freezing cycles of water, which induce frost heaving.

## Introduction

In the northern latitudes, the snow-covered area and the thickness of the snowpack are declining because of climate warming (IPCC 2007). As the snowpack is a good insulator, its thickness can effectively regulate the intensity of soil frost (Solantie 2000, Sutinen *et al.* 2008, Repo *et al.* 2011). At snow-free sites, the maximum depth of soil frost in southern and central Finland can be 100–150 cm, and in northern Finland, as low as 100–300 cm (Venäläinen *et al.* 2001). In a snow

manipulation study in central Finland, the forest soil temperature as low as –15 °C was reached at the depth of 5 cm when snow accumulation was prevented through the winter by shovelling (Repo *et al.* 2011). If the insulating snow cover becomes thinner or disappears in the future, soil frosts deeper than the present ones may occur in the northern latitudes, where winter air temperature may still be very low (Groffman *et al.* 2001, Hardy *et al.* 2001, Venäläinen *et al.* 2001, Kellomäki *et al.* 2010). On the other hand, the wintertime warming may increase the proportion

of precipitation that falls as rain rather than snow (Räisänen 2008), and episodes of snowmelt will be more frequent.

Air temperatures may change suddenly, whereas soil temperatures change more slowly (Jyske *et al.* 2012, Sutinen *et al.* 2008, 2012). Thus rain may fall on frozen soil, as was noted in May and June 1996 when soil frost remained well after the shallow snow cover was melted away in northern Sweden (Nyberg *et al.* 2001). The melt water during mild events in winter may infiltrate the frozen soil and freeze there during the subsequent cold periods, and this freezing cycle can be seen as increased and then decreased volumetric soil water content (Sutinen *et al.* 2008). In forested areas, rain on frozen soils or snowmelt water does not normally form ponds on the ground surface, as may happen on finer-textured agricultural fields, but it may infiltrate onto the ground water through contraction cracks and pathways of decomposed roots or freeze in the upper soil layers (Stadler *et al.* 2000, Sutinen *et al.* 2009).

The sensitivity of conifer roots to low wintertime soil temperatures depends on the level of frost hardiness of the roots, which varies from autumn to winter and spring (Sutinen *et al.* 1998). Furthermore, the frost hardiness depends also on the age of the roots, tree species, and geographical location of the tree (Bigras and Dumais 2005 and references therein). Root damage may occur as a direct effect of low temperature and consequent ice crystal formation and dehydration within the roots, or it may occur indirectly, such as root breakage caused by frost heaving (Goulet 1995, Sutinen *et al.* 1998, de Chantal *et al.* 2006, 2007). In snow removal experiments, deleterious effects of low soil temperatures on fine roots were found for *Picea abies* (Gaul *et al.* 2008), *Picea glauca*, *Picea mariana*, *Pinus banksiana* (Coursolle *et al.* 2002), and *Pinus sylvestris* (Repo *et al.* 2005, 2008). In many cases, however, recovery of the root damage was noted during the next growing season.

This study simulates infiltration of melt water and/or rain onto frozen soil and consequent freezing of the infiltrated water in early spring conditions. To our knowledge, there are no studies on the reactions of conifer roots to that kind of soil frost events. It is not known either, how

the above-ground parts of trees respond during the next few growing seasons to such a situation, which may become frequent in boreal forests under future climates. It was therefore our aim to find out how watering before and after soil thawing affects the physiology, anatomy, phenology, and the growth of shoots and roots in Scots pine saplings during the subsequent follow-up season. We measured water potential, chlorophyll fluorescence, gas exchange and concentrations of soluble sugars, starch and chlorophyll in the needles as well as needle inner structure. In addition, we used electrical impedance spectroscopy (EIS) that measures extracellular and intracellular resistance, and indicates cell membrane damages. EIS has been found to detect sensitively the effects of abiotic stresses, such as freezing damage, on plants (Zhang and Willison 1992, Repo *et al.* 1994, 2000). We hypothesized that infiltration of liquid water in early spring could either benefit saplings by increasing water availability during early summer or be harmful due to the possible damage on roots during winter.

## Material and methods

### Saplings and treatments

Sixteen four-year-old Scots pine (*Pinus sylvestris*) saplings (seeds from seed orchard SV124, Suokanta, Iitti, Finland, 60°52'N, 26°26'E, 73 m a.s.l.) were lifted from a plantation stand in eastern Finland (62°29'N, 30°25'E, 130 m a.s.l.) in May 2004 after the snow had melted and the soil was not frozen any longer. The plantation had been established in a *Calluna* type (CT) of forest (Cajander 1949) with one-year-old seedlings in 2001. The mean height of the saplings at the time of lifting was 89 cm, and their condition was good, i.e., they had green needles in three age classes and the buds showed slight elongation. To avoid damage to the roots, the root systems with soil were carefully lifted onto a large plastic sheet. The saplings were transferred to dasotrons (RTR48, Conviron, Winnipeg, Canada) at Joensuu (Finér *et al.* 2001) and planted into cylindrical containers (volume 0.19 m<sup>3</sup>, height 0.5 m, diameter 0.7 m) onto a 20-cm layer of filler sand. The containers around the roots were filled with

a 30-cm layer of mineral soil from the lifting stand (pH 5.2) and on the surface a 5-cm organic layer (pH 4.0) from the stand was laid.

To control the soil temperature, each pot was equipped with two glycol circulation coils, one at the bottom and the other above the organic layer (for details, see Finér *et al.* 2001). A sheet of insulating material was placed over the upper coil. The texture of the mineral soil was 6% silt (0.002–0.02 mm), 61% fine sand (11% 0.02–0.06 mm, 50% 0.06–0.2 mm), 31% medium and coarse sand (0.2–2 mm), and 2% gravel (> 2 mm). The mineral layer was classified into three 10-cm layers down from the soil surface as follows: mineral layer 1 = 5–15 cm, mineral layer 2 = 15–25 cm, and mineral layer 3 = 25–35 cm from the surface of the container. In each of the four dasotrons, there were four root containers, in which the air and soil conditions were controlled independently. The soil temperature (105T Thermocouple, Campbell Scientific, Shepshed, UK) and the volumetric water content (Theta Probe, ML 2x, Delta-T Devices, Cambridge, UK, and CS615, Campbell Scientific, Shepshed, UK) were logged at 20-minute intervals at the depth of 2.5 cm from the surface of the organic layer and at the bottom of the first mineral layer (15 cm from the surface; Table 1 and Fig. 1).

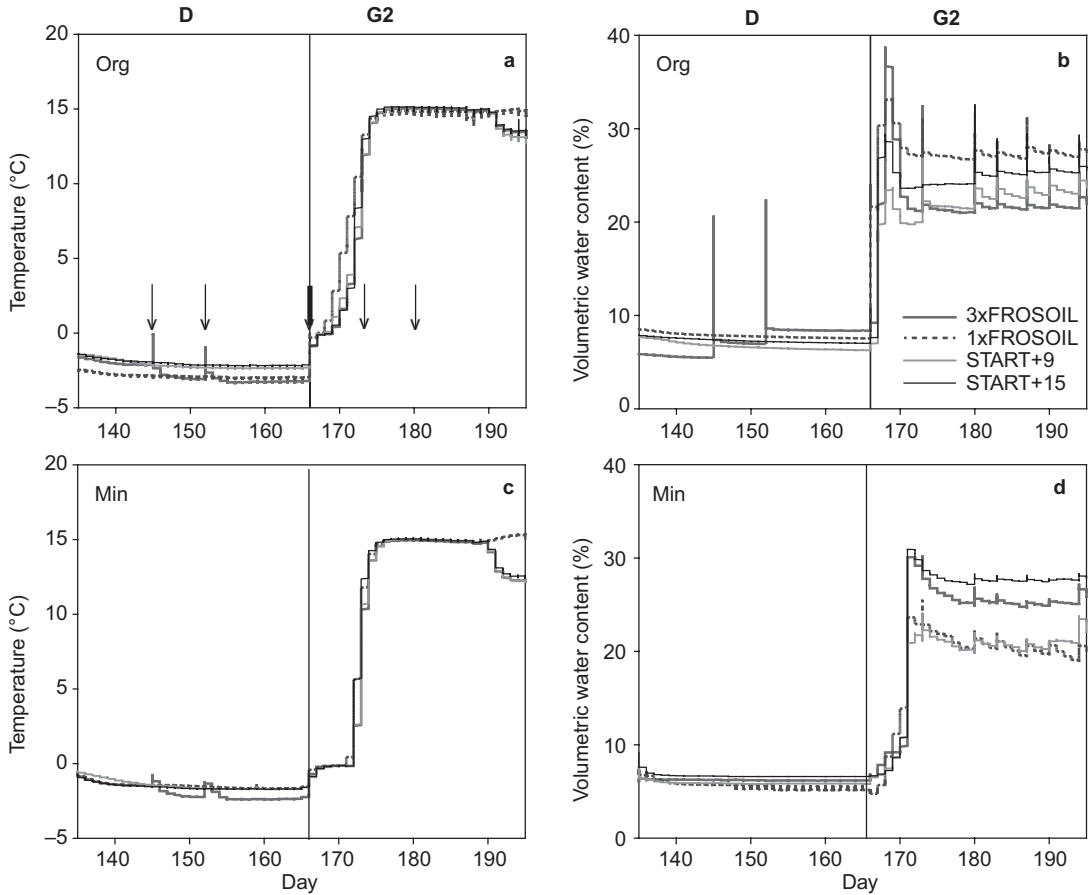
Before the start of the treatments, the saplings had the first growing season (G1) to adjust to the chamber conditions. This was followed by dormancy (D) with the soil frozen (Table 1, Fig. 1a and c). The treatments were as follows: In 3×FROSOIL, water was applied three times and

in 1×FROSOIL, once onto frozen soil during dormancy. In START+9 and START+15, water was applied when the soil temperature reached 9 and 15 °C, respectively (Fig. 1a). In 3×FROSOIL, the temperature of the organic layer was raised before the first and the second watering, but the soil temperature never rose above 0 °C (Fig. 1a and c). Then, 5 l of water at 1 °C was applied evenly onto the surface of the pot, and the soil temperature was lowered to –3 °C again. The watering corresponded to approximately 13 mm of rain. The first two waterings in 3×FROSOIL can be seen as short-lived peaks in the soil temperatures of the organic layer and the upper mineral layer (Fig. 1a and c) as well as peaks in the volumetric water content of the organic layer (Fig. 1b) but not of the mineral layer (Fig. 1d).

At the beginning of the follow-up season (G2), soil thawing was started simultaneously in all containers. In all dasotrons, the air temperature and the light conditions were changed gradually over five days until favourable conditions for growth were met (G2 in Table 1). The increase in soil temperature from –3 °C to +15 °C took about two weeks (Fig. 1a and c), whereas the soil moisture increased from around 8% to over 20% in just a few days (Fig. 1b and d). During the growing seasons the soil moisture was kept close to the field capacity (Table 1, Fig. 1b and d) by watering the saplings regularly in all treatments. The temperature of the irrigation water was adjusted according to the soil temperature and the chemical composition according to the precipitation in southern Finland (Sallantausta 1992). The volume of the irrigation water and

**Table 1.** Chamber conditions during the experiment. Day indicates the time from the start of the first growing season G1, and G2 is the follow-up season. RH indicates relative air humidity and PAR photosynthetically active radiation. Soil temperature and soil volumetric water content (WC), refer to organic and uppermost mineral soil layers.

	G1 long day	G1 short day	Dormancy	G2 long day	G2 short day
Day	12	89	110	166	241
Duration, weeks	11	3	8	11	3
Day/night air temperature (°C)	20/15	20/15	4/4	20/15	20/15
Day/night RH (%)	70/80	70/80	90/90	70/80	70/80
PAR ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	400	200	200	400	200
Day/night photoperiod (h)	18/6	6/18	6/18	18/6	6/18
Soil temperature (°C)	15	15	–3	15	15
Soil WC (%)	20–30	20–30	5–7	20–30	20–30



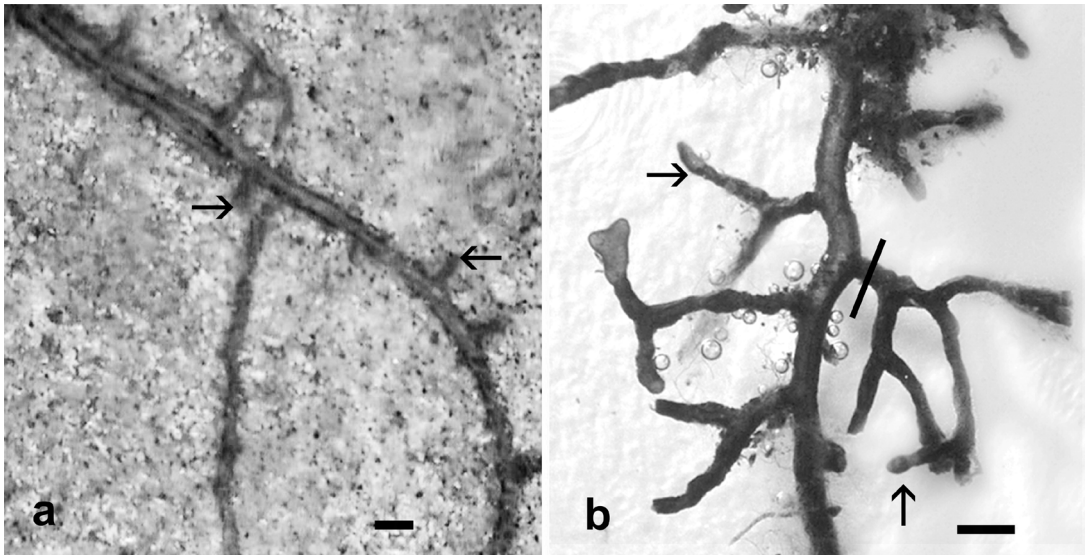
**Fig. 1.** (a and c) Mean hourly soil temperature, and (b and d) soil water content in the organic (Org) and the uppermost mineral layer 1 (Min), respectively, during dormancy (D) and the follow-up season (G2). Day indicates the time from the start of the first growing season (G1, see Table 1), when the soil temperature in the organic and the uppermost mineral layer was +15 °C, and the volumetric water content between 20%–30%. Days of simulated rain treatments are shown with arrows in a. The 3xFROSOIL included three waterings onto the frozen soils on days 144, 152 and 166 and 1xFROSOIL one watering on the day 166, when dormancy was completed. The START+9 was done on the day 173 and the START+15 on the day 180 when soil temperatures were +9 and +15 °C, respectively. The temperature of the water applied was 1 °C in 3xFROSOIL and 1xFROSOIL, 9 °C in START+9, and 15 °C in START+15. Each line represents the mean value of four root containers.

that of the water drained through the bottom valves of the pots were measured. The air conditions, i.e., the driving force of evapotranspiration, were similar in all the chambers (Table 1).

### Phenology and shoot growth

The start and the cessation of shoot and needle elongation and the diameter growth of trunk were determined during the follow-up season. The shoot and the needle elongation were measured with a ruler and the trunk diameter with a

slide gauge three times a week. Each time, the relative value of the growth attained was calculated, with 0 corresponding to no growth and 1 to completed growth. To determine the times of the initiation and the cessation of growth, 10% and 90% of the total growth for the season were used, respectively. The total biomass of the stems and the needles was determined by whorl and shoot-age category in the final harvest at the end of the follow-up season. Live and dead needles, as well as live branches, and trunks were separated, and the dry mass of each cohort was determined after drying for 4 days at 60 °C.



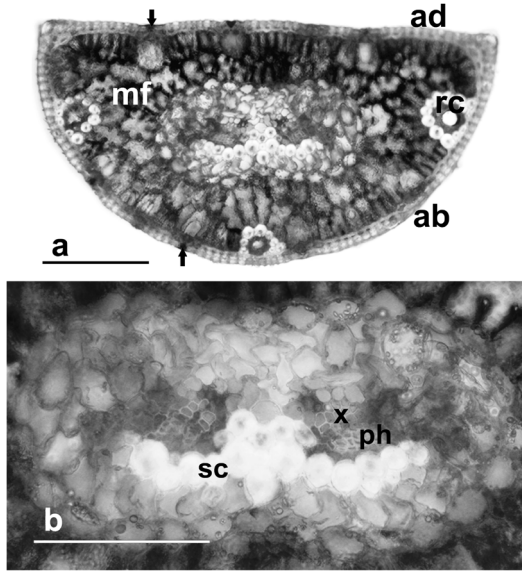
**Fig. 2.** (a) Fine roots of pine saplings from minirhizotron imaging, and (b) photographed with a digital camera under a stereomicroscope in a droplet of water showing long and short fine roots, which definition was used when the minirhizotron images were analysed by means of the RootView software. Arrows in both pictures show mycorrhizal first-order fine roots that represent the short roots. The line in **b** separates the lateral fine root branch from a long fine root. Second- or bigger-order fine roots are classified as long roots. The length of the bar in both pictures is 1 mm.

## Root growth

Root growth was recorded by means of minirhizotron imaging (Bartz BTC-100X Camera System, Bartz Technology Company, Santa Barbara, CA, U.S.A.) four times during the first growing season, twice during dormancy (data not shown), and at two-week intervals during the follow-up season. The imaging tube (exterior diameter 60 mm) was set horizontally at the bottom of the upper mineral layer (15 cm deep from the surface). Digital images of the roots were taken in an upward direction along the entire extension of the tube, with a total of 46 frames ( $13 \times 18$  mm) taken. Thus the level of the root images was approximately 9 cm from the surface and thus at about the middle of the upper mineral layer. To determine fine root elongation and the formation of new root tips, the images were analysed by means of the RootView software (Aphalo and Simonic 1999). The length of live and dead short and long fine roots were recorded (Repo *et al.* 2008). In the calculation of the cumulative growth for each seedling during the follow-up season, the number of root tips and

the length of the short and the long roots at the end of dormancy were used as points of reference. The short roots represent the first-order fine roots, where the ectomycorrhizas form, and the long roots represent branched, longer fine roots (Fig. 2; *see also* Pregitzer *et al.* 2002).

In the final harvest at the end of the follow-up season, two soil sector samples (the surface area of one sector being 385 cm<sup>2</sup>) were cut from the opposite sides of the trunk for separating the roots. Central cylinders (diameter 10 cm) including the root stump were excluded. The sector samples comprised the organic layer (mean thickness 5 cm) and the three mineral layers, each of which was 10 cm thick. The length, surface area, and volume of the fine roots and number of root tips of each layer were measured by means of scanning (WinRhizo 3.1.2, Québec, Canada) using five diameter categories (< 0.5, 0.5–1.0, 1.0–1.5, 1.5–2.0 and 2–4.0 mm). To determine the total dry mass of each sector the roots were dried for 4 days at 60 °C. Different degrees of decomposition of dead roots caused much uncertainty in their biomass assessment and thus it was excluded.



**Fig. 3.** C needle cross-section at the end of the follow-up season. (a) ad = adaxial surface, ab = abaxial surface, mf = mesophyll, and rc = resin canal; the lighter area in the middle of the needle is the central cylinder surrounded by endodermis; arrows indicate stomatal rows on the adaxial and abaxial sides. (b) sc = sclerenchyma, x = xylem, ph = phloem. The length of the bar is 200  $\mu\text{m}$  in a and 100  $\mu\text{m}$  in b.

### Needle anatomy

Needle samples were taken from the lateral shoots in the third whorl and from different sides of the sapling. At the end of the follow-up season, five current-year needles (C needles) were detached from the upper side of each shoot. To avoid any changes before further procedures, the needles were immediately put into a test tube containing a fixative solution (2% glutaraldehyde in cacodylate buffer, pH 7.0, 0.05 M). A piece of about 1 cm was removed from the tip of each needle, after which five fresh cross-sections per needle were prepared with a razor blade under a stereomicroscope in a drop of cacodylate buffer. The cross-sections were placed onto an objective plate in a drop of cacodylate buffer, covered with a cover plate, and photographed with a digital camera (Leica CD Camera, Switzerland) under a light microscope (Leica DM2500, Germany) with a 2.5 $\times$  objective. Measurements were made of the areas of the whole cross-section, the

mesophyll tissue, the central cylinder, the area and number of the resin ducts, the number of stomatal rows in the adaxial and abaxial sides, and the length of adaxial and abaxial surfaces (Fig. 3a). To measure the areas of the sclerenchyma, phloem, and xylem tissues and to count the number of tracheids, 25 $\times$  objective was used (Fig. 3b). From the measurements, the ratios of different tissue types were calculated. In all these analyses and measurements, tools of Adobe Photoshop (ver. 6.0) were used.

### Needle and shoot physiology

The water potential of ten previous year's needles (C + 1 needles), sampled equally from the second and the third whorls and from different sides of the sapling, was determined once during dormancy and five times during the follow-up season by means of a pressure chamber (Scholander *et al.* 1964). The measurements were made within 10 minutes of the sampling. Electrical impedance spectroscopy (EIS) was used for measuring properties of C + 1 needles which were sampled in the same way as was done for the water potential measurements (for details, see Repo *et al.* 2005). For analyses of the impedance spectra, a distributed circuit element model (single-DCE) was used. In the model, the complex impedance ( $Z$ ) is

$$Z = R_{\infty} + \frac{R_0 - R_{\infty}}{1 + (i \times \tau \times \omega)^{\psi}}$$

where  $R_{\infty}$  ( $\Omega$ ) is the resistance at very high frequency and  $R_0$  ( $\Omega$ ) the direct current resistance,  $i$  is an imaginary unit,  $\tau$  is the relaxation time (s) of polarization,  $\psi$  is the distribution coefficient of the relaxation time, and  $\omega = 2\pi f$  ( $f$  = frequency in Hz) is the angular velocity. The parameters of the model were estimated by means of the complex non-linear least squares (CNLS) curve-fitting program LEVM ver. 6.0 (J.R. Macdonald, Department of Physics and Astronomy, University of North Carolina, Chapel Hill, U.S.A.).

Photosynthesis was measured during the follow-up season in ambient (ca. 270  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) and saturation light (800  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) by means of ADC LCPro+ (ADC BioScientific Ltd., Herts, UK), twice from C + 1 needles (on days 174

and 189) and once from C needles (on day 254). Dark-adapted chlorophyll fluorescence ( $F_v/F_m$ ), and chlorophyll *a* and *b* contents were measured during dormancy and the follow-up season, nine times from C + 1 needles and once from C needles at the end of the follow-up season (for details, *see Repo et al.* 2005).

To determine the soluble sugar and starch contents, the C + 1 and C needles were dried at 40 °C to constant weight and ground as proposed by Hansen and Møller (1975). The C needles could be analysed only at the end of the follow-up season, when they were fully developed.

The sap flow of the trunk was measured in two saplings for each treatment with a Dynamax Flow32 Stem-Flow Gauge system as described in Repo *et al.* (2008). The flow rate was logged at 15-minute intervals and converted into daily rates. The water consumption of each sapling was calculated on the basis of the volume of water applied and that of the excess water drained through the bottom valves of the pots.

## Statistical methods

The treatment effects on chlorophyll fluorescence, chlorophyll *a* and *b* content, soluble sugar and starch content, EIS parameters, root growth, and root tip formation were analysed by means of a linear mixed model (procedure MIXED in SPSS 17, SPSS Inc., Chicago, IL, USA). The model was  $y = \mu + \text{treatment} + \text{time} + \text{treatment} \times \text{time} + \text{chamber} + \text{sapling} + \varepsilon$ , where  $\mu$  is a constant and  $\varepsilon$  is an error term. The ‘treatment’ (i.e., different starting times of watering) and ‘time’ (i.e., sampling time) were regarded as fixed factors, and the chamber and sapling were random terms. The time correlation of the error terms within a sapling was described by a heterogeneous AR(1) structure. Dominator degrees of freedom of the *F*-statistics were computed using Satterthwaite’s method (IBM Statistics, Algorithms). To account for multiple comparison effect, a Bonferroni correction was applied, i.e., the *p* value of each contrast was multiplied by the number of comparisons at a sampling time. The normality and homogeneity of the variance of the residuals were checked graphically, and the selection of the covariance structure

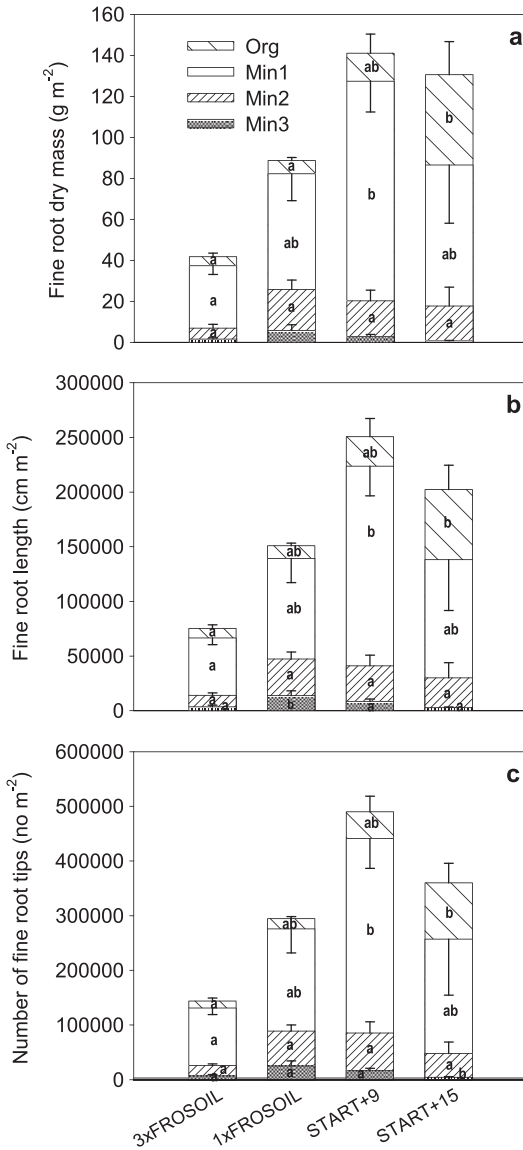
was based on Akaike’s (Akaike 1973) information criteria. The data were arctan- or log-transformed when necessary to fulfil the requirement of normality and homogeneity of the variance of the residuals. In the analysis of the minirhizotron data, the length of the short and the long roots and the number of roots tips prior to the start of the follow-up season were taken as covariates. The fine root morphology and biomass data of the saplings (harvested at the end of the follow-up season) were analysed by means of the linear mixed model similar to the one described above, except that ‘time’ was replaced by ‘soil layer’ and within-sapling correlations were described by means of an unstructured model. The differences among the treatments at the start and the cessation of shoot elongation, trunk diameter growth, and the cessation of needle elongation during the follow-up season were analysed with the Mann-Whitney *U*-test. The effects of the different treatments on needle anatomy and the total shoot and root biomass were subjected to one-way ANOVA followed by Tukey’s test.

## Results

### Shoot phenology and growth

During the follow-up season the trunk diameter began to increase in all saplings between days 166 (day 166 was the start of the follow-up season, G2; *see* Fig. 1a) and 172, i.e., before the first waterings in START+9 and START+15. Elongation of leader shoot began in all treatments between days 180 and 188, i.e., around the start of the watering in START+15. Needle elongation started on day 197, when watering started in all of the treatments. Trunk diameter growth, shoot elongation, and needle elongation ceased around days 201–229, 197–208, and 247, respectively (for the days, *see* Fig. 1a). Relative needle and shoot elongation advanced similarly during both first growing and follow-up seasons. Even though some irregularities were found during the follow-up season, no significant differences were found among the treatments (data not shown).

The total shoot dry biomass at the end of the follow-up season was  $245.3 \pm 27.6$ ,  $348.5 \pm 68.7$ ,  $360.5 \pm 60.7$  and  $295.4 \pm 32.5$  g in 3×FROSOIL,



**Fig. 4.** (a) Mean biomass, (b) length, and (c) number of root tips in fine roots (diameter  $\leq 2$  mm), of pine saplings by treatment, in a 5-cm-thick organic layer (Org) and three 10-cm-thick mineral soil layers at the end of the follow-up season. Order of mineral layers is as follows: Min1 = the uppermost, Min2 = middle and Min3 = lowermost mineral layer. For the treatments, see Fig. 1a. Bars indicate standard errors ( $n = 4$ ). Different letters indicate significant differences between the treatments (all Bonferroni-corrected  $p$  values are  $< 0.05$ ).

1x FROSOIL, START+9, and START+15, respectively, and it did not differ significantly among the treatments (ANOVA:  $F_{3,12} = 1.00$ ,  $p = 0.425$ ).

## Root growth and morphology

Repeated watering onto frozen soil was harmful to the fine roots (diameter category  $\leq 2$  mm) of pine saplings, especially in the organic layer and in the uppermost mineral layer where most of the roots were growing. In the organic layer, the fine root biomass was lower in 3x FROSOIL and 1x FROSOIL than in START+15 (Fig. 4a, MIXED: Treatment:  $F_{3,12} = 5.70$ ,  $p = 0.012$ ; Layer:  $F_{3,12} = 76.38$ ,  $p < 0.001$ ; Treatment  $\times$  Layer:  $F_{9,12} = 4.13$ ,  $p = 0.010$ ). Accordingly, the length of the fine roots (MIXED: Treatment:  $F_{3,12} = 4.65$ ,  $p = 0.022$ ; Layer:  $F_{3,12} = 128.70$ ,  $p < 0.001$ ; Treatment  $\times$  Layer:  $F_{9,12} = 4.63$ ,  $p = 0.008$ ) and the number of root tips in the organic layer (MIXED: Treatment:  $F_{3,12} = 4.56$ ,  $p = 0.024$ ; Layer:  $F_{3,12} = 99.59$ ,  $p < 0.001$ ; Treatment  $\times$  Layer:  $F_{9,12} = 3.57$ ,  $p = 0.022$ ) were lower in 3x FROSOIL than in START+15 (Fig. 4b and c). In the uppermost mineral soil layer (5–15 cm from the surface), 3x FROSOIL had a negative effect on all measured fine root parameters in comparison with START+9 (biomass, length and number of root tips shown in Fig. 4;  $p$  for main effects shown above). In the middle mineral layer (15–25 cm from the surface), no differences were found among the treatments in the biomass or morphology of the roots (Fig. 4). In contrast, in the deepest mineral soil layer ( $> 25$  cm from the surface), the fine root length was greater in 1x FROSOIL than in other treatments (Fig. 4b;  $p$  for main effects shown above). The total root length consisted extensively of fine roots. The pattern of distribution was similar in all studied soil layers: proportions of the root lengths were 54% for diameter  $< 0.5$  mm, 33% for diameter between 0.5–1.0 mm, 9% for 1.0–1.5 mm, 3% for 1.5–2 mm and 1% for diameter 2–4 mm. The thicker roots (diameter 2–4 mm) responded to the treatments in a similar manner as the thinner roots, but the effects were not significant (for biomass, ANOVA:  $F_{3,12} = 1.68$ ,  $p = 0.225$ ) (data not shown).

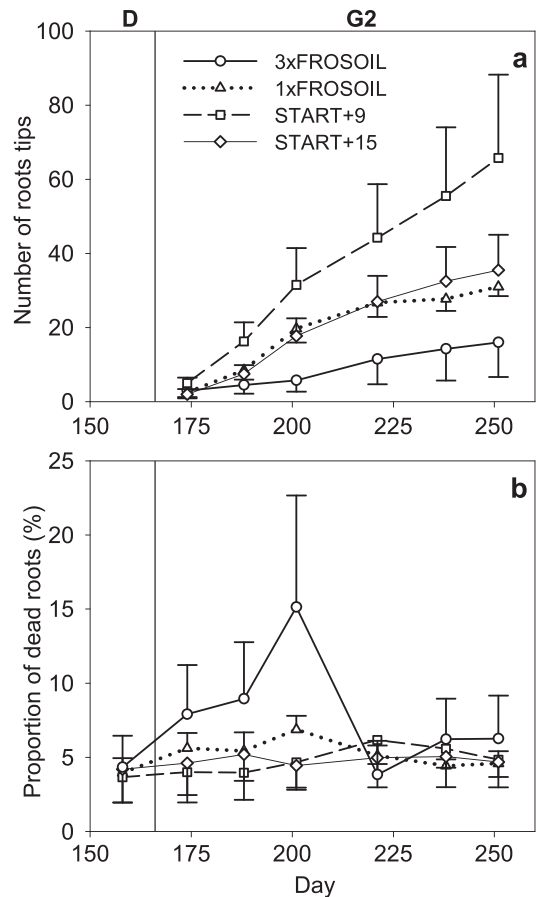
Minirhizotron imaging from the middle of the uppermost mineral soil layer showed an increase in the number of root tips and the elongation of fine roots during the second half of the follow-up season. For the number of root tips, both the main effect of treatment and the



interaction with time were significant (MIXED: Treatment:  $F_{3,11.7} = 8.61, p = 0.003$ ; Time:  $F_{5,16.9} = 0.37, p = 0.862$ ; Treatment  $\times$  time:  $F_{15,17.7} = 3.01, p = 0.014$ ). The number of root tips increased less in 1xFROSOIL than in START+9 on days 221, 238, and 251 (Bonferroni-corrected  $p$  values: 0.020, 0.001 and  $p < 0.001$ , respectively), less in 3xFROSOIL than in START+9 on days 238 and 251 (Bonferroni-corrected  $p$  values: 0.029 and 0.015, respectively), and less in 1xFROSOIL than in START+15 on days 238 and 251 (Bonferroni-corrected  $p$  values: 0.013 and 0.010, respectively) (Fig. 5a). Treatment had a significant effect on short root elongation (MIXED: Treatment:  $F_{3,12.4} = 5.20, p = 0.015$ ; Time:  $F_{5,21.3} = 1.16, p = 0.363$ ; Treatment  $\times$  time:  $F_{15,21.8} = 1.80, p = 0.102$ ). Elongation of the short fine roots was smaller in 1xFROSOIL than in START+9 on days 238 and 251 (Bonferroni-corrected  $p$  values: 0.032 and 0.022, respectively) (data not shown). The proportion of the length of the dead short roots to the total length of the short roots was higher in 3xFROSOIL than in the other treatments during the first half of the follow-up season, after which the dead short roots started to disappear from the images. However, the differences among the treatments were not significant (MIXED: Treatment:  $F_{3,11.5} = 0.23, p = 0.872$ ; Time:  $F_{5,32.0} = 0.44, p = 0.814$ ; Treatment  $\times$  time:  $F_{18,32.4} = 0.67, p = 0.791$ ). During the rest of the follow-up season we found about 5% of dead roots in all treatments (Fig. 5b). There was no treatment effect on elongation of the long fine roots (MIXED: Treatment:  $F_{3,11.8} = 0.194, p = 0.898$ ; Time:  $F_{5,21.9} = 0.29, p = 0.912$ ; Treatment  $\times$  time:  $F_{15,21.9} = 0.55, p = 0.881$ ) (data not shown).

### Water flow and needle physiology

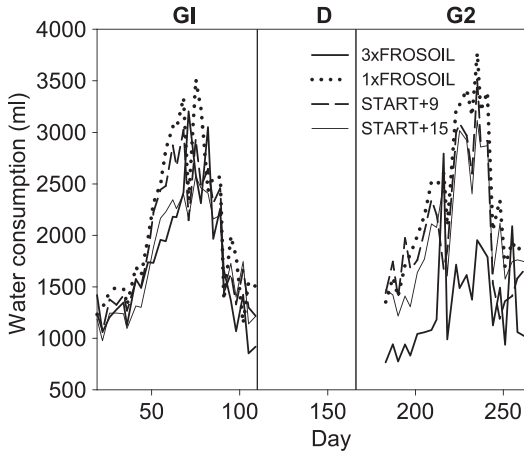
There had been no differences in water consumption among the saplings during the first growing season, but during the follow-up season it remained at a constantly lower level in 3xFROSOIL than in the other treatments (Fig. 6). Furthermore, the daily mean sap flow in 3xFROSOIL was 42 g day<sup>-1</sup> in the beginning of the follow-up season and 361 g day<sup>-1</sup> in the middle of the follow-up season, as opposed



**Fig. 5.** (a) Mean number of root tips, and (b) proportion of the length of dead short roots out of the length of all short roots of pine seedlings, by the treatment, in the follow-up season (G2), from minirhizotron imaging. D = dormancy. For significant differences, see the text, and for the treatments and days, see Fig. 1a. Bars indicate standard errors ( $n = 4$ ).

to the corresponding values of 181–301 and 513–668 g day<sup>-1</sup> in the other treatments. Sapflow during dormancy was so low that it could not be accurately measured. Due to the low number of measured saplings ( $n$  was 1 or 2), the statistical significance of the sap flow results could not be calculated.

The mean water potential of C + 1 needles varied from approximately -1.8 MPa during dormancy up to approximately -0.5 MPa at the end of the follow-up season (Fig. 7a). During the follow-up season it was the lowest in 3xFROSOIL, and a significant difference from that in START+15 was noted on day 235

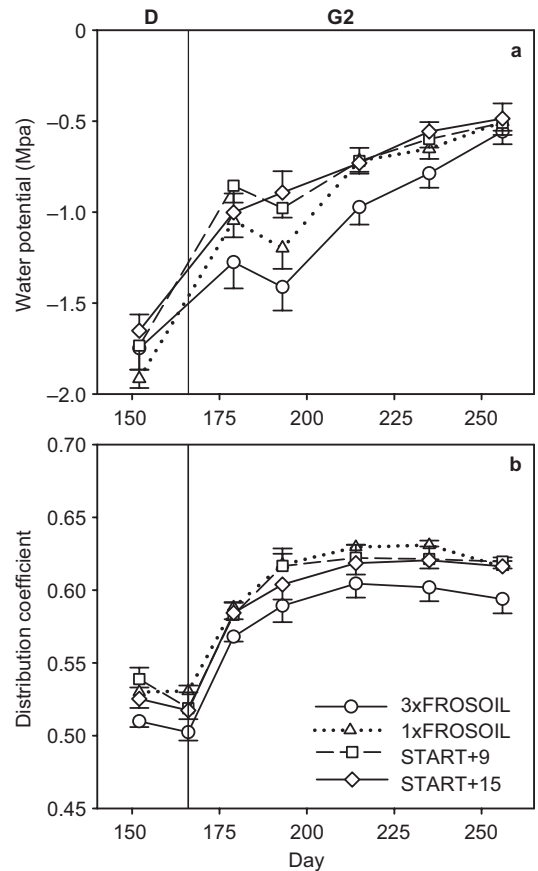


**Fig. 6.** Mean water consumption (ml) of pine saplings during the first growing season (G1) and follow-up season (G2). The gap between the growing seasons is the dormancy (D), with the soil frozen (see Fig. 1a and c). Regular watering was started on day 0 in G1 and on day 187 in G2, after the simulated watering treatments.

only (Bonferroni-corrected  $p = 0.034$ ) (MIXED: Treatment:  $F_{3,12.4} = 3.38$ ,  $p = 0.054$ ; Time:  $F_{5,30.3} = 85.28$ ,  $p < 0.001$ ; Treatment  $\times$  time:  $F_{15,30.3} = 1.49$ ,  $p = 0.172$ ).

Electrical impedance spectroscopy (EIS) revealed a significant treatment and time effect on the distribution coefficient of the relaxation time of the needles (MIXED: Treatment:  $F_{3,13.6} = 4.02$ ,  $p = 0.030$ ; Time:  $F_{6,43.4} = 144.68$ ,  $p < 0.001$ ; Treatment  $\times$  time:  $F_{18,43.4} = 1.15$ ,  $p = 0.339$ ). On days 152, 194, 235, and 256 the coefficient was the lowest in 3x FROSOIL, and significant differences from that in 1x FROSOIL on day 235 (Bonferroni-corrected  $p = 0.029$ ) and from that in START+9 on days 152 and 256 were noted (Bonferroni-corrected  $p$  values: 0.025 and 0.028, respectively) (Fig. 7b).

Dark-adapted chlorophyll fluorescence was high in both growing seasons and low during dormancy (MIXED: Treatment:  $F_{3,13.6} = 0.863$ ,  $p = 0.480$ ; Time:  $F_{6,25.9} = 56.10$ ,  $p < 0.001$ ; Treatment  $\times$  time:  $F_{18,25.9} = 1.22$ ,  $p = 0.313$ ) (Fig. 8). Soluble sugars were high during dormancy and decreased towards the growing season, and the starch and chlorophyll contents were high in the growing season and low during dormancy. The treatments did not show significant differences (data not shown).



**Fig. 7.** (a) Mean water potential, and (b) distribution coefficient of the relaxation time in C + 1 needles during the follow-up season (G2). D = dormancy. The horizontal lines indicate the start of the follow-up season. For significant differences, see the text, and for the treatments and days, see Fig. 1a. Bars indicate standard errors ( $n = 4$ ).

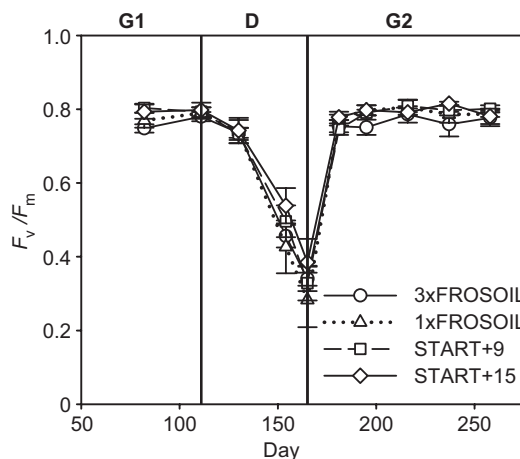
## Needle anatomy

The structure of the C needles, as analysed from fresh cuttings, was intact (see Fig. 3a and b). The phloem area was insignificantly smaller in 3x FROSOIL than in START+15 and the xylem area insignificantly smaller in 3x FROSOIL than in START+9. In other respects the tissue areas or their ratios did not show any significant differences (Table 2). The number of stomatal rows on the abaxial side (convex surface) was significantly lower in 3x FROSOIL than in START+9 and START+15, and on the adaxial side (straight surface) it was significantly lower in 3x FROSOIL than in START+15. The number

of stomatal rows on the entire circumference was significantly lower in 3×FROSOIL than in START+9 and START+15. The length of the adaxial side of the needle cross-section and the total circumference of the cross-section were insignificantly smaller in 3×FROSOIL than in START+9. When the number of stomatal rows was counted per 1 mm of the circumference, it was significantly lower in 3×FROSOIL than in START+15 both on the abaxial side and on the entire circumference (Table 2).

## Discussion

Watering the frozen soil reduced the root biomass, root length, and number of root tips of pine saplings in comparison with watering thawed soils. Increased fine root mortality and reduced root tip formation during the follow-up season was found especially in 3×FROSOIL, which indicated that repeated addition of water, simulating snowmelt events or wintertime rain, and



**Fig. 8.** Mean chlorophyll fluorescence ( $F_v/F_m$ ) during the first growing season (G1), follow-up season (G2) and dormancy (D) of the needles born during the first growing season. For the treatments see Fig. 1a. Bars indicate standard errors ( $n = 4$ ).

freezing of the added water in soil was deleterious to the fine roots. The freezing events here were seen as an immediate increase in volumetric

**Table 2.** Characteristics of the C needles (see Fig. 3) of *Pinus sylvestris* at the end of the follow-up season, with the standard errors ( $n = 4$ ) indicated. For the treatments, see Fig. 1a. The values followed by different letters are significantly different ( $p < 0.05$ ) according to ANOVA, followed by Tukey's test.

Treatment	3×FROSOIL	1×FROSOIL	START+9	START+15	$F_{3,12}$	$p$
<b>Area (mm<sup>2</sup>) of</b>						
whole needle	0.389 ± 0.020	0.480 ± 0.033	0.553 ± 0.075	0.527 ± 0.045	2.259	0.134
mesophyll	0.296 ± 0.013	0.356 ± 0.022	0.407 ± 0.054	0.385 ± 0.029	2.090	0.155
central cylinder	0.093 ± 0.008	0.124 ± 0.011	0.147 ± 0.022	0.142 ± 0.007	2.547	0.105
<b>Area (μm<sup>2</sup>) of</b>						
resin canals	6638.3 ± 400.0	7941.3 ± 626.7	8507.7 ± 1004.4	8091.7 ± 576.8	1.376	0.297
sclerenchyma cells	0.013 ± 0.003	0.019 ± 0.003	0.021 ± 0.003	0.023 ± 0.005	1.121	0.379
phloem	655.0 ± 60.5	814.6 ± 120.2	1002.4 ± 119.4	1061.7 ± 119.0	2.951	0.076
xylem	600.0 ± 45.0	798.1 ± 68.9	995.3 ± 140.9	876.9 ± 89.7	3.399	0.054
<b>Number of</b>						
resin canals	2.9 ± 0.5	3.4 ± 0.3	3.8 ± 0.8	3.9 ± 0.6	0.612	0.620
tracheids	46.9 ± 11.3	50.5 ± 13.9	53.0 ± 11.3	46.8 ± 15.4	0.054	0.983
<b>Needle circumference(μm)</b>						
adaxial	794.0 ± 24.5	909.8 ± 20.5	938.2 ± 54.0	905.2 ± 32.2	3.035	0.071
abaxial	1236.0 ± 35.0	1337.8 ± 57.9	1449.8 ± 96.1	1389.9 ± 27.0	2.437	0.115
entire	2030.1 ± 52.7	2247.6 ± 65	2388.0 ± 141	2295.1 ± 31.8	3.280	0.059
<b>No. of stomata on</b>						
adaxial side	4.5 ± 0.3 <sup>a</sup>	6.1 ± 0.5 <sup>ab</sup>	5.9 ± 0.7 <sup>ab</sup>	6.9 ± 0.5 <sup>b</sup>	4.153	0.031
abaxial side	5.5 ± 0.3 <sup>a</sup>	6.9 ± 0.5 <sup>ab</sup>	7.6 ± 0.6 <sup>b</sup>	7.9 ± 0.6 <sup>b</sup>	4.904	0.019
total circumference	10.0 ± 0.6 <sup>a</sup>	13.0 ± 0.9 <sup>ab</sup>	13.5 ± 0.8 <sup>b</sup>	14.8 ± 1.0 <sup>b</sup>	5.912	0.010
1 mm adaxial length	5.7 ± 0.4	6.7 ± 0.4	6.3 ± 0.5	7.6 ± 0.5	3.063	0.069
1 mm abaxial length	4.4 ± 0.2 <sup>a</sup>	5.1 ± 0.3 <sup>ab</sup>	5.2 ± 0.2 <sup>ab</sup>	5.7 ± 0.4 <sup>b</sup>	4.367	0.027
1 mm total length	4.9 ± 0.2 <sup>a</sup>	5.7 ± 0.3 <sup>ab</sup>	5.6 ± 0.2 <sup>ab</sup>	6.4 ± 0.4 <sup>b</sup>	4.798	0.020

water content followed by a sudden decrease of moisture content, which indicates freezing of the applied water. Similar, but longer lasting freezing cycles in moisture content were reported from the field, when warm spells in winter caused snowmelt followed by cold weather and thus freezing of the infiltrated water in the frozen soil (Sutinen *et al.* 2008). There was no difference in any of the measured characteristics between the saplings which were watered for the first time 9 days or 15 days after the thawing of the soil was started. This is understandable, as the soil moisture content was next to the field capacity immediately after thawing and there was apparently no drought stress despite the delayed watering.

In our study, the soil temperature was not lower than  $-3$  °C in any treatment, so that the root damage observed in 3×FROSOIL could not have been caused by low temperatures as such. At their most hardy state, the fine roots of conifer trees can tolerate this temperature without serious damage in northern latitudes (Sutinen *et al.* 1998, Repo *et al.* 2011). Instead of direct freezing damage, it is more likely that the movement of soil particles due to freezing of added water to the frozen soil broke part of the long lateral fine roots, which was seen as significantly smaller length of the fine roots in 3×FROSOIL as compared with that in the other treatments. This kind of damage is characteristic of frost heaving, which typically occurs in frozen soils with a fine texture and a reasonably high water content before the freezing (de Chantal *et al.* 2006, Michalowski and Zhu 2006). In addition, xylem embolism due to freeze–thaw events might have contributed to the results, as has been found in excised conifer shoots (Pittermann and Sperry 2006, Mayr *et al.* 2007).

Reduced formation of new root tips in 3×FROSOIL during the follow-up season indicated lack of full recovery, which may be connected to the loss of long, lateral fine roots and thus reduced places for new growth of lateral root branches with new root tips (cf. Pregitzer *et al.* 2002). Our result differs from those of snow-removal experiments carried out in northern hardwood forests in New Hampshire, USA, (Tierney *et al.* 2001) and on *Picea abies* in SE Germany (Gaul *et al.* 2008), where elevated fine-root mortality in winter was followed by an earlier peak in fine root production during

the subsequent growing seasons. It seems that increased soil freezing in those soils and experimental conditions damaged mainly the lateral short branches, and thus the survival of lateral long roots makes possible the rapid recovery by production of new short roots.

For early stages of boreal forest regeneration, root damage, slow recovery, and even root death caused by frost heaving have been reported for conifer seedlings with small root systems and incomplete anchoring to the soil (Goulet 1995, de Chantal *et al.* 2003, 2007). According to our study, it seems that the larger saplings, which have quite extensive root systems but still not many coarse roots — the diameter of the roots was less than 1.0 mm in nearly 90% of root length — may also suffer from frost heaving. Even though the root damage here was not as serious as that reported for small seedlings (*see, e.g., de Chantal et al.* 2007), it was serious enough, especially in 3×FROSOIL, to hamper root recovery during the follow-up season. This finding suggests that the roots of larger saplings in the field may suffer somewhat even at present if the climatic and soil conditions favour repeat freezing cycles of soil water.

The finest roots (diameter < 1 mm) and the root tips, including mycorrhizas, are the most important structures for the uptake of nutrients and water. In our case, there was no soil water deficit as the soil water content was kept near field capacity during both growing seasons. Therefore, it seems that loss of fine roots and lack of root recovery during the follow-up season reduced the water uptake, which consequently appeared as diminished consumption of water and reduced trunk sap flow in 3×FROSOIL. Furthermore, the reduced water transport in the saplings caused the observed low water potential (with the cells having less water and more solutes) and the low distribution coefficient of the relaxation time (with only a thin water layer around the cell; *see Zhang et al.* 1995) in C + 1 needles during the follow-up season. The reduced availability of water for the shoots apparently caused structural xeromorphic acclimation, rounding of the needles, and decrease in the number of stomatal rows (Toole and Toole 1999) in the needles developed in 3×FROSOIL saplings during the follow-up season.

Even though the saplings in our study grew in artificial conditions, their needle physiology, e.g., low chlorophyll fluorescence and starch content during dormancy and high starch accumulation in the growing seasons, followed a seasonal rhythm similar to that described in field studies of conifer needles (Repo *et al.* 2011, Sutinen *et al.* 2000). Still, no treatment effects were found on these physiological parameters. This is consistent with the results of root-freezing studies, in which only slight responses in shoot physiology were found in 2-year-old seedlings of *Picea mariana* and *Pinus banksiana* during the next growing season despite extensive root damage (Coursolle *et al.* 2002).

In conclusion, freezing of infiltrated soil water damaged fine roots and reduced root growth during the follow-up season. The root responses were the strongest when watering treatments were done three times before soil thawing. It is possible that repeated infiltration of water onto frozen soil and following freezing caused mechanical breakage, and thus loss of the long lateral fine roots. These events would explain lack of root recovery during the follow-up season. However, the above-ground parts of the saplings, showed only slight and statistically not significant reduction in growth. Yet, it appears that the root damage and consequent decrease in water uptake induced low water potential in older needles and structural acclimation in the needles developed during the follow-up season. If repeated infiltration and freezing occurs during several years, the root damage may lead to damage to above-ground parts as well. If the climate warming scenarios will lead to decreased snow cover and more rain instead of snow in boreal areas in winter, it will also affect soil freezing processes, which may harm to growth and survival of young saplings in the field.

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